Summary and concluding remarks

Introduction

All living cells are separated from their external environment by a lipid membrane. The integrity and barrier function of this membrane is essential for the survival of the cell. This barrier not only prevents hazardous substances to enter the cell, but it also functions in energy transduction in which the electrical and chemical gradients across the membrane energise various cellular processes. To enable the cell to grow, nutrients have to pass the membrane, and proteins, which have their function outside the cell, have to be secreted across this membrane. Furthermore, several proteins function within this membrane environment, and thus need to be inserted in the membrane. Translocation of proteins across the membrane and the integration of membrane proteins into the membrane have to occur without compromising the integrity of this membrane. In bacteria, both processes involve a pore-like integral membrane protein complex consisting of three proteins, SecY, SecE and SecG. The heterotrimeric subunit organisation of this pore-like complex is conserved in all organisms. Homologues of SecY and SecE have been found in eukaryotes and archeae. The third component of this complex, SecG, is not conserved but other membrane proteins replace it. Although the heterotrimeric organisation of the protein-conducting channel is conserved, different mechanisms exist that drive protein translocation across this pore. In *E. coli*, post-translational protein translocation is driven by the large (100 kDa) homodimeric SecA protein that couples the hydrolysis of ATP to the stepwise translocation of the preprotein across the membrane. In mammals, protein translocation is mostly co-translational and thus coupled to the chain-elongation at the ribosome. A detailed overview of the insight obtained during recent years in the mechanism of protein translocation across the inner membrane of bacteria and across the endoplasmatic reticulum membrane of eukaryotes is presented in **Chapter 1**.

The goal of this thesis work was to analyse the structure and function of translocase that mediates the translocation of proteins across the cytoplasmic membrane of *E. coli*.

Functional overexpression of SecYEG

In wild-type cells, the SecYEG complex constitutes less than 0.1% of the total membrane protein. To enable the purification of large quantities of SecYEG complex, a system for the high level over-expression of SecY, SecE and SecG was developed which is described in **Chapter 2**. The genes of *secY*, s*ecE* and *secG* are not contained in an operon but located at various sites within the chromosome of *E. coli*. Therefore, these genes were cloned into a synthetic operon and placed under control of different inducible promoters. Various strains and growth conditions were tested for optimal overproduction. This finally yielded two highly effective combinations, which allow overproduction of the SecYEG complex to up to 40% of the total membrane protein. Membranes which contained high levels of the SecYEG complex ($SecYEG^+$) were tested for activity and showed a large increase in both precursor protein stimulated ATPase activity of SecA and in *in vitro* translocation of proOmpA. Overexpression of SecY, SecE and SecG resulted in an increased number of high-affinity binding sites for SecA. Moreover, the

high level of overproduction of the SecYEG complex caused a shift of the cellular SecA pool towards a tightly membrane-bound SecA fraction, which resists extraction with urea.

Purification and reconstitution of SecYEG

In **Chapter 3**, a method to purify the overexpressed SecYEG is described. SecYEG was expressed with a $(His)_6$-enterokinase tag on SecY, but attempts to use Ni^+-NTA column chromatography were unsuccessful due to presence of interfering levels of mixed micelles of detergent and phospholipids. The presence of a his-tag on SecYEG, however, allowed the purification of large amounts of homogeneously pure SecYEG by a single conventional anion exchange chromatography step starting from octylglucoside-solubilised membranes. The SecYEG was reconstituted into proteoliposomes using the rapid dilution method, and was highly active in both precursor protein stimulated SecA ATPase activity and the *in vitro* translocation of proOmpA after reconstitution. Using the amino-terminally fused $(His)_6$-enterokinase-tag as topology marker, it was established that SecYEG reconstitutes in a random orientation. The purified SecYEG present in octylglucoside solution was further characterised. Circulair dichroism measurements showed that the complex is largely α-helical. Solubilised SecYEG bound SecA with high affinity and supported the AMP-PNP driven conformational change of SecA.

Lipid dependency of bacterial preprotein translocation

In **Chapter 4**, the lipid dependency of the reconstitution of protein translocation was determined. When purified in octylglucoside, the presence of phospholipids is needed to stabilise the SecYEG complex in an active state. To study the lipid dependency of protein translocation, a method was developed to delipidate the SecYEG complex. For this purpose, octylglucoside was replaced for dodecylmaltoside, yielding a SecYEG complex that is stable in the absence of phospholipids and could be re-activated after reconstitution into proteoliposomes. The reconstitution studies demonstrated that negatively charged phospholipids are essential for activity, while non-bilayer forming lipids stimulate protein translocation. In the absence of SecG, the SecYE complex showed a dramatically reduced activity, but the lipid requirement for translocation remained unaltered. To determine if the requirement for negatively charged and non-bilayer lipids is common to the bacterial translocase, reconstitution experiments were performed with the *B. subtilis* translocase using prePhoB as the substrate precursor protein. This system showed an even stronger requirement for negatively charged and non bilayer phospholipids. The *E. coli* and the *B. subtilis* translocase are most optimally active when they are reconstituted into lipid mixtures that most closely resemble the 'native' lipid composition.

Interactions between SecY and SecE

Chapter 5 describes a study of the interaction between SecY and SecE by Cysteine scanning mutagenesis. SecY forms a stoichiometric complex with SecE. To identify sites of interaction a Cysteine-less SecYEG complex was constructed by replacing 2 Cysteines in SecY by serines. This enzyme, which is fully active was used to re-introduce Cysteines in transmembrane segment (TMS) 2 of SecY and TMS 3 of SecE. Putative interacting sites were selected based on genetic studies, which indicate that specific pairs of mutations in these regions may result in a syntheticcally lethal phenotype. Constructs containing single and/or

pairs of Cysteine mutants were tested for overexpression, precursor protein stimulated SecA ATPase activity, *in vitro* translocation of proOmpA and *prl* suppressor activity, *i.e.*, the ability of these mutant SecYEG complexes to allow translocation of precursor proteins with a defective signal sequence. All individual mutants and pairs of mutants showed an activity that was comparable to that of the wild-type SecYEG complex. Oxidation of the pairs of Cysteine-mutants in TMS 2 of SecY and TMS 3 revealed a periodic contact between both transmembrane segments confined to a specific α-helical face. Strikingly, one of the Cysteine mutants in TMS3 of SecE was found to interact with a neighboring SecE molecule. Oxidation of this particular Cysteine mutant reversibly blocked protein translocation, but it did neither interfere with the high affinity binding of SecA to the SecYEG complex nor inhibit the SecA ATPase activity. These data suggests that SecE not only functions in the stabilisation of SecY, but that it also fulfills a catalytic role in protein translocation.

Structure of the SecYEG complex

In **Chapter 6**, the structure of the SecYEG complex was analysed by high-resolution negative stain electronmicroscopy, single particle alignment and averaging. The detergent solubilised SecYEG complex was found to be present mainly as a dimeric species. The size of this dimer was approximately 8.5 by 6.5 nm. After incubation of SecYEG proteoliposomes with SecA and AMP-PNP, and re-purification of the SecYEG complex, a significant fraction of the SecYEG was found in a larger complex with a diameter of approximately 10.5 nm and a stain-filled indentation of about 5 nm. The size of these particles and determination of their molecular mass by scanning transmission electron microscopy indicate that these large particles represent tetramers of the SecYEG complex. These data suggests that the AMP-PNP-induced conformational change of SecA allows the recruitment of SecYEG complexes to yield a stable tetramer of SecYEG possibly forming the protein-conducting channel.

Interactions between SecA and SecYEG

Several methods were used to examine the functional aspects of the interaction between SecA and SecYEG. In **Chapter 2**, membranes containing the overproduced SecYEG complex were used to examine the membrane topology of the SecYEG-bound SecA protein. It was shown that the carboxy terminal tail of SecA is protease accessible from the periplasmic site suggesting that SecA exposes domains to the external face of the membrane.

During the process of protein translocation SecYEG-bound SecA undergoes conformational changes. These conformational changes were studied in further detail in **Chapter 3**. In the presence of ATP and precursor protein, or with the non-hydrolysable ATP analog AMP-PNP alone, the SecYEG-bound form of SecA changes its conformation resulting in the formation of a proteinase K protected 30 kDa fragment that can be detected as I^{125} labeled fragment of SecA. It has been suggested that the formation of the protease resistance of this fragment is caused by membrane insertion of SecA. However, the formation of the fragment can also be found when SecA and SecYEG interact in micellar solution, provided that a detergent is used that does not disrupt this interaction. In detergent, however, the SecYEG complex was proteolysed to remnants smaller that 6 kDa, indicating that the 30 kDa SecA fragment is neither protected by the lipid phase nor by the SecYEG complex. These data suggests that the 30 kDa SecA fragment represents a SecYEG and nucleotide-induced stable conformation rather than a membrane-protected form of a SecA domain.

The SecYEG complex is not merely a passive pore, but undergoes conformational changes during protein translocation. This was demonstrated by using the formation of a disulfide bond between two neighboring SecE molecules as a molecular ruler under translocation conditions. This is described in **Chapter 5**. Conditions preventing the de-insertion of SecA from its "membrane-integrated" state after insertion of the signal sequence strongly stimulated the interhelical contact between two SecE molecules. This demonstrates that SecA-mediated insertion of the signal sequence is transiently sensed by the SecYEG complex resulting in a re-arrangement of the subunits.

These SecYEG subunit re-arrangements induced by the action of SecA were further studied by high resolution electron microscopy, as is described in **Chapter 6**. SecA triggers the formation of a tetrameric structure of the SecYEG complex. To investigate the structure of this SecA-SecYEG complex in the presence of a preprotein, proOmpA was trapped in this channel-like structure by utilising a stable disulfide bridge in the carboxy-terminus of proOmpA that prevents further translocation when the system is kept under oxidising conditions. This proOmpA translocation intermediate stabilised the SecA-SecYEG complex in micellar solution. Quantitative immunoprecipitation experiments showed that translocase consists of four SecY molecules per SecA dimer. The proOmpA-SecA-SecYEG complex was isolated by sucrose gradient centrifugation and analysed by high-resolution negative stain electronmicroscopy. Although the particles appeared heterogeneous in size, orientation and distribution, a substantial amount of particles showed the characteristic shape of tetrameric SecYEG complex. These data, therefore, demonstrate that the SecYEG heterotrimers assemble into a tetrameric supercomplex during translocation.

Concluding remarks and future work

Major progress has been made in the last decade in the field of protein translocation. The proteins involved in this process have been identified, cloned, overexpressed, purified and in case of the membrane proteins, the complexes have been reconstituted in an active state. Individual steps in the targeting and translocation process have been identified and studied. However, there are several important questions that still need to be answered. First, what is the atomic structure of the proteins involved? Knowledge of these structures may facilitate our understanding of how ATP hydrolysis is coupled to the translocation of polypeptide-segments of 2.5 kDa in size. Further information is needed about the interacting domains of the various Sec-proteins, and how the process of channel assembly occurs. Also the function of the SecD and SecF proteins is unclear.

In future years, studies will be focussed on solving the structure of the various Sec-proteins and understanding the dynamics of the interactions between these components. The three-dimensional structure of SecA is close to completion, but still much may be gained from structures representing different nucleotide bound states. Two- and three-dimensional crystallization studies with the SecYEG complex are also underway, but a major challenge will be to obtain snap-shots of the various states of this complex either with or without SecA and precursor protein. Since structure elucidation of membrane proteins is a tedious task, also other techniques need to be employed to obtain structural information. In this respect, the Cysteine scanning mutagenesis described in chapter 5 will help to define sites of interactions between the different membrane proteins, and with the aid of modeling techniques; detailed information on the structure can be obtained. In addition, this

technique also records dynamic changes such as pore assembly that may also be analysed by means of fluorescence studies. Finally, information on the force generation and thermodynamics of protein translocation can be obtained with atomic force and microcalorimetric techniques. These techniques will also be used to study the dynamics of the process of protein translocation.

Another interesting problem in bacteria is how membrane proteins integrate into the cytoplasmic membrane. Targeting of membrane proteins is dependent on the signal recognition particle, but the actual membrane insertion mechanism seems to involve the translocase. It will be important to establish an efficient reconstituted co-translational membrane protein insertion assay using purified components to reveal the intimate details of this process. An exciting road is in front of us to elucidate the mechanisms of how preproteins are translocated across or inserted into the membrane.