Identification and characterization of stable membrane protein complexes

Identificatie en karakterisatie van stabiele membraaneiwit complexen

(met een samenvatting in het Nederlands)

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Contents

Abbreviations

CL	cardiolipin
DDM	dodecylmaltoside
DOPE	1,2-dioctadecanoyl-sn-3-glycerophosphoethanolamine
DOPG	1,2-dioctadecanoyl-sn-3-glycerophosphocholine
DSP	dithiobis(succinimidyl)propionate
DTT	dithiothreitol
E.coli	*Escherichia coli*
ECL	enhanced chemoluminescence
ESI-MS	electrospray ionisation mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
IEF	isoelectric focussing
IM	inner membrane
IPTG	isopropyl β-D-glycopyranoside
LC-MS/MS	nano-flow liquid chromatography coupled to tandem mass spectrometry
LDS	lithiumdodecylsulphate
LI	Lipid I
LII	Lipid II
LUV	large unilamellar vesicle
MALDI	matrix-assisted laser desorption ionisation
Mr	relative mobility
MurNAc	N-acetylmuramic acid
Ni-NTA	Nickel-nitrilotriacetic acid
OG	octylglucoside
OM	outer membrane
PBP	penicillin-binding protein
PBP1A	penicillin-binding protein 1A
PBP1B	penicillin-binding protein 1B
PBP2	penicillin-binding protein 2
PBP3	penicillin-binding protein 3
PC	phosphatidylcholine
PE	phosphatidylethanolamine
pI	isoelectric point
PLE	polar lipid extract
PMF	peptide mass fingerprinting
PTS	phosphotransferase system
SDS	sodiumdodecylsulphate
SDS-PAGE	sodiumdodecylsulphate-polyacrylamide gel electrophoresis
TFE	1,1,1-trifluoroethanol
TM	transmembrane
TX-100	Triton X-100
UDP-glucNac	undecaprenylpyrophosphate N-acetylglucosamine

Chapter 1

Introduction

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Introduction

Membranes

Every living creature is composed of one or more cells. These cells are separated from their environment and are often compartmentalized by barriers known as membranes. Every membrane is composed of a combination of lipids and protein, held together by non-covalent interactions. Lipid molecules are amphiphatic, containing both a hydrophilic and a hydrophobic part. In biological membranes, lipids are arranged in a bilayer in which the hydrophobic parts are clustered together in the interior and the headgroups face outward to the watery environment. This bilayer forms a barrier that prevents molecules inside the cell from leaking out and unwanted molecules from outside the cell from flowing in. In addition to providing a barrier, membranes are involved in a broad variety of processes, including oxidative phosphorylation, photosynthesis, signal transduction and transport. These functions are performed by the protein components of the membrane.

The lipid bilayer has often been described as "fluid mosaic", a two-dimensional fluid in which the individual lipids and protein components are free to diffuse (1). More recent models however indicate that non-random codistribution of protein and lipid components is common (2) and that "patchiness" is the rule rather than the exception (3). Despite their common architecture, membranes differ significantly in composition of both the lipid and protein components between different cells and subcellular compartments (4,5).

Membrane Proteins

Generally, membranes are rich in protein although the actual amount may vary based on the type and function of the membrane in question. In myelin sheaths, which primarily provide electrical insulation, as little as 18% (w:w) protein may be present, whereas up to 75% (w:w) is present in mitochondrial inner membranes (6), which perform vast arrays of functions in oxidative phosphorylation and other metabolic tasks.

The importance of membrane proteins is clearly illustrated by their abundance in genomes. As much as 20 to 30 percent of all open reading frames encode for a protein that resides in or on the membrane (7). Membrane proteins are often implicated in syndromes and a majority of prescription drugs have membrane proteins as their targets. Furthermore, membrane interaction domains are among the most common protein domains (8).

Proteins can associate with the membrane in several distinct manners. Integral membrane proteins span the lipid bilayer one or more times by transmembrane (TM) segments. To prevent exposure of the polar groups of the peptide backbone in such a transmembrane segment to the hydrophobic interior of the bilayer many proteins typically assume an α-helical structure, exposing only the amino acid side chains to the lipid acyl chains. Only the more hydrophobic amino acids are common in membrane-exposed transmembrane α-helices, although polar or charged amino acids can be found as well if they are compensated for by a sufficient amount of hydrophobicity (9). The helices often contain aromatic residues located at the lipid headgroup region (10-12), where they have been suggested to serve as membrane

anchors (13). The region flanking a transmembrane segment often contains positive charges on the intracellular side of the protein, a phenomenon known as the "positive-inside rule" (14).
Outer membrane proteins make use of β-sheets to span a lipid bilayer, in the form of a so-called β-barrel that often contains a central aqueous pore. In this arrangement individual β-strands are arranged in an anti-parallel pattern where the backbone of each individual strand forms hydrogen bonds with its neighbors. The amino acid side-chains point to the inside and outside of the barrel in an alternating pattern, the hydrophobic residues pointing outward to the interior of the bilayer, whereas the hydrophilic ones point inwards towards the pore. This type of transmembrane protein only occurs in the outer membranes of Gram-negative bacteria like *E.coli*, of mitochondria and potentially of chloroplasts

Furthermore, it is possible for a protein to associate with the membrane in a peripheral manner, without traversing the membrane. This association may be based on hydrophobicity, charge effects, or a combination thereof: hydrophobic patches of a protein can insert partly into the hydrophobic part of the membrane, and/or positively charged amino acids may interact with negatively charged phospholipids, resulting in membrane association. Besides interacting with the membrane directly, peripheral membrane proteins may associate with the membrane through interaction with an integral membrane protein. Alternatively, a membrane protein can associate with the membrane via a covalently attached lipid.

Examples of some of these types of membrane proteins are shown in Figure 1. These proteins are common in the cell envelope of the Gram-negative bacterium *E.coli*, the organism that this thesis will focus on.

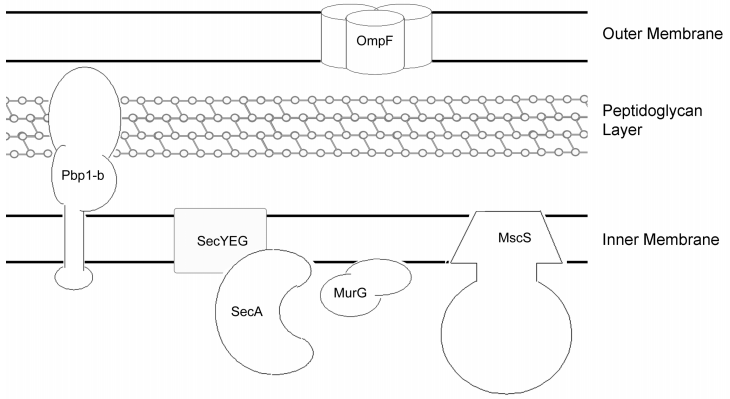

Figure 1: Schematic representation of the Gram-negative cell envelope. The envelope consists of three layers, the inner membrane, the peptidoglycan layer and the outer membrane. Several proteins that interact with the membrane in different ways are depicted. Integral membrane proteins in the inner membrane like MscS and PBP1-B are attached to the membrane through α-helical transmembrane segments. Peripheral membrane proteins like MurG interact with the membrane via a combination of hydrophobic and electrostatic interactions. Peripheral membrane proteins may also associate with the membrane by interacting with an integral membrane protein, as is shown here for SecA residing on the SecYEG complex. Transmembrane proteins in the outer membrane like OmpF have a fundamentally different structure from proteins in the inner membrane. These proteins are composed of adjacent β-strands forming a β-barrel structure with a hydrophobic outer surface.

Chapter 1

The Gram-negative cell envelope consists of an inner membrane, a peptidoglycan layer and an outer membrane. The outer membrane contains lipid-anchored proteins and β-barrel proteins like OmpF, a homotrimeric protein that allows the passage of small solutes through the outer membrane via a central aqueous pore. The peptidoglycan structure is vital for cell survival. It is made up out of linear glycan strands that are cross-linked by peptides, giving strength and rigidity to the peptidoglycan meshwork. It protects the cell from lysis due to shearing forces and differences in pressure across the inner membrane by providing structural stability The penicillin-binding protein PBP1-B is an example of a protein that passes the membrane via a TM helix. It is one out of a variety of inner membrane associated proteins involved in the synthesis of the peptidoglycan layer where it attaches new disaccharide-pentapeptide subunits onto growing peptidoglycan strands. It has an N-terminal transglycosylase domain in the periplasm close to its transmembrane segment that is involved in the formation of linear glycan strands. In addition, the protein contains a periplasmic, penicillin-sensitive transpeptidase C-terminal domain further away from the TM segment that cross-links the peptide subunits.
Another protein involved in the peptidoglycan synthesis pathway is MurG, an example of a peripheral membrane protein, shown on the cytoplasmic side of the inner membrane. MurG's natural substrate is Lipid I, an undecaprenyl-pyrophosphate chain coupled to a muramyl pentapeptide. The protein catalyzes the transfer of N-acetylglucosamine from UDP-GlucNac onto Lipid I, thereby forming Lipid II (15). This is the last step on the cytoplasmic side of the peptidoglycan synthesis pathway prior to the translocation of Lipid II across the inner membrane into the periplasm (16), where the disaccharide pentapeptide is removed from the lipid carrier chain and incorporated into nascent peptidoglycan strands. MurG is associated with the membrane peripherally via a combination of hydrophobic and electrostatic interactions (17).

The peptidoglycan layer is not the only way bacteria use to protect themselves against lysis due to osmotic stress. A class of proteins known as the mechanosensitive channels provides additional protection. The mechanosensitive channel of small conductance, MscS, is shown as an example in the inner membrane in Figure 1. Functional MscS channels are formed by seven identical subunits each spanning the membrane three times. These channels sense stress across the membrane and open an aselective pore in the membrane as a response, allowing the passage of small solutes. This relieves osmotic stress across the membrane, thereby protecting the cell from lysis.

Mechanosensitive channels like MscS are representatives of a large fraction of homooligomeric membrane proteins. Another example of a homo-oligomeric membrane protein is KcsA, which forms a voltage-gated potassium channel composed out of 4 identical subunits. However also many heterooligomeric membrane protein complexes occur in nature. A good example is the bacterial preprotein translocase SecYEG (18) which consists of the three proteins SecY, SecE and SecG. The translocase is involved in transporting newly synthesized soluble proteins and protein domains from the cytoplasm into the periplasmic space in *E.coli*. Two main components of the translocase are the SecYEG part that is integral to the inner membrane and the peripheral SecA protein. The SecYEG part forms the protein-

conducting pore, while SecA provides the driving force through the hydrolysis of ATP. While SecA can associate with the membrane directly, during translocation it resides on the SecYEG part of the translocase. SecA exists as a dimer in solution, but its oligomeric state during translocation has been the subject of debate, with various studies supporting either a monomeric or a dimeric state during its catalytic cycle (see (19) and references therein). In addition to secreting proteins, the translocase is also involved in inserting membrane proteins into the inner membrane. If it encounters a stretch of amino acids of sufficient hydrophobicity, the translocase can undergo a lateral opening, allowing this hydrophobic amino acid chain to escape into the membrane to form a transmembrane segment.

The presence of so many types of integral membrane proteins is the result of the differences in translocation, integration and assembly of proteins in the inner membrane. Little is known on the processes that govern this activity.
Membrane protein folding is described by the so-called two-stage model, in which individual helices are first inserted into the membrane (stage 1), after which helix association takes place (stage 2) (20). In the second stage, individual helices associate to form higher order structures (21). For proteins that do not function as single entities but as components of larger structures, like the MscS channel and the SecYEG complex, it is necessary that they interact with the other subcomponents to assemble into a functional structure. Oligomerisation of membrane proteins likely takes place via the same mechanism as helix-helix association within a single protein (21). Such membrane protein oligomers are held together by protein-protein interactions and by interactions between the protein components and the surrounding lipids.

Membrane lipids
The lipid components of the membrane show great variation in structure and properties. While the three main groups of lipids are sphingolipids, glycerophospholipids and sterols, this thesis will focus on glycerophospholipids because all major lipid classes in the *E.coli* inner membrane belong to this group.
Glycerophospholipids are composed of a glycerol backbone to which one or two hydrophobic fatty acids are attached via ester bonds. The glycerols third hydroxyl group is attached to a phosphate moiety carrying an alcohol, which together form the lipid headgroup. The nature of the alcohol determines the lipid headgroups properties like charge, size, shape and capacity for hydrogen bonding.

E.coli forms an attractive model to study interactions between proteins and lipids for a number of reasons. Its genome if fully sequenced and it is easy to introduce new genes via plasmid-based transformation. Moreover, it grows rapidly and is well studied. In addition, *E.coli* inner membranes are relatively simple in lipid composition. It contains only three main classes of lipids, comprised of roughly 75% of the zwitterionic lipid phosphatidylethanolamine (PE), and 20% and 5% of phosphatidylglycerol (PG) and the dimeric glycerophospholipid cardiolipin (CL) respectively. Both these latter lipids carry a negative charge under physiological pH. These three classes of lipids perform a broad variety of functions in *E.coli* membranes and influence membrane proteins in different manners based on their specific properties.

Lipid-protein interactions

Both the structure and function of membrane proteins can be exquisitely sensitive to the membrane environment. For example, the activity and stability of membrane protein complexes can be affected by specific lipid species (22) and lipids can display a chaperone-like function in insertion, topogenesis and folding (23,24). Indeed, the crystal structures of a number of membrane proteins show lipids tightly associated with them, suggesting important direct interactions (25-27). Also, the biogenesis of membrane proteins is influenced by lipids in many ways. The activity of the preprotein translocase itself is dependent on the lipid environment it resides in (28,29), and even the insertion of integral membrane proteins that do not use the SecYEG complex is influenced by the composition of the lipid bilayer (30). Once inserted, the presence of specific phospolipids may be required to induce correct folding of a protein (24) or mediate the assembly of a membrane protein complex (30).

A well-studied example of an integral membrane protein that is influenced in multiple ways by its lipid environment is the voltage-gated bacterial potassium channel KcsA, shown in Figure 2.

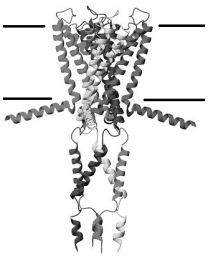

Figure 2: Structure of the tetrameric potassium channel KcsA. This structure was based on crystal structure data from (48) and spin-labeling data (49). Each monomer is displayed in a different color.

KcsA has been shown to undergo a gating transition only when negatively charged lipids are present (31). In addition, its reported crystal structure seems to contain bound lipids at the junction of the subunits (32). Electrospray experiments revealed that KcsA preferentially binds the anionic lipid PG over zwitterionic lipids (33). Also the incorporation of KcsA into membranes is enhanced by the presence of specific phospholipids (34). In addition, the resistance towards dissociation of tetrameric KcsA against both exposure to TFE (35) and heat (34) is improved by the presence of PE. Besides such specific interactions, protein function may be influenced non-directly by structural properties of the lipid bilayer like curvature stress, hydrophobic thickness and packing properties (36). The lateral pressure profile within the membrane has been suggested to serve as a unifying principle for such properties.

What are lateral pressure profiles?

A lipid bilayer is a stable, self-assembled structure, free to adjust its molecular area to minimize the total free energy. In this stable bilayer the sum over all forces acting in the lateral plane of the bilayer must be equal to zero. However, there may be large differences in lateral pressure at different depth in the bilayer. A large attractive

component at the polar-apolar interface arises through interfacial tension. This component is compensated for by repulsive interactions between the lipid acyl chains. Further compensation for the negative component is provided by repulsion between the lipid head groups. These different contributions to the lateral pressure at different depth in the bilayer result in the lateral pressure profile (Figure 3A).

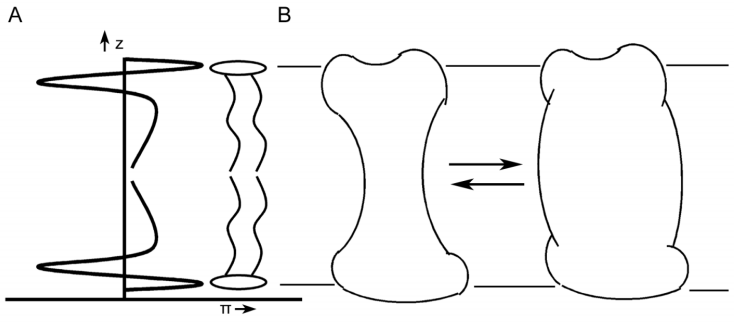

Figure 3: A: Schematic representation of the lateral pressure profile in a lipid bilayer. Adapted from (47). The lateral pressure (π) is indicated as a function of the depth (Z). A tension at the polar-apolar interface is compensated for by repulsion between the lipid headgroups and between the lipid acyl chains. B: Lateral pressure profiles affect integral membrane proteins that undergo a depth-dependent change in cross-section as they change between different conformational states. The protein needs to perform work against the membrane environment to change between states. A change in the lateral pressure profile changes the amount of mechanical work necessary for the conformational change and can therefore influence the proteins distribution among these states.

These lateral pressure profiles will also affect integral membrane proteins. Proteins that can occur in different conformational states will be affected providing they undergo a depth-dependent change in cross-section as they change between conformations. Such a change in cross-section is accompanied by mechanical work depending on both the change in volume and the lateral pressure density against which it expands or contracts. A change in the lateral pressure profile will change the amount of work necessary to undergo a conformation change and therefore will affect the distribution between those conformational states (Figure 3B).

Small alcohols

Lateral pressure profiles can be changed by varying the lipid composition. The addition of lipids with shorter or more unsaturated acyl chains or of lipids with weaker headgroup interactions can have a profound effect on the lateral pressure profile (37). However, small membrane active alcohols like trifluoroethanol (TFE) can also influence the lateral pressure profile by partioning into the lipid headgroup region. Hereby, these alcohols increase the lateral pressure in this area, leading to a reduced lateral pressure at the polar-apolar interface and an overall change in lateral pressure profile. When high amounts of membrane active alcohols are added to lipid vesicles, the bilayer can even become disrupted. Vesicles composed purely of the phospholipids PC are disrupted at lower concentration than vesicles composed of a combination of PC and PE. This is explained by the difference in headgroup size between PC and PE. Because the PE headgroup is smaller, more space is available to

allow partitioning of TFE. This allows for more insertion of the alcohol into the headgroup region before the resulting decrease in lipid packing causes leakage.

It was found that TFE does not only affect the lipid bilayer itself but also proteins present in it. The bacterial potassium channel KcsA (Figure 2) can be visualized in intact, tetrameric form on SDS-PAGE. Exposing KcsA to increasing amounts of TFE prior to electrophoresis revealed that this treatment dissociates the protein into its monomeric subunits. Interestingly, this dissociation occurs at a higher percentage of TFE when PE is present in the bilayer. Clearly, in the case of KcsA, the bilayer plays an important role in stabilizing the protein against TFE-induced dissociation. To explain these observation the following model was suggested (Figure 4): When tetrameric KcsA resides in a bilayer containing non-bilayer lipids (Figure 4B) a higher lateral pressure in the acyl chain region occurs compared to a bilayer containing only bilayer lipids (Figure 4A). This higher lateral pressure, indicated as dark coloring in the figure, results in an increase in KcsA stability. When TFE is added it partitions into the headgroup region, increasing lateral pressure there. This results in a lower lateral pressure in the acyl chain region. This then destabilizes the KcsA tetramer, eventually resulting in dissociation into monomeric subunits (Figure 4C). Because more space is available in a bilayer containing PE, more TFE is required before the lateral pressure has decreased enough to allow for the dissociation of KcsA (Figure 4D).

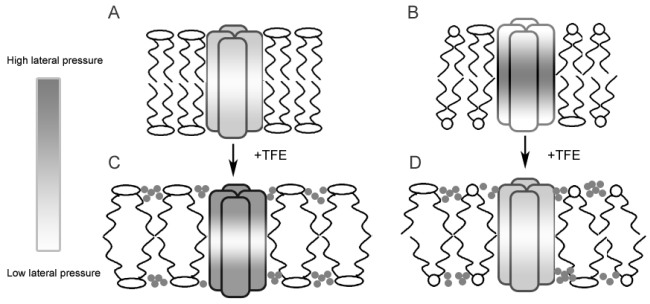

Figure 4: Suggested model for the effect of surrounding lipids and TFE on lateral pressure acting on KcsA, adapted from (50). For simplicity KcsA subunits are shown as rectangles. The color scale on the cylinders indicates lateral pressure, dark color corresponds to high pressure while light color corresponds to low lateral pressure acting on the protein. For the sake of clarity, the negative lateral pressure at the polar-apolar interface is omitted. The effect of addition of 20% v:v TFE is shown for KcsA in a membrane composed solely of PC (left) and a membrane containing both PE and PC (right).

Besides affecting the membrane, alcohols like TFE can exert a direct influence on proteins and peptides as reviewed in Buck, 1998 (38). This effect can be stabilizing in some cases but destabilizing in others. TFE has also been known to accelerate the folding of soluble proteins. Several mechanisms are possible that mediate the effect of TFE on proteins. First, direct binding of TFE to the carbonyl oxygen of the amino acid backbone can lead to enhanced intramolecular hydrogen bonding as the solvent exposure of the amide is minimized. Second, the lower dielectric constant of the alcohol-water mixture could enhance electrostatic interactions within the protein

molecule. Third, TFE can associate preferentially with hydrophobic sites on the protein, weakening hydrophobic-hydrophobic interactions. In fact, TFE seems to weaken interactions between residues distant from each other in the primary sequence, whereas structures that rely on local sequence information like helices are stabilized. Finally, indirect mechanisms in which secondary structures are stabilized by affecting their hydration shells may play a role.
The actual effect of TFE on proteins or peptides is probably a combination of these and possibly other factors whose relative contribution varies depending on the specific conditions. Nevertheless, it is clear that small membrane-active alcohols can have a large effect on membrane protein complexes. Conceivably, these alcohols, in combination with proteomics techniques, can form a generic tool to study such complexes.

Proteomics
Many membrane proteins take part in oligomers. However, it is unclear how many membrane protein complexes exist, what the exact protein composition of these complexes is or what kinds of interactions play a role in keeping them together.
One method that seems particularly well-suited to address such questions is proteomics. Proteomics research focuses on the identification and quantification of the complete proteome present in cells, organisms or tissue. It encompasses the characterization and functional analysis of all proteins that are expressed by a genome as well as their interactions. Because the expression levels of proteins strongly depend on complex regulatory systems, the proteome is highly dynamic. This also holds true for *E.coli*. In various studies, *E.coli* cells have been exposed to many different environmental perturbations resulting in changes in the observable proteome by anywhere from a few proteins to nearly half of the proteins in the cell (39).
Most of the initial proteomics work was done by gel-based methods like 2-dimensional electrophoresis (40). Briefly, it consists of an initial separation based on isoelectric point (pI) by isoelectric focusing (first dimension), followed by a separation based on relative mobility (Mr) in a sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) gel (second dimension). Upon completion of the run the proteins must be identified and if possible quantified. One method that has found widespread use is identification by mass-spectrometry (MS). Usually this is performed by excising the spots from the gel and digesting the proteins present with a mixture of proteases. The peptides acquired in this way are subsequently extracted from the gel. A mass-spectrometer is used to determine the masses of the peptides. For mass spectrometry to work it is necessary to ionise the peptides, which can be achieved by a number of methods. The most common methods are matrix-assisted laser desorption ionisation (MALDI) (41), achieving ionisation with short bursts of laser radiation, and electrospray ionisation (ESI) (42) which works by desorbing peptide ions from the surface of highly charged droplets of a suitable solvent. The masses obtained in this way are then checked against a database of possible peptides masses that can be formed by the used proteases out of the proteins present in the organism of interest. This usually gives sufficient information to identify the protein or proteins present in the spot. If this method, commonly known as peptide mass fingerprinting (PMF)(43) is not capable of identifying the peptides unambiguously the peptide can be sequenced by a process called tandem-MS (or MS/MS). In MS/MS a quadrupole or ion-trap is set to allow only one single mass-over-charge value to pass through at any given time. This serves to let only one kind

of peptide through at a time. These peptides can be fragmented in-flight by collisions with an inert gas, resulting in a fragmentation spectrum. Here too the obtained masses are checked against a database to identify the peptide the fragmentation spectrum originates from, again resulting in a protein identification (Figure 5).

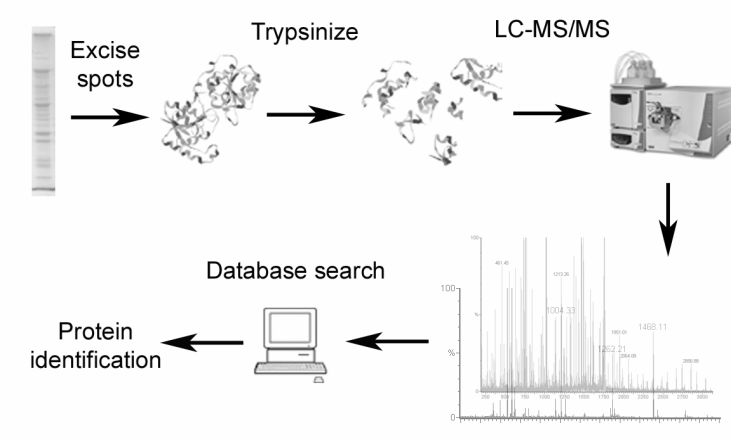

Figure 5: A common proteomics workflow for identifying proteins by their peptide fragmentation spectra. A protein sample is separated on SDS-PAGE gel after which bands are excised and exposed to trypsine. The originating peptides are extracted from the gel and introduced into a mass spectrometer after separation by liquid chromatography. The mass spectrometer (an LCQ Ion Trap in this case) produces fragmentation spectra by collision-induced dissociation of peptides. By matching the observed peptide fragments on a database of proteins by the SEQUEST algorithm the proteins from which the peptides originate can be determined. This scheme represents the proteomics method applied in chapter 2.

Unfortunately, while this generally works well for soluble proteins as witnessed by the studies on the soluble *E.coli* proteome (44), analysis of membrane proteins by these methods have lagged behind. This is because their pI's are generally higher than the range of common IEF strips and their intrinsically hydrophobic nature makes them prone to precipitation upon focusing. Because IEF requires that the intrinsic surface charge of the protein be maintained, the use of strong ionic detergents like SDS is generally avoided. In addition, membrane proteins are not very abundant compared to soluble proteins, making them harder to detect on gel. Furthermore, membrane proteins contain large stretches of hydrophobic amino acid residues. Peptides containing such stretches extract poorly from the gels and are more difficult to ionize, hampering detection.

To avoid these problems new methods have been developed which generally avoid the use of gels altogether. In these approaches a complete proteome is enzymatically digested and introduced into a mass spectrometer via an LC column, an approach referred to as "shotgunning". Because the enormous complexity of such a sample would interfere with the analysis, it is often fractionated into a subproteome. This can be achieved either prior to enzymatic treatment or afterwards by targeting only cysteine-containing or phosphorylated or glycosylated peptides.
Recently, several large-scale proteome analyses aimed at the identification of interacting proteins have been performed on *E.coli* and yeast. In these approaches tags

are cloned onto bait proteins. The proteins are then purified via the tags and any co-purifying proteins are identified. These approaches are typically restricted to the soluble proteome because the presence of detergent may disrupt less stable interactions.
To determine interactions between membrane proteins specific methods have been developed that typically rely on gel electrophoresis under non-denaturing conditions. One that has seen much use recently, especially in the analysis of supercomplexes in mitochondria, is Blue-Native electrophoresis. This method relies on the binding of the dye Coommassie Brilliant Blue to solubilize membrane proteins and separate them in an electric field. Sometimes this is combined with the use of mild detergents like octylglucoside or digitonin. One important drawback of this method is the limited resolution, especially in lower mass regions, and a narrow dynamic range. Differences in delipidation of protein complexes may also hamper resolution. In addition, the mild conditions used often give rise to artefactural associations (45). These drawbacks make it desirable to have a method available that allows for high resolution at lower molecular weight. While SDS is typically considered a denaturing detergent, several protein complexes are known to retain their tertiary structure in room-temperature SDS, suggesting that regular SDS-PAGE can be used to study stable membrane protein complexes.

Scope of this thesis

Proteomics technology provides interesting perspectives in addressing fundamental questions regarding membrane protein oligomers. In this thesis a novel method is presented that allows for the identification of stable membrane protein complexes in the presence of ionic detergents like SDS (chapter 2). The method is based on the ability of small membrane-active alcohols to disrupt the tetrameric potassium channel KcsA. Via diagonal electrophoresis combined with LC-MS/MS it is shown that many integral and peripheral membrane proteins in the *E.coli* inner membrane take part in complexes that are stable in SDS but become dissociated by TFE.

Several proteins that were identified in this manner were subjected to further study. Among them is the preprotein translocase SecA. We show in the appendix to Chapter 2 that on SDS-PAGE two pools of SecA occur, one in dimeric form and one in monomeric form. These two pools seemingly correspond to the translocase-bound and membrane-associated pools reported previously (46), supporting the notion that SecA is functional as a dimer in protein translocation.
Another protein that was identified and examined in detail is the glycosyl transferase MurG. This is the first time evidence has been found to suggest MurG interacts directly with other membrane proteins. To identify a possible interaction partner the complex was extracted from an overexpression system and subjected to protein identification analysis. A third protein that was studied is the mechanosensitive channel MscS. The effect of small membrane-active alcohols on the oligomeric state of the protein was examined in different lipid and detergent environments and the results are reported in chapter 4.

In chapter 5 the method presented in chapter 2 is discussed in relation to pre-existing ones. The additional data obtained on specific protein complexes is summarized and discussed in the context of membrane protein interactions and function.

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Chapter 1

Chapter 2

Detection and identification of stable oligomeric protein complexes in *E. coli* inner membranes. A proteomics approach

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Chapter 2

Abstract

In this study we present a new technology to detect stable oligomeric protein complexes in membranes. The technology is based on the ability of small membrane-active alcohols to dissociate the highly stable homotetrameric potassium channel KcsA. It is shown via a proteomics approach, using diagonal electrophoresis and nano-flow liquid chromatography coupled to tandem mass spectrometry, that a large number of both integral and peripheral *E. coli* inner membrane proteins are part of stable oligomeric complexes that can be dissociated by small alcohols. The study gives insight into the composition and stability of these complexes.

Introduction

Approximately one out of four open reading frames encodes for a protein that resides in or on a membrane (1). This abundance signifies the importance of membrane proteins for cellular function. For several classes of these proteins it is known that they function as oligomeric complexes. Examples are solute channels (2) and protein translocators (3). Although extensive networks have been established of interaction partners of water soluble proteins in for example *E. coli* (4) and yeast (5), very little is known about the interaction partners of membrane proteins or of the stability of the complexes that are formed in membranes. This lack of insight is largely the result of the difficulties in handling membrane proteins and the lack of suitable tools to study them.

The bacterial potassium channel protein KcsA is a well-studied example of a protein that functions as a stable oligomeric complex. It is a small homotetrameric protein of known structure (6) that is representative of many related channel proteins (2,7-9). When KcsA containing membranes are treated with SDS, the protein retains its tetrameric form, even at elevated temperatures (2). Very recently it was discovered that small alcohols like trifluoroethanol (TFE) can dissociate the KcsA tetramer into monomers in a manner that is dependent on both the concentration and the type of alcohol (10,11). This observation suggests that small alcohols may be used as a generic tool to detect and characterize stable oligomeric membrane protein complexes. Here we explored this possibility for proteins in *E. coli* inner membranes. For this we developed a new proteomics approach using diagonal SDS-based electrophoresis. In this approach non-heated protein samples are first separated by SDS-polyacrylamide gelelectrophoresis, followed by *in-gel* exposure to the alcohol and subsequent electrophoresis in a second dimension. All proteins or protein complexes that are not affected by the alcohol are expected to migrate on the diagonal of the gel, whereas protein complexes that are stable in room temperature SDS but are dissociated by the alcohol will migrate off the diagonal. Off-diagonal spots are then digested by trypsin and identified by LC MS/MS. Using this method we observed many off-diagonal spots that contained membrane proteins, either integral with one or more transmembrane segments or peripheral with a membrane interactive domain. We thus readily identified a subproteome consisting of membrane proteins that are part of very stable homo- or hetero-oligomeric complexes. The method is expected to be applicable to a variety of membrane preparations of different cells or organelles, because it does not require labeling of the proteins with specific tags, as used in other

studies to detect interaction partners of proteins (4). The method is complementary to traditional 2-dimensional electrophoresis methods of blue-native PAGE in combination with SDS-PAGE (12), which allows detection of protein complexes that are not stable in SDS.

Materials and methods

Materials

Electrophoresis setups and electrophoresis buffer (25 mM Tris/HCl pH 8.3, 192 mM glycine, 0.1% (w/v) sodium dodecyl sulphate) were purchased from BioRad Laboratories B.V. (The Netherlands). Other sources of materials were: Ni^{2+}nitrilotriacetic acid (Ni-NTA) (Qiagen Benelux N.V. (The Netherlands)), N-dodecyl-β-D-maltoside (DDM) (Anatrace Inc. (Ohio, USA)), 2,2,2-trifluoroethanol (Merck (Germany)), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Acros Organics (The Netherlands)), Coomassie brilliant blue G-250 (ICN Biomedicals Inc (Ohio, USA)), Bocillin FL (Invitrogen B.V. (The Netherlands)), enhanced chemiluminescence detection kit (Amersham-Pharmacia, Freiburg, Germany) and bovine trypsin, sequencing grade (Roche Diagnostics (The Netherlands)). SecA antibodies were a kind gift of Dr. Hans de Cock. The pHK2414 plasmid was a kind gift of Dr. Martine Nguyen-Distèche.

Isolation of membrane vesicles and purification of KcsA

Crude *E. coli Bl21 (DE3)* membrane vesicles from cells overexpressing KcsA were prepared as described (13). Membranes of HMS174 cells overexpressing penicillin binding protein 1B from the pHK2414 plasmid were obtained similarly. Vesicles were either resuspended in 100 mM NaCl, 5 mM KCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH7.5 for use in the 2D gel-system, or used for KcsA purification according to (14). Resuspended membrane vesicles were stored at -20^0C.

Purified KcsA was stored at 4^0C in buffer containing 100 mM NaCl, 5 mM KCl, 10 mM HEPES pH 7.5 and 1 mM N-dodecyl-β-D-maltoside.

Purified inner membrane vesicles from late-exponential BL21(DE3) *E. coli* cells were prepared as in (15). Cells were collected and lyzed in a French press. Inner- and outer membrane vesicles were separated via sucrose density centrifugation. The purified inner membrane vesicles were stored at -80^0C.

Fluorescence detection of penicillin binding protein 1B

A crude preparation of membrane vesicles from HMS174 cells expressing PBP1-B was labelled with 10 μM of bocillin FL for 30 minutes at 35^0C. Vesicles were subsequently dissolved in SDS gel-loading buffer (12 mM Tris/HCl pH 6.8, 5% (v/v) glycerol, 0.4% (w/v) SDS, 14 mM dithiothreitol and 0.02% bromophenol blue) and either kept at room temperature, heated in sample buffer for 5 minutes or incubated with 20% TFE for 1 hour. The samples were then run on regular 1-dimensional 11% SDS-PAGE gels. After electrophoresis the gels were rinsed with water. The labelled proteins were visualised by scanning the gels on a Typhoon 9400 imaging system (Amersham) using an excitation wavelength of 488 nm and an emission wavelength of 526 nm.

Immunodetection of SecA
Preparations of inner membrane vesicles were solubilized in SDS-gel loading buffer and either kept at room temperature, heated in sample buffer for 5 minutes or incubated with 25% TFE for 1 hour. These samples were run on an 11% SDS-PAGE gel. Proteins were blotted onto a nitrocellulose membrane. SecA was detected by a specific antibody and by the use of the enhanced chemiluminescence detection kit.

2D SDS-PAGE

SDS-PAGE in the first dimension
The membranes were solubilized at room temperature in SDS-gel loading buffer and the samples were run in electrophoresis buffer in two different setups. A BioRad mini-Protean II setup was used for gels for either purified KcsA or a crude *E. coli* inner membrane preparation. These samples were run on a lane of a regular 0.75 mm thick 11% SDS-PAGE gel of 7.2 cm by 8.2 cm at a voltage of 180 V. Purified *E. coli* inverted inner membrane vesicles were run on a BioRad Protean II setup on a 0.75 mm thick 11% gel of 16 cm by 18 cm at a current of 30 mA.

In-gel TFE-exposure and SDS-PAGE in the second dimension
Lanes of either 5.5 cm by 1 cm or 15 cm by 2 cm were excised from the first dimension gels and run on a BioRad mini Protean III or Protean II setup respectively. In order to dissociate membrane protein complexes the excised gel-strips were incubated for 1 hour in electrophoresis buffer containing the indicated percentage (v/v) of TFE or HFIP at room temperature. After incubation the gel-strips were washed twice for five minutes with water, and twice with electrophoresis buffer to reduce the amount of TFE, which interferes with electrophoresis.
The gels for the second dimension consisted of a 5 cm by 8.2 cm separating gel with a 0.8 cm by 8.2 cm stacking gel on top for use in the BioRad mini Protean III setup or a 15 cm by 18 cm separating gel with a 1.2 cm by 18 cm stacking gel on top for the BioRad Protean II system. Room was left on top of the gels to fit in the TFE-treated gel strips. An overlay of isopropanol was used during polymerisation of the stacking gel as this gave a smoother surface than a watery overlay. After polymerisation the isopropanol was decanted and the surface of the stacking gel was rinsed with water to remove traces of isopropanol. The gel strips were placed on top of the second dimension gels. Empty regions between the excised gelstrip and the glass plates were filled with 0.3% agarose in electrophoresis buffer. The gels were run at the same voltage or current as the first dimension gels. Gels were stained using Coommassie brilliant blue G250.

In-gel tryptic digestion of off-diagonal spots
Off-diagonal protein spots were excised and *in-gel* digested with trypsin with a slightly modified protocol as described by Wilm et al. (16). In brief, the gel pieces were destained using 50% (v/v) methanol followed by reduction (6.5 mM DTT in 50 mM ammonium bicarbonate pH 8.5) and alkylation (54 mM iodoacetamide in 50 mM ammonium bicarbonate pH 8.5.) Subsequently, the gel pieces were washed and dehydrated using 50 mM ammonium bicarbonate and acetonitrile, respectively. Proteins were digested overnight at 37^0C by adding trypsin at a concentration of 10 ng/µl. The digestion was stopped by adding 1 µl of acetic acid.

Nano-LC-MS/MS

Nano-LC-MS/MS analysis was performed by coupling an Agilent 1100 Series LC system (vacuum degasser, auto sampler, and one high-pressure-mixing binary pump without static mixer) to a LCQ^{tm} Classic Quadrupole Ion Trap mass spectrometer. (Finnigan, San Jose, CA, USA) basically as described by Meiring et al (17). Briefly, peptide mixtures were delivered to a trap column (Aqua^{TM} C18RP (Phenomenex); 15mm × 100 µm, packed in-house) at 5 µl/min 100% A (A = 0.1 M acetic acid). After reducing the flow to approximately 150 nl/min by a splitter, the peptides were transferred to the analytical column (PepMap C18 (LC Packings); 15 cm × 75 µm, packed in-house) with a linear gradient from 0-50% buffer B (B = 0.1M acetic acid in 80% acetonitrile) in 60 minutes. The column eluent was sprayed directly into the ESI source of the mass spectrometer *via* a butt-connected nano-ESI emitter (New Objectives). The LCQ operated in positive ion mode and peptides were fragmented in data dependent mode. One MS survey scan was followed by three data dependent MS/MS scans.

Identification of MS/MS spectra by database searches

The SEQUEST algorithm (18) was used to interpret the obtained MS/MS spectra. The SEQUEST algorithm was run against the complete non-redundant proteome database in FASTA format of *Escherichia coli* from the European Bioinformatics Institute (EBI). The algorithm was set to use trypsin as enzyme, allowing at maximum for two missed cleavages and assuming carbamidomethyl as a fixed modification, and oxidized methionine as a variable modification. The program DTAselect (19) was used to select the peptide identifications and to assemble the peptides into proteins. The following selection criteria were used to filter the peptide identifications: the minimum Xcorr was set at 1.9, 2.2 and 3.75 for 1+, 2+ and 3+ peptides, respectively and the ΔCns was 0.1 or higher for each peptide.

Results

The principle of the presented proteomics approach is shown in fig. 1 for purified KcsA. One-dimensional SDS-PAGE (fig. 1A) confirms that KcsA migrates as a stable tetramer (2), which is dissociated into monomers by incubation with 25% TFE (v:v). In the absence of TFE only a minor band of the monomer is observed. When the sample is run on a 2D gel under identical conditions in both dimensions and in the absence of TFE, the major spot of the tetramer and the minor spot of the monomer are expected to lie on the diagonal of the gel, as is indeed observed (fig. 1B). However, when after running the gel in the first dimension, the gel strip is exposed for 1 hour to 25% TFE, the tetramer dissociates and migrates off-diagonally on the monomer position in the second dimension (fig. 1C).

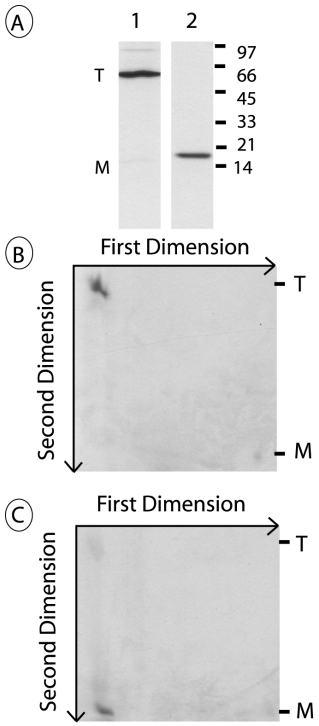

Figure 1: TFE-induced dissociation of the KcsA tetramer as shown on SDS-PAGE. Panel A shows a one-dimensional (9) SDS-PAGE gel of KcsA before (lane 1) and after (lane 2) exposure to 25% TFE. Monomeric (M) and tetrameric (T) KcsA are indicated and a protein size marker (in kDa) is shown on the right. The lower two panels show a 2D SDS-PAGE gel of KcsA without exposure to TFE (B) and with exposure to 25% TFE after running the first dimension (C). Monomeric (M) and tetrameric (T) KcsA are indicated as well as the direction of electrophoresis in the first and second dimensions.

The same behaviour of KcsA is observed for a crude *E. coli* membrane preparation obtained from cells over-expressing KcsA (fig. 2). A prominent KcsA spot that runs off-diagonal at its monomer position shows up in addition to a heavy stained diagonal corresponding to *E. coli* proteins that are not affected by TFE.

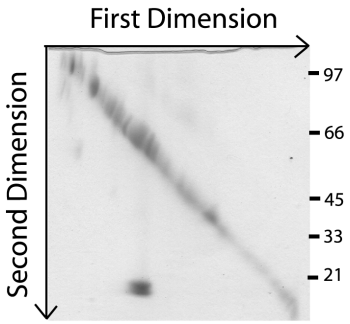

Figure 2: TFE-induced dissociation of proteincomplexes in a crude *E. coli* inner membrane preparation of cells overexpressing KcsA, as shown on SDS-PAGE. The directions of migration in the first and second dimension are indicated. The intense protein spot under the diagonal corresponds to the KcsA monomer.

Interestingly, also some other spots at higher molecular weight run off-diagonally, indicating that besides KcsA also several other *E. coli* proteins form stable complexes in SDS that are dissociated by the alcohol. To better visualize these off-diagonal proteins, purified inner membrane vesicles obtained from wild-type *E.coli* cells were applied to larger gels. A representative gel is shown in fig.3A.

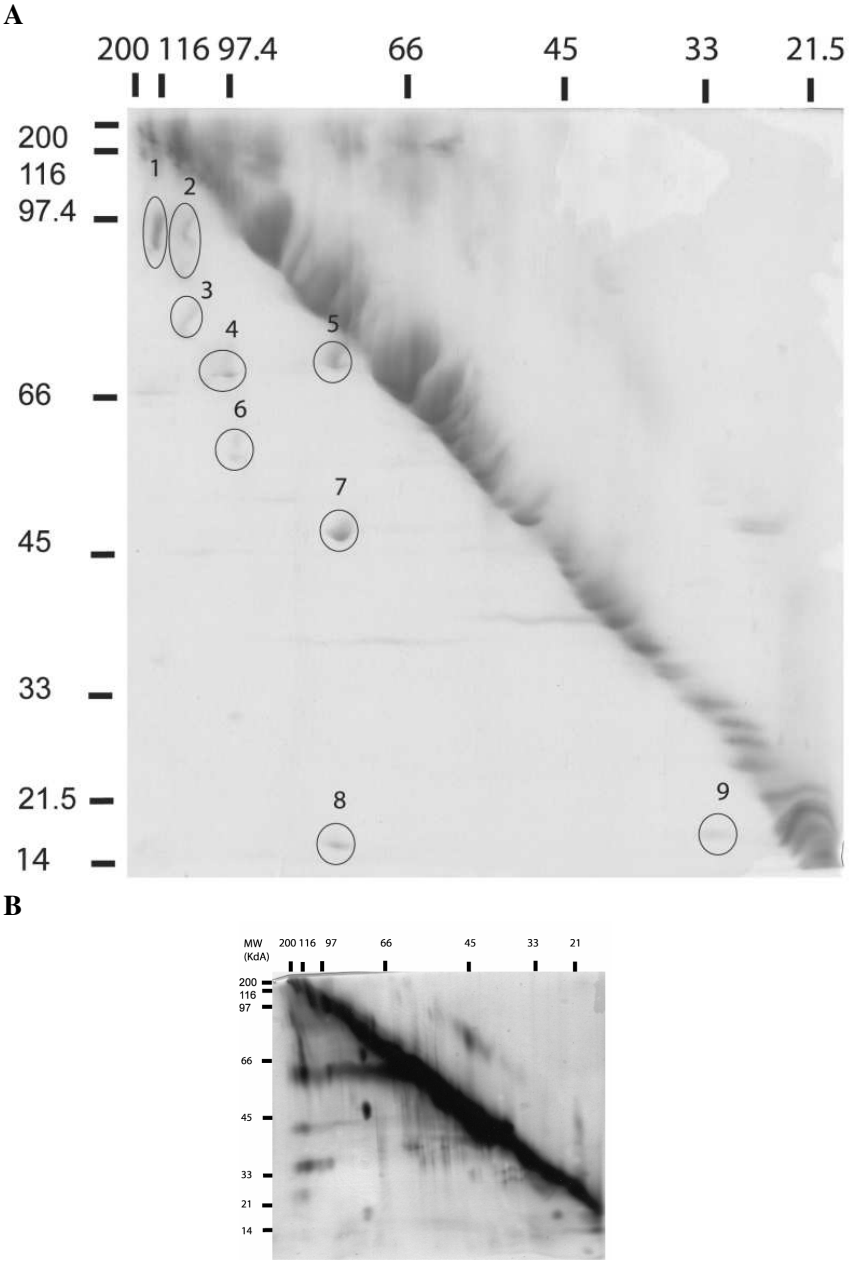

Figure 3: Dissociation of protein complexes from a preparation of *E. coli* inverted inner membrane vesicles by 25% TFE as shown on SDS-PAGE gel stained with Coomassie (A) or silver stained (B). The numbers in fig. 3A correspond to the spot-numbers shown in table 1. MW markers are shown in both the first and second dimension.

An intensely stained diagonal is observed as well as a number of off-diagonal spots. The stain above the diagonal is most likely the result of smearing. This interpretation is supported by the observation that analysis of such an off-diagnonal spot that corresponded to an approximate molecular weight of 70 kDa was found to contain protein with molecular weights ranging from 20 to 55 kDa (fig. 3B). Furthermore, when the gel was silver stained the smearing was even more obvious (not shown). The spots under the diagonal were excised and in-gel digested with trypsin, after which the resulting peptides were analyzed by mass spectrometry. In this way 58 proteins could be identified (Table 1).

Table 1. *E.coli* inner membrane proteins that are dissociated from complexes in SDS at roomtemperature by TFE.

Type[a]	Spot[b]	Gene	Swissprot accession number	TMs[c]	Gene product	No. of peptides[d]	Coverage[e]	MW (kDa)
Integral	3	nagE	Q8X9H9	10	PTS system, N-acetylglucosamine-specific enzyme IIABC	1	2 %	68
	6	ptsG	P05053	10	PTS system, glucose-specific IIBC component	4	9 %	51
	6	treB	Q8XCE0	10	PTS system enzyme II, trehalose specific	4	9 %	51
	4	mtlA	Q8XDH1	8	PTS system, mannitol-specific enzyme IIABC components	7	9 %	68
	1	mrcB	Q8X903	1	Penicillin-binding protein 1B	26	32 %	94
	2	mrcA	Q8X809	1	Penicillin-binding protein 1A	7	10 %	94
	3	mrdA	P08150	1	Penicillin-binding protein 2	3	6 %	71
	4	secD	P19673	6	Protein-export membrane protein secD	8	17 %	67
	9	secG	P33582	2	Protein-export membrane protein secG	1	16 %	11
	4,5	cydA	Q8X979	9	Cytochrome d terminal oxidase, polypeptide subunit I	11	17 %	58
	1	mdoH	P62517	6	Glucans biosynthesis glucosyltransferase H	3	5 %	97
	4	cydD	Q8X5I1	5	ATP-binding component of cytochrome-related transport, Zn sensitive	6	10 %	65
	5	oxaA	Q8FBV4	4	Inner membrane protein oxaA	17	23 %	62
	1	yggB	P11666	3	MscS mechanosensitive channel	5	18 %	31
	3,4,6	cyoA	Q8XE63	3	Cytochrome o ubiquinol oxidase subunit II	3	18 %	35
	5	hflB	Q8X9L0	2	Cell division protease ftsH	15	23 %	71
	4	tolA	Q8X965	1	Membrane spanning protein TolA	8	20 %	41
	5	pqiB	Q8XDA7	1	Paraquat-inducible protein B	6	15 %	60
	5	yfgA	Q8XAA6	1	Putative membrane protein	4	13 %	36
	6	yibP	Q8XDE2	1	Putative membrane protein	7	14.4%	47
	6	yrbD	P45391	1	Hypothetical protein yrbD precursor	10	57 %	20
	8	yibN	P37688	1	Hypothetical protein yibN - Escherichia coli	12	62 %	16
	9	yhcB	P39436	1	Hypothetical protein yhcB	6	52 %	15
Peripheral	3	nuoC	Q8XCW9	0	NADH dehydrogenase I chain C, D	48	65 %	69
	6	nuoF	Q8XCX1	0	NADH dehydrogenase I chain F	4	13 %	49
	2	nuoG	Q8XCX2	0	NADH dehydrogenase I chain G	7	10 %	100
	4	atpA	P00822	0	ATP synthase alpha chain	28	51 %	55
	9	atpH	P00831	0	ATP synthase delta chain	10	55 %	19
	5	atpD	P00824	0	ATP synthase beta chain	5	12 %	50
	3	dld	Q8X666	0	D-lactate dehydrogenase, FAD protein, NADH independent	4	4 %	65
	7	lldD	Q8XDF7	0	L-lactate dehydrogenase	9	29 %	43
	5	frdA	Q8XDQ0	0	Flavoprotein subunit of fumarate reductase FrdA	2	5 %	66
	2	plsB	P58130	0	Glycerol-3-phosphate acyltransferase	24	29 %	91
	4,5	glpD	Q8X6Y5	0	Sn-glycerol-3-phosphate dehydrogenase (Aerobic)	46	59 %	57
	7	manX	P08186	0	PTS system, mannose-specific IIAB component	34	73 %	35
	4	hybC	P37181	0	Hydrogenase-2 large chain precursor	3	9 %	62
	4	mglA	Q8X5D9	0	ATP-binding component of methyl-galactoside transport system	4	10 %	56

	2	lepA	P60787	0	GTP-binding protein lepA	4	8 %	67
	2	secA	Q8X996	0	Preprotein translocase SecA	65	52 %	102
Outer Membrane	1	ompF	Q8XDF1	OM	Outer membrane protein 1a (Ia,b,F)	12	27 %	39
	2	imp	Q8XA13	OM	Organic solvent tolerance protein precursor	7	13 %	90
	1,2	lamB	Q8X5W7	OM	Maltoporin precursor	8	20 %	50
	4,5	TolC	Q8XBP7	OM	Outer membrane channel TolC	20	42 %	54
	4	z2268	Q8X9X1	OM	Putative outer membrane receptor for iron transport	2	4 %	77
Ribosomal	9	rplI	P02418	0	50S ribosomal protein L9	5	36 %	16
	9	rplJ	P02408	0	50S ribosomal protein L10 (L8)	7	47 %	16
	9	rplO	P66071	0	50S ribosomal subunit protein L15	3	26 %	15
	9	rplQ	P02416	0	50S ribosomal protein L17	3	21 %	14
Unknown	1	sucA	P07015	0	2-oxoglutarate dehydrogenase E1 component	19	24 %	105
	4,5	sucB	P07016	0	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase	6	15 %	44
	1	aceE	P06958	0	Pyruvate dehydrogenase E1 component	53	54 %	100
	4,2	aceF	Q8X966	0	Pyruvate dehydrogenase Dihydrolipoyltransacetylase component	14	23 %	66
	2	malP	Q8X708	0	Maltodextrin phosphorylase	5	10 %	91
	2	speA	Q8XCX9	0	Biosynthetic arginine decarboxylase	3	6 %	74
	6	srmB	Q8XA21	0	ATP-dependent RNA helicase	8	18 %	50
	2	ydiJ	Q8X5Y8	0	Putative oxidase	6	8 %	113
	9	ybjP	Q8X6N7	0	Putative enzyme	2	14 %	19
	2	ECs3386	Q8XA93	0	Orf, hypothetical protein	9	8 %	181

(a) Type of protein or sub-cellular localization. (b) Number of the spot in figure 3A (c) Number of transmembrane segments as calculated from the primary structure of the proteins by the TMHMM 2.0 program (38). The designation OM indicates an outer membrane protein. (d) Total number of peptides used for protein identification. (e) Percentage of the amino acid sequence covered by the detected peptides.

The large majority (39) were proteins that are known to be present in or are associated with the inner membrane. Five outer membrane proteins were found which most likely originate from the remnants of outer membranes known to be present in these preparations (15). The remaining proteins were four ribosomal proteins, possibly originating from ribosomes that are attached to the membrane and ten proteins of unknown location.

From an analysis of the monomeric molecular weights of the proteins that we identified and from the apparent molecular weight of the corresponding protein complex on the diagonal, several homo-oligomeric protein complexes could be identified. For instance, the penicillin-binding protein IB that is encoded by the MrcB gene (fig. 3A. spot 1) undergoes an apparent molecular weight shift from approximately 200 to 100 kDa, suggesting that in room temperature SDS the protein is at least partially present as a dimer that becomes dissociated by TFE. This is indeed the case as shown by 1D gel electrophoresis of *E. coli* inner membranes obtained from cells overexpressing PBP1-B that are solubilized in cold SDS and stained with a fluorescent penicillin analogue (fig. 4).

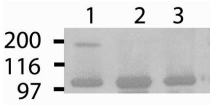

Figure 4: Fluorescence image of an 11% SDS-PAGE gel with Bocillin FL-labeled PBP1-B. The figure shows membrane vesicles from HMS174 without the pHK2414 plasmid (lane 1) as well as membrane vesicles from HMS174 carrying the pHK2414 plasmid, run without heating the sample in loading buffer (lane 2), after heating to 100^0C in loading buffer for 5 minutes (lane 3) and after exposure to 20% TFE in loading buffer for 1 hour (lane 4). Molecular weight markers are shown on the left hand side.

Clearly, two bands are visible with molecular weights corresponding to the dimer and monomer (lane 2). In the absence of the plasmid carrying the PBP1-B gene no staining is observed (lane 1). When the SDS solubilized sample is either heated (lane 3) or treated with 20 vol% TFE (lane 4) the dimer band vanishes and only a monomeric band remains.
Next to homo-oligomeric integral membrane proteins we identified homo-oligomers of proteins that do not contain transmembrane helices but that are more peripherically bound to the membrane. For instance, we identified the SecA protein in an off-diagonal spot (fig. 3A, spot 2). The off-diagonal position of SecA on the 2D gel corresponds to a TFE-induced dimer-monomer transition. We verified this, using 1D gel electrophoresis identifying the SecA protein with a SecA antibody (fig. 5).

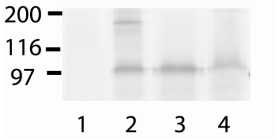

Figure 5: Western-blot with anti SecA antibodies of an 11% SDS-PAGE gel. The figure shows purified *E.coli* inner membrane vesicles run without heating the sample (lane 1), after boiling in sample buffer for 5 minutes (lane 2), and after being exposed to 25%TFE for one hour in sample buffer (lane 3). Molecular weight markers are shown on the left hand side.

In wild-type *E. coli* inner membranes, dissolved in cold SDS two spots are visible in lane 1, one corresponding to the monomer (MW 100 kD) and one to the dimer (MW 200 kD). The band at 200 kD ran at exactly the same position as a chemically linked dimer (data not shown). Either heating the solution (lane 2) or incubation of the sample with 25% TFE (lane 3), results in disappearance of the dimeric band fully consistent with an interpretation for the 2D experiment that some SecA protein is present as a dimer that becomes dissociated by TFE. The implication from the 1D gel experiment on SecA in fig. 5 is that in the 2D gel experiment of fig. 2 next to the off-diagonal SecA spot, SecA should be present as monomer on the diagonal. This we could confirm with immunoblotting (data not shown).

In this study we concentrated only on protein spots that were visible after staining with Coomassie. However, most spots were found to contain several different proteins. Moreover, some gelpieces outside the stained regions were found to contain additional membrane proteins (data not shown). How can we explain this? The most likely interpretation is that only a selected number of proteins is detected by Coomassie blue staining, while a much larger number of proteins can be detected by mass spectrometry. This would imply that the proteins that we detect in the spots are a significant underestimation of the total number of proteins that are stable in SDS but become dissociated by TFE.

In four cases (cydA, glpD, sucB and tolC) the protein was detected in two spots that were well separated in the first dimension, suggesting that these proteins were present in a larger complex that was dissociated by SDS in different hetero- or homo-oligomeric complexes. Finally, it should be noted that some proteins were also detected in neighbouring spots, suggesting some smearing, mostly in the second dimension as we also observed for KcsA (e.g. figure 2). This would imply that the molecular weights of some of the proteins, as determined from this second dimension, may be somewhat overestimated.
It should be noted that all off-diagonal spots we identified are caused by the treatment with TFE. In a control experiment in which the gel strip that was excised after electrophoresis in the first dimension was incubated in alcohol free buffer, no Coomassie-stained spots below the diagonal spot were observed. Moreover, when 5 spots that corresponded in position to the Coomassie-stained spots in fig. 3A were analyzed by LC-MS/MS none of them were found to contain a protein.

By varying the concentration of the alcohol, it was possible to gain insight into the stability of membrane protein complexes by analyzing their resistance against alcohol-induced dissociation. Thus, differences in stability were investigated by monitoring the off-diagonal appearance as function of the alcohol concentration of selected proteins for which it was established they were solely or dominantly present in particular spots. As shown in fig. 6, dissociation of KcsA occurs at 15% TFE. Less TFE is needed to dissociate the complex containing manX, a non-membrane spanning inner membrane protein of the PTS family and more TFE is needed to dissociate the complex containing yibN, a single membrane spanning protein of unknown function. This figure also shows that dissociation of *E. coli* inner membrane proteins is not a specific effect of TFE but that it can also be induced by the alcohol HFIP with similar protein specificity but with much higher efficiency.

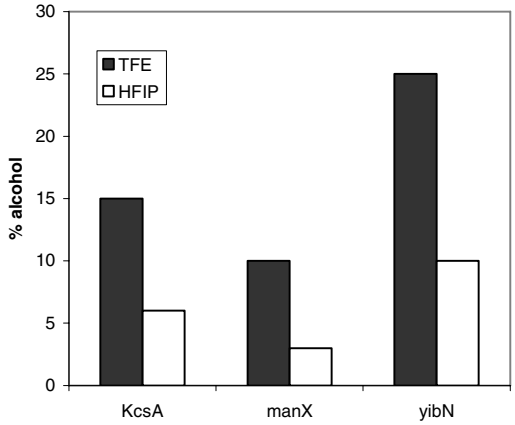

Figure 6: Minimum concentration of TFE (black bars) and HFIP (open bars) required to visualise dissociation of different *E. coli* inner membrane proteins from oligomeric complexes as off-diagonal spots.

Discussion

Although the search for protein interaction partners has received much attention recently, and although extensive interaction networks have been established for water soluble proteins (4,5), little attention has been focused sofar on the search for interaction partners of membrane proteins. In this study we developed a new and relatively simple membrane protein proteomics approach using diagonal electrophoresis to identify a subproteome consisting of membrane protein complexes that are stable in SDS and become dissociated by small membrane active alcohols. Next to KcsA, close to sixty *E. coli* inner membrane proteins were identified that became dissociated from larger complexes by TFE (table I). Because the analysis was limited to protein spots visible after staining with Coomassie, this is probably a gross underestimation of the total number of TFE-dissociable membrane protein complexes. Thus we can conclude that a large number of *E. coli* membrane proteins are part of highly stable oligomeric complexes that withstand solubilization into monomers by room temperature SDS.

We have a number of arguments that support our view that the protein complexes we detect in our assay are complexes that reside in the *E. coli* membrane and are not an artifact of the procedure we apply. Firstly, we have detected a substantial number of proteins that are already known from published data to be present in complexes in membranes. This applies not only to the KcsA tetramer on which we based the technology, but also to the large number of subunits of members of the PTS family that are known to occur as dimers (20,21) in membranes and which we identified as such. For instance, the trehalose specific enzyme II encoded by treB (spot 6) undergoes a molecular weight shift of approximately 100 to 60 kDa upon TFE treatment. Also from the structurally very different penicillin binding protein 1b we identified a dimer in room temperature SDS consistent with the suggested dimeric

state in the membrane (22,23). It should also be realized that within the range of uncertainty of determining the molecular weight of a membrane protein from SDS-PAGE it is clear that most of the protein that we identified in off-diagonal spots run at their monomeric position. This demonstrates that TFE fully dissociates these proteins from a complex they were part of. This makes it extremely unlikely that TFE causes the formation of artificial oligomeric membrane protein complexes.

The apparent molecular weight of proteins on an SDS-gel is influenced by many factors. For example, proteins that form stable homo-oligomeric complexes will often run at a position that is different from that expected from their monomer position. Nevertheless, inspection of the differences in apparent molecular weight before and after treatment with alcohol led to several interesting observations for different membrane protein complexes.
Next to homo oligomeric membrane proteins also proteins were identified that seemed to undergo only a small shift in molecular weight upon TFE treatment, such as the penicillin binding protein IA encoded by MrcA (spot 3). This protein undergoes an apparent shift in molecular weight from 110 to 90 kDa, suggesting it is part of a hetero-oligomeric complex. Consistent with this, several candidate interacting proteins have been reported with a molecular weight of approximately 20 kDa (23).

We furthermore observed four outer membrane proteins that run off-diagonal and most likely originate from the small amount of outer membranes that contaminate the purified inner membranes (15). These proteins form oligomeric β-barrels, which run in the second dimension at their oligomeric molecular weights of about 100 kDa (LamB) or 80 kDa (OmpF). Their off-diagonal position on the gel suggests that they are present as oligomer in larger complexes that are stable in room temperature SDS, but become dissociated by TFE. This interpretation is consistent with the finding that in room temperature SDS the porins ompF and lamB remain associated with other proteins and cell wall components (24). Apparently they are liberated out of these complexes by TFE as stable oligomers. This interpretation we could substantiate for OmpF using one-dimensional SDS-PAGE and OmpF specific antibodies (data not shown). This sharply contrasts their behaviour upon heating in SDS which dissociates the oligomers into monomers (25,26). The resistance of the oligomers to alcohol-induced dissocation demonstrates an interesting difference in stability between β-barrel and α-helical membrane protein complexes.

Strikingly, also many proteins that do not contain transmembrane segments are released from larger complexes. These include the SecA component of protein translocase (spot 2), several components of the PTS system, different subunits of the ATPase and many components of large *E. coli* inner membrane complexes involved in bioenergetics.
The result for SecA is particularly intriguing. The off-diagonal position of SecA on the 2D gel corresponds to a TFE-induced dimer-monomer transition of part of the proteins. Experimental evidence has been presented that the protein indeed is a dimer (27), but recently the oligomeric state of SecA has been questioned (28,29). Our results demonstrate that there are two pools of SecA that behave differently. What are these two pools? SecA is an integral part of the membrane embedded protein translocase, but it is present in a large excess over the translocase (30). This pool of excess SecA will be in interaction with the membrane lipids and is expected to

migrate as monomer, because purified SecA migrates on SDS PAGE (non-boiled) as monomer, both by itself and in interaction with lipids (data not shown). The simplest interpretation for the off-diagonally localized SecA is then that it represents a pool of SecA which is part of the translocase complex to which it thus must be bound as a dimer. SDS would dissociate this hetero-oligomeric SecYEG complex, thereby liberating the SecA dimer that after TFE exposure runs at its monomeric molecular weight.

The examples illustrated above demonstrate that alcohol-induced dissociation can occur for a large structural variety of integral membrane proteins. How can we understand the strong effects that small alcohols have on all these different membrane protein complexes? One possibility is that the alcohols perturb protein-protein contacts. It is known for soluble proteins that TFE can cause weakening of such contacts (31). However, alternative possibilities may be related to effects on lipid packing in the SDS micelles. For KcsA we provided evidence that the perturbation of the lipid packing in the membrane is responsible for dissociation of the membrane-embedded protein by alcohols (11). These alcohols partition into the head group region and loosen the acyl chain packing, thereby altering the lipid-protein interaction, resulting in tetramer dissociation. For KcsA we proposed that in particular it is the change in lateral pressure profile in the membrane that is sensed by the tetramer (11). It is possible that a similar mechanism occurs for dissociation of the different *E. coli* inner membrane protein complexes in SDS by alcohols. The alcohols are expected to partition into the water-micelle interface and thereby to alter the interactions of the oligomeric protein with the surrounding SDS and/or the endogenous membrane lipids that will be present in the micelle. This interpretation is consistent with the observation that HFIP is much more efficient than TFE in dissociating a particular protein, because it is known that HFIP perturbs lipid packing more strongly than TFE (10,11). Furthermore, for several of the protein complexes that were found to become dissociated by TFE like the mannitol PTS system (32,33) and protein translocase (30,34,35), it was demonstrated that lipid-protein interactions are important for function. Our results suggest that this may be related to the effects of the lipids on oligomer stability, possibly via effects on the lateral pressure profile. We propose that the resistance of the various protein complexes against alcohol-induced dissociation in SDS micelles reflects the stability of these complexes in intact membranes. This proposition is supported by our finding that when *E. coli* inner membranes that contain penicillin-binding IB are incubated with increasing concentrations of TFE prior to solubilization in SDS, a dimer-monomer transition around 20% TFE could be observed in subsequent SDS-PAGE in room temperature SDS (data not shown).

According to theory (36), membrane proteins can only be sensitive to changes in lateral pressure profile, when destabilization or dissociation would be accompanied by a change in shape of the protein, as we also proposed for KcsA (37). In this respect the results that we obtained with the β-barrel forming porins are particularly intriguing. We observed a lack of effect of TFE on the stability of these trimeric proteins, whereas heating clearly dissociates the trimers. Because these rigid β-barrel forming proteins are not expected to be able to change their shape, these results are fully consistent with an important role for lateral pressure profiles in stabilization of membrane proteins.

In conclusion, we have shown that small alcohols dissociate many *E.coli* inner membrane protein complexes that are stable in cold SDS. The technology provides a powerful and simple tool to detect and characterize stable membrane protein complexes. In principle, it is possible to obtain similar information by boiling the samples prior to running the second dimension in order to dissociate complexes. However, the use of small alcohols offers several important advantages. First, by varying the concentration and nature of the alcohol it allows to gain insight into differences in stability of the membrane protein complexes (see e.g. fig. 4). Second, since the alcohols will act differently on the proteins and the lipids (10), insights may be obtained on the importance of protein-protein and protein-lipid interactions that hold the complexes together. Third, since these alcohols act as local anaesthetics and since for KcsA the potency to dissociate the tetramer was shown to correlate with the anaesthetic potency (10), it may allow to obtain new insights into the mode of action of anaesthesia in relation to their effect on lateral pressure profiles.

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Chapter 2

Chapter 2 Appendix

Translocase associated SecA is dimeric

Adapted from Jeanine De Keyzer, Eli O. van der Sluis, Robin E.J. Spelbrink, Niels Nijstad, Ben de Kruijff, Nico Nouwen, Chris van der Does and Arnold J.M. Driessen (2005)

Journal of Biological Chemistry 280, 35255-35260

Abstract

The oligomeric state of SecA during the translocation cycle has been the topic of controverse lately. Recently SecA was detected in a proteomics approach designed to identify stable membrane protein oligomers as a homodimer in SDS solution. Analysis revealed that two pools of SecA exist on SDS-PAGE, resulting in the hypothesis that the dimeric SecA observed in the assay originates from SecA that is present on the translocase prior to solubilization. Here we show that the relative amount of dimeric SecA in a strain overexpressing translocase is higher than that in an empty vector control. This supports the hypothesis SecA binds to the translocase as a dimer.

Introduction

The same membrane that allows a cell to maintain its internal organization forms a barrier for proteins that work outside of the cytosol. To allow proteins to cross the membrane without compromising the barrier function, cells have evolved several transport systems, the most ubiquitous being the sec-machinery. This machinery is composed of a membrane-embedded part made up out of the SecYEG protein that forms the protein-conducting pore and a peripheral part. This peripheral part is formed by SecA and functions as a molecular motor, driving the translocation through the pore by hydrolyzing ATP (for review see de Keyzer et al, 2003 (1)).
This SecA protein exists in solution as a homodimer in equilibrium with monomers (2). Because heterodimers of active and inactive SecA are not functional in protein translocation, it was suggested SecA is active as a dimer on the translocase (3). In addition, an *in-vitro* study using a monomeric SecA variant showed that monomeric SecA is entirely non-functional in preprotein translocation(4,5). contrast, functional studies on another SecA mutant that lacks the ability to dimerize have shown that monomeric SecA introduced from plasmid can partially complement growth defects in SecA-depleted cells (6).
Thus, the oligomeric state of SecA during translocation is a topic of controversy.

Recently we reported a method to detect and identify oligomeric membrane proteins that are stable in SDS- gel loading buffer (Chapter 2) (7). This method relies on the ability of trifluoroethanol (TFE) to dissociate membrane protein complexes. Briefly, a membrane protein mixture is separated by SDS-PAGE in one dimension and exposed to TFE. Then, electrophoresis is performed identically in a second dimension. All proteins that are not affected by TFE will migrate the same distance in the second dimension as they did in the first dimension, ending up on a diagonal line. Because all protein complexes that are stable in SDS but become dissociated by TFE will migrate differently in the second dimension then they did in the first, they will appear as off-diagonal spots, standing out clearly from the diagonal line. In this screen we found that some of the SecA present in *E.coli* is present as part of a 200 kDa oligomer, presumably a homodimer. This was surprising because purified SecA migrates as a monomer suggesting the occurance of two different oligomeric forms of the protein. Specific detection using αSecA antibodies allowed us to visualize SecA on a western-blot. This revealed that under wild-type conditions there are indeed two pools of SecA on two-dimensional SDS-PAGE. One variety migrates at 100 kDa on the diagonal line corresponding to SecA that is monomeric in SDS solution and one migrates at

100 kDa below the diagonal line. The SecA below the diagonal line originates from the 200 kDa position in the first dimension, strongly suggesting that it dissociates from a dimer into a monomer upon exposure to TFE.
These two pools can be visualized on normal one-dimensional SDS-PAGE as well, allowing for easier and quicker analysis (see also figure 5 in Chapter 2).
Previous research has shown that there are two pools of SecA, one in interaction with the inner membrane, and associated with the translocase (8). Is it possible that the two bands observed in western-blotting correspond to these two different pools? If that is the case it is expected that overexpression of the transmembrane part of the translocase will result in a higher amount of the dimeric variety of SecA. To test this we have studied the relationship between amounts of dimeric and monomeric SecA under wild type conditions and in a strain overexpressing the translocon.

Materials & Methods

Materials
SecA antibodies were a kind gift of Dr. Hans de Cock. The SecY antibody, the pET605 plasmid for SecYEG overexpressing and the pET324 plasmid as a control were kind gifts of Dr. Jeanine de Keyzer. Enhanced chemoluminesence kit was purchased from Amersham. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Calbiochem (Cal., USA). All other chemicals were of the highest purity commercially available.

Isolation of membrane vesicles
The pET605 and pET324 plasmids were transformed into competent *E.coli* BL21(DE3) cells. Cells were grown from overnight culture to an OD_{600} of 0.6 and induced with 1 mM IPTG for 3 hours. Cells were pelleted in a HSA6000 rotor (20', 5000 rpm, 4^0C) and resuspended in 100 mM NaCl, 5 mM KCl and 20 mM HEPES pH 7.5. The suspension was passed through a cell disrupter operating at 1.38 kbar and cleared of unbroken cells and debris by low speed centrifugation (SS34, 5', 5000 rpm, 4^0C). The vesicles were collected by ultracentrifugation (Ti60, 45', 45k rpm, 4^0C) and resuspended in 250 mM sucrose, 20 mM HEPES pH 7.5. The protein concentration was determined according to Bradford. Vesicles were stored at -80^0C until use.

Immunodetection of SecA and SecY
Vesicles of the SecYEG overexpressing strain were loaded onto 11% SDS-PAGE gel without boiling the sample and blotted onto nitrocellulose. Blots were developed using either SecA or SecY antibodies and quantified by densitometry using BioRad Quantity 1 software.

Results and Discussion

Based on previous results, we suggested the dimeric pool of SecA on gel originates from SecA residing on the translocase, whereas the amount of SecA interacting with the membrane results in monomeric SecA on gel (7).
We investigated whether cells overexpressing the genes for SecYEG contained more SecA in dimeric form than did control cells. Vesicles were obtained from these cells

and loaded onto SDS-PAGE gels. The gels were then subjected to western-blotting using both αSecY antibodies to monitor the overexpression of the translocase and αSecA antibodies to assess the amount of dimeric SecA compared to monomeric SecA.
Figure 1 shows western-blots of both antibodies for gels loaded with vesicles in which SecYEG was overexpressed (right lane) and vesicles from a strain containing an empty vector control (left lane). Clearly, induction of the pET605 plasmid results in an increased amount of SecY by roughly a factor of 10 as detected by western-blotting (lower panel).

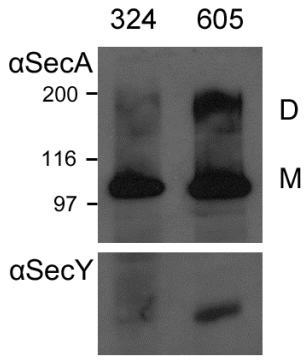

Figure 1: Overexpression of SecYEG results in an increased amount of dimeric, SDS-resistant SecA. Membrane vesicles containing 4 µg of total protein of a strain overexpressing SecYEG (right lane, pET 605) and a negative control (left lane, pET 324) were loaded onto 11% SDS-PAGE gel without boiling the sample and subjected to westernblotting using either αSecA antibodies (upper panel) or αSecY antibodies (lower panel). In the SecA panel both monomeric (M) and dimeric (D) bands are visible. A molecular weight marker is shown on the left.

Western-blotting by SecA antibodies shows bands corresponding to both monomeric SecA at 100 kDa and dimeric SecA at 200 kDa (upper panel). Induction of the pET605 plasmid shows an increase of roughly a factor 2 in the relative amount of dimeric SecA as compared to induction of an empty vector control. Although the increase in the dimeric form of SecA is not linear with the increase in the amount of translocase it is clear that overexpression of the translocase results in an increase in the SecA dimers.
These data suggest that SecA occurs on the translocon in a dimeric form. It is likely that the dimeric form of SecA corresponds to the pool of translocon-bound SecA reported by van Voorst et al. (8), whereas the monomeric form of SecA originates from the membrane-bound pool. In regards to the controversy revolving around the oligomeric state of SecA, we believe that in combination with studies published in De Keyzer et al. (9) there is a strong case that the dimer is SecA's physiologically relevant state in protein translocation.

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Chapter 2 Appendix

Chapter 3

MurG takes part in an oligomeric membrane protein complex in *E.coli*

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Chapter 3

Abstract

We found that approximately 30% of the MurG present in *E. coli* cells occurs in the form of a 120 kDa oligomeric complex that can be dissociated either by heating or by exposure to 25% trifluroethanol. This oligomer most likely represents a homogeneous tetramer, as based on its molecular weight relative to the monomer (32 kDa) and on the observations that no interacting proteins could be detected by mass spectrometry, N-terminal sequencing or gel electrophoresis. The oligomer, like the monomer, was found to be associated with the inner membrane, but was attached to it much more tightly. Oligomerisation is not an intrinsic property of MurG, since in purified form MurG could only be detected as monomer, even in the presence of model membranes of defined lipid composition either with or without its Lipid I substrate. We conclude that additional components in the *E.coli* inner membrane are involved in oligomerisation and we speculate that oligomerisation may play a regulatory role in determining the activity of these enzymes.

Introduction

It is becoming increasingly clear that many membrane proteins, like their soluble counterparts, are present in oligomeric complexes. The majority of the high resolution structures of membrane proteins contain more than one protein (1) and many membrane protein complexes have been identified by different forms of electrophoresis (2-4). For example, in a recent proteomics study on *E.coli* inner membranes, using a novel 2D gel electrophoresis approach, several proteins involved in the peptidoglycan biosynthesis pathway were identified as part of oligomeric complexes that were stable even in SDS. ((5), Chapter 2).

The peptidoglycan layer confers shape and strength to bacterial cells and protects them from lysis. This structure has no mammalian counterpart, therefore its synthesis pathway is a major target for antibiotics (6-9) like penicillin(10,11), bacitracin (12,13) and vancomycin (14) as well as antimicrobial peptides like nisin (15). Many of the membrane proteins involved in this pathway are known to function as part of larger oligomeric complexes. This applies for instance to the penicillin-binding proteins involved in the final steps of peptidoglycan biosynthesis. Penicillin-binding protein 1A (PBP1A) and PBP1B are known to homo-dimerize (16) as was also found via the 2D gel approach (Chapter 2). In addition, PBP1B forms a complex with PBP3 (17). These proteins also take part in multi-enzyme complexes with several other proteins, including other PBPs (18,19).

Other proteins in this pathway however have generally been thought of as monomeric. One such protein involved in this peptidoglycan biosynthesis pathway (20) is MurG, an enzyme of known structure (21,22). The natural substrate of MurG is lipid I, an undecaprenyl- chain coupled via a pyrophosphate to a muramyl pentapeptide. The enzyme catalyzes the transfer of N-acetylglucosamine from UDP-GlucNAc onto lipid I, thereby forming lipid II (23). This is the last step on the cytoplasmic side of the peptidoglycan synthesis pathway in *E.coli* prior to the translocation of lipid II across the inner membrane into the periplasm (24).

There, the disaccharide pentapeptide is removed from the lipid carrier and incorporated into growing peptidoglycan strands by the penicillin binding proteins.

Cell fractionation experiments suggest that MurG interacts with membranes (25). In addition, lipids co-purify with MurG in the absence of detergent and modulate its enzymatic activity, indicating direct interactions between MurG and the lipids of the bacterial membrane (26). The protein is thought to associate with the cytoplasmic side of the inner membrane via a hydrophobic patch surrounded by positively charged residues (21). However, nothing is known about the oligomeric state of the protein or its potential proteinaceous interaction partners.

In this study we investigate whether MurG associates with other proteins in the *E.coli* membrane using SDS-PAGE at room temperature and western blotting. We found that MurG (38 kDa) migrates at a 32 kDa position but also as an oligomer of 120 kDa under both wild-type and overexpression conditions. The oligomeric form was found to be more firmly membrane associated than the monomeric form and could be enriched using detergent treatment and affinity purification. No other proteins were identified by either mass spectrometric analysis or Edman-degradation, suggesting that MurG forms a homo-tetramer or interacts with a protein that is undetectable by gel electrophoresis combined with silverstaining, mass spectrometry or N-terminal sequencing.

Materials and methods

Materials
Ni^{2+}nitrilotriacetic acid (Ni-NTA) was obtained from Qiagen Benelux N.V. (The Netherlands). Coomassie brilliant blue G-250 and vancomycin were purchased from ICN Biomedicals Inc (Ohio, USA). Dithiobis(succinimidyl)propionate (DSP) was bought from Pierce Biotechnology (Il, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Calbiochem (Cal, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and *E.coli* Polar Lipid Extract (PLE) were purchased from Avanti Polar Lipids Inc (Al, USA). Bacitracin was from Sigma. Nitrocellulose membranes and TGS buffer (25 mM Tris, 192 mM glycine and 0.1% (w:v) SDS) were from BioRad (The Netherlands). All other chemicals were of the highest quality commercially available. αMurG antibodies were a gift from Tanneke den Blauwen (Swammerdam institute of life sciences, University of Amsterdam). Lipid I was obtained as described (27).

Strains and plasmids
E.coli strain Bl21(DE3) was used to investigate the oligomeric state of endogenous MurG. Overexpression was performed in Bl21(DE3)/pLysS carrying IPTG-inducible plasmids containing the gene for either wild-type MurG (pET21b-MurG) or MurG with a C-terminal LEHHHHHH tag (pET21b-MurGhistag).
E.coli strain MHD52, a kind gift of Ute Bertsche (Microbial Genetics, Tuebingen University), was used to test the sensitivity of the oligomeric form of MurG towards bacitracin and vancomycin.

The pET21b-MurGhistag plasmid was a kind gift of Dr. Susan Walker (Department of Microbiology and Molecular Genetics, Harvard Medical School). The pET21b-MurG plasmid was cloned from the pET21b-MurGhistag plasmid as described (26).

Gel electrophoresis and western-blotting

Gel electrophoresis was performed using the Leammli system (28). Samples were not boiled prior to electrophoresis unless specified. When required MurG-containing samples were incubated with TFE (25% v:v) for one hour at ambient temperature before being loaded onto gel. After electrophoresis the gels were stained using Coomassie Brilliant Blue G-250. When specific protein detection was necessary, the separated protein sample was transferred from gel onto a nitrocellulose membrane in TGS buffer with 20% v:v methanol. MurG was detected using specific antibodies and visualized by the use of chemoluminescence.

The amounts of MurG on the gel were determined by scanning the blots and quantifying the bands intensities using BioRads Quantity One software. Molecular weights were determined by comparison to BioRad All-Blue Precision Plus protein standards.

Determination of the effect of antibiotics on oligomeric MurG

The *E.coli* MHD52 strain, which is sensitive to antibiotics targeting the lipid intermediated involved in the peptidoglycan synthesis pathway, was used to determine the effect of vancomycin and bacitracin on the oligomeric state of MurG. Cells were grown at 37^0C to an OD_{600} of 0.3 and exposed to 10, 20, 30 or 40 μg/ml of either bacitracin or vancomycin. The exposed cells were grown until the antibiotics took effect and caused the cell density to drop back to 0.3. Cells were dissolved in loading buffer and loaded onto SDS-PAGE gel without boiling. The gel was blotted on nitrocellulose after which MurG was detected by αMurG antibodies and chemoluminescense.

Overexpression of MurG

E.coli Bl21 (DE3)/pLysS cells carrying the pET21b-MurG or the pET21b-MurGhistag plasmid were grown from 5% overnight culture in LB medium at 37^0C. At an OD_{600} of 0.6 the cells were induced with 1 mM IPTG and grown for 3 hours. Cells were harvested by centrifugation for 20 minutes at 7,200g at 4^0C and washed once with physiological salt solution. MurG-containing material was kept at 4^0C during all subsequent steps. The cell pellet (9 g of wet weight) was resuspended in 50 ml of icecold Tris-HCl pH 8.0 buffer and passed twice through a refrigerated cell disruptor (Basic Z, Constant Cell Disruption Systems, UK) operating at 20,000 PSI. Unbroken cells and large debris were removed by low-speed centrifugation (5', 3000 g). Membrane vesicles were pelleted by ultracentrifugation at 200,000 g for 45 minutes. Membrane pellets (1.2 grams of wet weight) were resuspended in 32 ml of 20 mM of Tris-HCl pH 8.0 and stored at -80^0C. Membranes intended for DSP crosslinking were resuspended in 20 mM of phosphate at pH 8.0 and stored in the same way.

Affinity-purification of MurG-histag
MurG-histag was purified essentially as described (26). Briefly, 25 ml of the soluble fraction of a lysate of MurG-histag overexpressing cells was incubated with 1 gram of Ni-NTA for one hour on ice. The Ni-NTA was poured into a column and washed once with 10 column volumes of 20 mM Tris-HCl pH 8.0 with 5 mM of imidazol and once with the same buffer with 50 mM of imidazol.
The column was then eluted with 10 ml 20 mM Tris-HCl with 100 mM of imidazol. The fractions obtained in this way were analyzed for the presence of MurG by SDS-PAGE with coomassie staining and stored at -20^0C.

Determination of the effect Lipid I on MurG
LUVs were prepared essentially as described (29). Briefly, stock solutions of phospholipids in 1:1 methanol/chloroform were mixed. Volatiles were removed by evaporation under a nitrogen stream. The films obtained in this way were dried overnight in a vacuum exsiccator and rehydrated in 50 mM Tris-HCl pH 8 during 10 cycles of freeze-thawing for a final phospholipid concentration of 2.5 mM. These vesicles were passed ten times through a 200 nm filter (Anatop 10, Whatman, UK).
100 µl of Ni-NTA purified MurG (0.23 mg/ml) was exposed to 100 µl of either LUVs of DOPC (2.5 mM), or LUVs of PLE (2.5 mM) containing Lipid I (65 nM), or mixed micelles of Triton X-100 (TX100) (0.1% w:v) and PLE LUVs containing lipid I, or Lipid I in TX100 micelles for 30 minutes on ice and loaded onto SDS-PAGE gel without boiling the samples.

Enrichment and analysis of oligomeric MurG-histag
To minimize the amount of monomeric MurG-histag in the membranes the vesicles were incubated in the presence of 0, 0.1, 0.5, 1 or 5% of TX100 on ice. After 1 hour the vesicles were spun down at 200,000 g. The pellets and supernatants were run on an 11% SDS-PAGE gel and subjected to western-blotting using αMurG antibodies to determine the relative amounts of oligomeric and monomeric MurG.
Vesicles exposed to 0.1% TX100 showed the highest oligomer-to-monomer ratio and were used for further enrichment. These vesicles were solubilized in 20 mM Tris-HCl pH 8.0 containing 5% of TX100. The resulting solution was cleared from insoluble material by ultracentrifugation (45' 200,000 g). The supernatant was incubated overnight with 1 g of Ni-NTA slurry at 4^0C. The slurry was poured into a column and washed thrice with 10 ml of 20 mM Tris-HCl pH 8.0 containing 5, 1 and 0.1% TX100 respectively. To elute MurG-histag from the Ni-NTA material imidazol was added to the Tris-buffer containing 0.1% of TX100 in a gradient in 6 steps. The imidazol concentrations used were 10, 25, 35, 50, 100 and 200 mM. Fractions were tested for the presence of MurG by coomassie-stained SDS-PAGE and westernblotting followed by specific detection using αMurG antibodies. A mock purification was performed in the same way on cells containing an empty Pet21b plasmid.

Protein identification
The preparation enriched in oligomeric MurG was run on SDS-PAGE gel and stained using Coomassie Brilliant Blue G-250. The 120 kDa oligomer band was analysed for protein content via in-gel tryptic digestion followed by mass spectrometry using the methods described in (5) [Chapter 2]. The 120 kDa band was also analysed by N-terminal sequencing at the Eurosequence facility, Groningen.

DSP-crosslinking on MurG-containing membrane vesicles
E.coli membrane vesicles containing MurG-histag were washed with 0.1% of TX100 in 20 mM phosphate pH 8.0. The washed vesicles were exposed to 2.5 mM DSP in 20 mM phosphate buffer pH 8.0 for one hour on ice. 10 mM of Tris-HCl pH 8.0 was added to quench the crosslinking reaction. Vesicles were dissolved in 5% TX100 and DSP-crosslinked MurG-histag was purified similar to regular oligomeric MurG-histag.

Results

MurG is part of an SDS-resistant membrane protein complex
To investigate whether MurG occurs as part of an SDS-resistant oligomer we checked the migration of endogenous MurG from a preparation of *E.coli* inner membrane vesicles on SDS-PAGE through western-blotting using αMurG antibodies. When such a preparation is loaded on gel without boiling, two bands become visible (Fig 1 lane1), a band at 32 kDa, in fairly good agreement with the theoretical molecular weight of monomeric MurG (38 kDa), and a band at roughly 120 kDa. The ratio in intensity between the bands is approximately 3:1. Either boiling the sample or incubating it with 25% of TFE prior to electrophoresis results in the disappearance of this 120 kDa band, while the 32 kDa band remains present (Fig 1 lanes 2 and 3). We conclude that the 32 kDa band corresponds to monomeric MurG while the 120 kDa corresponds to a protein oligomer containing one or more copies of the MurG protein.

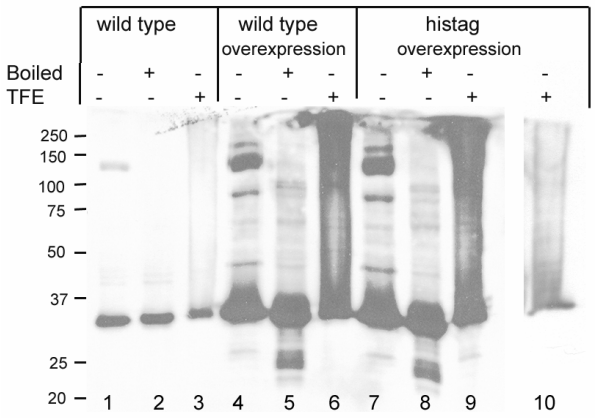

Figure 1: αMurG westernblot of an 11% SDS-PAGE gel loaded with *E.coli* membrane vesicles. The membrane preparations were loaded at room temperature, either directly, after boiling for 5', or after 1 hour of exposure to 25% (w:v) TFE, as indicated. Lanes 1 trough 3 contain Bl21(DE3) inner membrane vesicles, representing the wild-type situation. Lanes 4 – 6 were loaded with total membranes of Bl21(DE3)/pLysS cells overexpressing wild-type MurG, while lanes 7 through 9 were loaded with total membranes of Bl21(DE3)/pLysS overexpressing MurG-histag after similar treatment as the wild-type vesicles. Lane 10 was loaded with a lower amount of TFE-treated vesicles of cells overexpressing Murg-histag to better illustrate the disappearance of the oligomer band upon treatment with TFE. A molecular weight marker in kDa is shown on the left.

To gain insight into the nature of the proteins taking part in the oligomer, the protein was overexpressed from plasmid in *E.coli* strain Bl21(DE3)/pLysS. When samples of a membrane preparation of these cells were subjected to western-blotting both the 32 and 120 kDa bands were observed at higher intensities than in the wild-type situation. In addition, several other bands were observed at lower intensities (Figure 1, lane 4). The ratio of monomer to oligomer was not strongly affected by overexpression, remaining at approximately 3:1. Boiling the sample prior to loading resulted in the disappearance of the 120 kDa band as well as the lower intensity bands at 80 and 160 kDa (Figure 1, lane 5) and the appearance of an additional band at approximately 25 kDa, possibly corresponding to a heat-induced degradation product of MurG. Also incubating the vesicle preparation with 25% TFE resulted in dissociation of the oligomer (Figure 1, lane 6), but in this case extensive smearing was observed, in line with results from previous experiments ((5), Chapter 2).

Very similar results were obtained when a membrane preparation of cells overexpressing the histagged protein was loaded on gel. Again, boiling for 5 minutes resulted in the disappearance of the oligomeric band and the appearance of a band at 25 kDa (Figure 1, lanes 7 and 8), while exposure to 25% TFE resulted in dissociation of the oligomer, but caused smearing. The same experiment performed with a lower protein load shows the disappearance of the oligomeric band more clearly (Figure 1, compare lanes 9 and 10).

The observation that both the wild-type and histagged proteins show a temperature- and TFE-sensitive band at 120 kDa, strongly suggests that this band corresponds to the same protein complex in both situations. Attempts to increase detection of the MurG-containing oligomer by changing from an SDS-PAGE to a milder LDS-based gel system (30) did not show any significant difference between the two approaches (data not shown).

MurG oligomerisation is not the result of the presence of lipids or substrate

To test whether oligomerisation is an intrinsic property of the protein, MurG-histag was purified from the soluble fraction of a lysate of cells overexpressing the protein as described (26). When this preparation was subjected to western blotting with αMurG antibodies, the band corresponding to the oligomer could not be detected (Fig 2, lane 2).

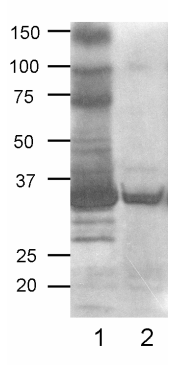

Figure 2: αMurG western blot of purified MurG-histag. Lane 1 contains a crude lysate of cells overexpressing MurG-histag. Lane 2 was loaded with 4 µg of MurG-histag that was purified according to (26). A molecular weight marker in kDa is shown on the left.

This suggests that either the oligomer becomes dissociated during purification, or that it is not present in the soluble fraction of the lysate. When purified MurG was brought into contact with membrane vesicles composed of DOPC or of an *E.coli* lipid extract no oligomerisation was observed. Neither did the presence of Lipid I, a substrate of MurG, affect the monomer-oligomer ratio regardless of whether Lipid I was presented in TX100 micelles, mixed micelles or *E.coli* PLE vesicles (data not shown).
Furthermore, when growing *E.coli* MHD52 cells were exposed to either bacitracin or vancomycin, no difference in the ratio between the monomeric and oligomeric forms of MurG was observed relative to a control situation (data not shown). These compounds block the cell wall synthesis by binding to the lipid intermediates involved in the pathway, making them unavailable as substrate to MurG. This suggests that also in the biological system the presence of substrate or product does not directly affect the monomer to oligomer ratio. Apparently, neither the presence of a membrane environment nor the availability of products or substrate directly causes or disrupts oligomerisation.

Oligomeric MurG associates more tightly with the membrane than does monomeric MurG
To establish where the oligomeric form of MurG-histag resides, pellet and supernatant fractions of a lysate of *E.coli* cells overexpressing MurG-histag were analysed for the presence of the oligomer. This revealed that the oligomeric form of MurG-histag is mainly present in the pellet fraction of a cell lysate after ultracentrifugation (Fig 3, lanes 1 and 2).

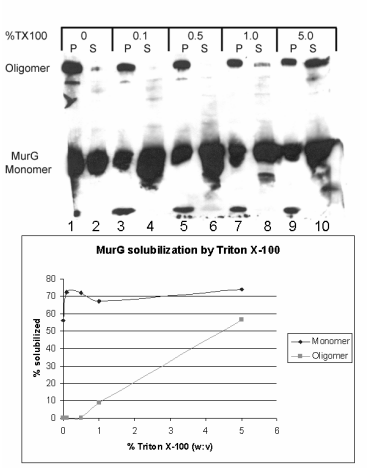

Figure 3: Effects of TX-100 on membrane association of MurG. Membrane vesicles of cells overexpressing MurG-histag were incubated with different concentrations (w:v %) of TX-100 and spun down. An SDS-PAGE gel was loaded with the pellets (P) and supernatants (S) and subjected to western-blotting using αMurG antibodies. The lower panel shows a graph of the relative amounts of monomeric and oligomeric MurG in the supernatant as a function of the TX100 concentration.

To allow for affinity-based purification, membrane vesicles of a strain overexpressing MurG-histag were exposed to varying amounts of TX-100 to determine at what concentration solubilization was achieved. The addition of low amounts of detergent caused monomeric MurG-histag to be released from the membrane. In sharp contrast, the oligomeric form of MurG-histag remained attached to the membrane under these conditions. Some oligomeric MurG could still be spun down at even 5% of TX-100. The band intensities obtained for MurG in the pellet and supernatant fractions for all tested percentages of TX-100 can be converted into fractions of MurG-histag in the supernatant. Plotting these fractions against the percentages of TX-100 clearly shows the difference in membrane association between the monomeric and the oligomeric form of MurG-histag (Figure 3, lower panel). We conclude that the oligomeric form of MurG-histag is more strongly associated with membranes than the bulk of the monomeric variety.

Affinity-based enrichment of oligomeric MurG-histag

The observation that monomeric MurG-histag is released from the membrane at lower detergent concentration than the oligomeric form allowed for the specific enrichment of oligomeric MurG-histag in the vesicle preparation. The vesicles were washed once with 0.1% TX-100 and subsequently solubilized in 5% TX-100. The solution obtained in this manner was exposed to Ni-NTA to bind the histagged protein. Because the high amounts of detergent necessary to dissolve the MurG-containing vesicles interfere with SDS-PAGE, the detergent concentration was lowered in a stepwise manner during a series of washing steps. The fractions obtained in this way were then analyzed by SDS-PAGE and western-blotting for the presence of oligomeric MurG-histag.

The MurG-histag solution prepared in this way was run on SDS-PAGE gel followed by silverstaining (Figure 4).

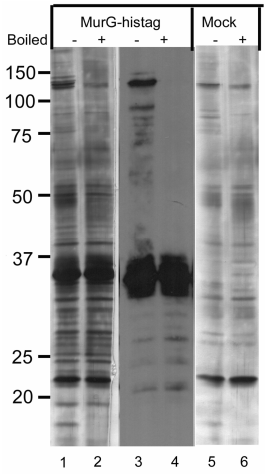

Figure 4: A silverstained 11% SDS-PAGE gel and αMurG westernblot of Ni-NTA purified MurG histag obtained from cells overexpressing MurG-histag. Lanes 1 and 2 show MurG-histag purified from vesicles washed with 0.1% TX-100 prior to solubilisation in 5% TX100. The sample in lane 2 was boiled prior to loading, while the sample in lane 1 was not. Lanes 3 and 4 are identical to lanes 1 and 2, but were subjected to western-blotting using αMurG antibodies instead of silverstaining. Lanes 5 and 6 were loaded with material from a mock-purification of cells carrying an empty vector control. A molecular weight marker in kDa is shown on the left.

A large band corresponding to monomeric MurG-histag is clearly visible, as well as a large number of co-purifiying bands at much lower intensities. A series of bands occur between the 100 and 150 kDa marker bands (Figure 4, lane 1), the most intense of which seems to correspond to the 120 kDa MurG-histag containing oligomer band on αMurG western-blot (Fig.4 lane 3). Boiling the sample causes this band to disappear from both the silverstained gel and the corresponding western-blot (Fig 4. lanes 2 and 4). No new bands of comparable intensity but at lower molecular weight appear on the silverstained gel upon boiling (Fig. 4 lane 2), and hence there is no evidence for the presence of another protein besides MurG in the oligomer. While silverstaining shows another band between 100 and 150 kDa, this latter band does not seem to be affected by heat treatment and it also occurs in a mock purification performed on IPTG-induced cells containing an empty plasmid (Fig. 4 lanes 5 and 6).

When the band corresponding to the MurG-containing oligomer was excised and submitted for mass-spectrometric identification only the MurG protein itself could be detected (data not shown). Subjecting the oligomer band to N-terminal sequencing showed that 96% of the amino acid signal of this band had the sequence: MMSGQGKRLMV corresponding to MurG itself (NCBI sequence identification number GI|9955024). The remaining 4% of intensity of the amino acids revealed no other protein sequences, and probably correspond to internal chain cleavage during the Edman procedure.

Given the fact that no bands other than monomeric MurG appear upon dissociation and that neither mass spectrometry nor N-terminal sequencing showed the presence of protein besides MurG, it seems reasonable to conclude that the 120 kDa oligomer is homogenous in nature.

Chemical crosslinking and purification of MurG-histag

To further improve the analysis of the molecular nature of the MurG-histag containing oligomer the purification procedure was repeated on a preparation of TX100-washed membrane vesicles that were treated with the reducable crosslinking agent DSP. This molecule covalently crosslinks proteins by reacting with lysine residues. Figure 5 shows the effect of DSP-crosslinking on MurG-histag. While MurG-histag purified from untreated vesicles migrates mainly in its monomeric form, MurG-histag that was purified from membranes that were exposed to DSP was found to migrate as a complex at a molecular weight exceeding that of the 120 kDa MurG oligomer. Boiling the DSP-treated MurG in sample buffer containing beta-mercaptoethanol released monomeric MurG-histag from this complex, as well as a number of lower molecular weight bands of lower intensity.

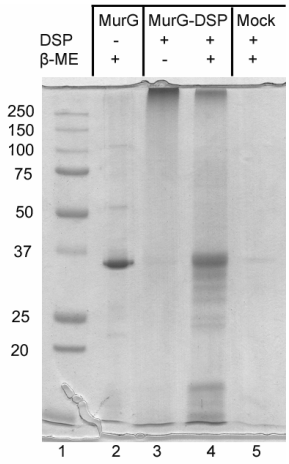

Figure 5: The effect of DSP crosslinking on MurG-histag. TX100-washed vesicles overexpressing MurG-histag were subjected to DSP-crosslinking. Lane 1 contains a molecular weight marker. Lane 2 was loaded with MurG purified from vesicles that were not treated with DSP. Lane 3 was loaded with Ni-NTA purified MurG from DSP-treated vesicles in sample buffer without a reducing agent. Lane 4 contains the same sample as lane 3, but was boiled in β-mercaptoethanol (βME) containing sample buffer prior to loading. Lane 5 is from a mock purification on an empty vector control. The gel was stained with Coomassie Brilliant Blue.

Protein bands that were detected in the MurG-histag containing preparation but not in a mock-purification were again subjected to tryptic digestion followed by LC-MS/MS. This resulted in the detection of 132 proteins involved in a broad variety of processes (Appendix I).

Discussion

MurG is partially present in an oligomeric complex

In this study we show using conventional SDS-PAGE and western blotting that MurG migrates mainly to two locations on the gel: one at 32 kDa and one at 120 kDa. While other bands are visible their relatively low intensity precluded detailed investigation. The presence of the 120 kDa band demonstrates that MurG is partially present in an oligomeric protein complex. We observed that heating the sample prior to electrophoresis causes dissociation of the 120 kDa protein complex, resulting in the presence of only the 32 kDa protein. Based on these results we conclude that the 32 kDa band corresponds to the monomeric form of MurG. Interestingly, exposing the sample to 25% TFE prior to electrophoresis also results in dissociation of the 120 kDa MurG-containing protein complex, similar to TFE and heat-induced dissociation of PBP1-B (5) and SecA dimers (5,31), KcsA tetramers (32-34) and MscS oligomers (Chapter 3). Both treatments apparently weaken protein-protein contacts, resulting in dissociation of the oligomer.

The 120 kDa band can either represent a homo-oligomer or a hetero-oligomer. In support of the first possibility, both mass spectrometric and Edman-sequencing based identification of the affinity-purified oligomer band identified no other proteins besides MurG. It may be argued that proteins are present that do not become detected by the mass spectrometer because of poor solubility or ionizability and are undetected by Edman sequencing because of their blocked N-termini. However, this is unlikely because boiling samples prior to electrophoresis showed no additional protein bands appearing upon the dissociation of the 120 kDa band as would be expected upon the dissociation of a hetero-oligomeric complex. Further support for a homo-oligomeric complex is found in the observation that overexpression of the protein does not result in a significant change in the monomer to oligomer ratio. Such a change might be expected in the case of a hetero-oligomeric complex because the additional protein component is unlikely to be available in the required amounts. These observations strongly suggest that the 120 kDa band represents a homo-oligomeric form of MurG. Interestingly, in both available MurG crystal structures (21,22) the protein is present as a homo-dimer in the unit cell, indicating that MurG has the ability to self-associate.

However, the observed oligomer is too large to correspond to a homodimer. The MurG protein itself runs at 32 kDa, slightly below its calculated molecular weight and therefore it can be suggested that the 120 kDa oligomer corresponds with a tetramer. Nevertheless, care should be taken in calculating oligomerisation orders from gel positions, as protein complexes have a tendency to migrate faster than would be expected based on their molecular weights. In addition, both a monomeric and oligomeric form of MurG are observed, rather than just an oligomer, like in the case of KcsA. It is conceivable that MurG occurs exclusively in complexed form in the cell, but is partly dissociated upon the addition of sample buffer. However, electrophoresis under milder conditions using LDS shows the same 3:1 monomer to oligomer ration as conventional SDS-based electrophoresis (data not shown). This suggests that the observed ratio between the different forms of MurG is not an artifact introduced by SDS-PAGE.

Both forms of MurG occur in the vicinity of additional proteins under overexpression conditions, as can be concluded from experiments in which membrane vesicles of cells overexpressing MurG were incubated with the reducable crosslinker DSP. Exposure to DSP induces all MurG present in the vesicles to take part in a larger structure that migrates to a higher molecular weight than the 120 kDa oligomer.

To determine what additional proteins are present in this complex, it was dissociated by reducing the crosslinker and the products were analysed on SDS-PAGE gel. This gel did not seem to contain any bands of comparable intensity to the MurG-band. Instead, a number of lightly stained bands became visible, containing a broad variety of soluble and inner membrane proteins. A recent proteomics study by Butland et al. on the soluble proteome of *E.coli* indicated that MurG interacts with several other soluble proteins (35). Only minor overlap was found with the proteins detected in this study, in the form of 4 ribosomal proteins. Apparently, rather than specifically crosslinking a preexisting complex, DSP crosslinks proteins to MurG in a stochastic manner.

Localization of the oligomer

Both monomeric MurG and oligomeric MurG associate with membrane vesicles. Although low amounts of detergent can dissociate the monomer from the membrane, the oligomer resists solubilisation even at high concentrations of TX-100, indicating that the oligomer is more strongly associated with the membrane than the monomer.

This is in good agreement with previous studies which have shown that MurG associates with the cytoplasmic side of the inner membrane (25), although a proteomics study (35) focusing on soluble proteins detected MurG in the cytoplasm. In agreement with these studies, our own data suggests that MurG occurs in both membrane-associated and soluble form, whereby the oligomer is clearly found exclusively on membranes (Fig 3A, lanes 1 and 2).

Interestingly, when his-tagged MurG is purified from an overexpression system (26) the oligomeric form cannot be detected despite the fact that the MurG is associated with *E.coli* phospholipids that are present in the preparation as bilayer based vesicles. These vesicles most likely originate from the intracellular vesicles that are present near the cell poles during overexpression of the protein (26). This strongly suggests that the ability of MurG to form oligomers that are stable in SDS at room temperature is not an intrinsic property of MurG. Even adding additional phospholipid vesicles with or without Lipid I or detergent/phospholipids micelles containing Lipid I to the MurG protein do not induce oligomer formation. Likewise, lowering the available pool of Lipid I *in-vivo* by blocking the lipid intermediates involved in peptidoglycan biosynthesis with vancomycin or bacitracin does not alter the ratio between the monomeric and the oligomeric forms of MurG. Apparently one or more protein components of the *E.coli* inner membrane are required for oligomer formation.

Biological significance

What is the biological function of the MurG-containing oligomer?

Interestingly, in both MurG crystal structures, the interface between the subunits differs. While MurG proteins with GlcNAc bound to them interact through the hydrophobic patches at their N-termini (22), free MurG molecules interact through the C-termini (21). The observation that the interactions between MurG enzymes differ in

the presence and absence of substrate makes it tempting to speculate on a regulatory strategy involving oligomerization.
Furthermore, a clear parallel is visible between MurG and the SecA protein discussed in Chapter 2. Here also a peripheral membrane protein appears to occur in two distinct forms on SDS-PAGE gel, a monomer and a dimer apparently representing two distinct pools of the protein. In the case of SecA the dimer seems to represent the active form of the protein, docked on the membraneous part of the translocase. Increasing the relative amount of the translocase results in an increase in the amount of dimeric SecA, strongly suggesting that the interaction with this integral membrane component induces stable homo-oligomerization. A similar mechanism can be envisioned for MurG, where one pool of the protein resides in the cytosol or in interaction with the lipids of the inner membrane, while a different pool of the protein is induced to form a putative tetramer by another protein component involved in peptidoglycan biosynthesis.
This could hypothetically serve as a mechanism to modulate MurG activity, offering a potential explanation for the observed difference between the *in-vitro* and *in-vivo* enzymatic activities of MurG (25,36).

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Chapter 3

Appendix: List of proteins identified by analysis of MurG-DSP crosslink products.

Protein name	Protein accession numbers
Chain A, Ftsj Rna Methyltransferase Complexed With S-Adenosylmethionine, Mercury Derivative,	gi|10120641,gi|24053650
peptidyl-tRNA hydrolase	gi|1093598,gi|3114515,gi|581202
ribosomal protein S12	gi|11120595,gi|22121665,gi|56383865,gi|83754106
glycerol kinase	gi|1134966,gi|12518827,gi|4389228,gi|5107585, gi|6729905,gi|75238369,gi|89110103,gi|91213467
succinate dehydrogenase, flavoprotein subunit	gi|12513638,gi|75189745,gi|75256433,gi|89107581
phosphoribosylpyrophosphate synthetase	gi|12514920,gi|147379,gi|26107931,gi|75195360, gi|75255057,gi|89108052
orf, hypothetical protein	gi|12515444,gi|15831155,gi|26108060,gi|75259434
PTS system, mannose-specific IIAB component	gi|12515860,gi|24051725,gi|75241314
NADH-quinone oxidoreductase chain G	gi|12516633,gi|3183546,gi|38704071,gi|74312801, gi|75187065,gi|75238066,gi|75259741,gi|75512755, gi|89109101,gi|91211575
NADH dehydrogenase I chain A	gi|12516641,gi|24052725,gi|38704072,gi|75238071
N-acetylmuramoyl-l-alanine amidase I	gi|12516816,gi|24052857,gi|26109217,gi|75196449, gi|75227766,gi|89109249,gi|91211773
putative membrane protein	gi|12516910,gi|15832632,gi|26109286,gi|75187268, gi|75257940 ,gi|75515855,gi|89109322,gi|91211842
ATP-dependent RNA helicase	gi|12516997,gi|226216,gi|24053020,gi|42996,gi|75228849 ,gi|75237678
protein chain initiation factor IF-2	gi|12517779
protease	gi|12517856,gi|24053711,gi|26110244,gi|49259536 ,gi|56966066,gi|91212655
unnamed protein product	gi|12517946,gi|42832,gi|83754097
putative membrane protein	gi|12518179,gi|15833614,gi|24053964,gi|75197411, gi|75242719,gi|75516049,gi|82545848,gi|89110525 ,gi|91212982
PTS system, mannitol-specific enzyme IIABC components	gi|12518343,gi|15833729,gi|26110667,gi|74314167, gi|75211354,gi|89110413,gi|91213110
transcription termination factor Rho; polarity suppressor	gi|12518646,gi|2611095l,gi|56383986
twin arginine translocation protein; sec-independent protein export	gi|12518713,gi|148236,gi|24054404,gi|3123496, gi|91213385
orf, hypothetical protein	gi|12518743,gi|15834041,gi|26111058,gi|67462332, gi|75237830,gi|89110163
CDP-diacylglycerol phosphotidylhydrolase	gi|12518819,gi|15834097,gi|26111115,gi|31562988 ,gi|75197790,gi|75230439,gi|75513896,gi|89110111, gi|91213459
orf, hypothetical protein	gi|12518890,gi|56384047,gi|75197836,gi|82592559
Chain C, Elongation Factor Complex Ef-TuEF-Ts From Escherichia Coli,	gi|12518902,gi|1942723,gi|229896,gi|229900,gi|24054557 ,gi|26110363,gi|26111180,gi|49258331,gi|75188710, gi|75766400,gi|91212815,gi|93279787
glycerol-3-phosphate acyltransferase	gi|12518990,gi|14285669,gi|15834278,gi|26111254, gi|4426885,gi|89110762
Chain B, The CapCRP VARIANT T127LS128A	gi|13096635,gi|2098303,gi|24053823,gi|2769262, gi|33357050,gi|89110652,gi|93278614
30S ribosomal protein S6	gi|133976,gi|24054885,gi|75515437
Chain A, Ompf Porin Mutant Kk	gi|14488509,gi|14488510,gi|15830266,gi|1943073, gi|1943074,gi|1943076,gi|223138,gi|26107356,gi|6729727 ,gi|89107779,gi|999673,gi|999982
heat shock protein 70 precursor	gi|145774,gi|75194239,gi|75229475,gi|75235743
acetylCoA carboxylase, beta (carboxyltranferase) subunit	gi|146019,gi|146364,gi|26109110,gi|89109136,gi|91211613
succinate dehydrogenase, iron sulfur protein	gi|146200,gi|24050816
dihydrolipoamide succinyltransferase	gi|146202,gi|26107093,gi|46015765,gi|75194885, gi|75256436,gi|91209760
H+ ATPase F1 alpha subunit	gi|146323,gi|148137,gi|24054296,gi|41030,gi|43264
extragenic suppressor	gi|147899,gi|26109307,gi|75240442
ATP synthase gamma subunit	gi|148138,gi|24054295
proton-translocating ATPase b subunit (uncF; gtg start codon)	gi|148145,gi|24054298
possibly rffM [	gi|148197,gi|26110965,gi|75197682,gi|75230760,gi|75512645 ,gi|83585953,gi|89110222,gi|91213318
o251	gi|148231,gi|24054401,gi|26111032
Chain B, Crystal Structure Of Murg:glcnac Complex,	gi|15829348,gi|26106420,gi|28948962,gi|40858,gi|42055, gi|75234327,gi|82542694
dihydrolipoamide acetyltransferase	gi|15829373,gi|434011,gi|75233842,gi|75236918,gi|75258892 ,gi|91209183
COG1622: Heme/copper-type cytochrome/quinol oxidases, subunit 2	gi|15829740,gi|75238760,gi|75512493
short chain dehydrogenase	gi|15829810,gi|26106906,gi|75239011,gi|75257862,gi|91209574
COG1252: NADH dehydrogenase, FAD-containing subunit	gi|15830741,gi|75255624,gi|75511402,gi|83584876,gi|89107955
hypothetical protein ECs1672	gi|15830926,gi|26107892,gi|89108023
putative sugar transferase	gi|15832396,gi|75238042,gi|89109071
hypothetical protein ECs3144	gi|15832398,gi|75187042,gi|89109073

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NADH dehydrogenase I chain C/D	gi|15832424,gi|24052722,gi|26109078,gi|397900,gi|89109104
putative sugar nucleotide epimerase	gi|15832442,gi|75187086,gi|75196347,gi|75227200 ,gi|75515669,gi|89109124
FtsH	gi|15833311,gi|24053649,gi|746401,gi|75239255, gi|75240799,gi|75260106
FtsX	gi|15833565,gi|26110506,gi|56383899,gi|75237061,gi|75516073
zinc-transporting ATPase	gi|15833572,gi|74314069,gi|75188241,gi|75197395,gi|75226950, gi|75237068,gi|75516066, gi|83588669,gi|89110543,gi|91212958
essential cell division protein	gi|15834114,gi|24054512,gi|26111131,gi|290448,gi|75230454, gi|75238376,gi|75240326 ,gi|75258084,gi|75513881,gi|89110096
lipoate-protein ligase A	gi|15834599,gi|24054954,gi|26111711,gi|91214103
50S ribosomal subunit protein L20	gi|16502855,gi|43068,gi|83754133
50S ribosomal subunit protein L11	gi|16504608,gi|396322,gi|42814,gi|75238524,gi|83584813,gi|83754147
hypothetical protein	gi|1657507,gi|75194528,gi|75237287,gi|89107182
Chain C, Crystal Structure Of Tem-1 Beta-Lactamase	gi|16975251,gi|3882023,gi|60594090,gi|67464385,gi|9257166
Chain A, Structure Of Rsua	gi|20664194,gi|24052623,gi|26108971,gi|75237979
5-aminolevulinate synthase	gi|216524,gi|24051513,gi|34811366,gi|75226808,gi|75255055,gi|968928
30S ribosomal protein S9	gi|223001,gi|26110238,gi|83754103
ribosomal protein L10	gi|223035,gi|24054563
ribosomal protein L10	gi|223035,gi|24054563
polymerase beta,RNA	gi|223154,gi|24054564,gi|46015535,gi|46015541,gi|85544273
protein L12	gi|223571
lipoprotein mutant	gi|223967,gi|24052039,gi|75241442,gi|9256992
fruR gene	gi|227854,gi|24050280
protein S21	gi|229549,gi|24053529,gi|75511251
50S ribosomal subunit protein L6	gi|229631,gi|24053779,gi|42984,gi|83754124
acetyl CoA carboxylase, carboxytransferase component, alpha subunit	gi|24050389,gi|26106529,gi|91209255
orf, conserved hypothetical protein	gi|24050572,gi|26106810,gi|42932
membrane protein	gi|24050573,gi|26106811,gi|75238735,gi|75512469
protein export protein SecD	gi|24050573,gi|26106811,gi|75238735,gi|75512469
cell division inhibitor	gi|24051458,gi|75227593
putative protease	gi|24051584,gi|26108003,gi|290470,gi|75515116
putative ATP-binding protein of peptide transport system	gi|24051606
putative outer membrane protein	gi|24051999,gi|26108291
NADH dehydrogenase I chain B	gi|24052724,gi|397899
30S ribosomal subunit protein S16	gi|24053054,gi|26109378,gi|75236031
putative ATP-binding component of a transport system	gi|24053675,gi|551337,gi|75188007
50S ribosomal subunit protein L17	gi|24053768
30S ribosomal subunit protein S11	gi|24053771,gi|83754105
30S ribosomal subunit protein S13	gi|24053772,gi|83754107
50S ribosomal subunit protein L15	gi|24053775,gi|42988,gi|75236329
30S ribosomal subunit protein S5	gi|24053777,gi|83754099
50S ribosomal subunit protein L18	gi|24053778,gi|75207949
30S ribosomal subunit protein S8, and regulator	gi|24053780,gi|42983,gi|83754102
50S ribosomal subunit protein L14	gi|24053784
50S ribosomal subunit protein L2	gi|24053793,gi|33357930
50S ribosomal subunit protein L23	gi|24053794
50S ribosomal subunit protein L3	gi|24053796
30S ribosomal subunit protein S10	gi|24053797
30S ribosomal subunit protein S7	gi|24053804,gi|83754101,gi|89110669
L-lactate dehydrogenase	gi|24054112,gi|26110678,gi|466743,gi|75255823,gi|75514326, gi|89110406,gi|91213119
50S ribosomal subunit protein L28	gi|24054148
membrane-bound ATP synthase, F1 sector, beta-subunit	gi|24054294,gi|75197628
HemY protein	gi|24054361,gi|75512639
protease specific for phage lambda cII repressor	gi|24054858
orf, conserved hypothetical protein	gi|24054863

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50S ribosomal subunit protein L9	gi|24054887
DnaJ-like protein djlA	gi|26106378,gi|54035858,gi|56383142,gi|75189159,gi|75194276, gi|75229092,gi|75234296 ,gi|75236946,gi|75256069,gi|89106939
Hypothetical ABC transporter ATP-binding protein yadG	gi|26106464,gi|89107008
30S ribosomal protein S2	gi|26106512,gi|42842,gi|75512181,gi|83754115
Hypothetical protein ybjQ	gi|26107289
Lipid A biosynthesis lauroyl acyltransferase	gi|26107594,gi|48957,gi|75240651
Lipoprotein releasing system ATP-binding protein lolD	gi|26107662,gi|7445930,gi|89107963
D-amino acid dehydrogenase small subunit	gi|26107904,gi|89108034,gi|91210402
Hypothetical lipoprotein ydcL precursor	gi|26108119,gi|91210674
ElaB protein	gi|26109061
Hypothetical lipoprotein yfiO precursor	gi|26109364,gi|75236019
sensor protein QseC	gi|26110004,gi|75240519,gi|75515180,gi|83587532, gi|89109796,gi|91212443
Hypothetical protein yrbD precursor	gi|26110207,gi|89109956,gi|91212618
30S ribosomal protein S9	gi|26110238,gi|83754103
50S ribosomal protein L24	gi|26110325,gi|42980,gi|83754137
60 kDa inner-membrane protein	gi|26110876,gi|75188461,gi|75210684,gi|75236428, gi|75258244,gi|83588430,gi|89110307
HflK protein	gi|26111498,gi|75255901,gi|89110894,gi|91213723
Chain L, The Crystal Structure Of Dps, A Ferritin Homolog That Binds And Protects Dna,	gi|3660186,gi|384221,gi|440182,gi|56383283, gi|75230656,gi|91209847
30S ribosomal subunit protein S4	gi|42798,gi|83754098
rplA (L1)	gi|42815,gi|75208782,gi|75236714
ribosomal protein L4	gi|42828
30S ribosomal subunit protein S3	gi|42832,gi|83754097
histone-like protein	gi|52695974
sigma-E factor of RNA polymerase	gi|56383694,gi|75209497,gi|75237408
orf, conserved hypothetical protein	gi|56383810,gi|75240867
50S ribosomal subunit protein L13	gi|56383843
ATP-binding component of a membrane-associated complex involved in cell division	gi|56383900,gi|75197389
fumarate reductase, anaerobic, iron-sulfur protein subunit	gi|56384131,gi|90108811,gi|91213705
unnamed protein product	gi|581098
Chain A, Solution Structure Of Ribosomal Protein L25 From Escherichia Coli	gi|6980397
23S rRNA pseudouridine synthase	gi|75197880,gi|89110743
COG1622: Heme/copper-type cytochrome/quinol oxidases, subunit 2	gi|75512493
outer membrane protein A (3a;II*;G;d)	gi|89107807

Chapter 4

Small alcohols dissociate oligomeric MscS in a lipid-independent manner

Adapted from Bradley Akitake & Robin E.J. Spelbrink, Andriy Anishkin, J. Antoinette Killian, Ben de Kruijff and Sergei Sukharev (2007)

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Chapter 4

Abstract

The *E.coli* inner membrane contains a broad variety of SDS-resistant membrane protein complexes that become dissociated by exposure to 2,2,2-trifluoroethanol (TFE) [Spelbrink et al, Journal of Biological Chemistry 280, 28742-28748, 2005]. Among these complexes is the mechanosensitive channel of small conductance, a part of the bacterial turgor-control system.
We report here the conditions under which MscS retains its oligomeric state in detergent-based electrophoresis. This allowed us to study in a systematic manner the stability of the protein in different membrane environments. Exposure of MscS to either 10% (v:v)TFE or 2% (v:v) 1,1,1,3,3,3-hexafluroisopropanol (HFIP) results in dissociation of the complex into monomers irrespective of the detergent or membrane-environment in which the protein was present. This contrasts with the behaviour of the tetrameric potassium channel KcsA, which was previously shown to dissociate at different concentrations of TFE depending on the environment it resides in. The differences between the influence of TFE on KcsA and MscS are discussed in the light of the effect of alcohols on protein-protein and protein-lipid interactions.

Introduction

Many membrane proteins are known to function as oligomeric complexes. These complexes can be remarkably stable, as exemplified by the bacterial potassium channel KcsA which forms a stable tetramer even in SDS at elevated temperature (1). However, these protein complexes can often readily be dissociated into theirmonomeric forms by exposure to small membrane active alcohols like 2,2,2,-trifluoroethanol (TFE) (2,3). This was shown by the results of a novel 2-dimensional electrophoresis approach applied to the subproteome of membrane proteins in *E.coli* inner membranes (4). The approach involved two electrophoresis steps in perpendicular directions, with an incubation with the small alcohol TFE after the first step. Proteins that were unaffected by the alcohol migrated identically in both dimensions, forming a diagonal line. However, proteins that were dissociated from SDS-resistant complexes by exposure to the alcohol migrated faster in the second dimension, ending up at off-diagonal positions. The method identified many membrane proteins that are part of stable oligomeric complexes.

The mechanism of TFE dissociation remains speculative but most likely involves both effects on protein-lipid and protein-protein interactions. For KcsA several findings pointed toward a role of the membrane lipids in the alcohol induced dissociation of the protein. First, the tetramer became dissociated at much lower alcohol concentrations when present in detergent micelles than when present in a biomembrane or when reconstituted in a bilayer of defined composition (5). Furthermore the presence of the lipid phosphatidylethanolamine (PE), which has a relatively small head group and by itself prefers to organize in non-bilayer structures, was found to protect the tetramer against dissociation as compared to the exclusive presence of the bilayer-forming lipid phosphatidylcholine (PC).
These findings were interpreted within the framework of the lateral pressure profile that occurs in bilayers (5).

The pressure in a bilayer is location specific as a result of the balance of the interfacial tension and the inter-headgroup repulsion and inter-chain repulsion. Partitioning of the alcohol in the interfacial region would change the lateral pressure profile and result in a decreased pressure in the acyl chain region. This in turn was proposed to mediate dissociation of the oligomer. An implication of such a mechanism would be that the transition from oligomer to monomer results in a change in depth dependent area of the membrane-embedded part of the protein (6).
To explain the stabilising effect of PE it was proposed that due to the relatively small headgroup of PE more space is available at the lipid/water interface for TFE to partition into before the lateral pressure in the acyl chain region drops sufficiently to allow for the dissociation of KcsA.

As of yet it is unknown whether such a dissociation mechanism is specific for KcsA or whether it applies to other proteins as well. We selected the mechanosensitive channel MscS as a protein to test the hypothesis formulated for KcsA. This protein was one of 59 proteins present in the subproteome of stable oligomeric membrane protein complexes in the *E. coli* inner membrane that become dissociated by TFE (4).
MscS is a stretch-activated (7,8) voltage-sensitive (8,9) ion channel of known structure (10). Functional channels are formed by seven individual subunits, giving the protein a different oligomeric design than KcsA. The MscS channel gates in response to tension across the inner membrane (11). By doing so it serves as an "emergency relief valve" in the bacterial osmoregulation system, preventing cell lysis due to osmotic pressure (12). The gating mechanism was suggested to be influenced by the lipid composition (13). This is supported by the observation that the presence of specific lipids in the membrane induces MscS to take on an open conformation (14,15).

The MscS protein was exposed to two membrane active alcohols that differ in their capacity to dissociate KcsA and several other membrane protein oligomers. Surprisingly, we observed that the composition of the bilayer had little effect on the concentration of the alcohols that is required to dissociate the oligomeric complexes, nor did solubilising the protein in detergent micelles.
The effect of membrane active alcohols on the oligomeric stability of MscS will be discussed and compared to their effect on KcsA

Methods

Materials
Electrophoresis setups and SM-2 Bio Beads were purchased from BioRad Laboratories B.V. (The Netherlands). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc (USA). Lithium dodecyl sulphate (LDS) was purchased from USB Corp. (Ohio, USA). Octylglucoside (OG) was obtained from Labscientific Inc. (New Jersey, USA). Ni^{2+} nitrilotriacetic acid (Ni-NTA) was obtained from Qiagen Benelux N.V. (The Netherlands). Anti-his6-C-terminal antibodies were purchased from Invitrogen (The Netherlands). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Calbiochem (California, USA). 2,2,2-Trifluoroethanol was purchased from Merck (Germany). 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was purchased from Acros Organics (The Netherlands). Coomassie brilliant blue G-250

was purchased from ICN Biomedicals Inc (Ohio, USA). LDS-PAGE gradient gels were cast using a Hoefner SG30 gel maker while non-gradient LDS gels were cast on BioRad Protean III casting systems. All other chemicals were of the highest quality commercially available.

Preparation of membrane vesicles
PB111, a plasmid construct containing MscS with a C-terminal 6His tag, was transformed and expressed in MJF465 cells (mscL-. mscS-, mscK-) (12). Cells were grown from overnight culture in 800 ml LB medium at 37^0C to an OD600 of 0.6 and induced with 0.8 mM IPTG. Cells were induced for 1½ hour and collected by centrifugation. The cell-pellet was washed with 50 ml of 50 mM potassium phosphate buffer pH 8 containing 5 mM $MgCl_2$ and resuspended in the same buffer. The suspension was passed twice through a cell disruptor (Basic Z, Constant Systems Ltd, UK) operating at 16.000 PSI. Unbroken cells were removed by low-speed centrifugation (10' 3.000 g) and membrane vesicles were collected by ultracentrifugation (200.000 g, 45', 4^0C), resulting in approx. 0.6 g of cell membranes (wet weight). Membrane pellets were stored at -80^0C until either being resuspended in 50 mM phosphate buffer pH 8 or used for the purification of MscS-his6.

Purification of MscS-his6
Histagged MscS was purified essentially as in (11).
0.6 g of membrane pellet was dissolved in 8 ml of 50 mM potassium phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole and 3% (w:v) octylglucoside. This solution was cleared from insoluble particles by ultracentrifugation (200.000 g, 45', 4^0C). The resulting solution was incubated with 0.5 ml Ni-NTA slurry on ice for 1 hour. The nickel-slurry was poured into a column and drained by gravity. The gelbed was washed with 10 volumes of 300 mM NaCl, 50 mM potassium phosphate buffer pH 8, 20 mM imidazol and 1% (w:v) octylglucoside. Elution was performed stepwise with buffers containing 50, 75 and 200 mM imidazol, using 2 gelbed volumes for each step. Aliquots were run on an 11% SDS-PAGE gel and stained with Coomassie G-250. Fractions containing purified MscS were pooled and supplemented with 0.1% (w:v) Triton X-100. The protein solution was stored at 4^0C.

Reconstitution of MscS into lipid vesicles.
A method was developed based on the methodology proposed by Rigaud and co-workers (16). Stock solutions of phospholipids in 1:1 methanol/chloroform were mixed. Volatiles were removed by evaporation under a nitrogen stream. The films obtained in this way were stored overnight in a vacuum exsiccator for further drying. The resulting lipid films were rehydrated in 300 mM NaCl, 50 mM of potassium phosphate pH 8 for a final phospolipid concentration of 2.5 mM. Large Unilamellar Vesicles (LUVs) were prepared as described previously (17). LUVs were solubilized by the addition of 18 µl of a 10% (w:v) octylglucoside solution. 200 µl of purified 0.3 mg/ml MscS solution were added and the resulting mixture was incubated on ice for 30'. The resulting solution was incubated overnight under light agitation at 4^0C with 50 µg of SM-2 bio beads to remove detergent. Vesicles were spun down at 200.000 g for 45' at 4^0C and resuspended in 200 µl of 20 mM imidazole, 50 mM potassium phosphate pH 8.0 and 300 mM NaCl.

Alcohol-induced dissociation of MscS
20 µl samples of MscS in detergent solution (0.3 mg/ml) or MscS in lipid vesicles (0.3 mg protein/ml) or a membrane preparation from MJF465 cells containing roughly 4 mg/ml total protein were added to solutions of either TFE or HFIP in water for a total volume of 30 µl. The samples were incubated at ambient temperature for 1 hour. Samples were cooled on ice before addition of ice-cold LDS-PAGE gel loading buffer prior to electrophoresis on either 9.5% continuous or 8-18% gradient LDS-PAGE gels.

LDS-PAGE
To facilitate detection of oligomeric MscS, electrophoresis was performed at low temperature. Replacing sodium dodecylsulphate with lithium dodecylsulphate in the gels and buffers prevented precipitation of dodecylsulphate. Otherwise, the gels and buffers were identical to those commonly used in SDS-PAGE. Electrophoresis setups, gels and buffers were chilled prior to use and cooled continuously throughout each run. Gels were run at 120V until the blue dye-front reached the edge of the gel. Gels were stained with Commission Brilliant Blue G-250 in the case of purified and reconstituted protein or subjected to western-blotting with anti-his6-COOH antibodies in the case of inner membrane vesicles.

Results

To assess the stability of MscS oligomers, the protein-containing samples were incubated with varying concentrations of TFE prior to separation by LDS-PAGE. The use of LDS allowed electrophoresis at low temperature, offering improved detection of MscS oligomers. Electrophoresis was performed on *E.coli* membranes overexpressing MscS using a gradient-gel to allow for adequate resolution in the high MW region. LDS-PAGE followed by western-blotting with anti-his-6-C-term antibodies revealed three bands: one at 200 kDa, one at an estimated 300 kDa, and a lightly stained band at 25 kDa (Fig 1, lane 1). Boiling this sample prior to electrophoresis resulted in a single band at 25 kDa (Fig 1, lane 2), indicating that this 25 kDa band corresponds to monomeric MscS. Using a double-cysteine mutant of MscS (S95C/I97C), which spontaneously cross-links under ambient atmospheric oxygen, allowed us to compare the migration of the 200 and 300 MW bands to those of covalent homo-oligomers of MscS. This double mutant has cysteine residues at opposite sides of the TM3 helix, allowing the formation of intermolecular crosslinks between individual MscS subunits. Fig 1, lane 3, shows that the covalent oligomers migrate mainly as two bands at the same location as the regular MscS oligomers. When the sample containing the double-cysteine mutant was boiled prior to loading, a ladder of denatured covalent oligomers was observed (Fig 1, lane 4). Based on the relative migration of the MscS bands compared to the ladder bands we propose that the 300 kDa band corresponds to folded, heptameric MscS. The lower MW band then seems to be a homotetramer. Apparently, both these oligomeric forms are relatively stable in cold LDS. The slightly slower migration in its native state compared to its denatured state is the opposite of what one would normally expect and may be caused by the fact that native MscS has a large hollow domain which may increase its apparent molecular weight. As expected, boiling the preparation with the double-cysteine mutant in the presence of DTT caused most of the higher MW bands to disappear and the monomer-band at 25 kDa to increase in intensity (Fig 1 lane 5).

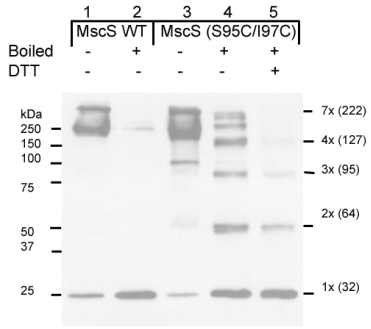

Figure 1: Migration of MscS-6his and MscS-6his S95C/I97C in E.coli membranes as visualized on anti-his6-C-term western-blot of an 8-18% LDS-PAGE gradient gel. Lanes 1 and 2 were loaded with membrane vesicles of MJF465 overexpressing MscS-6his. The sample in lane 1 was kept on ice after exposure to LDS gel loading buffer while the sample in lane 2 was boiled for 5 minutes prior to loading on the gel. Lanes 3-5 were loaded with membranes of MJF465 overexpressing MscS-6his S95C/I97C. The samples were either kept on ice (lane 3), boiled in the absence of DTT (lane 4) or boiled in the presence of 14 mM DTT (lane 5). Positions of soluble molecular weight markers are indicated on the left. Marker positions based of disulfide-crosslinked MscS subunits are shown on the right. Calculated molecular weights in kDa are indicated between parentheses. For reasons of legibility the pentamer and hexamer have been omitted.

To test whether MscS-oligomers can be dissociated by exposure to TFE, membrane vesicles of a strain overexpressing MscS were incubated with this alcohol for 1 hour at ambient temperature before being subjected to electrophoresis on continuous LDS-PAGE gels. Fig. 2A shows that the 200 and 300 kDa bands corresponding to the putative heptamer and tetramer disappear from the gel upon exposure to increasing amounts of TFE while simultaneously an intense band corresponding to monomeric MscS at 25 kDa appears. In addition, several lightly stained bands that seemingly correspond to higher order oligomers between the 200 kDa band and the 25 kDa band appear (Fig 2A). Both the 200 and 300 kDa bands disappear almost completely at more than 10 percent of TFE, although some signal at high molecular weight, well above 300 kDa remains visible at higher concentrations. This may be caused by aggregates of MscS.

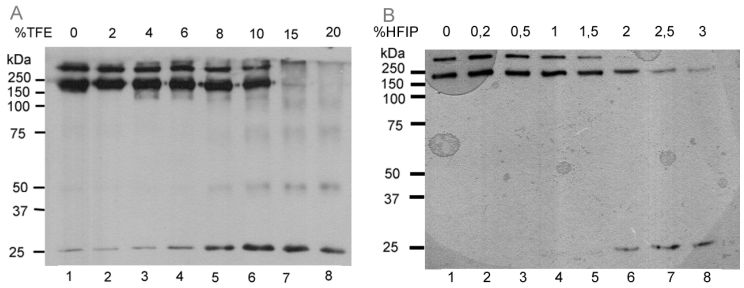

Figure 2: Alcohol-induced dissociation of MscS-6his in E.coli membrane vesicles as visualized by anti-his6-C-term western blotting on a continuous 9.5% LDS-PAGE gel. The gel was loaded with membrane vesicles of MJF465 overexpressing MscS-6his that were incubated for 1 hour at room temperature with the indicated percentages of TFE (Panel A) or HFIP (Panel B). Molecular weight markers are shown on the left of each blot.

In order to check whether the observed decomposition of MscS oligomers to monomers is specifically due to the presence of TFE, and not a result of the combined action of TFE and LDS, in a separate experiment we pelleted the TFE-exposed membranes and carefully removed the TFE-containing buffer prior to adding the LDS sample buffer. This procedure led to a dilution of the residual TFE by at least 10 times. The resultant pattern of bands in the gel was similar to that in Fig. 2A showing dissociation between 10 and 15 vol% TFE (data not shown). This suggests that TFE present around and inside the membrane is by itself capable of disrupting intersubunit interactions in MscS.

The dissociation of MscS by TFE is not a specific effect of this alcohol. A similar effect is observed upon the addition of HFIP to a preparation of MscS containing vesicles, although the concentration required to induce dissociation lies around 2.5 % rather than 10 % (Figure 2B). The observation that HFIP is more efficient than TFE at dissociating MscS is consistent with their respective effects on other membrane protein complexes including KcsA (4,18).

To establish whether sensitivity to TFE of the MscS protein is dependent on the membrane context or whether it is an intrinsic property of the protein, preparations of purified protein in octylglucoside were also subjected to TFE-induced dissociation. The addition of minor amounts of Triton X-100 (0.1% w:v) was found to improve the stability of the purified protein in LDS-PAGE. Affinity-purified MscS migrated to the same 200 and 300 kDa band positions, similar to MscS that was loaded directly from a membrane preparation as verified by western-blotting (data not shown). Exposing MscS to an increasing concentration of TFE caused the dissociation of MscS into monomers at 12% TFE (Fig 3A, lanes 5-7), similar to the concentration required for MscS embedded in the *E.coli* inner membrane.

Affinity-purified MscS was also exposed to HFIP (Figure 3B). The ratio of intensity between the oligomers differs somewhat compared to Figure 3A because a different preparation was used. Furthermore, lightly stained bands are visible here at low alcohol concentrations apparently corresponding to oligomers between the 200 and 300 kDa bands and the 25 kDa monomer band. Raising the HFIP concentration to 2% results in the disappearance of the tetramer and heptamer bands and the appearance of the monomer band.
Occasionally more complex behaviour of the protein is observed upon the addition of alcohols. In several experiments the protein migrates as a series of bands slightly below and in between the 200 and 300 kDa bands. These additional bands disappear upon the addition of even minor amounts of TFE (2% v:v) (Fig. 3C) or HFIP (0.4% v:v, data not shown) respectively. This behaviour is likely a consequence of complex conformational behaviour at low alcohol concentration.

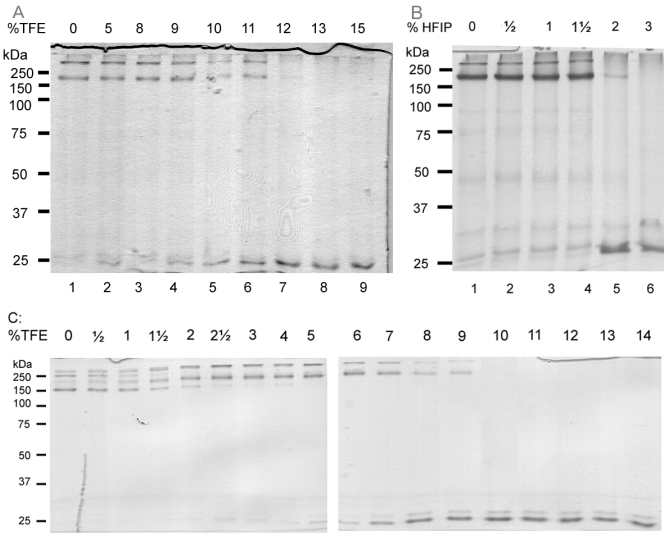

Figure 3: Alcohol-induced dissociation of purified oligomeric MscS-6his in 1% (w:v) octylglucoside and 0.1% w:v TX100 on a continuous 9.5% LDS-PAGE gel. Aliquots of purified MscS-6his protein solution (0.3 mg/ml) were incubated with the indicated percentages of TFE (Panel A) or HFIP (Panel B) for 1 hour at room temperature and analyzed by LDS-PAGE. Panel C shows a more complex band pattern that is occasionally observed when the purified MscS protein is exposed to TFE. The gels were stained with Coomassie Brilliant Blue G-250. Molecular weight markers are shown on the left of each gel.

Quantification of the band intensities of the 25 kDa and the 200 and 300 kDa bands allowed for the determination of the percentage of oligomeric MscS as a function of alcohol concentration (Figure 4).

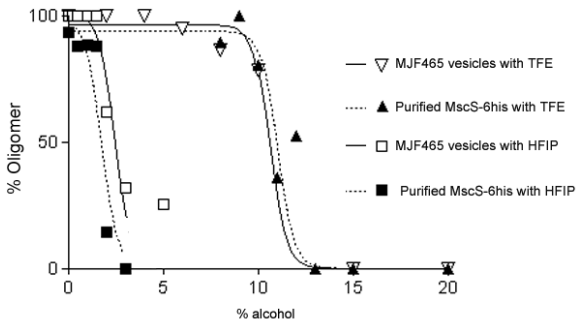

Figure 4: Quantification of the alcohol-induced dissociation of MscS-6his in E.coli membranes (open symbols, continuous lines) or detergent micelles (black symbols, dotted lines). The figure shows the percentage of oligomeric MscS-6his out of the total amount of MscS-6His visible on gel as a function of the alcohol concentration (v:v) for either TFE (triangles) or HFIP (squares). The lines indicate sigmoidal curve fits over the datapoints, assuming complete dissociation at high alcohol concentration. Western-blots or Coomassie-stained gels were quantified by densitometry. The relative intensity of the two MscS-6his oligomer bands relative to the total amounts of MscS-6his were calculated.

Like in the case of TFE, the HFIP concentration required to induce dissociation of purified, detergent solubilized MscS is similar to that required for the dissociation of membrane-embedded MscS. Clearly, the presence of a lipid bilayer does little to influence the stability of oligomeric MscS against dissociation. This suggests that the alcohol works directly on the protein. To test this, MscS was reconstituted in lipid vesicles composed of DOPC and of mixture of DOPC and DOPE (7/3, molar ratio). KcsA that is present in vesicles containing PE is known to be more resistant towards TFE-induced dissociation (5).
The vesicles were subjected to TFE-induced dissociation and analysed on LDS-PAGE gel (Figure 5).

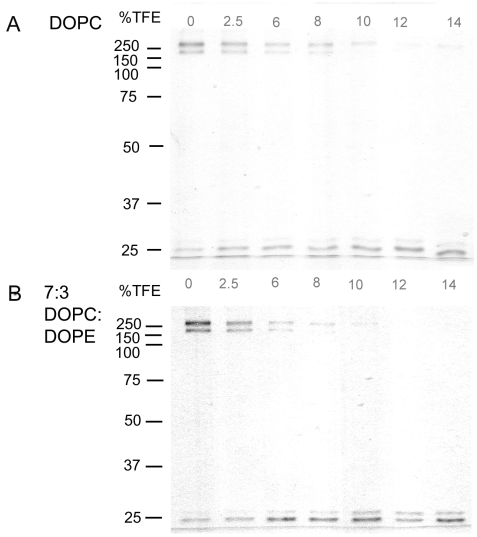

Figure 5: The effect of TFE on purified oligomeric MscS reconstituted in DOPC vesicles (Panel A) or DOPC:DOPE in a 3:7 molar ratio (panel B) as analysed by 9.5% LDS-PAGE. Samples were incubated with the indicated amount of TFE for one hour at room temperature. Molecular weight markers are shown on the left hand side of each gel.

These preparations show the same 200 and 300 kDa bands in the high molecular weight region as purified MscS. However, at low molecular weight a faint additional band is visible above the band corresponding to monomeric MscS. Because no other protein besides MscS was present we hypothesize that this band represents a different conformation of MscS. For purposes of quantification both these lower molecular weight bands were considered to correspond to monomeric MscS.
The band intensities were quantified and plotted as a function of alcohol concentration (Figure 6).

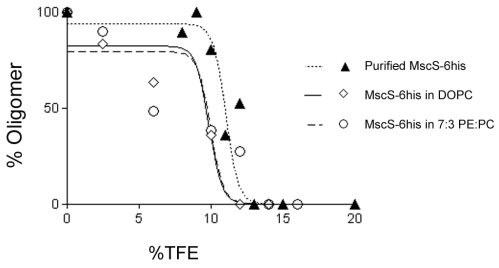

Figure 6: The relative amount of oligomeric MscS-6his as percentage of the total amount of MscS-6his after TFE exposure as determined by densitometry. MscS-6his in DOPC is indicated by open diamonds, MscS-6his in 7:3 DOPE/DOPC by open circles. The black triangles indicate the dissociation of MscS-6his in detergent micelles. The lines show sigmoidal curve fits over the datapoints. The relative intensity of the two MscS-6his oligomer bands relative to the total amounts of MscS-6his were calculated.

The reconstituted protein starts to dissociate and displays complete dissociation at a somewhat lower concentration than MscS that has not been exposed to the reconstitution protocol. However, overall no major difference is observed in TFE sensitivity between the different lipid environments.

Discussion

Low-temperature electrophoresis experiments using LDS revealed two high-molecular weight bands of MscS. The native structure of MscS is heptameric, as shown by gel-filtration experiments (11). The crystal structure of the protein also shows a heptameric organization (10). Hence, in all likelihood one of the two high-MW bands represents the heptameric form of the protein. The two bands migrate at approximately 200 and 300 kDa respectively. MscS is a 31 kDa protein, while the heptamer is expected to migrate at 242 kDa. Since this is in fairly close agreement with the 200 kDa band and since folded proteins generally migrate somewhat faster than unfolded proteins, one possibility would be that this band represents the heptamer. The 300 kDa band may then represent a “dimer of heptamers”. This band assignment is in reasonable agreement with the theoretical molecular weights determined by marker bands. Nevertheless, we favour a different interpretation. We suggest that the 300 kDa band represents the heptameric form of the protein, and the 200 kDa band a tetramer. This interpretation is based on the fact that protein migration may deviate significantly from that of protein markers. Hence, the best expected marker for the MscS protein is MscS itself. The 200 and 300 kDa bands migrate closer to the tetramer and heptamer bands of the cysteine-crosslinked mutant, supporting this interpretation. The reason why these bands migrate more slowly compared to their denatured counterparts may be the presence of a large hollow domain in MscS which will result in slower migration when the protein is in its native state. Another argument in favour of this interpretation is that during gel-filtration experiments no peak corresponding to a possible “dimer of heptamers” was observed (11).

What causes the dissociation of MscS upon exposure to TFE? It might be argued that a cumulative effect occurs between both TFE and LDS, but removing the free TFE from the system prior to membrane solubilization in LDS does not change the outcome of the incubation. This indicates that TFE by itself dissociates MscS, without the need for LDS.

The observation that neither the presence of detergent nor of lipids affects the TFE requirements for MscS dissociation suggest that MscS is affected directly by the alcohol. How might this process occur?

Several mechanisms are known that mediate the effect of TFE on proteins (Reviewed in (19)). First, direct binding of TFE to the protein backbone may enhance intermolecular hydrogen bonding of the amide groups by excluding water molecules from their presence. This could cause a change in conformation in the MscS protein that would cause its dissociation into monomeric subunits. Second, because the dielectric constant of a TFE-water mixture is lower than that of pure water, TFE could enhance electrostatic interactions within the protein molecule or within subunits. However, this would probably strengthen subunit interactions rather than weaken them. Third, TFE may affect water structure, resulting in the stabilization of helices by affecting their hydration shells. If the C-terminal basket is important for oligomeric stability of MscS this mechanism may be of influence in the dissociation process. This would be consistent with the absence of an effect of the lipid bilayer in dissociation. Finally, TFE can associate preferentially with hydrophobic sites on the protein through its CF_3 group, weakening hydrophobic interactions. Considering the fact that in membrane proteins the hydrophobic areas are presented towards the lipid phase, this mechanism may be a major factor in dissociating membrane protein oligomers like MscS. The observation that the more hydrophobic alcohol HFIP affects MscS more strongly than TFE is consistent with this mode of action.

HFIP is more efficient than TFE at dissociating a variety of membrane protein complexes including KcsA. The required HFIP concentration is about one fifth of that of TFE, in good agreement with literature values that range from one third to one fifthteenth of the required TFE concentration (4,18).

Both KcsA and MscS are stable oligomeric ion channels, KcsA as a tetramer, MscS as a heptamer. Both protein structures contain tilted transmembrane helices as well as a C-terminal basket hanging in the cytoplasm. Yet, while the presence of a lipid bilayer, especially one containing PE, protected KcsA from the effect of TFE, no such effect was observed for MscS. Why then does the effect of TFE differ between KcsA and MscS?

Several explanations are conceivable. The most plausible one is as follows. The MscS heptamer has hydrophobic crevices separating the TM1-TM2 from the TM3 helices that form the contacts between the individual subunits. While the actual MscS channel structure will likely be more compact than the crystal structure reported by Bass and coworkers (10) it does demonstrate that under proper conditions large hydrophobic binding pockets are available for TFE to occupy. Figure 7 shows the crystal structure of heptameric MscS, with a monomeric subunits one the right. A hydrophobic binding pocket between the transmembrane helices of the monomer is clearly visible, indicated by the arrow. KcsA does not have such distinct hydrophobic patches, but a relatively smooth hydrophobic, lipid-exposed exterior (20). Accumulation of TFE in these crevices in MscS may cause its structure to perturb resulting in dissociation of the heptamer, a process that could not occur in KcsA.

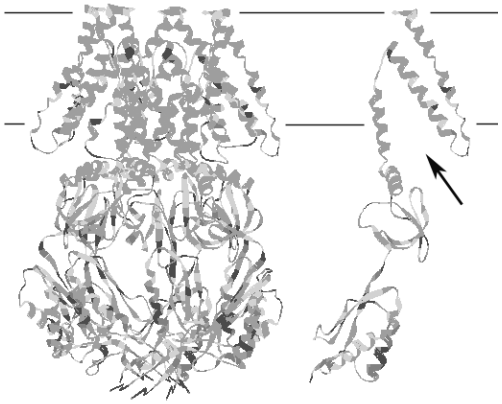

Figure 7: Crystal structure of heptameric MscS (10). One of the individual MscS subunits is shown on the right-hand side. The arrow indicates a hydrophobic pocket between the transmembrane helices. The dark lines indicate the proposed position of the membrane.

Support for this comes from electrophysiology measurements that suggest that even minor amounts of TFE can occupy these hydrophobic binding pockets in MscS, causing the channel to adopt an inactivated conformation [Akitake & Spelbrink, Biophys J in press].

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Chapter 5

Summarizing discussion

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Many membrane proteins exist as oligomers. Such oligomers play an important role in a broad variety of cellular processes such as ion transport, energy transduction, osmosensing and cell wall synthesis. Despite years of study many questions remain open in the study of membrane protein oligomerization. Specifically it remains unclear how many membrane proteins take part in larger complexes, how they are formed and what interactions keep them together.

The oligomeric potassium channel KcsA has proven to be an attractive model for addressing such questions. It forms highly stable tetramers, even in SDS at 80^{o}C (1). The structure of the tetramer is known (2) and tetramer formation can be studied both *in vitro* as well is *in vivo (3)*. It was discovered that exposing tetrameric KcsA to TFE efficiently dissociates the tetramer into its monomeric form (4,5). Other small alcohols of similar structure were found to share this property (6). This observation opened the way for an electrophoresis-based method of identifying oligomeric membrane proteins that, like KcsA, are stable in SDS-based electrophoresis but become dissociated upon exposure to small alcohols (7). In this method proteins are first separated on gel in one dimension, then they are incubated with alcohol and next the gel is run in a perpendicular direction under the same electrophoresis conditions. Any off-diagonal spots then represent proteins that initially were part of oligomeric complexes. This method and its results are described in detail in chapter 2.

When this method was applied to a preparation of *E.coli* inner membrane vesicles, nine of such off-diagonal spots were detected which were found to contain 58 different proteins. These proteins included both integral and peripheral membrane proteins with a large variety of different functions. The list of proteins that became detected is shown in chapter 2, table 1. From this list, three proteins were selected for further studies. Studies on the preprotein translocase SecA indicated that this protein is associated with the translocase as a dimer, a subject under dispute in the scientific literature (8,9). This is discussed in detail in the appendix to chapter 2. For the cell wall synthesis enzyme MurG it was found that it occurs as an oligomer on the inner membrane, most likely in the form of a homotetramer. These findings and its implications are reported in chapter 3. Finally, as described in chapter 4, the mechanosensitive channel of small conductance MscS was found to dissociate upon exposure to membrane-active alcohols in a manner independent of the lipid environment, suggesting a different mechanism of dissociation for MscS than for KcsA.

Mechanism of effect of membrane-active alcohols on proteins

How do membrane-active alcohols like TFE influence proteins? This has been most extensively studied with soluble proteins. The effect of TFE on such proteins may vary wildly depending on the specific protein in question, the pH of the solution (10,11) and the possible presence of cofactors (12) . In some cases TFE stabilizes proteins, while in other it destabilizes them. Nevertheless, several general mechanisms for the influence of TFE on soluble proteins are known, reviewed in Buck, 1998 (13). First, TFE can bind directly to the protein backbone. This excludes water molecules

from the presence of the amino acids' amide groups and therefore enhances intramolecular hydrogen bonding. Second, TFE enhances electrostatic interactions within the protein molecule because the dielectric constant of a TFE-water mixture is lower than that of pure water. Third, TFE may affect water structure, resulting in the stabilization of helices by affecting their hydration shells. Finally, TFE can associate preferentially with hydrophobic sites on the protein through its CF_3 group, interfering with hydrophobic interactions. The overall effect of TFE on a given protein is most likely a combination of these factors.

The effects of other small alcohols, such as ethanol or hexafluoroisopropanol (HFIP) can be expected to be qualitatively similar to those of TFE. Indeed, for KcsA it appeared that only the concentration of alcohol required for tetramer dissociation varied with the nature of the alcohol (6).

It can be expected that the various mechanisms by which small alcohols can influence soluble proteins should apply equally well to the soluble parts of integral membrane proteins. Nevertheless, different rules may apply to the integral parts of membrane proteins that are exposed to the lipid phase. Thus, proteins that have large extramembranous domains, like the penicillin binding proteins that were identified in the screen, may behave more like soluble proteins while different mechanisms may dominate the overall effect of TFE for proteins that are almost completely membrane-embedded, like SecG and the glucose-specific enzyme IICB part of the phosphotransferase (PTS) system. Several such potential mechanisms by which membrane-spanning parts of proteins may be affected by small alcohols are discussed below.

The mechanism of dissociation of KcsA by TFE and other small alcohols was suggested previously to involve a change in the lateral pressure profile of the bilayer the proteins reside in (4,6). This was based on the observation that the presence of lipids that give rise to different lateral pressure profiles also change the alcohol-requirements to induce dissociation (4). TFE and other alcohols are known to associate with the membrane by occupying the headgroup region of the lipid bilayer, resulting in a change in the lateral pressure profile (6). This in turn would result in a change in the required amount of work to dissociate a protein complex, provided that dissociation would result in a depth-dependent change in cross-sectional area of the proteins involved (14). Interestingly, alcohols with a higher octanol-water partitioning coefficient are more capable of disrupting tetrameric KcsA, suggesting that the ability to associate with the membrane is directly related to the capacity to disrupt oligomeric structures (6). This concept of lateral pressure-induced dissociation can be used to explain the anomalous behavior of the porins that were detected in the screen described in chapter 2. The porins OmpF and LamB were detected off-diagonally, indicating that these proteins were liberated from oligomeric complexes. However they migrated at their oligomeric molecular weights. This can be rationalized by the fact that beta-barrel proteins are rigid, incompressible tubes, that will not display a depth-dependent change in cross-sectional area upon dissociation and will therefore be unaffected by an alcohol-induced change in the lateral pressure profile. The nature of the complex they become dissociated from remains unclear.

While this model adequately explains the observations made for KcsA, it fails to explain the alcohol-induced dissociation of MscS, which was found to be not dependent on the nature of the surrounding lipids. Hence, in this case other mechanisms are expected to play a role. As a likely explanation we propose the following. The X-ray crystal structure of the MscS protein shows large hydrophobic pockets that are exposed to the lipid bilayer (15). Alcohols might partition into these crevices and thereby weaken the interactions between the individual protein subunits or force them apart (Chapter 3). KcsA does not have such crevices (2), thereby excluding such a potential mechanism.

Another mechanism that needs to be considered when discussing the differences in behaviour of MscS and KcsA is related to the tryptophan residues that often anchor transmembrane helices in the lipid-water interface. Recent studies on model transmembrane peptides indicate that TFE causes a loss of this anchoring ability (16). If such an effect would occur in oligomeric membrane proteins then it could be expected that the loss in anchoring function would destabilize the protein oligomer. This implies that proteins that are anchored in the bilayer primarily via tryptophans would lose more readily their lipid-mediated anchoring by exposure to alcohols than proteins that are anchored otherwise, for instance via basic amino acids. Also this hypothesis is consistent with our observations of a difference in response between KcsA and MscS. MscS contains no anchoring tryptophan residues, while KcsA contains five such residues on each subunit. A cursory investigation of the proteins detected by the 2D gel approach suggests several candidates for which this hypothesis may be tested. The penicillin-binding proteins 1A and 2, SecG and the hypothetical protein yibN do not contain anchoring tryptophan residues. In contrast, other SDS-resistant proteins do contain anchoring tryptophans, like subunit 1 of the cytochrome d terminal oxidase, PBP1-B and many components of the PTS system.

Another effect to be considered of importance for dissociation of membrane-embedded proteins is related to the recent observation that certain negatively charged lipids like phosphatidic acid (PA) have a strong stabilizing effect against alcohol-induced dissociation of the KcsA tetramer (Raja et al, unpublished observations). It has recently been shown that PA interacts directly with basic amino acids in model peptides via hydrogen bonds. These hydrogen bonds affect the charge of the PA molecule, allowing it to carry two negative charges, thereby enforcing the electrostatic interaction (17). Because TFE strengthens hydrogen bond and electrostatic interactions in soluble proteins rather than weakening them, the presence of PA may result in a stabilizing interaction at the membraneous parts of proteins. The differences in susceptibility of membrane protein oligomers towards alcohols might then be related to the occurrence of electrostatic/hydrogen bond interactions between positively charged amino acids in the membrane-water interface and specific anionic lipids.

Comparison to existing methods

How does the method presented here compare to existing methods of identifying membrane protein oligomers? The work-horse in identifying and analyzing membrane protein oligomers is the so-called Blue-Native PAGE method developed by Shagger and coworkers (18). In BN-PAGE, protein complexes are solubilized by binding of the charged dye Coomassie Brilliant Blue, allowing their solubilization under quite mild conditions. Solubilized protein complexes are then separated by electrophoresis. Subsequently, a second separation is performed, typically using regular SDS-PAGE. Under these much more denaturing conditions many membrane protein complexes dissociate, allowing the identification of their constituent components. It should be noted that the ways by which the proteins are dissociated in BN-PAGE or by the alcohols are very different, and thus that these methods identify essentially different subproteomes. While BN-PAGE typically identifies protein complexes that are dissociated by SDS, alcohol-induced dissociation targets a subproteome of exceptionally stable complexes: those that are stable in SDS, but become dissociated by small alcohols.

Although BN-PAGE has been remarkably successful in analyzing protein complexes in mitochondria it suffers from a number of drawbacks. It has limited resolution, especially in the lower molecular weight region and the mild conditions that are used in the first dimension can give rise to poor delipidation and artefactural associations (19). The method we present, while restricted to analyzing alcohol-sensitive SDS-stable oligomers, is unaffected by these drawbacks. The use of SDS allows for better resolution, although the incubation with TFE does cause some smearing. Overall, our method can be seen as complementary to the traditional BN-PAGE method. Indeed, when we compare the set of 39 inner membrane proteins identified by our method with that of 42 inner membrane proteins identified by a BN-PAGE approach on the same system, the overlap exists of only 9 proteins (The peripheral membrane proteins atpA, atpD, nuoCD, nuoG, manX, cyoA and the integral membrane proteins MscS, SecD and yhcb) (20).

The method presented in chapter 2 has the additional advantage that it provides a direct mechanism to test the stability of the detected complexes by varying the concentration of the alcohol. In addition, observing a protein via this 2D gel approach immediately provides a convenient and rapid protocol for investigating the proteins oligomeric state via 1-dimensional electrophoresis simply by running the same preparation before and after alcohol incubation, on a gel that is identical to the gel used in the first dimension of the 2D gel system.

Implications for oligomeric peripheral membrane proteins

Somewhat surprisingly, also 12 peripheral membrane proteins were identified in addition to the 23 integral membrane proteins, among them the preprotein translocase SecA (discussed in the appendix to chapter 2). The cell wall synthesis protein MurG (Chapter 3) was also found to take part in a stable membrane protein complex. We showed that SecA, while a dimer in aqueous solution, migrates on SDS gel at room temperature in both a dimeric and a monomeric form. The dimeric form is most likely associated with the membrane-embedded SecYEG complex, while the monomeric form probably originates from cytosolic or lipid-bound SecA that dissociates upon addition of SDS.

A similar observation was made for MurG, which also migrates as a combination of oligomers and monomers. These oligomers are much more tightly associated with the membrane than the monomers, quite conceivably by interacting with an integral membrane protein.

Based on these two examples we propose the following hypothesis: Oligomeric peripheral membrane proteins may occur in different forms that display different behaviour on SDS-PAGE gel, migrating as monomers and as oligomers respectively. The monomeric form may either be monomeric *in-vivo* or become dissociated from an oligomer by exposure to SDS. The oligomeric form on gel, however, is unaffected by SDS but becomes dissociated when it is exposed to TFE. We propose that this oligomeric form originates from peripheral membrane proteins that interact with another membrane component and that it is this interaction which protects them from dissociation by SDS, possibly by inducing a different conformation.

The table listing the observed proteins suggests several candidates for further investigation, as any observed peripheral membrane protein that is known to dock onto an integral membrane component may display this behaviour. For example, the mannose-specific enzyme IIAB component of the PTS system occurs both in free form as well as bound to the membrane-embedded part of the PTS system. If the data obtained for SecA and MurG extend to this protein as well, the free form is expected to dissociate upon exposure to SDS, while a pool of the protein docked on its integral membrane protein partner resists such treatment.

Another protein that became detected as a stable oligomer is the ATP-binding component of the methyl-galactoside transport system, which is also expected to dock onto an integral membrane protein. Also here a straightforward SDS-PAGE-based investigation of the oligomeric state may reveal behaviour similar to that of SecA and MurG, furthering our understanding of membrane protein oligomerization.

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Chapter 5

Nederlandse samenvatting

Alle organismen bestaan uit een of meerdere cellen. Alle cellen zijn omgeven door een beschermend omhulsel: het celmembraan. Dit celmembraan scheidt de inhoud van de cel van de omgeving. Naast het celmembraan kunnen cellen tevens interne membranen bevatten die interne compartimenten omsluiten. Sommige cellen en sommige interne compartimenten worden omsloten door meerdere membranen. Dit is ook het geval bij de darmbacterie *Escherichia coli* (*E.coli*), het organisme waaraan gewerkt is in het onderzoek waarop dit proefschrift gebaseerd is. Bij *E.coli* spreken we dan ook van een binnen- en buitenmembraan.

Een membraan is opgebouwd uit vele individuele moleculen. Deze moleculen kunnen worden ingedeeld in twee types: lipiden en eiwitten. De lipiden zijn moleculen met een wateroplosbare kop en een vetachtige staart. In een membraan vormen de lipiden een dubbellaag, waarbij de vetachtige staarten van beide lagen naar binnen toe wijzen, terwijl de hydrofiele koppen in contact staan met een waterige omgeving. Er bestaat een grote verscheidenheid aan lipiden, zowel in de koppen als in de vetachtige staarten.

Hoewel het membraan een barrière vormt die het inwendige van cellen van de buitenwereld afsluit, is het noodzakelijk om materiaal en informatie met de buitenwereld uit te wisselen. Voedingsstoffen moeten door het membraan naar binnen getransporteerd worden, terwijl afvalstoffen juist naar buiten moeten. Daarnaast moeten cellen reageren op signalen uit de omgeving. Deze functies worden uitgevoerd door eiwitten.

Ieder eiwit is verantwoordelijk voor een specifieke taak, zoals bijvoorbeeld het transport van materiaal of informatie over het membraan. Deze taak kan in vele gevallen beïnvloed worden door de lipiden die aanwezig zijn in het membraan.
Ieder eiwit is een lange keten van aan elkaar gekoppelde aminozuren. Zo een keten neemt een ingewikkelde, opgevouwen structuur aan die voornamelijk bepaald wordt door de aminozuursamenstelling.
Eiwitten kunnen op verschillende manieren met het membraan geassocieerd zijn. Sommige eiwitten steken dwars door het membraan heen. Deze eiwitten worden integrale membraaneiwitten genoemd. Daarnaast is het mogelijk dat eiwitten op het

membraan liggen zonder er doorheen te gaan. Dit noemen we perifere membraaneiwitten.

Veel membraaneiwitten vervullen hun specifieke rol niet alleen, maar werken samen in grotere complexen (oligomeren) tezamen met andere eiwitten. Dit kunnen zowel andere exemplaren van hetzelfde type zijn, ofwel volledig andere eiwitten. In het eerste geval spreken we van een homogene oligomeer, in het tweede geval van een heterogene oligomeer.

Een goed bestudeerd voorbeeld van een homogene oligomeer is het bacteriële kaliumkanaal KcsA. Dit eiwit vormt een stabiel complex. Dit complex bestaat uit 4 exemplaren van het KcsA eiwit, een zogeheten tetrameer. Zelfs bij hoge temperatuur onder omstandigheden waaronder de meeste eiwitten hun vouwing verliezen blijft KcsA een intacte oligomeer. Verassend genoeg echter valt de KcsA tetrameer uit elkaar bij blootstelling aan een 25% oplossing van de alcohol 2,2,2,-trifluoroethanol (TFE).
Het is onaannemelijk dat KcsA het enige eiwit is dat dit gedrag vertoont. Indien het mogelijk is een methode te ontwerpen die het toelaat specifiek te zoeken naar eiwitten die zich soortgelijk gedragen, kunnen hieruit nieuwe inzichten worden verkregen over de identiteit en samenstelling van stabiele membraaneiwit-complexen.
Zo een methode wordt beschreven in hoofdstuk 2. Deze methode is gebaseerd op een bestaande technologie waarin een mengsel van vele eiwitten door middel van een elektrisch veld langzaam door een acrylamide gel loopt, resulterend in een scheiding op basis van grootte. Dit staat bekend als SDS-PAGE. Hoewel deze methode zeer destabiliserend werkt op vele eiwitcomplexen, zijn stabiele oligomeren zoals KcsA hier goed tegen bestand.
Indien echter de gel, met daarin de gescheiden eiwitten, behandeld wordt met een oplossing van TFE, vallen ook deze stabiele complexen uit elkaar. Hoewel dit niet direct zichtbaar is, is het eenvoudig te visualiseren. Wanneer de gel met daarin de gescheiden eiwitten 90^0 gedraaid wordt, kan er opnieuw een scheiding worden uitgevoerd, loodrecht op de eerste scheiding. In feite wordt dan gebruik gemaakt van een tweedimensionale gel (2D gel). Alle eiwitten die geen deel uitmaken van stabiele complexen die uiteenvallen in TFE, zullen zich tijdens de tweede scheiding identiek gedragen aan de eerste scheiding. Al deze eiwitten zullen vervolgens op een rechte, diagonale lijn komen te liggen. Echter, de eiwitten die tijdens de eerste scheiding deel waren van stabiele complexen zijn inmiddels uiteengevallen, en gedragen zich als kleinere eenheden. Omdat de scheiding gebaseerd is op grootte, zullen deze eiwitten nu niet op de diagonale lijn terechtkomen. Vervolgens kan een kleurmethode worden toegepast die de delen van de gel die eiwit bevatten laat verkleuren, maar die lege delen van de gel kleurloos laat.
Deze methode stelt ons dus in staat om uit een mengsel van vele eiwitten exclusief die eiwitten op te pikken die deel uitmaken van stabiele complexen.
Toen dit werd toegepast op een preparaat van de binnenmembranen van *E.coli* werd een sterk gekleurde diagonale lijn waargenomen. Tevens werden een aantal gekleurde vlekken (spots) waargenomen die afweken van deze diagonale lijn. De negen meest intens gekleurde spots werden onderzocht op de identiteit van de aanwezige van eiwitten.
De spots bleken vrijwel allemaal meerdere eiwitten te bevatten, in totaal achtenvijftig. Van een aantal van deze eiwitten was bekend dat ze onderdeel vormden van een

complex dat SDS-PAGE doorstaat. Echter, van een aantal andere eiwitten was onbekend dat ze tezamen met andere eiwitten in het membraan kunnen worden aangetroffen. Een aantal van deze geïdentificeerde eiwitcomplexen werd nader onderzocht op samenstelling, functie en invloed van de lipide omgeving.

Het perifere membraaneiwit SecA was een van de eiwitten die werd aangetroffen. Dit eiwit speelt in de cel een rol bij het transporteren van ongevouwen eiwitketens vanuit het binnenste van de cel, het zogeheten cytosol, naar de ruimte tussen het binnen- en het buitenmembraan, het periplasma. SecA vervult deze functie door zich vast te zetten op een integrale component van het binnenmembraan, het porievormende complex SecYEG. SecA duwt vervolgens een eiwitketen door deze porie heen. Er bestaan tegenstrijdige studies over de vraag of SecA als een enkel exemplaar op het SecYEG complex aanwezig is, of als een combinatie van twee eenheden.

Verassend genoeg werd SecA aangetroffen op een plaats op de gel die duidt op een overgang van een homogeen complex bestaande uit twee SecA-moleculen (dimeer) naar een enkel SecA molecuul (monomeer). Hoewel SecA in de cel een dimeer is, is deze niet bijzonder stabiel. Op SDS-PAGE wordt SecA alleen aangetroffen als een monomeer. Waar komt dan de op gel aangetroffen dimeer vandaan? Het is bekend dat SecA in twee vormen kan voorkomen in de cel. In de ene vorm zit het vast op het SecYEG complex, waar het zijn translocatiefunctie vervult. In de andere vorm is het inactief, en is het geassocieerd met lipiden van het binnenmembraan. Deze tweede vorm is de meest voorkomende vorm van SecA.
Wanneer nu een preparaat van *E.coli* binnenmembranen wordt geanalyseerd op het aanwezige SecA blijkt dat de een groot deel van het SecA als monomeer aanwezig is, terwijl een kleiner deel aanwezig is als dimeer.

Dit leidde tot de volgende hypothese: De vorm van SecA die in aanraking is met de lipiden van het binnenmembraan geeft aanleiding tot de monomeer die op SDS-PAGE wordt waargenomen. Echter, zodra SecA op het SecYEG complex gaat zitten ondergaat het een verandering waardoor het bestand is tegen SDS-PAGE. Dit is de vorm die we vervolgens op SDS-PAGE waarnemen als dimeer.

Indien deze hypothese correct is, zou men verwachten dat als we in een cel kunstmatig de hoeveelheid van het SecYEG complex verhogen, ook de hoeveelheid stabiel SecA zal toenemen. Toen dit experiment werd uitgevoerd bleek inderdaad dat de hoeveelheid stabiel SecA toenam met de hoeveelheid van het SecYEG complex. Dit gaf ondersteuning voor de stelling dat SecA op het translocase aanwezig is als een dimeer, in plaats van als een monomeer.

Een ander eiwit dat een stabiel complex vormt, is het perifere membraaneiwit MurG. Dit enzym is betrokken bij het aanmaken van de bacteriële celwand. Deze celwand bevindt zich tussen het binnen- en buitenmembraan in en vormt daar een rigide laag die structuur en bescherming biedt. De celwand is essentieel voor het overleven van veel bacteriën. Om deze reden zijn de eiwitten die betrokken zijn bij de aanmaak hiervan belangrijke doelwitten voor antibiotica.
Van het MurG eiwit is bekend dat het in complexen kan voorkomen met wateroplosbare eiwitten. Echter, er zijn geen gevallen bekend van andere membraaneiwitten die samen met MurG in een complex aanwezig zijn. Om deze

reden werd MurG geselecteerd voor nadere studie om te bepalen of het een heterogeen of homogeen complex vormt. We vonden dat in *E.coli* MurG ten dele voorkomt in een homogeen complex, dat waarschijnlijk uit 4 identieke eenheden bestaat. Dit complex is, net zoals vrij MurG geassocieerd met het binnenmembraan. Het complex is echter veel sterker met het membraan geassocieerd dan MurG dat in vrije vorm voorkomt.
Hoewel ongeveer een vierde deel van het in de cel aanwezige MurG als deel van dit stabiele complex voorkomt, bleek het niet mogelijk om gezuiverd MurG *in-vitro* in de gecomplexeerde vorm om te zetten. Zowel de aanwezigheid van een lipide bilaag of van substraat of een combinatie hiervan gaf geen aanzet tot oligomerisatie. Ook het verwijderen van substraat in groeiende *E.coli* cellen had geen invloed op de oligomerisatie van MurG.
Dit suggereert dat een andere component van het *E.coli* binnenmembraan nodig is om een stabiel MurG complex te vormen.
Hier ligt een interessante parallel met het eerder besproken perifere membraaneiwit SecA. Zowel MurG als SecA verschijnen voor een deel als een stabiele oligomeer op SDS-PAGE gel, terwijl een ander deel van het aanwezige eiwit als monomeer verschijnt. In het geval van SecA bleek de oligomeer band op SDS-PAGE gel te corresponderen met die eiwitten die op het SecYEG complex gebonden zitten, terwijl de monomeer band correspondeerde met SecA dat geassocieerd is met de lipiden van het binnenmembraan. In het geval van MurG is de oligomere vorm sterker geassocieerd met het binnenmembraan dan de vorm die als monomeer op gel voorkomt.
Wellicht bindt MurG aan een integrale eiwitcomponent van het binnenmembraan, hetgeen aanleiding geeft tot de vorming van een stabiele oligomeer.
Als dit het geval blijkt, is er mogelijk sprake van een algemeen model voor de oligomerisatie van perifere membraaneiwitten die in interactie zijn met integrale membraaneiwitten. Oligomere, perifere membraaneiwitten komen voor in verschillende vormen op SDS-PAGE gel, als monomeer en als oligomeer. De monomere vorm komt of als monomeer in de cel voor, of als oligomeer die niet bestand is tegen SDS-PAGE. Echter, de oligomere vorm is bestand tegen SDS-PAGE maar dissocieert zodra deze wordt blootgesteld aan TFE. Deze oligomere vorm is afkomstig van eiwit dat in interactie is met een integraal deel van het membraan en deze interactie beschermt het tegen SDS-PAGE, mogelijk door het perifere eiwit complex in een stabielere vorm te brengen.

In de lijst met door middel van de 2D gels gedetecteerde eiwitten bevindt zich een aantal perifere membraaneiwitten, die zich uitstekend lenen om dit model verder te testen. Voorbeelden zijn het mannose-specifieke enzym EIIAB van het fosfotransferase systeem en het ATP-bindende deel van het methyl-galactoside transport systeem.

Behalve de twee eerder genoemde perifere membraaneiwitten werd ook een integraal membraaneiwit aan verdere experimenten onderworpen. Van het integrale membraaneiwit MscS is het bekend dat het voorkomt als een homoheptameer, een complex opgebouwd uit zeven identieke eenheden. Dit eiwit is deel van een systeem dat een gat vormt in het binnenmembraan van *E.coli* zodra er een te sterke druk over dit membraan staat. Zo wordt voorkomen dat het binnenmembraan kan scheuren. MscS functioneert dus als een overdrukventiel.

Deze functie van MscS blijkt afhankelijk te zijn van de samenstelling van de lipide bilaag waarin het zich bevindt. MscS was ook een van de eiwitten die geïdentificeerd werden door de 2D gel methode, en vormt dus een stabiel complex dat gedissocieerd kan worden door blootstelling aan TFE. Op gewone SDS-PAGE komt MscS dan ook voor als een combinatie van tetrameren en van heptameren, die gedissocieerd kunnen worden in monomeren onder invloed van TFE. Hoewel van KcsA, dat ook dissocieert door TFE, bekend is dat de lipide bilaag een sterk effect op de dissociatie heeft, vonden wij verassend genoeg dat dit voor MscS niet het geval was.

Hoe werkt TFE dan op MscS? Deze alcohol kan integrale membraaneiwitten op verschillende manieren beïnvloeden. Het kan effect uitoefenen op de wateroplosbare delen van integrale membraaneiwitten, maar het kan ook inwerken op de delen van het eiwit die integraal in het membraan zitten.

Bij de wateroplosbare delen kan TFE ervoor zorgen dat elektrostatische- en waterstofbrug interacties in een eiwit versterkt worden. Ook kan TFE helices stabiliseren door de structuur van de waterige omgeving te beïnvloeden. Verder kan TFE hydrofobe interacties in een eiwit verstoren door aan de hydrofobe delen te binden.
Het totale effect van TFE op een eiwit is waarschijnlijk een combinatie van deze factoren.

Over het effect van TFE op de delen van eiwitten die in het membraan zitten, is minder bekend. Hoewel TFE een pakkingeigenschap van het membraan, het laterale drukprofiel, kan beïnvloeden weten we dat dit geen effect heeft op MscS omdat de dissociatie van dit complex ongevoelig is voor de lipide samenstelling. In plaats hiervan lijkt het waarschijnlijk dat TFE bindt aan hydrofobe plekken van MscS die zijn blootgesteld aan het membraan, en hierdoor dissociatie bewerkstelligt. Dit is in overeenstemming met observaties aan de activiteit van MscS dat is blootgesteld aan kleine hoeveelheden TFE.

Verder is het mogelijk dat TFE de aminozuren die de transmembraan helices aan het membraan verankeren, beïnvloedt. Aangezien MscS deze verankerende residuen niet heeft, mag verwacht worden dat hier de interacties met het membraan minder belangrijk zijn. Dit is consistent met het waargenomen geringe effect van de lipide bilaag op de stabiliteit van MscS. Als dit mechanisme algemeen voorkomt, valt het te verwachten dat ook andere eiwitten die geen membraan-ankerende residuen bezitten, weinig effect van de samenstelling van het membraan zullen ondervinden op de door TFE veroorzaakte dissociatie. Tegenovergesteld valt te verwachten dat zo een effect juist wel zal optreden voor eiwitten die veel membraanverankerende residuen bevatten. De tabel met geïdentificeerde eiwitten geeft ook hiervoor een aantal geschikte kandidaten voor verdere experimenten. Eiwitten die weinig of geen van zulke residuen bevatten zijn YibN, SecG, deel van het eerder genoemde SecYEG complex en de penicilline-bindende eiwitten PBP-1A en PBP2. Eiwitten die daarentegen veel ankerende residuen bevatten zijn subunit 1 van het cytochrome d terminal oxidase, PBP1-B en vele onderdelen van het fosfotransferase systeem.
Sterk geladen lipiden oefenen mogelijk invloed uit op de stabiliteit van eiwitcomplexen. Aangezien TFE in staat is om ladingsinteracties te versterken kan het

zijn dat dit ook een mechanisme vormt waardoor het effect van TFE op membraaneiwit complexen beïnvloed wordt.
Uiteraard is het zeer wel mogelijk dat het totale effect van TFE op een membraaneiwit een combinatie van twee of meerdere van de bovenstaande mechanismen is.

Hoe verhoudt deze methode zich tot de al bestaande technieken om membraaneiwit complexen te analyseren? De meest gangbare methode om dit te bereiken is de zogeheten Blue-Native electroforese (BN-PAGE). BN-PAGE volgt echter een andere strategie. Waar de BN-PAGE methode zich richt op de analyse van instabiele eiwitcomplexen, richt onze methode zich juist specifiek op een uiterst stabiel onderdeel van alle membraaneiwit complexen. Hierdoor identificeert onze methode ook een andere selectie aan eiwitten dan wanneer men BN-PAGE zou toepassen op hetzelfde systeem. Wanneer de binnenmembranen van *E.coli* worden geanalyseerd via zowel onze methode als via BN-PAGE blijkt dat hoewel er vergelijkbare hoeveelheden eiwitcomplexen worden geïdentificeerd (42 voor BN-PAGE, 39 voor de hier gepresenteerde methode) de overlap slechts gering is (9 eiwitcomplexen).
Daarbij heeft onze 2D gel methode het voordeel dat doordat het ruwere condities toestaat, de methode eenvoudiger en sneller is uit te voeren. Verder geeft de SDS-PAGE techniek die wij gebruiken een betere resolutie dan BN-PAGE normaalgesproken geeft.
Over het geheel genomen kan onze technologie gezien worden als een aanvulling op de bestaande BN-PAGE methode en blijkt deze nieuwe inzichten te geven in de biochemie van membraaneiwit complexen.

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Robin

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Curriculum Vitae

Robin Spelbrink was born on the 12th of january 1979 in Rotterdam, the Netherlands. He moved to Middelburg, the Netherlands and graduated from the Christelijke Scholengemeenschap Walcheren in 1997. In september of the same year he started the study of Chemistry at Utrecht University.
These studies exposed him to a broad variety of chemical disciplines, including minors on organometalic synthesis in the department Organic Synthesis and on the folding behaviour of colicin proteins in the department Biomolecular Mass Spectrometry.
In september of 2000 he started his major in the department Biochemistry of Membranes on a project involving the study of the membrane interaction of the bacterial peptidoglycan synthesis enzyme MurG, supervised by Els van den Brink-van der Laan. Subsequently he enrolled in a PhD project in the same department under the supervision of Prof. Dr. Ben de Kruijff and Prof. Dr. Antoinette Killian. During this period he investigated lipid-protein interactions and membrane protein oligomerisation via a variety of biochemical and biophysical techniques. Throughout these activities he applied himself to the study of Aikido, a Japanese martial art. This led him to take up a position as treasurer in the Aikido foundation Jiki Shin Kan Utrecht. In february 2007 he moved to Groningen where he is currently employed as a protein chemist by BIRD Engineering at Solanic BV, an AVEBE group company.

Curriculum Vitae