universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

In vitro Reconstitution of DegP-Omp complexes from *Escherichia coli*

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, im
Dezember 2010

Zusammenfassung

Ein wichtiger Prozess in zellulärer Proteinqualitätskontrolle ist das Überwachen von Faltungszuständen von Proteinen, da ent- oder fehlgefaltete Proteine Zellen durch die Bildung von potentiell giftigen Aggregaten gefährden. Das bifunktionelle Hitzeschockprotein DegP, ein Mitglied der HtrA Familie, fungiert sowohl als Protease, als auch als Chaperon und hat eine Schlüsselrolle in der Proteinqualitätskontrolle im Periplasma von *E.coli* inne. Zusätzlich ist DegP in der Biogenese von Außenmembranproteinen (outer membrane proteins, Omps) impliziert. Gemeinsam mit den periplasmatischen Chaperonen Skp and SurA soll DegP Omps durch das wässrige Periplasma leiten, um schädliche Aggregation zu verhindern.

Obwohl DegPs Funktion als Protease schon eingehend charakterisiert wurde, ist über seine Funktion als Chaperon und seine Beteiligung in der Biogenese von Omps noch nicht viel bekannt. DegP muss in Komplexen mit gefalteten Substraten biochemisch und strukturell analysiert werden, um seine Rolle als Chaperon auf einem mechanistischen Level zu verstehen. In dieser Arbeit rekonstituierten wir Komplexe von DegP mit gefalteten und ungefalteten Substraten *in vitro* und gewannen biochemisch Einblicke in DegPs mögliche Rolle als Proteinfaltungsfaktor in der Biogenese von Omps.

Wir beobachteten, dass DegP Komplexe mit ungefalteten OmpA und OmpC/F bildete, konnten aber keine Faltungsaktivität nachweisen. Allerdings bindet und schützt DegP gefaltete Omps, die aus der äußeren Membran extrahiert wurden. Zusätzlich zu den DegP-Omp Komplexen generierten wir Komplexe aus den Chaperonen Skp und SurA mit OmpA und OmpC/F. Dabei fanden wir heraus, dass DegP die Porine OmpC und OmpF gegenüber dem Strukturprotein OmpA als Substrate bevorzugte, während für Skp und SurA das Umgekehrte galt. Wir untersuchten eine der potenziellen Rolle von DegP in einer zweiten Ablaufebene der Biogenese von Omps als Qualitätskontrollmechanismus, wobei wir allerdings keinen Transfer des Proteins OmpA von Skp/SurA zu DegP nachweisen konnten. Außerdem analysierten wir mutmaßliche DegP Schnittstellen in OmpC/F Signalpeptiden und gewannen weitere Einblicke in DegPs Proteaseaktivität.

Die in dieser Arbeit präsentierten Daten unterstützen eine Rolle von DegP als Faltungsfaktor in der Biogenese von Omps nicht, aber die stabile Assoziation von DegP und gefalteten Omps untermauert seine Rolle als wichtiger Qualitätskontrollfaktor.

Die DegP-Omp Komplexe, die in dieser Arbeit generiert wurden, stellen eine wichtige Basis für weitere strukturelle Untersuchungen von DegP-Omp Komplexen dar. Ausserden wurden Fortschritte, DegPs Funktion als Chaperon betreffend, gemacht und mögliche Substratspezifitäten von DegP, Skp und SurA aufgedeckt. Dies bietet eine experimentelle Basis für die weitere Aufklärung des Chaperon-Netzwerks in der Biogenese von Omps.

Abstract

Monitoring protein folding states is a crucial process in cellular protein quality control. Un- or misfolded proteins endanger cells by forming potentially toxic aggregates. The bifunctionial heat-shock protein DegP, a member of the HtrA family, functions both as a protease and a chaperone and plays a key role in protein quality control in the *E.coli* periplasm. Furthermore, it is implicated in outer membrane protein (Omp) biogenesis, together with the periplasmic chaperones Skp and SurA, which most likely shuttle Omps through the aqueous periplasm to prevent harmful aggregation.

Even though DegPs protease function is already characterized in depth, its chaperone function and its involvement in Omps biogenesis is not well understood. To mechanistically comprehend DegPs role as a chaperone we need structural and biochemical analyses of DegP complexed with folded substrates. In this work we reconstituted complexes of DegP with folded and unfolded Omp substrates *in vitro* and biochemically gained further insights into DegPs probable role as a folding factor in Omp biogenesis.

We found that DegP formed complexes with unfolded OmpA and OmpC/F, but observed no folding activity. However, DegP binds and protects folded Omps extracted from the outer membrane. Apart from DegP-Omp complexes, we generated complexes of the chaperones Skp and SurA with OmpA and OmpC/F. Thereby, we found that DegP preferred the porins OmpC and OmpF as substrate over the structural protein OmpA, while the opposite was true for Skp and SurA. We investigated a potential role of DegP as a second-step quality control mechanism in Omp biogenesis, however we detected no evidence of a transfer of OmpA from Skp/SurA to DegP. In addition, we analysed the putative DegP cleavage sites within the OmpC/F signal peptides and gained further insights into DegPs protease activity.

The data presented in this work does not support a role of DegP as a folding factor in Omp biogenesis, but its stable association with folded Omps fortifies DegPs role as an important quality control factor.

The DegP-Omp complexes generated in this work contribute an important basis for further structural investigation of DegP-Omp complexes. This work provides biochemical advancances on the chaperone function of DegP and suggests furthermore possible substrate specificities of DegP, Skp and SurA, offering an experimental basis

for future elucidation of the chaperone network involved in Omp biogenesis.

Acknowledgements

First of all, I would like to thank my supervisor Tim Clausen for the possibility to work on this cool project and for his guidance and trust.

Foremost, my heartfelt thanks go to 'my personal post-docs' Justyna and Alex. Thanks for your constant support, help and patience and for always taking the time to help me.

My thanks also go to all the past and present lab members, Alex, Anita, Bastian, Beata, Doris, Flavia, Linda, Linn, Markus, Jakob, Juliane, Justyna, Robert, Sonja and Ti for creating such a nice and helpful working environment. Thanks to Markus for invigorating discussions over lunch, Juliane for my birthday cake, and also the members of the Stolt Bergner lab, Barbara and Bettina, for sharing some fun with me.

Furthermore, I'd like to thank some friends who contributed in various ways. For advice on numerous programs: Bastian, Kira and Janette, Johi for the abstract translation and Sandra for proofreading it and for providing me with the critical brain-cell.

I want to thank all the friends who supported and encouraged me all the time and for whom I had far too little time, and the 'WG' and my brother especially for keeping me fed.

I want to thank my family and especially my parents who always trusted and encouraged me. I love you.

Finally, I want to thank Andi, for being who you are and for always being there for me.

Contents

1 Introduction

1.1 The cell-envelope of Gram-negative bacteria

The cell-envelope of Gram-negative bacteria consists of two membranes, the inner membrane (IM) and the outer membrane (OM). Between them an aqueous space, the periplasm, is enclosed (figure 1.1).

Although the IM and the OM are both membrane compartments composed of lipid bilayers, their composition and functions are dramatically different, as each of them contacts different environments. Both membranes contain membrane-spanning (integral) membrane proteins and membrane-anchored lipoproteins.

Within the cell-envelope crucial functions of the bacterial cell take place, such as importing nutrients and exporting effector proteins or toxic molecules.

The inner membrane

The IM forms a barrier between the cytoplasm on one side and the periplasm on the other. It forms an permeability barrier against most solutes, where traffic of ions, small molecules and ions is tightly regulated by integral or membrane associated proteins, which carry out most membrane-associated metabolic functions.

The composition of the IM is that of a symmetrical phospholipid bilayer, with the major components being phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

Integral IM proteins contain single or multiple membrane-spanning α-helices and are thought to be released laterally from the lumen of the translocation complex.

The periplasm

The periplasm is an aqueous compartment enclosed by the IM and the OM. In contrast to the controlled conditions in the cytosol, some conditions (e.g. salt, pH) in the periplasm are similar to those in the extracellular environment due to the selective permeability of the OM. It is generally oxidizing and does not contain adenosine triphosphate (ATP) as an energy source.

The periplasm is occupied by a layer of peptidoglycan and proteins in high concentration probably making it a highly crowded and viscous space (Mogensen and Otzen, 2005; Wülfing and Plückthun, 1994).

An important class of proteins in the periplasm are molecular chaperones and protein quality control factors. They are integral in folding, assembly and stabilisation of proteins and/or protein complexes, targeting proteins to their correct localisation and counteracting harmful misfolding and aggregation events (Allen et al., 2009). Periplasmic folding factors can be divided in three groups: Peptidyl-Prolyl-Isomerases (PPIases) (PpiD, PpiA, FkpA, SurA), which catalyse cis-trans-isomerisation of prolyl peptide bonds; Disulfide bond (Dsb) oxidoreductases (Dsb family) which promote correct disulfe bond formation; and molecular chaperones such as SurA, Skp and DegP (Mogensen and Otzen, 2005). Many periplasmic folding factors show functional duality, belonging to more than one group.

Molecular chaperones can be broadly divided into groups. Pili-specific chaperones from the PapD-like superfamily are responsible for chaperoning proteins of adhesive surface organelles (Hung et al., 1996). The LolA/LolB pathway is responsible for shuttling and incorporation of lipoproteins into the OM (Matsuyama et al., 1997). A group of chaperones generally upregulated in the σ^E and/or Cpx envelope stress response pathways consists of most notably SurA, Skp and DegP. They bind to a variety of mis- or unfolded proteins, protecting them from aggregation and sometimes proteolysis (Duguay and Silhavy, 2004; Sklar et al., 2007b).

All periplasmic proteins have the ability to carry out their function without ATP as an energy source.

The outer membrane

The OM forms the barrier between the periplasm and the extracellular space. It is an highly asymmetric lipid bilayer, where the inner leaflet is composed of the same phospholipds as the IM, but the outer leaflet is mainly composed of lipopolysaccharides (LPS). LPS comprises of Lipid A, core oligosaccharides and in O-(oligosaccharide-)antigen.

The OM forms a selective barrier, containing a high number of porins, pore proteins that allow the passage of small hydrophilic molecules, such as nutrients and water, while barring the entry for small hydrophobic molecules and macromolecules bigger than 600 Da (Nikaido, 2003).

In contrast to IM proteins, integral membrane-spanning outer membrane proteins (Omps) have a β-barrel architecture.

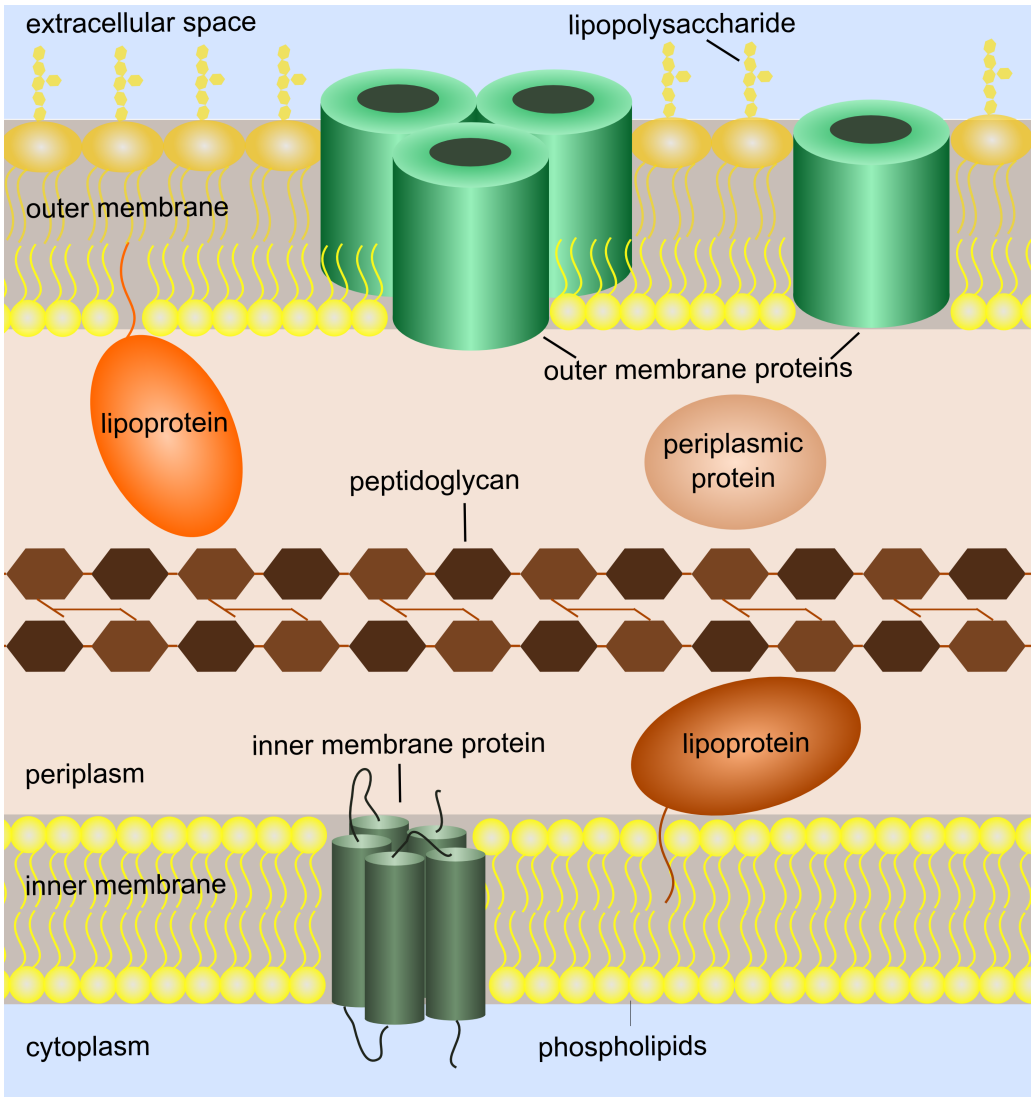


Figure 1.1: Envelope of E.coli. The cell envelope of gram-negative bacteria like Escherichia coli is composed of an inner (IM) and an outer membrane (OM). Enclosed between them is the periplasmic space. The inner membrane consists of a phospholipid bilayer, and α-helical transmembrane proteins (inner membrane protein). The OM contains lipopolysaccharide in its outer leaflet and β-barrel integral membrane proteins(outer membrane protein). The periplasmic space is a crowded environment occupied by membrane-anchored lipoproteins, periplasmic proteins, and a peptidoglycan layer. Figure reproduced from Ruiz et al. (2006).

1.2 Integral outer membrane proteins

The biological functions of Omps in the OM are diverse, ranging from OM structural proteins and adhesins, receptors and siderophore transporters, protein translocation pores and passive diffusion pores to enzymes like for example ligases, proteases and palmitoyl transferases (Mogensen and Otzen, 2005; Tamm et al., 2004).

Integral bacterial Omps form a β-barrel transmembrane architecture with an even number (between 8 and 22) of antiparallel β-strands (Schulz, 2002). Structure elucidation shows that these strands often are connected by short turns at the periplasmic side, in contrast to longer, often flexible, loops facing the extracellular side. The average length of a β-strand ranges between 11-14 residues, depending on barrel size and their inclination angle from the membrane normal (usually about 40°). The amino acid composition of the transmembrane strands is such that Tyr and Trp residues frequently localise on the outside of the barrel, mediating contact with the lipid bilayer by insertion of aromatic side chains at both ends of the barrels (Tamm et al., 2004). The interior contains largely hydrophilic amino acids. Smaller Omps (8 β-strands, e.g. OmpA) contain solid cores partially occupied with water, usually acting through soluble attached domains. Larger barrels (e.g. OmpC, OmpF, 16 β-strands) permit passage of small hydrophilic substances through their channel-like interiors.

Typically, Omps form mono-,di- or trimeric oligomeric states depending on the respective Omp. Omps with energetically weakly stable transmembrane regions need to stabilized by oligomerisation or by interaction with structural units like for example α-helical plug-ins which insert into a channel (Naveed et al., 2009).

A remarkable property of Omp β-barrels is their stability to sodium dodecyl sulfate (SDS). In the presence of SDS, the barrel structure can only be denatured after heating for several minutes. Folded and denatured Omps show a different apparent mass on SDS-PAGE, providing a convenient assay for monitoring Omp tertiary structure (termed semi-native SDS-PAGE) (Schweizer et al., 1978).

The major Omps in *E.coli* OM have been found to be OmpA and OmpC/F. OmpA is a structural protein and OmpC and OmpF are non-specific porins (Baslé et al., 2006; Chai and Foulds, 1977).

1.2.1 OmpA

OmpA (figure 1.2) is a highly expressed protein in enterobacterial OMs, with around 100 000 copies per cell (Koebnik, 1999). Its Gene expression is highly regulated in response to environmental conditions. Especially on mRNA level turnover of *ompA* mRNA is a paradigm of ribo-regulation, and has been researched in detail (Smith et al., 2007).

A number of versatile functions of OmpA have been described. It is needed for bacterial conjugation, for structural stability and maintenance of the OM. In addition, several bacteriophages and colicines use OmpA as a receptor. In meningitic strains of *E.coli*, it has been shown to operate as an adhesin/invasin and functions as an immune evasin, but also as an immune system target. Moreover, it has been implicated in biofilm formation (reviewed in detail in Smith et al. (2007)).

OmpA is a 35 kDa protein, composed of two domains, a β-barrel transmembrane domain (TMD) composed of 8 β-strands, and a soluble periplasmic domain. A high-resolution crystal structure of the OmpA TMD was solved in 2000 (Pautsch and Schulz, 2000). On the inside of the β-barrel several water-filled cavities were found, separated by extensive salt bridges, restricting passage of molecules through the core of the barrel. However, NMR studies showed high flexibility along the axis of the barrel, which could permit function as a channel (Arora et al., 2001). Further investigations showed that salt bridges observed in the crystal could form alternative bonds, opening a small pore in the OmpA barrel (Hong et al., 2006). The OmpA open channel form was shown to be present in a subset of 2-3% OmpA molecules (Sugawara and Nikaido, 1994). From channel conductance experiments, 2 different pore sizes of OmpA were proposed, where the 8 β-strand barrel with a small pore shifts to a 16-β-strand barrel by incorporating the OmpA periplasmic domain in a temperature induced shift mechanism (Zakharian and Reusch, 2005). This discussion remains controversial.

Folding of OmpA has been investigated in detail. Partial OmpA folding into lipid vesicles was observed spontaneously *in vitro*, for correct insertion and insertion *in vivo* however the need for chaperones is apparent (Kleinschmidt, 2003; Surrey and Jähnig, 1995).

Experimentally, OmpA folding state can be monitored by semi-native SDS-PAGE. Folded OmpA proteins show an apparent molecular mass of 30 kDa on SDS-PAGE,

unfolded ones 35 kDa. By this mobility-shift OmpA tertiary structure can be determined (Schweizer et al., 1978).

1.2.2 OmpC/F

OmpC (figure 1.2) and OmpF belong to the class of general diffusion pores, termed porins, and are expressed at high level in the *E.coli* OM. They are not specific for certain substrates, but allow passage of small hydrophilic molecules, where OmpC and OmpF show cation selectivity. OmpC and OmpF have 60% identity on sequence level and also a high similarity in their structures. However, they are regulated differently in response to environmental conditions (e.g. changes in osmolarity and nutrient composition) and differ in translocation efficiency towards different substrates. They are regulated by the Cpx envelope stress responses (Baslé et al., 2006; Batchelor et al., 2005).

OmpC and OmpF form β-barrels composed of 16 strands with the N- and C-terminus forming a salt bridge within the barrel. At the periplasmic side the strands are connected by short turns and on the extracellular (EC) side by long loops protruding into the extracellular space. Here, loop L3 extends into the pore and constricts it. Structurally, OmpC and OmpF mainly differ in the length, composition and arrangement of their loop regions, resulting in a different pore lining at the EC side, most probably responsible for substrate preferences (Baslé et al., 2006; Cowan et al., 1992). In the OM OmpC and OmpF typically form homotrimeric structures, which show high stability to denaturing agents and temperature (OmpF for example was shown resist denaturation in the presence of 5 M guanidinium hydrochloride or 2% SDS at 70°C) (Koebnik, 1999; Phale et al., 1998). The stability of the OmpC and OmpF trimeric β-barrels was exploited during semi-native SDS-PAGE, where folded trimers stay asoociated and can be separated from unfolded monomers.

1.2.3 Omp biogenesis

Outer membrane protein biogenesis involves three important steps: 1) targeting to the cytoplasmic side of the IM and transport across it 2) traversing the periplasm 3) incorporation into the OM (figure 1.3).

Here, we focus on Omp biogenesis, while different pathways for the biogenesis of Omps, lipoproteins and LPS have been found (reviewed in Ruiz et al. (2006); Tokuda (2009)).

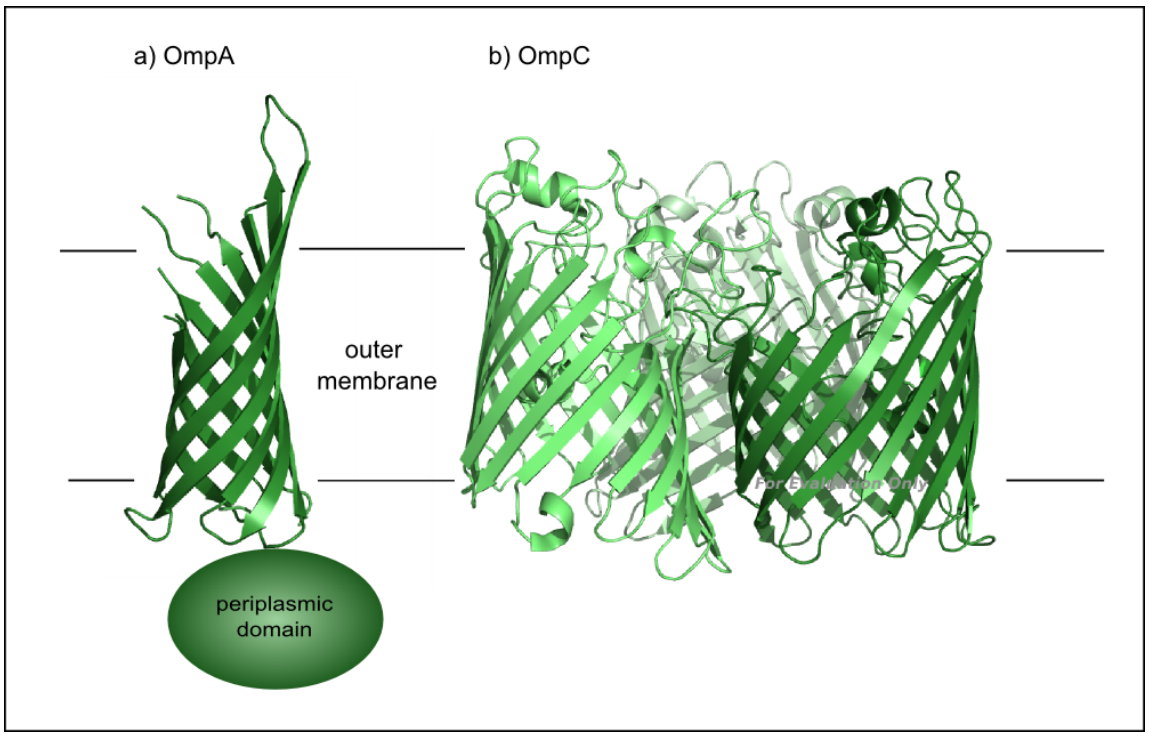


Figure 1.2: Outer membrane protein β-barrels of OmpA and OmpC. a. Structure of the *E.coli* transmembrane domain (PDB entry code: 1QJP (Pautsch and Schulz, 2000)), linked to its soluble periplasmic domain. **b.** Structure of the *E.coli* OmpC porin trimer, subunits colored in different shades (PDB entry code: 2J1N, (Baslé et al., 2006). Ribbon structures were done in PyMol.

Translocation through the IM

Omps (and all periplasmic proteins) are synthesised in cytoplasm as precursor proteins with an N-terminal localisation signal pepetide (SP), necessary for targeting the Omps to the Sec system, which translocates Omps through the IM (Bernstein, 2000). The SecB protein recognises the SP of newly synthesised proteins, protects them from aggregation and delivers them to the IM bound Sec complex in an unfolded form (Driessen, 2001). There, the Omps interact with the ATP-dependent motor protein SecA at the cytoplasmic face of the IM. The protein SecA pushes the unfolded preprotein through the aqueous protein-conducting channel formed by the proteins SecYEG (den Berg et al., 2004; Tomkiewicz et al., 2007). On the periplasmic side, the SP is cleaved off by the membrane-bound leader peptidase, releasing the mature, but still unfolded polypeptide chain into the periplasm (Dalbey, 1991).

Traversing the periplasm

After translocation through the Sec complex and cleavage of the SP, nascent Omps are immediately taken up by periplasmic chaperones (section 1.1), preventing misfolding and aggregation. In *E.coli*, the main chaperones implicated in this process are Skp, SurA and DegP. Additionally, they are thought to shuttle the Omps through the periplasm to the OM to deliver them to the BAM complex for membrane insertion (Sklar et al., 2007b). How chaperones participate in Omp folding and targeting remains to be fully understood.

Incorporation into the OM

Insertion and folding of Omps in the OM require the β-barrel assembly machinery (Bam) complex. In contrast to the Sec complex, the Bam complex is energy independent. Recently, the Bam complex was reconstituted *in vitro* (Hagan et al., 2010), however its structure and working principles remain elusive.

The Bam complex consists of 5 proteins, BamA, BamB, BamC, BamD and BamE. BamA consists of an integral β-barrel domain in the OM and five periplasmic POTRA (polypeptide transport-associated) domains. BamB-BamE are periplasmic lipoproteins, localising in the inner leaflet of the OM (Knowles et al., 2009).

BamA fulfills an essential role and is found in all Gram-negative bacteria (Voulhoux et al., 2003). Furthermore, homologues have been found in the OM of plastids and mitochondria. The structure of the POTRA domains has been solved (Kim et al., 2007; Knowles et al., 2008), and it was suggested that these domains bind substrates, e.g. the C-terminus of Omps, via β-augmentation.

BamB-BamE function as accessory components, forming tight interactions with BamA through its POTRA domains (Sklar et al., 2007a; Wu et al., 2005). BamB interacts with BamA independently of BamCDE, while the interaction of BamC with the complex relies on a direct interaction between BamD and BamA. All of these accessory components have a yet unclear role in Omp biogenesis, as Omp assembly defects occur upon their depletion. However, only BamD and BamA are essential proteins (Knowles et al., 2009).

For the insertion of Omps into the OM membrane several models have been proposed, suggesting that Omps are threaded into the membrane through or alongside a monomeric BamA barrel, or laterally between multimeric OmpA barrels. The exact

mechanism however, remains entirely unclear (Knowles et al., 2009).

So far, Bam complex interaction with periplasmic chaperones has only been shown for SurA, although it is not clear whether this interaction occurs directly or substrate-mediated (Knowles et al., 2009). Summarising, the manner of the interaction of chaperones with the Bam complex remains completely elusive.

SurA

The periplasmic chaperone SurA (**Sur**vival **A**) was first described in stationary phase cells as a survival factor under certain conditions (Tormo et al., 1990). Later studies implicated the main *in vivo* function of SurA in Omp biogenesis, as a folding and periplasmic shuttling factor. SurA deletion shows a strong similarity to OM defects such as increased permeability to hydrophobic substances (Justice et al., 2005). Furthermore, its deletion has an impact on specific Omps (Rouvière and Gross, 1996) and reduces Omp density in the OM (Sklar et al., 2007b). Substrate specificity studies demonstrated that it preferentially binds to unfolded Omps (over folded Omps or unfolded soluble proteins) (Bitto and McKay, 2004) and it was shown by a peptide library screening to preferentially recognise the Ar-X-Ar motif characteristic for Omp β-sheets (Ar: aromatic, X:polar residue) (Bitto and McKay, 2003; Hennecke et al., 2005).

SurA has dual functionality, it acts as a PPIase as well as a molecular chaperone. The crystal structure of mature SurA has been solved (figure 1.4a). The protein consists of four domains: 1) an N-terminal domain with no structural homologues, 2) two PPIase domains (P1 and P2), with a conserved parvulin fold, 3) a C-terminal domain (CTD) consisting of one long α-helix and a short β-strand (Bitto and McKay, 2002). Of the two PPIase domains only P2 is active (Rouvière and Gross, 1996) and it forms a satellite domain 30 Å away from the protein core. P2 deletion does not seem to greatly affect its chaperone activity (Behrens et al., 2001), indicating a structural separation between chaperone and PPIase function.

The substrate binding site is thought to be a deep groove in the N-terminal domain, which was occupied by crystal contacts (Bitto and McKay, 2002). Studies showing that the P1 domain in the core domain has negligible function (Behrens et al., 2001; Watts and Hunstad, 2008), were relativised by the finding of aromatic residues of consensus peptides binding into special pockets in the P1 domain (Xu et al., 2007). Longer peptides and proteins may be able to bind both domains simultaneously.

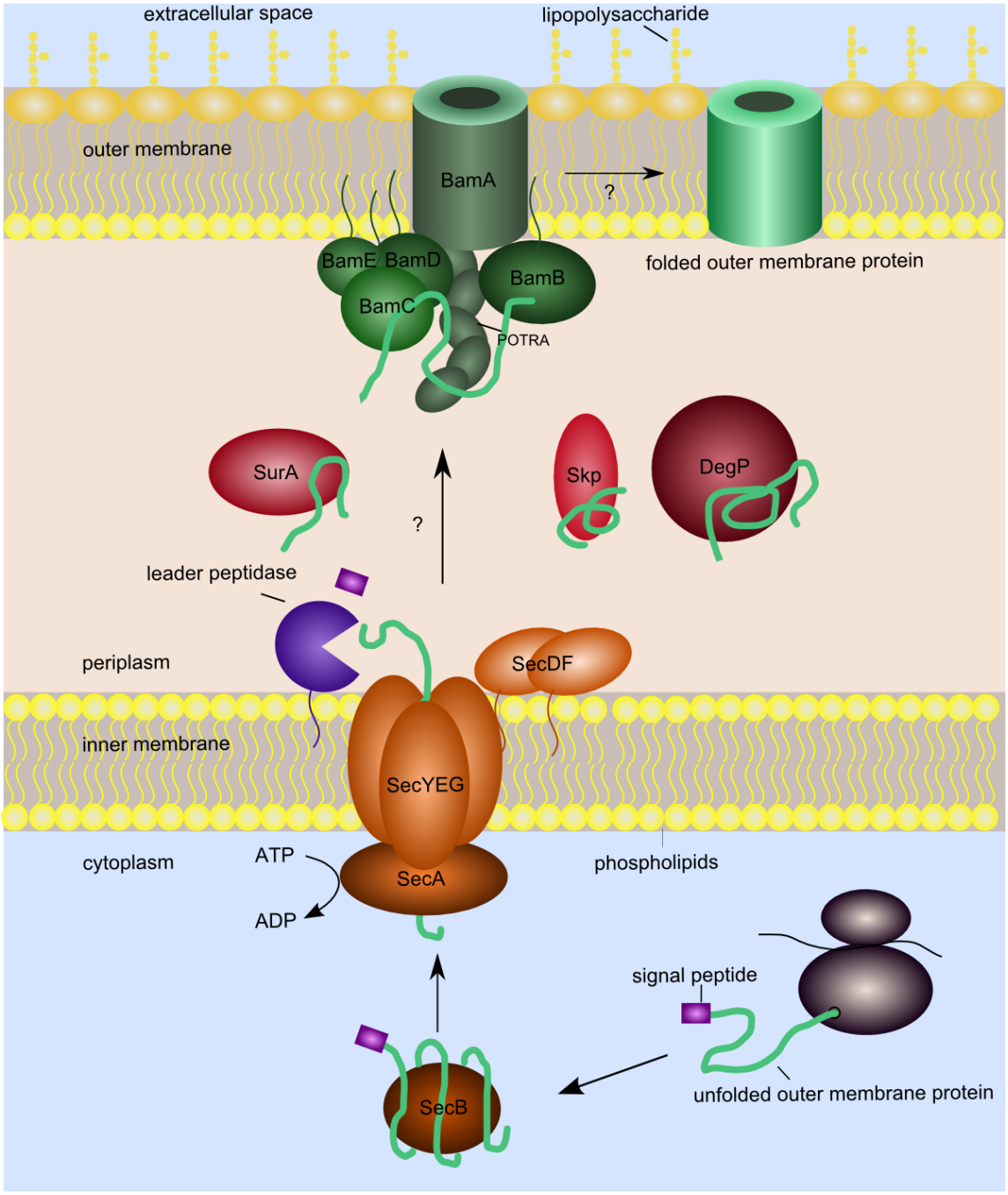


Figure 1.3: Outer membrane protein biogenesis. Omps are synthesised in the cytoplasm. A signal peptide sequence targets them via SecB to the SecYEG translocon where it is threaded through the inner membrane by ATP hydrolysis of SecA. The leader peptidase cleaves off the signal peptide and the unfolded Omps are sequestered by periplasmic chaperones to prevent premature folding and aggregation. Ultimately, unfolded Omps are given over to the Bam complex which promotes insertion in the outer membrane and folding therein. Figure adapted from Knowles et al. (2009); Ruiz et al. (2006).

The P1 domain has a proposed function as a recognition domain and the N-terminal domain as chaperone domain.

The CTD is structurally similar to the CTD of the trigger factor, a SurA homologue which also has chaperone function and contains an atypical PPIase domain (Martinez-Hackert and Hendrickson, 2009). For SurA, it was suggested that multiple SurA proteins bind to one unfolded substrate protein (Hagan et al., 2010).

SurA has been proposed to be the main chaperone responsible for Omp shuttling in the periplasm (Sklar et al., 2007b). A recent proteomics approach study however indicated that only 8 out of 23 β-barrel protein are negatively affected by SurA depletion. Among those are the major Omps OmpA and OmpF, whose decreased abundance might have been responsible for the dramatic effects on OM density (Sklar et al., 2007b; Vertommen et al., 2009).

However, the exact role and interactions of SurA in Omp biogenesis has not yet been defined.

Skp

Skp (**S**eventeen-**K**ilodalton-**P**rotein) is a periplasmic chaperone which was shown to bind to Omps with high selectivity (Chen and Henning, 1996). Its expression is regulated by the σ^E and/or Cpx envelope stress response pathways (Dartigalongue et al., 2001). Skp is implicated in Omp biogenesis and was found crosslinked to Omps in the the outer leaflet of the IM directly at the exit the Sec complex (Allen et al., 2009).

The crystal structure of Skp (figure 1.4b) revealed a trimeric protein whose overall structure reminds of a jelly-fish or a forceps. It is similar to Prefoldin, a cytosolic chaperone found in eukaria and archea (Korndörfer et al., 2004; Walton and Sousa, 2004). Skp trimerisation occurs in a rigid central domain predominantly composed of β-sheets, while the α-helical section forms tentacle-like protrusions, which are composed of two helices per monomer, interacting in 'knob into holes' and leucine-zipper like patterns. The tentacles are bent towards each other at the tips, creating a cavity where substrate binding occurs. At these tips, a high positive net-charge is observed, while the core domain is rather negatively charged, presumably to orient the protein tips toward negatively-charged lipid headgroups. There are furthermore positively charged residues on the outside of the tentacles, which are thought to be responsible for LPS binding (Korndörfer et al., 2004; Walton and Sousa, 2004).

At least 30 potential interaction partners of Skp were identified in a recent pull-down study, with a broad Omp substrate spectrum (Jarchow et al., 2008). This indicates a general role for Skp as an Omp chaperone. The binding mode of the Skp trimer with selected Omps was found to occur a 1:1 ratio, in a pH dependent manner. Interaction occurs via hydrophobic interactions of the β-barrel region, as well as via electrostatic interactions with Omp loop regions inside the Skp cavity (Qu et al., 2007).

Of all known Skp substrates, the best studied interaction is with OmpA. A recent NMR study showed the transmembrane domain of OmpA in a collapsed, unfolded form inside the Skp cavity, while the periplasmic domain protruded into the periplasm and folded independently (figure 1.4c). These results were further supported by cysteine-crosslinking experiments (Walton et al., 2009). Furthermore, binding sites of OmpA in Skp were mapped by a site-directed fluorescence study, showing that loops were as strongly bound as β-strands inside Skp. The addition of LPS to the Skp-OmpA complex strongly affected the binding of OmpA loops, indicating the function of LPS as a probable release factor competing with hydrophilic loops for binding sites (Qu et al., 2009).

A recent study showed that Skp mediates OmpA folding into lipid membranes with negatively charged headgroups, while positively charged headgroups prevent binding (Patel et al., 2009). This findings are in line with the suggestions that the positively charged tips of Skp interact with membranes to collect and release Omps.

Even though information on Skp and especially the Skp-OmpA interaction are already present, structures of complexes with bound Omps, the mediation of Omp release, the *in vivo* relevance of LPS binding and especially putative interaction of Skp with the Bam complex requires additional investigations.

From the collected information, Skp and SurA seem to act as chaperones, keeping Omps in an unfolded state to prevent aggregation until successful delivery to the Bam complex (Allen et al., 2009). Detailed insights into their workings and the interplay/redundancy of periplasmic chaperones remain to be discovered.

1.2.4 Chaperone interplay

Attempts have been made to investigate the individual roles of the main chaperones involved in Omp biogenesis, SurA, Skp and DegP.

Deletion studies showed a synthetic lethal phenotype for skp and surA, as well as

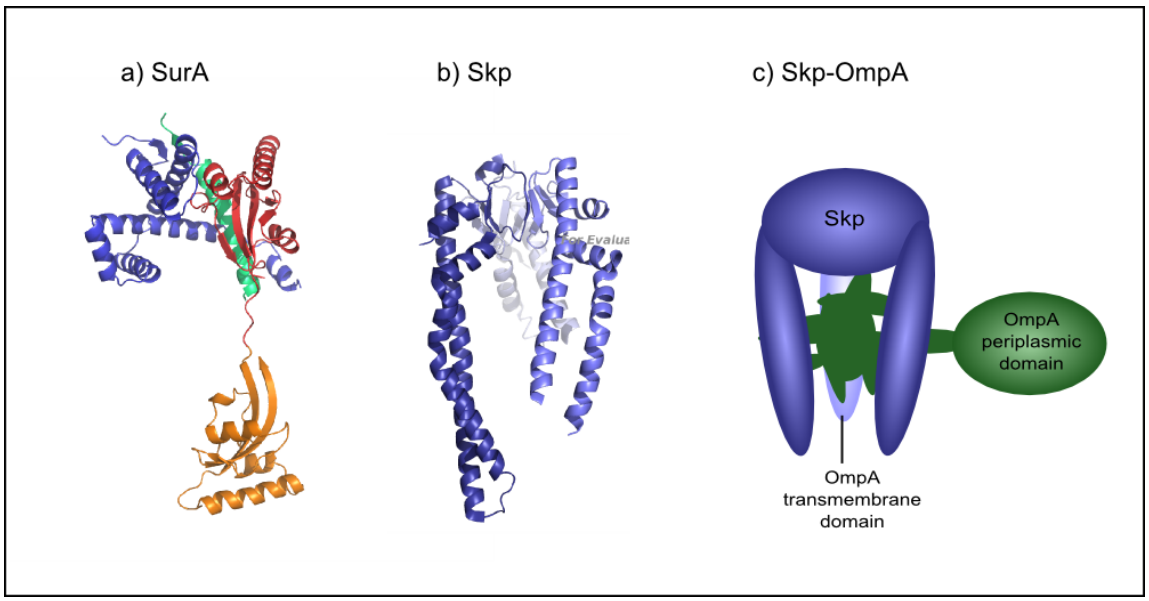


Figure 1.4: Structure of the chaperones Skp and SurA. a. Structure of the SurA monomer (PDB entry code: 1M5Y, Bitto and McKay (2002)). Domains are colored as follows: N-terminal domain: blue, PPIAse domain P1: red, PPIAse domain P2: orange, C-terminal domain: green. **b.** Structure of the Skp trimer (PDB entry code: 1U2M, Walton and Sousa (2004)). The individual monomers are colored in different shades of blue. **c.** Model of Skp binding to OmpA. The OmpA transmembrane domain is protected in an unfolded state inside the Skp trimer, while the OmpA periplasmic domain is folded and exposed to the environment. Reproduced and adapted from Walton et al. (2009). Ribbon structures were done in PyMol.

for degP and surA null mutations, whereas knock-out of the individual chaperones showed only minor effects, indicating a strong functional redundancy. Double knock-out of skp and degP showed no synthetic lethality, leading to the proposal of two chaperone pathways in Omp biogenesis: a mainly SurA dependent pathway and an Skp/DegP dependent pathway (see figure 1.5, right side model) (Rizzitello et al., 2001).

This model was further supported by depletion studies, which took into account the stress response and subsequent Omp down regulation caused by deleted chaperones (Sklar et al., 2007b). Depletion of SurA caused a notable decrease in OM density, producing effects reminding of a BamA (membrane insertion failure) deletion. This could not be observed for loss of DegP and Skp. This and the crosslinking of SurA to BamA led to the conclusion that SurA is responsible for shuttling the main bulk of Omps through the periplasm to the Bam complex. For Skp and DegP mutants no drastic effects on the OM were noticed, but the σE stress response was upregulated in their absence, so a minor role in Omp biogenesis was suggested. In

stress situations, like under depletion of SurA, Skp and DegP activity was highly amplified, leading to the speculation that they function as a rescue pathway for Omps that 'fall off' the SurA pathway (Sklar et al., 2007b).

This model was questioned by a recent proteomic study on SurA substrates, where only 8 out of 23 Omp substrates were shown to be mainly affected by a loss of SurA (Vertommen et al., 2009). In contrast, Skp is thought to non-specifically bind all unfolded Omps (see section 1.2.3), proposing a more general role in membrane protein shuttling.

A second model (figure 1.5, left model) suggests chaperone interplay in the periplasm as a two-step process. First, the chaperones Skp and SurA take up unfolded substrates. These substrates may then adopt a prefolded form and are subsequently transferred to DegP, which acts as a second-step quality control mechanism. DegP digests un- or misfolded proteins while protects prefolded ones. Subsequently DegP prefolds proteins in its cavity. As SurA also seems sufficient for mediating transfer of Omps to the Bam complex (Hagan et al., 2010), this second step might be optional or more favoured under heat stress conditions.

The models for chaperone interplay in the periplasm (summarised in figure 1.5) however are both not entirely congruent with all evidence available. Disentangling the roles of individual chaperones in Omp biogenesis proves a challenge. They seem to be part of a tight network of interaction between cell-envelope factors where disruption of one factor often causes highly pleiotropic phenotypes (Allen et al., 2009). Furthermore, additional factors may play a role in this process. Therefore the elucidation of the complex network of chaperone interplay in the periplasm is still ongoing.

1.3 Protein quality control and stress responses in the periplasm

Protein quality control (PQC) mechanisms ensure the correct folding state, and thereby the correct functionality of proteins. This is crucial as aberrantly folded, non-functional proteins must not accumulate in cells as they form potentially toxic aggregates. In mammals, malfunctioning PQC is implicated in a number of severe diseases linked to misfolding and aggregation of proteins, like Alzheimer's, Parkinson's and Huntington's (Macario and de Macario, 2005).

PQC is carried out by chaperones and proteases. Chaperones are responsible for

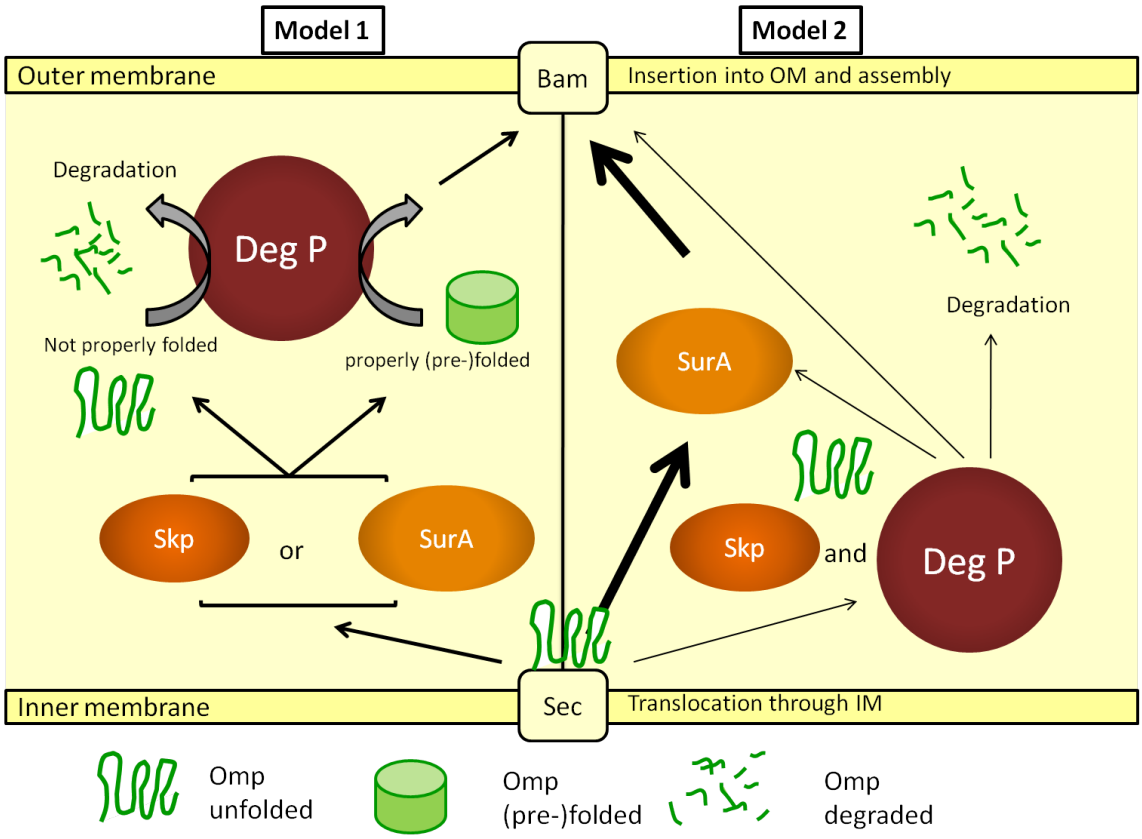


Figure 1.5: Two models for the roles of Skp, SurA and DegP in Omp biogenesis. In model 1 Skp (dark orange) and SurA (light orange) take up unfolded Omps (green) from the Sec translocon and transfer them in a second step to DegP (red) which acts as a quality control factor, degrading misfolded Omps, while protecting properly 'prefolded' Omps. In model 2, SurA transits the main bulk of Omps to the OM, while Skp and DegP act in a second pathway, protecting Omps that 'fall off' the SurA pathway (see text for details).

correct protein folding, whereas proteases remove damaged misfolded proteins (figure 1.6). Both usually recognise their un- or misfolded substrates by large exposed hydrophobic patches, which in natively folded proteins are buried (Wickner et al., 1999). In stress situations (e.g. heat-shock) the need for PQC is increased over normal level as misfolding occurs more frequently. Therefore PQC factors are often regulated by stress response pathways.

PQC in the cytoplasm

PQC factors in the cytoplasm are well studied and the best characterized examples are heat shock proteins, which convey tolerance against temperature, osmolarity and heavy metals. They consist of molecular chaperones (e.g. GroEL, GroES, DnaK and DnaJ, ClpB) and ATP-dependent proteases (e.g. ClpAP, ClpXP, ClpCP, HslUV

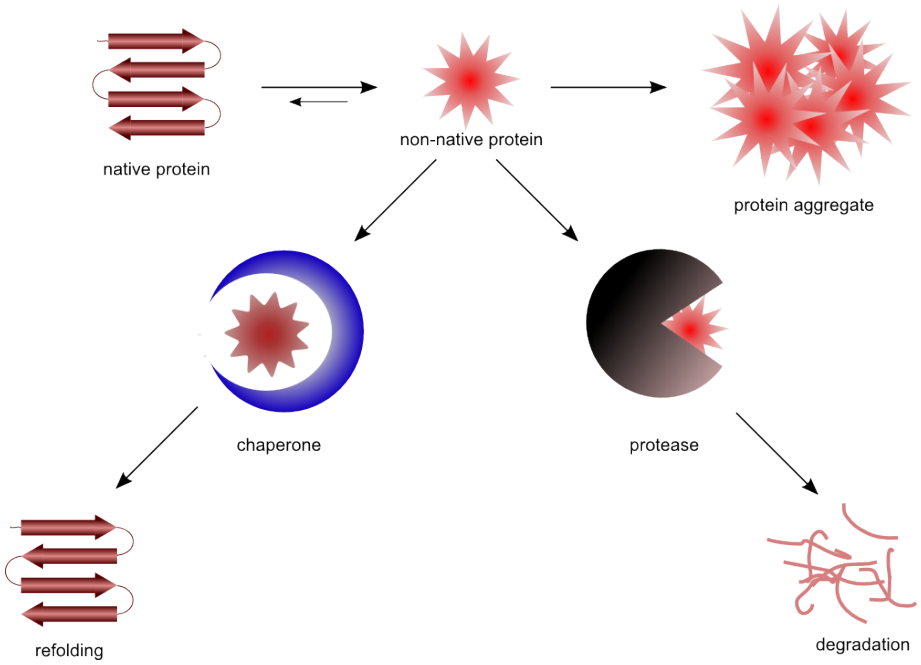


Figure 1.6: The principle of protein quality control. Non-native proteins, e.g. un- or misfolded proteins, are either taken up by chaperones to promote refolding into the native structure or degraded by proteases. Both pathways aim to prevent potentially toxic aggregation.

(ClpYQ), Lon and FtsH) which are important factors under normal conditions and under stress. For example, the essential chaperonin GroEL/GroES has been found to associate to 10-15% of newly translated proteins under normal conditions, under heat stress this increases to 30% (Wickner et al., 1999).

Many of the above mentioned chaperones and proteases share a multi-subunit structure, where stacked oligomeric ring structures enclose a cavity, in which the chaperone/protease activity is located (e.g. GroEL, ClpP). They depend on ATP activity for conformational change and/or substrate unfolding into the chamber and on smaller cofactors (e.g. GroES, ClpA) (Duguay and Silhavy, 2004; Wickner et al., 1999).

PQC and stress response in the periplasm

Periplasmic PQC differs remarkably from cytoplasmic PQC as the periplasm is 1) devoid of ATP and 2) due to the porous OM much more exposed to extracellular changes in conditions. Principles of cytoplasmic PQC factors are therefore not entirely applicable, making periplasmic PQC factors unique. They need to immediately sense and react to changes in the environment, protecting the cell envelope

without relying on ATP as energy source (Raivio and Silhavy, 2001).

In the *E.coli* cell envelope, the two stress response pathways are the σ^E and/or Cpx envelope signaling cascades, two distinct pathways with functional overlaps (figure 1.7) (Duguay and Silhavy, 2004).

While in *E.coli* the σ^E pathway is essential, the CpxAR pathway is not. Both pathyways regulate the expression of DegP, and it was recently shown that both responses are necessary to alleviate envelope stress caused by aberrant Omp assembly. Here, the Cpx regulon contributes principally by controlling degP expression (Gerken et al., 2010).

The Cpx stress response pathway consists of a two-component regulatory system, with CpxA as the sensor kinase bound to the IM and CpxR as the cytoplasmic response regulator. Upon periplasmic stress CpxA auto-phosphorylates and subsequently phosphorylates CpxR which then regulates gene expression influencing envelope protein folding (Gerken et al., 2010). The periplasmic protein CpxP regulates the CpxA activity most likely by keeping it in an inactive state (figure 1.7, left side) (Duguay and Silhavy, 2004).

The σ^E pathway consists of the σ^E factor inactively bound to the transmembrane anti-sigma factor RseA. Upon stress, the periplasmic IM-bound protease DegS cleaves RseA on its periplasmic side. Subsequently, RseP cleaves RsaA on its cytoplasmic side, freeing the RseA bound σ^E factor. The remains of RseA bound to σ^E are degraded by ClpXP, freeing the σ^E factor to activate gene transcription, e.g. to increase folding factors and decrease Omp synthesis (figure 1.7, right side) (Duguay and Silhavy, 2004; Gerken et al., 2010).

Taken together, both stress response systems ensure proper function of the bacterial envelope under normal and under stress conditions.

1.4 HtrA proteases

DegP belongs to the family of HtrA (Htr: **H**igh **t**emperature **r**equirement) proteases, and was discovered when degP mutant cells did not grow at elevated temperatures, classifying it as a heat-shock protein. Prokaryotic and eukaryotic HtrA family members are implicated in unfolded protein responses and related pathogenities and diseases.

HtrA proteases share a common fold architecture with a catalytic protease domain

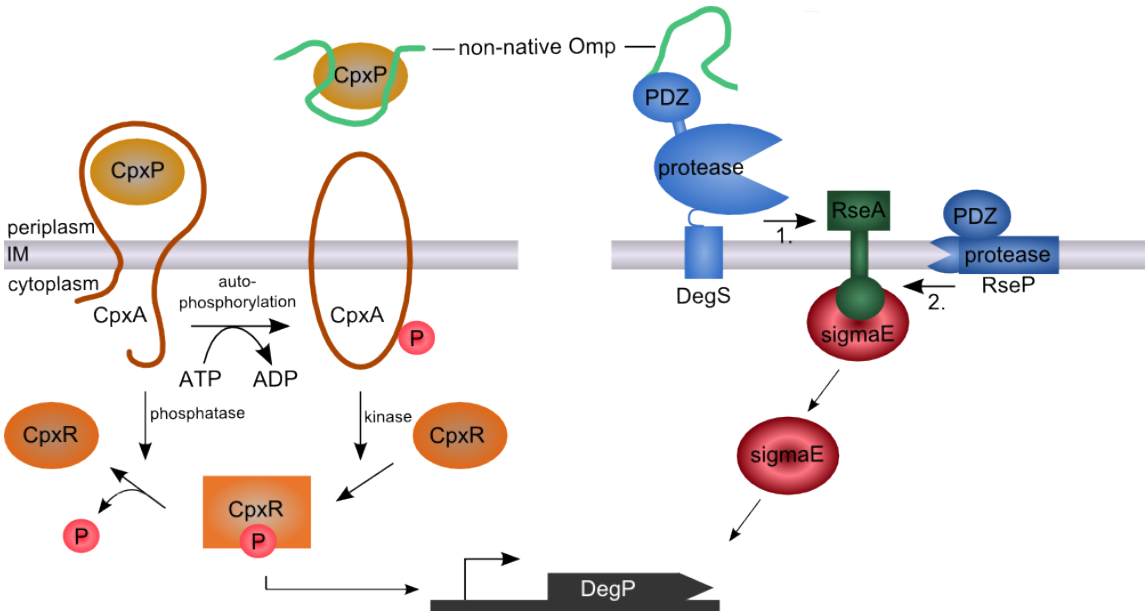


Figure 1.7: The Cpx and σ^E extracytoplasmic stress response signaling pathways. Unfolded proteins (non-native Omps, in light green) trigger two signaling cascades which lead to the regulation of envelope factors (see text for details), for example DegP. Figure adapted from Hasselblatt et al. (2007); Raivio and Silhavy (2001)).

and at least one C-terminal PDZ domain (see figure 1.8). Some family members possess an additional insulin growth factor-binding domain (IFGBP), and most of them contain a signal peptide targeting them to extracytoplasmic compartments or transmembrane segments. HtrA family proteases are oligomeric serine proteases, belonging to the trypsin-subgroup. The protease domain adopts a chymotrypsin-like fold and the catalytic triad is arranged as His-Asp-Ser. For substrate recognition and binding, one or more PDZ domains are responsible. PDZ domains are protein-protein interaction units that preferentially bind to 3-4 C-terminal residues of the substrate protein (Clausen et al., 2002; Krojer et al., 2008a; Meltzer et al., 2009). Crystal structures revealed that the basic oligomeric building block of all HtrAs seems to be a trimer stabilized by the protease domains. These form the center of the structure while the PDZ domains protrude outwards. Substrate binding to the PDZ domains causes reversible allosteric changes regulating the activity of HtrA enzymes (Meltzer et al., 2009; Wilken et al., 2004).

Two important HtrA family members in *E.coli* are the protein quality control factor DegP and the stress sensor DegS (figure 1.8).

For DegS, the activation mechanism has been elucidated in detail. Substrate bind-

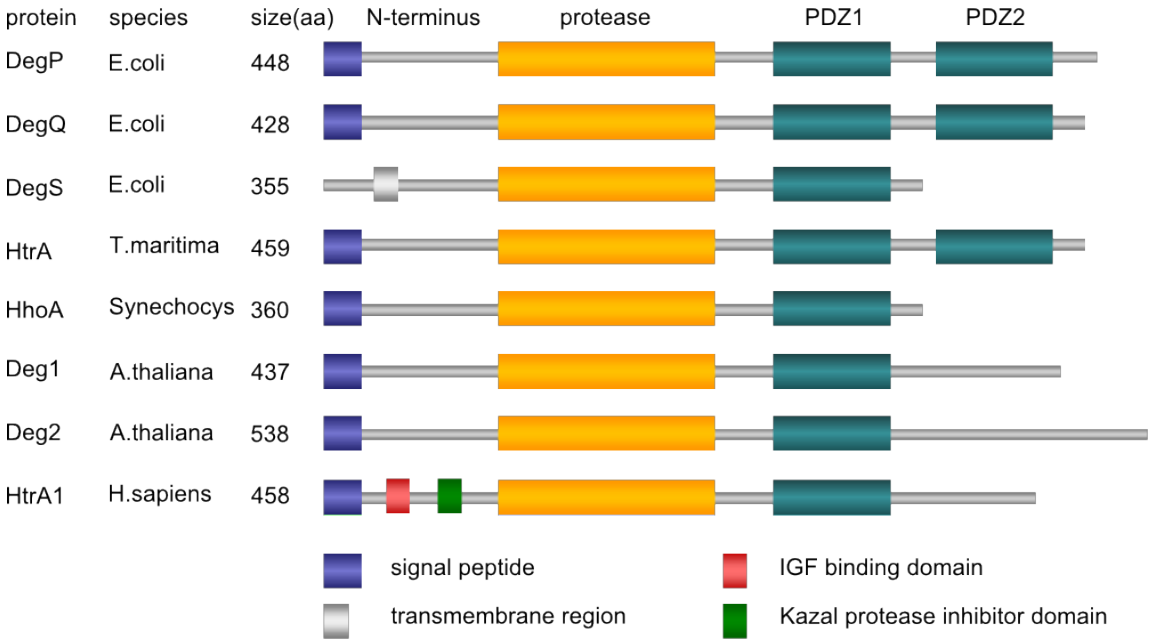


Figure 1.8: HtrA family members. Schematic representation of the domain organization of selected HtrA family members. The protease domain is colored in yellow, the PDZ1 and PDZ2 domains in dark turquoise, other colors are indicated in the figure. The sizes in amino acids (aa) refer to mature proteins without the N-terminal signal peptide. Figure adapted from Clausen et al. (2002).

ing to the PDZ domain by β-augmentation induces a series of precise conformational changes, arranging the active site of DegS in a proteolytically active conformation that allows it to carry out its function in the σ^E stress response. The reversibility of this activation allows flexible and immediate responses to folding stresses (Hasselblatt et al., 2007; Meltzer et al., 2009).

The activation and function of DegP will be discussed in detail in the next section.

1.4.1 DegP

DegP, as a *E.coli* periplasmic member of the HtrA protein family, is involved in PQC in the periplasm. It promotes survival of the cell at elevated temperatures using two separate mechanisms. Like all HtrA proteins it has a protease function, degrading un- or misfolded proteins before they accumulate and endanger the cell by forming toxic aggregates. Additionally, DegP can function as a chaperone, rescuing only slightly misfolded proteins (Clausen et al., 2002). At low temperature (28°C) the chaperone function has been shown to be prevalent over the protease function. This is reversed with increasing temperature (Spiess et al., 1999), which most likely

can be attributed to increased protease activity (as opposed to decreased chaperone function) (Skorko-Glonek et al., 2007). Under heat-shock (above 37 °C) or other stress conditions such as SurA deletion or Omp overexpression, DegP activity becomes critical for cell survival (Mogensen and Otzen, 2005; Spiess et al., 1999). DegP expression is regulated by the Cpx and σ^E stress responses.

As DegP has a general proteolytic endopeptidase activity and constantly undergoes self-cleavage, a proteolytically inactive DegPS210A mutant was used for structure elucidation studies.

DegP consists of an N-terminal trypsin-like serine protease domain, and two C-terminal PDZ domains, termed PDZ1 and PDZ2. Like all periplasmic proteins, it is synthesised as a pre-protein with an N-terminal SP which is cleaved off during Sec translocation. The basic building block of the DegP unit is a trimer, with the protease domains forming the contact via hydrophobic interactions. The flexibly linked PDZ domains point to the sides (Clausen et al., 2002; Krojer et al., 2002). The DegP protease domain is composed of two β-barrel lobes arranged perpendicular to each other, and a C-terminal helix. The catalytic residues His-Asp-Ser and the oxyanion hole are located between the lobes. The critical loops L1, L2 and LD are arranged around the active site (Krojer et al., 2008b).

Under physiological conditions, these trimers associate to form higher oligomeric complexes (Krojer et al., 2008b).

DegP protease activation

The inactive resting state of DegP is a 6-mer, composed of two trimers stacked upon each other (figure 1.9a). Two conformational states were observed and termed 'open' and 'closed'. In the open state, interactions between the trimers are solely mediated by the loops LA from opposing trimers, protruding into and distorting the active site of the protease. In the closed state, interaction is additionally mediated by PDZ domains (figure 1.9a) (Krojer et al., 2002, 2008b).

Upon unfolded substrate binding, the inactive DegP 6-mers shift to protease active DegP 12- or 24-meric cage-like structures (but also 15/18-mers have been observed (Shen et al., 2009)). These cages are composed of trimeric DegP units with interactions mediated by PDZ domains (figure 1.9b and c). The crystal structure and electron microscopic studies of DegP 24-mer revealed a cage with a diameter of 195 Å, creating a cavity of around 110 Å in diameter. The DegP 12-mer structure,

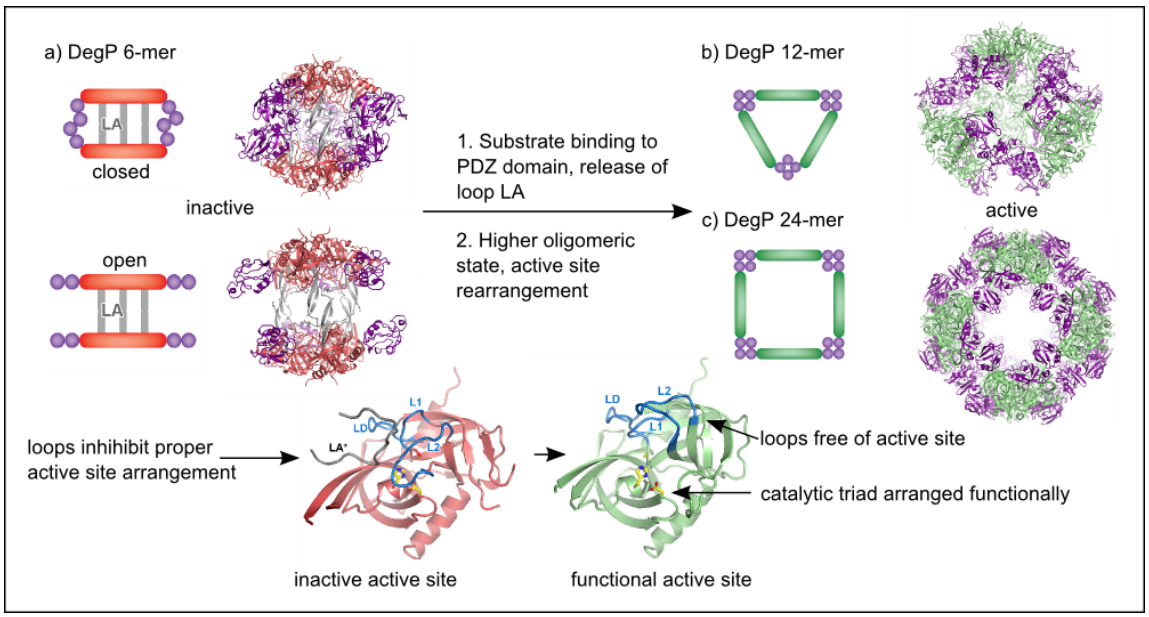


Figure 1.9: Structure of DegP oligomers. a. Schematic view and structure of the inactive DegP 6-mer in its open or closed state. Red color indicates the inactive proteolytic domain, violet the PDZ domains. The loops LA (in grey) connect the trimers by forming inter-subunit β-sheets. Below, a detailed view of the inactive active site (in red) is depicted. The loop LA (* means from the opposing unit) interacts with the loops L1, L2 and LD (in blue), blocking and distorting the catalytic triad (yellow). Unfolded substrates can enter the DegP 6-mer in the open form. **b and c.** DegP 12-mer and DegP 24-mer, respectively, in schematic and structural view. Green color indicates the active protease domain, violet the PDZ domains. Structures are not drawn to scale (Sizewise, the DegP 12-mer fits into the cavity of DegP 24-mer (Krojer et al., 2008b)). In the active DegP, high oligomeric cage-like structures are formed. Increased temperature greatly weakens the loop LA interactions, and binding of unfolded substrate to the PDZ domains allows rearrangement of DegP subunits so that loop L3 (not shown, see text) can bind to the PDZ domain and active site is remodeled into a functional state. Below, the active site in its functional arrangement is shown, where the loops L1, L2 and LD (in blue) are withdrawn from the catalytic triad (in yellow). PDB entry code for DegP 6-, 12-, and 24-mer structures are 1KY9, 2ZLE, and 3CS0, respectively (Krojer et al., 2002, 2008b). Figure is rearranged and adapted from Sawa et al. (2010).

solved by cryo-electron microscopy, shows a tetrahedral cage 160 Å in diameter, with a cavity of 78 Å (Jiang et al., 2008; Krojer et al., 2008b).

Activation of the inactive DegP 6-mer occurs by unfolded substrate binding to the PDZ1 domain via β-augmentation. C-terminal substrate residues bind to the PDZ domains in a shallow hydrophobic binding pocket favoring small hydrophobic residues. That initiates the rearrangement of the PDZ1 and PDZ2 domains. The change in PDZ1 is sensed by the protease domain loop L3 which associates with PDZ1 and allows remodeling of the active site to its functional position. PDZ1 and loop L3 rearrange into a precise conformation for active site activation, which is also found in the case of the DegS HtrA protease, indicating a common activation mech-

anism. Of further importance is loop LA, which is forming the pillars connecting the two trimeric units in the DegP 6-mer assembly. Loop LA binding to the active site of the opposing DegP unit is highly temperature-dependent, and is released with increasing temperature, promoting dissociation of the trimers and freeing the active site residues for further rearrangement by loop L3 and protease-active cage formation (overview in figure 1.9). This substrate and temperature dependent activation mechanism elegantly describes DegP regulation and behaviour, assuring immediate and reversible changes in the activation of DegP in response to environmental stresses (Krojer et al., 2002, 2010).

Degradation of substrates is done cooperatively between the PDZ1 and the catalytic domain in a processive fashion. PDZ1 recognises hydrophobic C-termini of unfolded substrate proteins. It anchors them, while they engage in a similar binding by β-augmentation and hydrophobic interactions near the active site for cleavage. DegP preferably cleaves after small hydrophobic residues (valine, alanine, threonine and isoleucine), which in turn can bind to the PDZ1 domain for the next cleavage step. By this interplay of protease and PDZ domain and the similar binding modes of the two sites, DegP ensures a processive digestion of substrate proteins. Binding to PDZ and protease domains reminds of a molecular ruler which assures a length of the digested peptides of 13-15 residues (mean value). The PDZ domain itself is not strictly necessary for cleavage, but greatly enhances efficiency thereof. The peptides cleaved off the substrate proteins in turn function as an allosteric activator of the DegP protease by binding to PDZ1 domains and ensuring correct arrangement of active site loops (Krojer et al., 2008a, 2010).

These activation mechanisms give an excellent example of PQC in the *E.coli* periplasm, where an enzyme of dual functionality is regulated on a molecular level by large-scale rearrangements concerning the oligomeric form, as well as on small-scale level in the active site. In an environment without the energy source ATP and without any cofactors, DegP can sense and respond to stress environmental stimuli in an immediate and reversible way.

The protease function seems to be partly differently regulated than the chaperone activity, as mutation of crucial amino acids for protease function do not disturb chaperone function (Krojer et al., 2010).

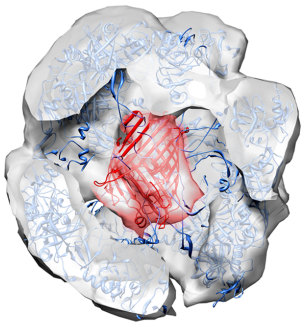


Figure 1.10: Cryo-EM DegP 12-mer structure with an Omp β-barrel modelled. EM densities are in gray, DegP structure is in blue, Omp β-barrel model is in red. Figure adapted from Sawa et al. (2010).

DegP chaperone in Omp biogenesis

The functional switch between chaperone and protease function depends on the substrate folding state (Krojer et al., 2008a). Unfolded substrates are immediately degraded by DegP, while in DegP 12/24-mers isolated from the *E.coli* periplasm folded Omps were bound. Monomeric OmpA and OmpC were found bound to DegP, however no trimeric OmpC, even though size-wise the cavity of DegP 24-mer could accommodate them. These folded Omps were stable to degradation from DegP suggesting that DegP acts as a genuine chaperone for Omps (Krojer et al., 2008b).

The involvement of DegP in Omp biogenesis was indicated by degP deletion, which severely affected the Omp content in the OM. Furthermore, DegP was shown to cosediment with and bind to liposomes with the positively charged regions provided by the PDZ domains (mutations of which abolished lipid binding). This led to the idea that DegP might deliver folded Omps to the membrane via large pores in the DegP 24-mer assembly (35 Å). Binding of DegP to membranes was also shown by a recent study, which instead of the cage-like structures in solution, observed bowl-shaped forms of DegP attached to lipid membranes, which showed increased proteolytic, but decreased chaperone function (Shen et al., 2009).

Binding of folded Omps inside DegP was furthermore supported by the cryo-EM structure of the DegP 12-mer, where a cylindrical density reminding of an Omp β-barrel could be modeled in (figure 1.10) (Krojer et al., 2008b). Due to the heterogeneity of substrates inside the DegP 12-mer the details on the Omp structure and on Omp orientation could not be determined. In the DegP 24-mer crystal structure, bound substrates were not detected but biochemically OmpA, OmpC, OmpF and LamB were detected in the crystals (Krojer et al., 2008b). Summarizing, folded

Omps seem to be present inside the DegP 12/24-mer cavity, but could not be visualised so far.

Reports concerning DegPs function as a chaperone in Omp biogenesis are controversial. On the one hand, it was shown that membrane-insertion deficient OmpC and OmpF mutants are lethal in DegP-deficient cells. However, upon expression of proteolytically inactive DegPS210A this phenotype could be partly rescued (CastilloKeller and Misra, 2003; Misra et al., 2000). In these cells, the Omps were found associated near the IM and the OM in an non-native folded state, indicating that chaperone activity of DegP might protect the cell from misfolded proteins rather than inserting them into the OM. On the other hand, DegP 12/24-mer particles have been shown to bind folded Omps inside their cavity and protect them from degradation. In the cryo-EM structure of the DegP 12-mer a additional density suggesting a folded Omp barrel was observed (Krojer et al., 2008b). This is in line with genetic studies in which the degp/sura double knock-out can be rescued by DegPS210A (Rizzitello et al., 2001), and the finding that knock out of DegP significantly reduces Omp levels in the OM indepently of the σ^E response (Krojer et al., 2008b). Furthermore, it was shown that DegP associates with lipid membranes via their PDZ domains (Krojer et al., 2008b; Shen et al., 2009). However, no apparent mechanism for Omp release towards membrane insertion or the Bam complex has been found yet.

Summarizing, the protease function of DegP is mechanistically well understood. However, concerning a potential DegP chaperone function many questions remain open. It is well understood how unfolded substrates are recognized for degradation, but how does DegP bind to folded substrates? Can DegP provide a folding chamber, where un- or partly folded substrates could complete their folding? Are folded substrates bound in an oriented manner or stochastically? How can folded substrates be released?

1.5 Aim of the study

The aim of this study is the *in vitro* reconstitution of complexes between the central protein quality control factor DegP and its outer membrane protein (Omp) substrates. DegP comprises both protease and chaperone function, and is implicated as one of the three main chaperones involved in Omp biogenesis in the *E.coli* periplasm. As DegP protease activity is already well understood, this study focuses on the DegP chaperone function and its role in Omp biogenesis.

To achieve homogeneous DegP-Omp complexes *in vitro*, different approaches were chosen. The first approach is to test complex formation of DegP with Urea-denatured Omps (OmpA, OmpC and OmpF) to reveal if complex formation occurrs and if unfolded Omps can be folded by DegP. This approach combines the scope of complex reconstitution with the question if the DegP 12/24-mer cavity might provide an environment favouring Omp folding. A further approach is to trap Omps in DegP 12/24-mer particles which are previously formed by incubation with Omp signal peptides.

Furthermore, we will test a potential role of DegP as a second-step quality control mechanism in Omp biogenesis. To gain insight into the chaperon interplay in the periplasm and DegPs role in Omp biogenesis we test *in vitro* the course of Omp transfer from SurA/Skp to DegP. First, complexes of Skp and SurA with Omps need to be generated and subsequent transfer of 'prefolded' Omps to DegP will be tested and folding state of Omps inside the DegP cavity monitored.

Moreover, characterising possible interactions of DegP with folded Omps, *in vitro* Omp refolding will be employed to investigate DegP-Omp interaction and to generate DegP complexed with a homogeneous *in vitro* refolded Omp species.

In another approach, complex formation between DegP and Omps directly extracted from *E.coli* outer membranes will be assayed and analysed for capacity of these Omps to interact with DegP *in vitro*.

The planned experiments should provide a solid base for *in vitro* reconstitution of DegP-Omp complexes, and to gain insights into potential folding activity of DegP and DegPs general role in Omp biogenesis.

2 Materials and Methods

2.1 General Buffers, Solutions, Media, Antibiotics

All general buffer, solutions, media and antibiotics are listed in table 2.1.

2.2 Enzymes and Reagents

Chemicals and antibiotics of highest grade were ordered from Merck, Sigma or Fluka unless otherwise noted. From New England Biolabs (NEB), Fermentas and Roche enzymes used in cloning protocols were used. For DNA preparation, Qiagen products were used. For purification, columns and other FPLC materials were purchased from GE Healthcare.

2.3 Constructs and Oligonucleotides

All constructs used were cloned into the pET21b vector (Novagen), a high copy plasmid, containing the T7lac promotor, a C-terminal His-tag, and resistance against ampicillin; *E.coli* BL21(DE3) cells used contain a chromosomal copy of the T7 RNA polymerase gene, which was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG).

All constructs were cloned into the vector using NdeI (CATATG, on the forward primer) and EcoRI (GAATTC, on the reverse primer) restriction sites. An exception are the OmpC constructs which use NdeI (on the forward primer) and HindIII (AAGCTT, on the reverse primer) restriction sites. The Strep tag was introduced on the primer, with a DNA sequence of TGGAGCCACCCGCAGTTCGAAAAG, translating into an amino acid sequence of TrpSerHisProGlnPheGluLys (W S H P Q F E L), followed by a stop codon to avoid the tag provided by the vector. His-tags, even though present on the vector, were also introduced on and only used from the primer (sequence CATCACCACCATCATCAC, amino acid sequence HisHisHisHisHisHis, His6). All tags are C-terminal (tag sequences were therefore used as their reverse complements.).

As all cloned and purified proteins are localised either in the periplasm or in the OM, they possess an N-terminal signal peptide (SP) in their propetide form. In constructs termed 'full-length' the SP was part of the sequence, in constructs

Name	Composition
Luria Bertani (LB) Medium	10 g tryptone, 5 g yeast extract, 10 g NaCl, adjusted to pH 7.0 with NaOH; filled up with H_20 to 1000 ml and autoclaved
LB Agar	15 g agar dissolved in 1000 ml LB medium
2x SDS sample buffer	100 mM Tris/HCl pH 6.8, 4% SDS, 20 % glycerol, 200 mM β-mercaptoethanol, 0.02 % bromophenolblue
1x SDS-PAGE running buffer	25 mM Tris/HCl pH 8.3, 200 mM glycine, 0.1% SDS
Staining Solution	250 ml ethanol, 80 ml acetic acid, 2.5 g Coomassie brilliant blue, filled to 1000 ml with H_20
Destaining Solution	250 ml ethanol, 50 ml acetic acid, filled to 1000 ml with H_20
10x TAE buffer	0.4 M Tris/HCl pH 8.0, 10 mM EDTA-Na_2-salt, 0.2 M acetic acid
Western blot (WB) transfer buffer	50 mM Tris, 280 mM glycine, 0.1% SDS, 20% methanol
WB TBS	20 mM Tris pH 8, 150 mM NaCl
WB TBS-T	20 mM Tris pH 8, 150 mM NaCl, 0.1% Tween-20
WB blocking buffer	3% BSA in TBS-T
Ampicillin	50 mg/ml in H_20 stock solution, stored at -20°C
Lysozyme stock	50 mg/ml in H_20 stock solution, stored at -20°C
DNAse stock	1 mg/ml in H_20 stock solution, stored at -20°C
PMSF	0.1 M stock solution, in DMSO stock solution, stored at -20°C

Table 2.1: Buffers, Media, Solutions

Primer Name	Primer Sequence
Skp_For	GCGCATATGAAAAAGTGGTTATTAGCTGCAGG
Skp_for_noSP	GCGCATATGGCTGACAAAATTGCAATCGTC
Skp_STREP_Rev	GCGGAATTCTTACTTTTCGAACTGCGGGTGGCTC CATTTAACCTGTTTCAGTACGTCGG
Skp_His_Rev	GCGGAATTCTTAGTGATGATGGTGGTGATGTTTA ACCTGTTTCAGTACGTCGG
SurA_STREP_Rev	GCGGAATTCTTACTTTTCGAACTGCGGGTGGCTC CAGTTGCTCAGGATTTTAACGTAG
SurA_His_Rev	GCGGAATTCTTAGTGATGATGGTGGTGATGGTTG CTCAGGATTTTAACGTAG
SurA_For	GCGCATATGAAGAACTGGAAAACGCTGCTTC
OmpA_full_noSS_For	GCGCATATGGCTCCGAAAGATAACACCTGG
OmpA_perip_STREP_Rev	GCGGAATTCTTACTTTTCGAACTGCGGGTGGCTC CAAGCCTGCGGCTGAGTTACAACG
OmpA_perip_His_Rev	GCGGAATTCTTAGTGATGATGGTGGTGATGAGCC TGCGGCTGAGTTACAACG
OmpA_TD_noTAG_Rev	GCGGAATTCTTAAACCGAAACGGTAGGAAACACCC
OmpC_full_noTAG_Rev	GCGAAGCTTTTAGAACTGGTAAACCAGACCC
OmpC_noSS_For	GCGCATATGGCTGAAGTTTACAACAAAGACG
OmpF_noSS_For	GCGCATATGGCAGAAATCTATAACAAAGATG
OmpF_full_noTAG_Rev	GCGGAATTCTTAGAACTGGTAAACGATACC

Table 2.2: Primer oligonucleotides were synthesised by Life Technologies

Constructs	Primer forward	Primer reverse
Skp-His full-length	Skp_For	Skp_His_Rev
Skp-Strep full-length	Skp_For	Skp_STREP_Rev
Skp-His no SP	Skp_for_noSP	Skp_His_Rev
Skp-Strep no SP	Skp_for_noSP	Skp_STREP_Rev
SurA-His full-length	SurA_For	SurA_His_Rev
SurA-Strep full-length	SurA_For	SurA_STREP_Rev
OmpA no SP	OmpA_full_noSS_For	OmpC_full_noTAG_Rev
OmpA-His no SP	OmpA_full_noSS_For	OmpA_perip_His_Rev
OmpC no SP	OmpC_noSS_For	OmpC_full_noTAG_Rev
OmpF no SP	OmpF_noSS_For	OmpF_full_noTAG_Rev

Table 2.3: Constructs

Host strain	Genotype
DH5α chemically competent cells	F-, j 80D lacZDM15 D(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk^-mk^+) phoA supE44 thi-l gyrA96 relA1
One shot BL21(DE3) chemically competent cells (Invitrogen)	F-, ompT $hsdS_B(r_B{}^-m_B{}^+)$ gal dcm (DE3)
One shot Mach1-T1 chemically competent cells (Invitrogen)	ΔrecA1398 endA1 tonA P80DlacM15DlacX74 $hsdR(r_K{}^-m_K{}^+)$

Table 2.4: *E.coli* competent cells

termed 'no SP' the SP was removed from the construct sequence. In table 2.2 primer sequences are depicted. Table 2.3 lists all constructs used with the respective primers.

2.4 Molecular Cloning

2.4.1 Bacterial transformation

Plasmids were transformed into chemically competent cells (table 2.4) by heat-shock transformation. For cloning and selection procedures at the beginning DH5α cells, and later the more efficient Mach1-T1 cells were used. For overexpression purposes, plasmids were transformed into BL21(DE3) cells. 100 µl cells were transformed with either 1 µl purified plasmid, or after ligation with around 20 µl of ligation mixture and incubated 20 min on ice. Heat-shock was done at 42°C for 90 seconds in a thermoblock unit, or for 45 seconds in a water bath. After 2 minutes on ice, 1 ml LB-medium was added and incubation at 37°C, at around 300 rpm followed for 1 hour. Depending on the purpose, cells were then either added in selective LB-media suspension cultures, or plated out on selective LB-media overnight at 37°C. For Mach1-T1 cells this protocol was slightly adapted to manufacturers instructions (Invitrogen).

2.4.2 Plasmid purification

For purification plasmids were transformed into DH5α cells (table 2.4). 100 µl of transformation were added to 4 ml of LB-Amp media and grown over night at 37°C.

Template (DH5α in H20)	1 µl
dNTPs 2.5 mM	1 µl
Phusion buffer HF 5x	10 µl
forward primer	0.5 µl
reverse primer	0.5 µl
Phusion Polymerase	0.5 µl
H_20	36.5 µl

Table 2.5: PCR reaction mixture

Step	Temperature [°C]	Time [min]
1. Denaturation (initial)	95°C	10
2. Denaturation	95°C	1
3. Annealing	60°C	0.5
4. Extension	72°C	2
5. Finished	8°C	forever

Table 2.6: PCR reaction conditions, Steps 2-4 were repeated 32 times.

Cells were harvested and plasmids purified with the Qiagen MiniPrep Kit, according to the provided protocol. With the NanoDrop (ND-1000) spectrophotometer, the concentration of DNA was determined at 260 nm, against an elution buffer blank. The samples were sent to the in-house sequencing facility for sequencing, and DNA sequence of the plasmid/desired insert was determined. Plasmids were stored at -20°C.

2.4.3 Polymerase chain reaction (PCR)

Above mentioned constructs (table 2.3) were cloned from *E.coli* DH5α genomic DNA as a template and amplified by PCR. For PCR, ingredients depicted in table 2.5 were used for an amplification protocol shown in table 2.6.

2.4.4 Restriction digestion

Restriction digestion was done mainly using enzymes and buffers from Fermentas, but also from NEB. For a 50 µl reaction mixture usually 1 µl enzyme was used with the appropriate buffer conditions. Both restriction enzymes were added at the same time, the ratio between them used according to manufacturer instruction. Reaction was usually performed overnight at 37°C.

2.4.5 Dephosphorylation and ligation

To avoid self-ligation of the vector and therby increase ligation efficiency, the cut vector was subjected to dephosphorylation of the 5'-phosphates of the vector by CIAP (Calf Intestinal Alkaline Phosphatase) in an approximate amount of 15U for 30 minutes at 37°C in the according buffer. Before ligation digested constructs and dephosphorylated vector were purified via gel extraction using a Gel Extraction Kit (Qiagen) according to manufacturer instruction.

2.4.6 Agarose gel electrophoresis

Agarose gel electrophoresis is generally performed to separate double-stranded DNA. Molecules are separated according their length in basepairs, but also according to structural factors, like closed, linear or coiled plasmids. DNA size, purity and approximated yield can be judged. After PCR and restriction enzyme digestion/vector dephosphorylation DNA was purified from the agarose gel. Gels were made of 1% (w/v) agarose in 1xTAE buffer with added ethidium bromide for visualition of DNA at 302nm UV-light and electrophoresis performed at 80V. Prior to loading sample was mixed with DNA loading dye (Fermentas) in appropriate amounts.

2.5 Protein expression in E.coli

Construct containing plasmids transformed into BL21(DE3) or glycerol stocks of according constructs in BL21(DE3) were inoculated into 50-100 ml LB-medium supplied with Ampicillin (LB-Amp) in a final concentration of 50 µg/ml and grown shaking with 220 rpm overnight at 37°C. (DegPSA and DegPwt plasmids pCS21 [ref] were transformed into CLC198 degp null strain (Krojer et al., 2008b).) 10 ml overnight culture were diluted into 1l LB-Amp, and incubated like before until an OD_{600} of around 0.8 (against an LB blank) was achieved. Expression was induced with 0.5mM IPTG. After around 4 hours cells were harvested by centrifugation (40 minutes, 3500 rpm in Sorvall RC 3B Plus). Cells pellets of 1 l culture were resuspended in 10 ml in the respective buffer A and stored at -20°C. Glycerol stocks were done by mixing 500 µl of overnight culture with 500 µl 87% sterile glycerol and vortexing. Glycerol stocks are stored at -80 °C.

2.6 Protein purification

Cells harvested as described above were thawed on ice in the presence of DNAse 1:100, Lysozyme 1:50, PMSF protease inhibitor 1:1000 (stock solutions see table 2.1) and 1 tablet complete protease inhibitor cocktail(Roche) / 50 ml cell suspension and subsequently incubated 30 minutes on ice. Cells were broken by sonication (Branson Sonifier 250) with a macrotip applying 50% pulse and 50% output for 4x1 minutes under constant cooling (for inclusion bodies sonication was done longer and not as gentle as for soluble proteins). To separate soluble proteins from unsoluble proteins and cell debris the suspension was centrifuged 30 minutes at 4 °C at 18 000 rpm in a Sorvall RC 3B Plus (Rotor: SS-34). For soluble proteins supernatant was taken and purified. For the inclusion body purificaiton supernatant was discarded and IB containing pellet was processed.

All purifications (exceptions noted) were performed using Äkta FPLC systems, monitoring the absorbance at 280 nm. After purification steps, fraction were analysed by SDS-PAGE. Pure protein fractions were concentrated, flash-frozen in liquid nitrogen and stored at -80°C.

2.6.1 Purification of DegPwt and DegPS210A

DegPwt and DegPS210A were C-terminally His-tagged and expressed as full-length proteins containing the periplasmic SP. Purification procedure was adapted from Krojer et al. (2008b). The supernatant after steps described in section 2.6 was purified with a NiNTA column (HiTrap HP column, GE Healthcare, column volume 5ml) equilibrated in buffer A using a sample pump (flow: 1ml/ml). DegP was eluted with 20% Buffer B, peak fractions collected and concentrated using vivaspin concentration devices to 5ml, and was further purified via loop injection by size exclusion chromatography (SEC) using HiLoad 26/60 Superdex 200 (GE Healthcare) in Buffer E, where DegP 6-, 12- and 24-mer particles were separated, the individual fractions collected and concentrated by vivaspins using MWCO 50.000 Da. A final concentration of 900 µM was reached.

Buffers used:

Buffer A: 200 mM NaCl, 50 mM Hepes pH8

Buffer B: 200 mM NaCl, 50 mM Hepes pH8, 150 mM Imidazole

Buffer E (Gelfiltration Buffer): 300 mM NaCl, 50 mM Hepes pH8

2.6.2 Purification of Skp

Purifications were adapted from Walton et al. (2009) for Skp-His and Schlapschy et al. (2004) for Skp-Strep. Purified C-terminally tagged Skp constructs were: Skp-His full-length, Skp-Strep full-length, Skp-Strep no SP. Skp-His full-length was purified the same way as DegP-His (section 2.6.1) except Vivaspin MWCO was 10,000 Da. Strep tagged constructs were first loaded on a 5 ml Streptactin Superflow cartridge H-PR (IBA GmbH) equilibrated with Buffer A until a stable baseline was reached, then eluted with 100% B. Column was regenerated with 10 CV regeneration buffer. Peak fraction was collected and concentrated using vivaspin concentration devices (MWCO 10 000 Da) to 5ml, which were further purified by size exclusion chromatography (SEC) using HiLoad 26/60 Superdex 200 (GE Healthcare) in Gelfiltration Buffer.

Buffer A: 150mM NaCl, 1mM EDTA, 100 mM Tris/HCl pH8

Buffer B: 150mM NaCl, 1mM EDTA, 100 mM Tris/HCl pH8, 2 mM D-Desthiobiotin

Gelfiltration Buffer: 150 mM NaCl, 50 mM Tris/HCl pH8

Regeneration Buffer: 150 mM NaCl, 50 mM Tris/HCl pH8, 1mM HABA (hydroxyazophenyl-benzoic acid)

Skp full-length constructs showed a double band already at expression trial level (see results 3.2.1, where only the upper one was soluble. We supposed that Skp was incompletely processed and only a part of the protein translocated to the periplasm, while the other part formed cytoplasmic inclusion bodies, probably due to the hydrophobic SP.

'Skp-Strep no SP' showed only a single, soluble protein band, and was purifiable in sufficient amounts and high quality.

2.6.3 Purification of SurA

C-terminally His-tagged SurA proteins were purified using the same buffers and steps as for DegP-His (section 2.6.1), except for a step-wise washing on the NiNTA column (15, 30, 45, 60 mM)), where SurA-His was then eluted at 150 mM Imidazole (probably more would have been better, because later it seemed some SurA was retained on the column).

2.6.4 Purification of OmpA from inclusion bodies

OmpA purification from inclusion bodies (IB) protocol was adapted from Walton et al. (2009). All Omp constructs were done without a tag as purification from inclusion bodies yields a sufficiently pure protein. OmpA was the most convenient Omp as the folding state can be determined, and also, anti-OmpA antibodies are available in the lab. OmpA-His was less soluble then untagged OmpA when subsequently added to incubation mixtures, but complex formation (with Skp) occurred as usual.

Buffer A: 20 mM Tris (pH 8.5), 0.1% Triton X-100, 5 mM EDTA, 5 mM 2-mercaptoethanol

Buffer B: 20 mM Tris (pH 8.5), 0.2% Triton X-100, 5 mM EDTA, 5 mM 2-mercaptoethanol

Buffer C: 20 mM Tris (pH 8.5), 1% deoxycholate, 5 mM EDTA, 5 mM 2-mercaptoethanol

Buffer D: 20 mM Tris (pH 7.5), 5 mM 2-mercaptoethanol, 5 M urea

Buffer E: 20 mM Tris (pH 9), 5 mM 2-mercaptoethanol, 5 M urea, 1 M NaCl

Cells were incubated with Lysozyme and DNAse and disrupted by sonication. IB pellets were harvested and washed 3 times with buffer B, then once with buffer C. The final pellet was resuspended in buffer D and yielded sufficiently pure OmpA.

2.6.5 Purification of OmpC and OmpF from inclusion bodies

First purifications of OmpC and OmpF full-length constructs were done using the OmpC purification protocol. This was then changed in favor of the OmpA purification protocol (section 2.6.4), which gave a better yield (the higher yield was not dependent on pH but on the urea concentration in the final buffers).

OmpC purification protocol

The OmpC purification protocol was adapted from Kumar and Krishnaswamy (2005).

Cells were incubated with Lysozyme and DNAse, and disrupted by sonication. IB pellets were harvested and washed 2 times with TTN buffer and with TN buffer, respectively. IBs were then solubilised for 5 hours, shaking at 37°C, in Tris-Urea buffer. The suspension was then centrifuged and the supernatant passed through a 0.2 µm filter to remove aggregates.

TTN buffer(Buffer A): 50 mM Tris, pH 8.5, 0.1 M NaCl, 2% Triton X-100

TN buffer: 50 mM Tris, pH 8.5, 0.1 M NaCl

Tris-Urea buffer: 50 mM Tris, pH 8.5, 0.1 M NaCl, and 4 M Urea

Ion Exchange Chromatography

Following the protcol of Kumar and Krishnaswamy (2005), Ion exchange chromatography (IEX) was performed and adapted. IEXC was performed following the usage of the OmpA purification protocol (section 2.6.4). Buffer pH was optimised. Increase of pH greatly enhanced binding efficiency of Omps to the column (pH 9 or 9.5 proved quite effective as OmpC/F have quite low theoretical pIs (around pH4.5)). Therefore, OmpA buffer D was adjusted to pH 9 and used to equilibrate IEX columns (HiPrep 16/10 QFF, CV: 20 ml). Sample was loaded and eluted with a gradient of OmpA buffer E (section 2.6.4). Samples were then concentrated using vivaspin columns to a concentration of around 10 mg/ml (around 250 - 300 µM), stopping often to mix them to avoid gelation.

2.6.6 Extraction of E.coli outer membrane

Extraction of *E.coli* outer membrane was adapted from Rouvière and Gross (1996). The *E.coli* strains DH5α and ΔOmpA strain MR58, respectively, were inoculated over night in LB-medium free of antibiotics. On the next day, cells were harvested and washed in 100mM Tris pH8. Cells were resuspended in 100mM Tris pH8 (usually around 500 µl/5ml overnight culture) and 0.1 mg/ml lysozyme, 1 mM EDTA and DNAse stock in a 1:100 dilution were added. After incubation between 15-60 minutes cell suspension was treated with sonication with a microtip and centrifuged on a table top centrifuge with rcf=10.000xg for 20 minutes to separate the viscous supernatant from the cell debris. Supernatant was carefully collected, separated into equal parts into eppendorf tubes and centrifuged for at least 30 min at 20.000xg in a table top centrifuge to separate the membrane fraction as a pellet. Supernatant was discarded and the pellet resuspended in 150 µl NaPi pH 7,5 + 0,5% sarcosyl and incubated for 30 minutes to resuspend the inner membrane fraction. The centrifuging step at 20.000 xg for 30 minutes was repeated, the supernatant with solubilised IM fraction was discarded and the whitish OM pellets stored at -20°C. Solubilisation of OM preps was done using the detergent SB-12 (0.5% for solubilisation, 0.25% final incubation), which was used in OmpC refolding procedures, thereby seeming a available and suitable choice for Omp solubilisation.

An amount of 5ml overnight culture gave a sufficient pellet for setup of at least 2 experiments. To test protein content, a pellet was resuspended in 1x SDS-Loading buffer, and applied to a SDS-gel in a heated and non-heated way. Strong OmpA and OmpC bands indicated good sample quality.

2.6.7 Refolding of OmpC

All OmpC refolding attempts are summarized in a table in figure 3.13 in section 3.2.5 and are parameters tested are shown here in order of appearance.

Refolding protocols

OmpCRF: OmpC Refolding (RF) buffer was stirred at 4°C, and unfolded OmpC was rapidly added in a 1:5 protein to buffer ratio. This solution was overnight incubated at the same temperature (Kumar and Krishnaswamy, 2005).

BamA: Unfolded OmpC was diluted in a 1:10 protein to buffer ration into BamA RF Buffer, incubated at at 4 °overnight, and then for 2 days at 30 °C. Small aggregates were removed by centrifugation (Robert et al., 2006).

OmpCRF(init): As explained in section 2.6.5 usually the OmpA purification protocol (section 2.6.4) was used for OmpC/F purification, in this case however the OmpC purification protocol was used (2.6.5) to more exactly follow the initial protocol for reproducibility.

Refolding buffers

OmpCRF: 50 mM Tris, pH 8.5, 0.1 M NaCl, 10% (v/v) glycerol, and 0.2% (v/v) polyoxyethylene-9-laurylether (C12E9, Sigma)

BamA: 50 mM Tris, pH 8.0, 0.5% SB-12

Detergent

C12E9: polyoxyethylene-9-laurylether, non-ionic.

SB-12: N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, zwitterionic.

Protein concentration (initial)

Concentration of protein sample before dilution into refolding buffer.

Protein concentration (final)

Concentration of protein sample after dilution into refolding buffer.

Folding state was monitored using SDS-PAGE and semi-native SDS-PAGE.

2.6.8 Small-scale purification of Skp-OmpA-His by NiNTA beads

Skp-OmpA-His complexes were purified using NiNTA Superflow beads resin (50 µl/reaction of 400 µg Skp). After each step were centrifuged 30 seconds at 13,000 rpm (tabletop centrifuge). Beads were stored in 20% ethanol which was taken off and beads were washed thrice with 200 µl Skp-Omp Gelfiltration (GF) buffer (20mM Phosphate pH6.5, 75mM Na_2SO_4). Sample was added and incubated at room temperature, gently shaking. Supernatant was removed and sample washed 4 times in GF buffer supplied with 15mM imidazole and eluted in 50 µl buffer GF supplied with 500 mM imidazole. After each step samples were taken and analysed with SDS-PAGE (Input sample: before loading on beads, Wash: after 15mM imidazole, Elution: eluted fraction, Pellet: beads loaded). Sucess was further monitored by analytical SEC.

2.7 Protein analysis

2.7.1 SDS-PAGE

Proteins were separated electrophoretically by SDS-Polyacrylamid gel electrophoresis (SDS-PAGE). SDS-gel composition is depicted in table 2.7 in amounts sufficient for 8 gels which were prepared simultaneously in an apparatus (MPI Martinsried, Germany) and stored at 4 °C in humid conditions. Samples were incubated with 2x SDS sample buffer, heated to 95°C for 5-10 minutes prior to loading on the gel. Per gel, 25mA were applied and it was run until the loading dye front reached the bottom of the gel. Markers that were used were PageRuler ™Unstained/Prestained/Prestained Plus Protein Ladders. Per gel, 5 µl were loaded.

semi-native SDS-PAGE

Semi-native SDS-PAGE was performed as SDS-PAGE (section 2.7.1) with the exception that samples were not boiled, but incubated on room temperature before loading (Schweizer et al., 1978).

Urea-SDS-PAGE

Urea-SDS-PAGE was performed as SDS-PAGE (section 2.7.1), with the gels prepared differently (containing Urea). For the separating gel Buffer, Protogel and

Component	Separating gel	Stacking gel
1.5 M Tris/HCl pH 8.8	20 ml	
0.5 M Tris/HCl pH 6.8		7.5 ml
10% SDS	800 µl	300 µl
20% Protogel [firma]	32 ml	4.5 ml
H_20	28 ml	17.4 ml
10 % APS	400 µl	300 µl
TEMED	40 µl	30 µl

Table 2.7: Recipe for 12% SDS-polyacrylamide gels

SDS (according to table 2.7) were mixed with 28.8 g urea, then 5 ml H_20, APS and TEMED were added. For the stacking gel, Buffer, Protogel and SDS were mixed with 10.8 g urea, then 5 ml H_20, APS and TEMED were added. Sample was taken up in 2x SDS-loading buffer containing 5M urea.

2.7.2 Coomassie blue staining

After SDS-PAGE gels were soaked in staining solution (table 2.1), heated in a microwave, then gently shaken at room temperature. After 15-30 minutes they were transferred to destaining solution (table 2.1), heated, and gently shaken at room temperature. Destaining was repeated until a satisfying reduction of background stain was achieved.

2.7.3 Western blot

Solution compositions are depicted in table 2.1. For western blot, polyacrylamide gels after SDS-PAGE were taken and proteins were transferred to a PVDF Immobilon-P transfer membrane (Millipore) pre-equilibrated with methanol using a semi-dry blotting method. The gel was equilibrated shortly in WB transfer buffer, which was also used for the transfer at 50mV / gel for 80 minutes. Transfer efficiency was judged by the intensity of prestained marker. The membrane was then blocked with WB blocking buffer for 2 hours at room temperature or overnight at 4°C gently shaking to occupy all possible protein binding sites and to inhibit background. Membrane was then incubated with the first anti-body (anti-OmpA) in a 1:50,000 ratio of antibody diluted into WB blocking buffer for 2 hours at room temperature or overnight at 4°C gently shaking. Subsequently the membrane was

washed 3 times with WB TBST and once with TBS for 5-15 minutes each. Then the second antibody, anti-rabbit HRP was diluted into WB TBST in a 1:25 000 ratio and incubated on the membrane gently shaking for around 2 hours at room temperature. The membrane was washed as before and incubated for 2 ml WB TBS and 1 ml Solution A and 25 µl Solution B of ECL Plus WB detection system (GE Healthcare) for one minute before it was brought to the darkroom and exposed to an Amersham Hyperfilm ECL (GE Healthcare) film which was developed there.

2.7.4 Protein quantification

Protein quantification was done with either the Bradford Protein assay for general measurements, whereas for exact measurements the NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific) was used.

For the Bradford assay (Bradford, 1976) 5 µl protein solution and 795 µl of H_20 were mixed and incubated with 200 µl Bradford-Solution (BioRad, Germany) in a plastic cuvette. Absorption was measured at a wavelength of 595 nm against a blank solution containing water and Bradford solution. The protein concentration was calculated from a BSA standard curve.

With the NanoDrop the absorption of the protein sample was measured at 280 nm and the protein concentration was calculated with the Lambert-Beer relation: $A = \epsilon \times c \times d$. A: Absorption 280 nm, ϵ: molar absorbance coefficient, c: sample protein concentration, d: cuvette cell length. ϵ-values were obtained with the ProtParam tool http://www.expasy.ch/tools/protparam.html.

2.8 Complex formation

Unfolded (urea-denatured) Omps were always added to a chaperone dilution, incubated with optional gentle shaking at 300rpm, and subsequently subjected to analytical SEC, unless otherwise noted. Usually a final volume of 40 µl was prepared and about 30 µl injected on the column.

For DegP-Omp complexes, 40 µM DegP were incubated with Omps in a 2:1 ratio (exceptions noted in figure captions) for 10 minutes at 37°C. For Skp-Omp complexes between 30 and 300 µM Skp were incubated with 10-100 µM Omps in a 3:1 ratio (= 1:1, Skp trimer:Omp monomer) for 10 minutes at room temperature. Initial SurA-Omp tries were incubated as DegP-Omp complexes, but with a 1:1 ratio (20-40 µM

of each protein). Optimised SurA-OmpA (40 µM SurA, 20 µM OmpA) complexes were incubated in a 2:1 SurA:OmpA ratio and 4°C for 30 minutes (but run at room temperature. Stability of the complex might be increased by SEC at 4°C.).

For transfer trials of OmpA from Skp to DegP incubation was done by preincubation of Skp and OmpA as above. Then DegP was added and incubated at 37°C for 10 minutes. Concentrations used: DegP 6-mer 15µM, Skp 20µM, OmpA 20µM.

For transfer trials of OmpA from SurA to DegP, SurA-OmpA was incubated 30 minutes at 4°C, then DegPSA was added and incubated at 37°C for 10 minutes. All controls, except SurA-OmpA, were incubated for 30 minutes at 37°C; SurA-OmpA was incubated 4°C. Concentrations used: DegPSA 6-mer 40µM, SurA 40µM, OmpA 20µM.

For interaction of DegP with DH5α or ΔOmpA strain MR58 OM preps, OM prep pellet was resuspended in DegP buffer E containing 0.5% SB-12. This was then mixed with DegPwt (40 µM) or DegPSA (45µM) to a final content of 0.25% SB-12 and incubated 10 minutes at 37°C and subsequently subjected to analytical SEC.

For large-scale Skp-OmpA complex formation samples were prepared with 300 µM Skp and 100 µM OmpA with a final volume of 500 µl for injection to a Superdex 200 10/300 GL column (GE Healthcare) via a 500 µl loop. Higher concentrations were in this case not advisable due to high urea contents in the OmpA sample.

2.9 Analytical size exclusion chromatography

Complex formation was analysed by analytical SEC, which was performed on a Superdex 200 10/300 column (GE Healthcare) at room temperature. For all experiments, except for Skp-OmpA complex formation trials, DegP Buffer E was used (section 2.6.1). For Skp-OmpA complex formation Skp-Omp Gelfiltration (GF) buffer (20mM Phosphate pH6.5, 75mM Na_2SO_4) was used. For analytical SEC in presence of SPs, DegP Buffer E was supplied with 100 µM of the respective SP solubilised in a final volume of 3% DMSO. Samples were applied with either a 25 µl or a 100 µl loop, with a flow rate of 0.7-0.9 ml/min (depending on column pressure). Before loading on SEC, samples were centrifuged 5 minutes at 13,000 rpm (tabletop centrifuge). Fractions were collected and analysed by SDS-PAGE. To calibrate the coloumn, the elution volumes of the following MW standards (Biorad) were determined. Thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44

kDa), equine myoglobin (17 kDa) and Vitamin B12 (1.35 kDa). A calibration curve of MW (logarithm) vs elution volumes was calculated and MW of the eluted proteins calculated.

2.10 ITC

The thermodynamic values of the interaction between DegP and the OmpF SP and its mutations were determined with an ITC microcalorimeter. All experiments were performed at 25°C. Approximately 300 µl DegP of a 30-60 µM solution was placed in the temperature controlled sample cell and titrated with different peptides (around 70 µl, 300-500 µM if possible) loaded in the mixing syringe. As buffer, 50 mM NaPO4 pH 7.5, 100 mM NaCl, 3% DMSO was used. Injections of peptide were dispensed into the sample cell by using a 120-s equilibration time between experiments and stirring at 300 rpm. The data was analyzed with ORIGIN software by following the instructions of the manufacturer. Peptides were dissolved in DMSO with a final concentration of 3%. Peptides were synthesised in-house by Mathias Madalinski.

2.11 Crystallization fine screens

Three fine screens were setup.

Skp01: 24-well (sitting drop): Ammoniumsulfate [500-3000 mM] (6 steps), isopropanol [0-20%] (4 steps)

Skp02: 96-well (sitting drop), 1 drop (2:1 protein:reservoir): Ammoniumsulfate [500-3000 mM] (12 steps), isopropanol [0-10%] (4 steps) / PEG400 2.5 + 5% (2 steps) / MPA 2.5 + 5% (2 steps)

Skp03: as Skp02, except that 2 drops / condition were done (1:1 and 2:1 protein:reservoir)

2.12 Mobility-shift assay of folded and unfolded Omps

For the controls of Omp-folding state, Omp mobility-shift assays were done.

For anti-OmpA Western blot controls, cells of *E.coli* wild-type strain MC4100 were harvested and lysed by incubation with 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% SDS and 1% 2-mercaptoethanol. To ensure the complete unfolding of OMPs, one sample was boiled at 95°C for 15 min in SDS loading buffer.

To maintain the folded states of OMPs, the second sample was incubated at room temperature. Mobility-shift of OmpA detected on WB served as control.

For general folding state determination, the sample was loaded once unheated/semi-natively and once boiled. Mobility-shift indicated folded Omp state in the native sample.

2.13 Digestion assay

Folding state of Omps inside the DegP-Omp complexes was determined by incubating the sample at 37°C. At selected timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C.

2.14 Electron Microscopy

DegP-OM prep complexes were prepared (section 2.8) and purified by analytical SEC. Complex containing fractions were collected, diluted to an appropriate concentration and were negatively stained with 2% (w/v) uranyl acetate on glow-discharged, carbon-coated grids (Agar Scientific).

Acknowledgements

We thank for the generous help of K. Mechtler and I. Steinmacher for mass spectrometry, M. Madalinki for peptide synthesis and G.Resch for electron microscopy.

3 Results

3.1 Chaperone activity of DegP

Due to the involvement of DegP in Outer Membrane Protein (Omp) biogenesis and the cage-forming structure of active DegP, we were interested whether the DegP cavity might provide an Omp folding chamber, in which the Omps could reach their mature structure. To investigate the chaperone activity of DegP, we tested if DegP forms complexes with unfolded Omps (ufOmps). We wondered if we thereby could generate complexes with folded Omps for the visualisation of Omps inside DegP.

3.1.1 Purification of ufOmpA/OmpC/OmpF from cytoplasmic inclusion bodies

Unfolded OmpA, OmpC and OmpF were purified from inclusion bodies, adapting published protocols (see section 2.6, Materials and Methods).

Expression of full-length OmpC from inclusion bodies results in the accumulation of two proteins of the approximate MW of OmpC (figure 3.1a), which could not be separated by subsequent Ion Exchange Chromatography (IEXC). This result suggested an inefficient translocation of OmpC to the periplasm, leading to the accumulation of periplasmic (translocated and processed), as well as cytoplasmic (unprocessed) OmpC, giving rise to an inhomogeneous OmpC species.

We then generated OmpC constructs lacking the SP. These accumulated in the cytoplasm and could be purified as homogeneous OmpC species (figure 3.1a, 'no SP'). Further purification by IEXC resulted in a pure sample (figure 3.1a, 'final').

OmpF was purified accordingly (final sample shown in figure 3.1b). For OmpA, IEXC was omitted, since purification from inclusion bodies already resulted in a pure sample (figure 3.1b). The final yield for all three proteins was approximately 100 mg/L culture, dissolved in buffer containing 5M Urea. They could be concentrated to at least 10 mg/ml.

Summarizing, Urea-denatured OmpA, C and F were obtained in pure and concentrated form from cytoplasmic inclusion bodies.

3.1.2 Interaction of DegP and ufOmps

The interaction of DegP and ufOmps was tested for potential complex formation.

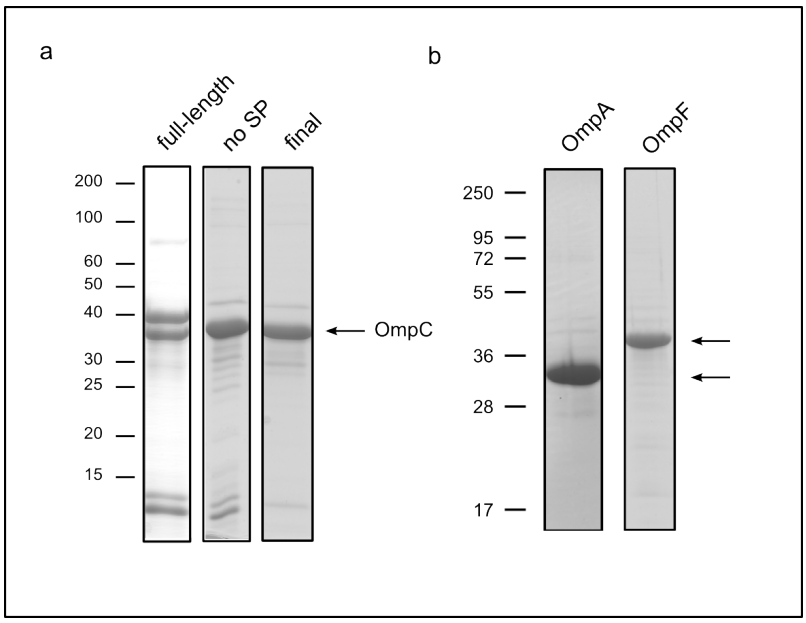


Figure 3.1: Purification of unfolded OmpA/C/F from cytoplasmic inclusion bodies. a. Purification of OmpC from inclusion bodies. The purified sample was loaded on a 12% SDS-PAGE gel and stained with Coomassie Blue. full-length: OmpC full-length construct. no SP: OmpC construct without SP. final: purified OmpC **b.** Final purified constructs of OmpA and OmpF. The individual Omps are indicated by arrows. OmpA was loaded after purification from inclusion bodies, while OmpF was additionally purified by IEXC.

DegP 6-mer and the respective Omps were mixed, incubated and subjected to analytical size exclusion chromatography (SEC). Complex formation was monitored by the elution volume of DegP, revealing the formation of a 12- or 24-meric state (Krojer et al., 2008b).

The folding state of OmpA was determined by semi-native SDS-PAGE, exploiting the fact that Omp β-barrels resist SDS denaturation, but not high temperature. Heating of the sample 10 minutes at 95°C melts the barrel secondary structure, resulting in a shift of OmpA 30 kDa (folded) to 35 kDa (unfolded) on SDS-PAGE. This provides a useful assay for determining OmpA folding states. For OmpC/F there are no reports for a shift in the monomeric state, however the trimeric form can be distinguished from the monomeric.

For DegPwt it is known that unfolded substrates are immediately digested. So this study uses mainly the protease inactive form of DegP, DegPS210A (active site serine mutated to alanine), further referred to as DegPSA, to test whether DegP displays any Omp folding activity by providing a suitable environment.

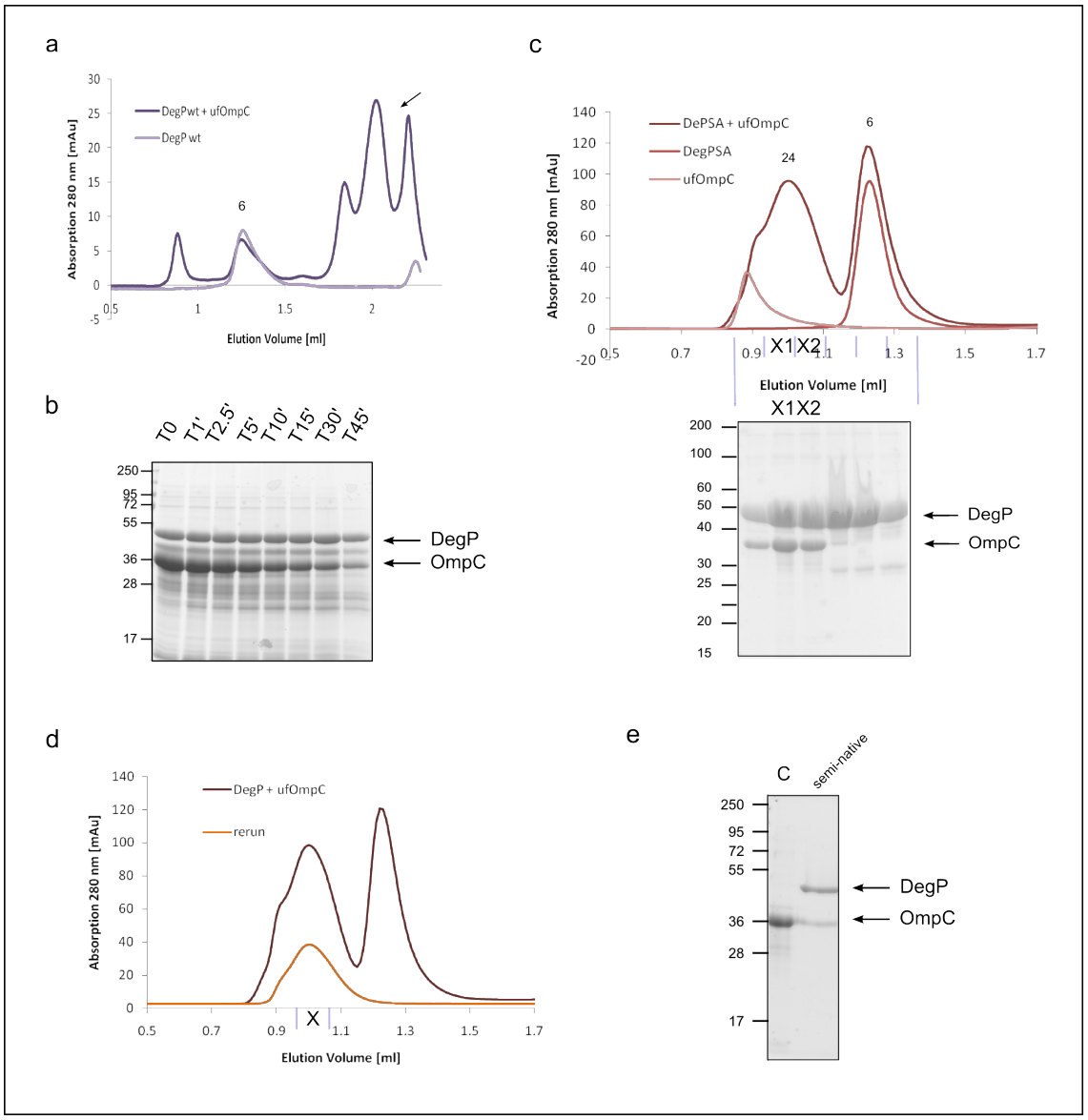


Figure 3.2: Analysis of complex formation between DegP and OmpC by analytical SEC. For the individual experiments DegP 6-mer (40 µM) and ufOmpC were incubated and subjected to SEC. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to DegP and OmpC are marked. Size exclusion chromatograms are labelled accordingly. DegP oligomeric states are indicated by numbers above the peaks. **a.** Equimolar amounts of DegPwt and of ufOmpC were used. A black arrow indicates peaks corresponding to degradation product. **b.** For the digestion assay, ufOmpC (40µM) was incubated with DegPwt (10µM) at 37°C. At the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C. **c.** DegPSA and OmpC were incubated in a 2:1 ratio and analysed by SEC (dark red line). Fractions were analysed by SDS-PAGE. **d.** DegPSA and OmpC were incubated in a 2:1 ratio and analysed by SEC (dark red line). Indicated peak fraction X was collected, concentrated, flash-frozen to 80°C, thawed and reinjected on the column (orange line). **e.** Semi-native SDS-PAGE of peak fraction X of (b). C: control, purified denatured OmpC.

3.1.3 Stable complex formation between ufOmpC and DegPSA, but not DegPwt

Incubation of DegPwt with ufOmpC does not lead to the formation of DegP 12- or 24-mers in these concentrations (figure 3.2a). The presence of degradation product indicates digestion of ufOmpC by DegPwt (indicated in figure 3.2a by a black arrow).

To test the digestion of unfolded OmpC by DegPwt, both proteins were incubated and at various timepoints aliquots were taken. After 45 minutes almost all ufOmpC is digested (figure 3.2b).

In contrast, when incubating DegPSA with ufOmpC the formation of DegP 24-mer is observed (figure 3.2b). Analysis by SDS-PAGE reveals a coelution of DegP and OmpC with an notable excess of DegP. The exact ratio between DegP and OmpC was not determined from the gel, but it is likely that in one DegP 24-mer 1 to 3 unfolded Omps are bound.

The shift of DegPSA to higher oligomers depends on concentration and incubation time. When lowering the overall concentration of DegP and OmpC while keeping the ratio constant, also a DegP 12-mer peak can be observed in addition to 24-mer. Reducing the incubation time (seconds) favours formation of DegP 12-mer, longer incubation time DegP 24-mer (Data not shown).

To test the stability of the DegP-OmpC complex after SEC, fraction 'X' (figure 3.2b) was concentrated, flash-frozen to 80°C, thawed and reinjected onto the column. The reinjection ('rerun') shows an identical peak profile, demonstrating the stability of the DegPSA-OmpC complex to the above named treatments.

To determine the folding state of OmpC, peak fraction 'X' (figure 3.2b) was loaded onto SDS-PAGE without prior heating (figure 3.2d). No shift of OmpC (lane labeled: semi-native) is observed compared to the Urea-denatured OmpC control (C). Such a shift would have indicated folding and trimerisation of OmpC inside the DegP cage. So, the only conclusion that can be made is that OmpC does not form folded trimers.

For OmpF the same experiments were done as for OmpC and analogous behaviour was observed.

In summary, ufOmpC induces complex formation with DegPSA, while it is digested by DegPwt. For the DegPSA-OmpC complex the folding state is not reliably determinable. However, semi-native PAGE and the fact that DegPwt immediately

degrades OmpC suggests that OmpC is not folded by DegP.

3.1.4 Rare complex formation between ufOmpA and DegPSA, but not DegPwt and no detection of folded OmpA species

In contrast to OmpC (see section 3.1.3), incubation of DegPSA with ufOmpA rarely induces complex formation (figure 3.3a). Comparison to control experiments with isolated DegP or ufOmpA reveals barely any differences. However, a slight increase in absorption at around 1.1 ml might indicate formation of DegP 12/24-mer and therefore a low capacity of complex formation.

Analysis of these fractions by SDS-PAGE revealed a small degree of coelution of DegP and OmpA, indicating complex formation. However, the amount of the observed DegP-OmpA complexes is very low, especially compared to complex formation between DegPSA and ufOmpC (figure 3.2b).

To detect the folding state of OmpA, semi-native PAGE was performed, followed by western blot (figure 3.3b). From the experiment we can see that the major portion of OmpA is in an unfolded state. There is also a prominent band visible at around 72 kDa, of unknown folding state.

We also performed the same experiment with DegPwt, like for DegPwt-OmpC (figure 3.2a) for DegPwt-OmpA, which also does not show any complex formation (Data not shown).

To summarise, DegPS10A-OmpA complex formation is less effective compared to DegPS10A-OmpC and OmpA bound to DegPSA was shown to be unfolded.

3.1.5 Influence of OmpC and OmpF signal peptides on DegP complex formation

It has been shown that peptides can stimulate the proteolytic activity of DegP, as well as induce higher oligomer formation (Krojer et al., 2008a, 2010). Upon translocation of proteins, including Omps, into the periplasm, their periplasmic localisation signal peptide (SP) is cleaved off. These SPs might signal the appearance of an Omp being translocated and in need of a chaperone, which in turn might facilitate DegP cage-formation to chaperone nascent Omps.

We analysed the effects of the OmpC and OmpF SP on inactive DegP 6-mer and we examined whether OmpC/F SPs induce higher oligomers of DegP, and if so, whether ufOmps can be trapped inside this complex.

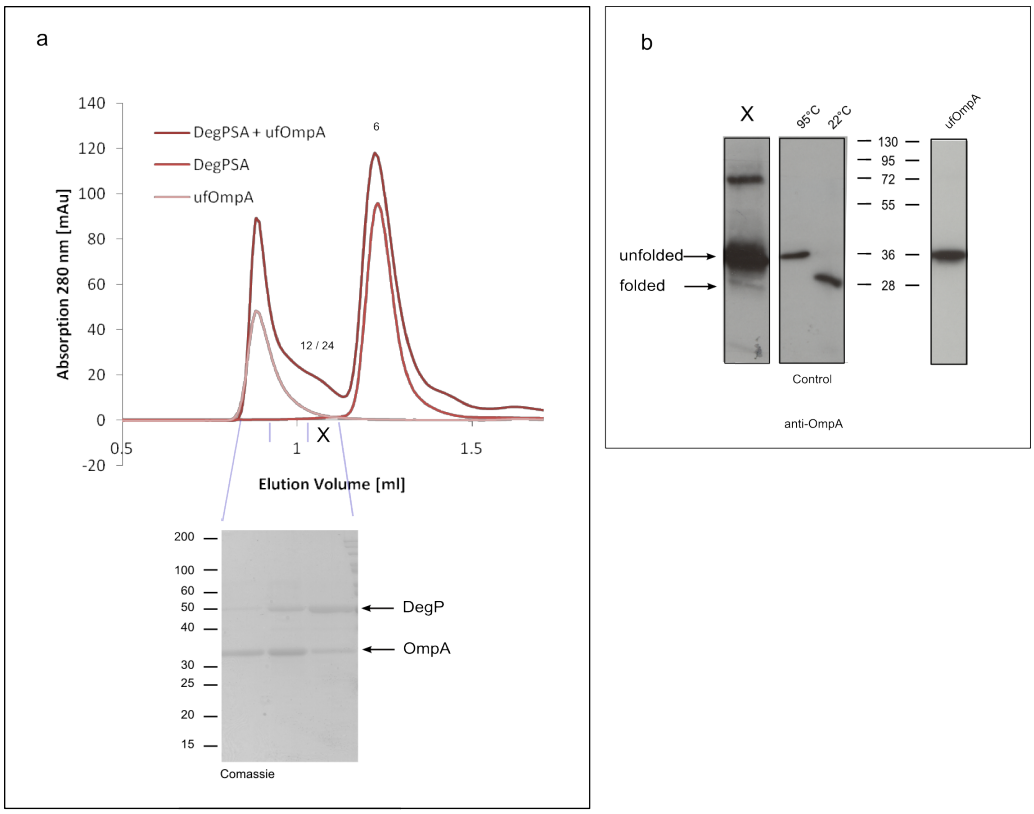


Figure 3.3: Analysis of complex formation between DegP and OmpA by analytical SEC. For the individual experiments DegP 6-mer (20µM) and ufOmpA were incubated and subjected to analytical SEC. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to DegP and OmpA are marked. Size exclusion chromatograms are labelled accordingly. DegP oligomeric states are indicated by numbers above the peaks. **a.** Equimolar amounts of DegPSA and ufOmpA were incubated and analysed by SEC and SDS-PAGE. **b.** Western blot against OmpA of fraction X (a). **c.** Western Blot of the ufOmpA control run from (a), elution volume corresponding to X (a).

OmpC SP induces DegP 12/24-mer formation in DegPwt but not in DegPSA

We first incubated DegP and Omps in the presence of SP prior to SEC experiments. However, no differences to the experiments described in section 3.1.4 could be observed (not shown). Therefore, we performed all experiments with 100 µM OmpC SP in the running buffer during SEC.

In figure 3.4a it is shown that the presence of SP leads to a shift of DegPwt (figure 3.4a, light). But, when DegPwt and ufOmpA are incubated and subjected to SEC (figure 3.4a, dark), no coelution of DegPwt and OmpA is detected by SDS-PAGE, indicating that OmpA may not be bound inside the DegP 12-mer cavity.

The same experiments were done with DegPSA (figure 3.4b). In contrast to Deg-

Pwt, isolated DegPSA does not show a shift from the 6-mer to a higher oligomeric state. When DegPSA is incubated with ufOmpA, a small peak corresponding to DegPSA 12-mer is observed (figure 3.4b) and analysis by SDS-PAGE shows a small degree of coelution in the peak fraction.

To detect if a fraction of the bound OmpA inside the DegP 12-mer was folded, semi-native SDS-PAGE followed by western blot was performed for the fractions of the experiments shown in figure 3.4a and b.

Western blot of the DegPwt - OmpA run is shown in figure 3.4c. The majority of OmpA is present in an unfolded form, but between 1.1 ml and 1.3 ml elution volume folded OmpA can be detected, corresponding to the elution volume of the DegP 12-mer.

In contrast, for the coelution of DegPSA - OmpA (figure 3.4d), the main portion of OmpA is unfolded, except between 1.3 ml and 1.4 ml a folded OmpA band can be observed. This elution volume corresponds to the DegP 6-mer peak.

As a control, isolated ufOmpA was subjected to SEC in the presence of SP and the corresponding fractions were analysed by semi-native SDS-PAGE followed by western blot (figure 3.4e). Here, the elution profile was not influenced by the SP (not shown). Western blot however showed a distinctly different pattern compared to the situation in the absence of SP (figure 3.4f). While in the experiments in the absence of SP OmpA is unfolded, presence of SP results in folded OmpA eluting in the area from 1.65 ml to 1.85 ml (figure 3.4f).

Interestingly, in all experiments described here, the presence of SP leads to the formation of folded OmpA, but elution volumes of these species were different in all experiments (figure 3.4c, d and e). However, folded OmpA is not observed in the absence of SP. These results are inconsistent and suggest that the SP introduces an experimental artifact.

Figure 3.4c suggests the formation of a folded OmpA inside a DegP 12-mer. To test if the DegPwt 12-mer peak harbours folded OmpA, we collected the putative complex fraction and subjected it to a digestion test (figure 3.4g). After 30 minutes all OmpA was digested, indicating that OmpA is not folded.

In conclusion, DegPwt does not seem to provide a folding chamber for unfolded Omps. Here, 12-mers induced by the SP bind OmpA inefficiently and do not induce folding. For DegPSA, incubation with OmpA in the presence of the SP shows no difference to the absence of the SP (figure 3.3a).

Taken together, the results from chapter 3.1 presented so far indicate that DegP does not provide a folding chamber for ufOmps. A detailed assessment of chaperone and folding activity is insofar difficult as for the DegP-OmpC complex the folding state of monomeric OmpC cannot be determined, while for the DegP-OmpA complex the efficiency of complex formation is low. However, western blot analyses support the conclusion that ufOmpA is not folded by DegP.

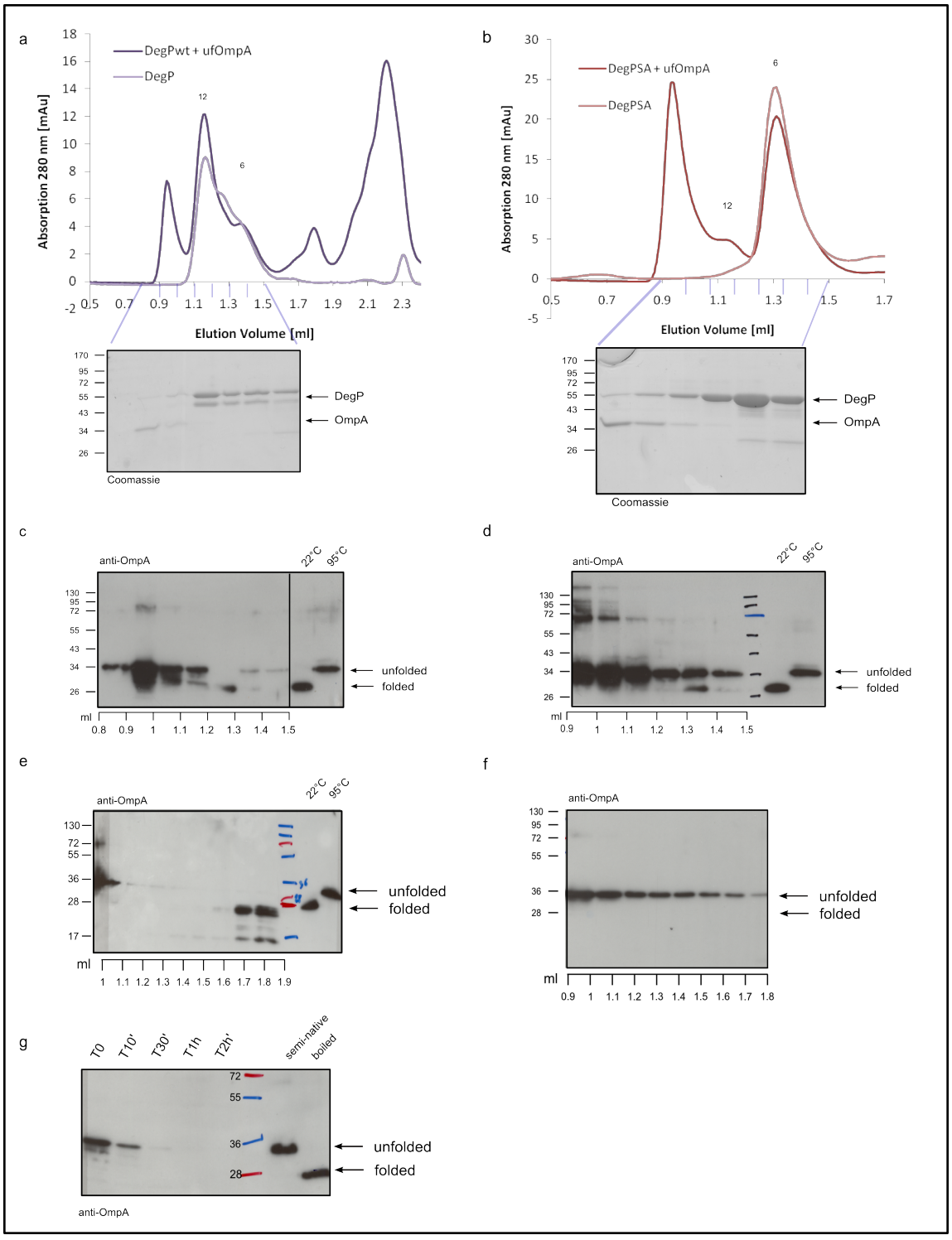
3.1.6 Follow-up: Mutants of putative cleavage site on the SP show different effects on higher oligomer formation of DegPwt

Analytical SEC in the presence of SP induced a shift of DegPwt 6-mer to 12-mer, while for DegPSA no effect was observed. This result suggests that the proteolytic activity of DegPwt might be coupled to higher oligomer formation. From previous studies it is known that DegPwt preferably cleaves after the residues Valine, Alanine, Threonine and Isoleucine (Krojer et al., 2008a). Therefore, we generated various versions of OmpF SP, in which we introduced Glutamate mutations on putative Valine cleavage sites (figure 3.5a).

As described in section 3.1.5 DegP 6-mer was subjected to analytical SEC in the presence of 100 µM of the respective signal peptides in the running buffer and the formation of DegP 12/24-mer was monitored.

Interestingly, the mutations had quite dramatic effects on the oligomeric state of DegPwt (figure 3.5b). While for the wt peptide (2308) almost all DegP forms 12-mers, for the peptides 2810 and 2381 no, or barely any higher oligomer formation

Figure 3.4 (facing page): Analysis of complex formation between DegP and OmpA on analytical SEC in the presence of OmpC SP. For the individual experiments DegP 6-mer ((40 µM)(wt), 45 µM (S210A)) and ufOmpA (20 µM) were incubated and subjected to analytical SEC in running buffer containing 100 µM SP. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to DegP and OmpA are marked. Size exclusion chromatograms are labelled accordingly. DegP oligomeric states are indicated by numbers above the peaks. Western blots are stained with anti-OmpA antibodies. Elution volume of the fractions is labelled under western blots for clarity. **a.** Incubation of DegPwt with ufOmpA (dark lilac line). **b.** DegPSA was incubated with ufOmpA (dark red line). **c.** Western blot of semi-native PAGE of fractions from (a) (dark lilac line). **d.** Western blot of semi-native PAGE of fractions from (b) (dark red line). **e.** Control: isolated, ufOmpA was subjected to SEC in the presence of SP, fractions analysed by semi-native SDS-PAGE followed by western blot. **f.** Control: isolated, ufOmpA was subjected to SEC in SP-free running buffer, fractions analysed by semi-native SDS-PAGE followed by western blot. **g.** Digestion assay of DegP 12-mer peak fraction of (a). Sample was incubated at 37°C and at the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C.


a
DegPwt + ufOmpA
DegP
Absorption 280 nm [mAu]
Elution Volume [ml]
12
6
DegP
OmpA
Coomassie
b
DegPSA + ufOmpA
DegPSA
Absorption 280 nm [mAu]
Elution Volume [ml]
12
6
DegP
OmpA
Coomassie
c
anti-OmpA
22°C
95°C
unfolded
folded
ml
d
anti-OmpA
22°C
95°C
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anti-OmpA
22°C
95°C
unfolded
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folded
ml
g
T0
T10'
T30'
T1h
T2h'
semi-native
boiled
unfolded
folded
anti-OmpA

is noticeable. For the peptides 2809 and 2808 the distribution of 6- and 12-mer is about 50:50.

In figure 3.5c the effects of the respective SPs on DegPSA are shown. As noticed in section 3.1.5 no effect of any peptide was observed for the DegPSA mutant, except for a slight peak broadening for the peptide wt (2308).

The question that appears is if this effect is caused by impairment of binding and/or cleavage due the SP mutations. For DegPSA it would be especially interesting if the peptides do bind, but do not induce 12-mer formation, or if they do not bind. To address this question, we planned to perform isothermal titration calorimetry (ITC) studies.

ITC studies. First, to evaluate the experimental setup, a positive control was done of DegPSA with peptide 2136 (PMFKGVLDMMYGGMRGYQV). The run showed the expected curve. For our experiments, it was however not possible to dissolve the wt peptide (2308) in the required concentration. So, we tried to analyse the interaction of peptide 2808 with DegPwt, since this peptide had a clear effect on analytical SEC. In the ITC experiment, however, the observed interaction was very weak, despite increasing the concentration almost two times compared to the positive control and could not be evaluated. Since experiments with the wt peptide failed due to solubility problems and the peptide 2808, which showed a strong interaction on SEC with the wt, was very weak here, we stopped the analysis at this point.

3.2 Prefolding of Omps by the chaperones Skp and SurA for subsequent transfer to DegP

During biogenesis, Omps have to cross the periplasm accompanied by chaperones that prevent their aggregation and successive damage to the cell. For this, several models about the roles of the individual chaperones Skp, SurA and DegP were proposed. For DegP a possible role as a second-step protein quality control factor in Omp biogenesis was suggested, where the chaperones SurA and Skp take up and prefold the nascent Omps (see section 1.2.4). In this scenario, SurA- and Skp-prefolded Omps can be subsequently transferred to DegP, which protects prefolded Omps, while digesting misfolded ones. The prefolded Omps would then either get the possibility to complete folding inside DegP or are further transferred to the BAM-complex for insertion into the outer membrane.

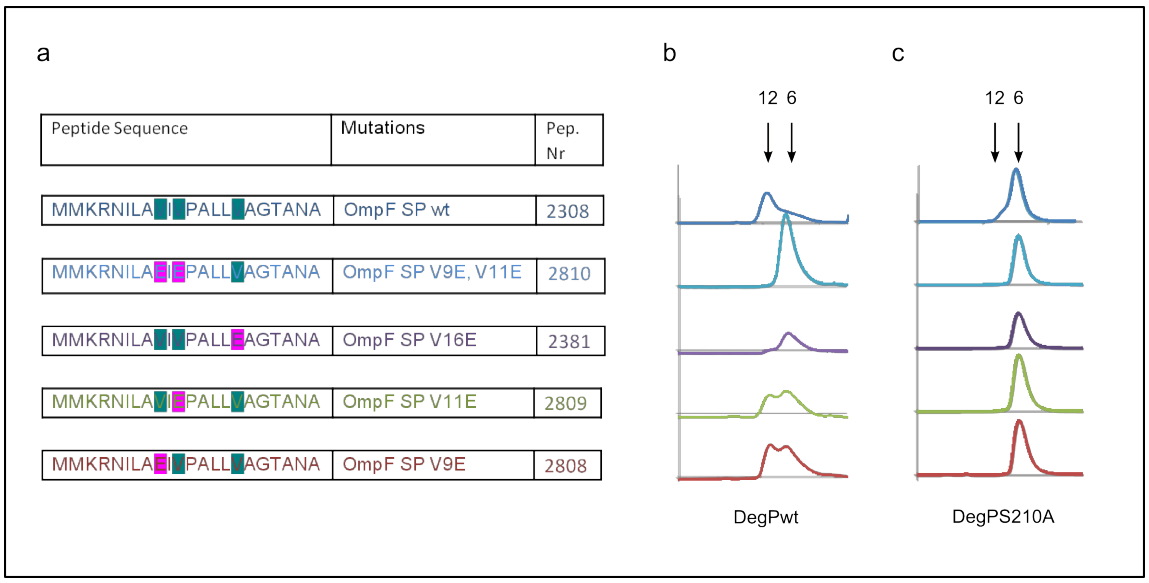

Figure 3.5: Effects of mutations of the putative cleavage sites in the OmpF SP on DegPwt and DegPSA. DegPwt and DegPSA 6-mer were subjected to analytical SEC in the presence of 100 µM SP in the running buffer. DegP oligomeric states are indicated by labelled arrows above the peaks. **a.** Table showing peptide sequence, mutations, and peptide number (Pep. Nr.). The mutated residues are highlighted with green (wildtype residue) and red (residue mutated). **b and c.** Analytical SEC of DegPwt and DegPSA, respectively, in the presence of the SP next to the corresponding peptide sequence in the table. Additionally, color code is consistent with the lettering.

The aim of my experiments was to find out if a transfer of Omps from Skp and SurA to DegP can be observed and if DegP complexes with folded Omps can be generated.

3.2.1 Purification of the periplasmic chaperones Skp and SurA

The first step was to establish the expression and purification of Skp and SurA based on published protocols (see section 2.6, Materials and Methods). Skp and SurA full-length constructs were designed with either a C-terminal His6- or Strep-tag. The full-length constructs contain a periplasmic targeting peptide (SP), which is recognized by the Sec-complex in the inner membrane. After translocation the SP is cleaved off and the mature protein is formed. The expression of the chaperones in the periplasm was chosen, since we planned to use the chaperones for pull-down assays to investigate their native substrates.

In the expression trials for Skp two distinct species were observed (figure 3.6a). These two proteins seem to correspond to processed Skp in the periplasm (without

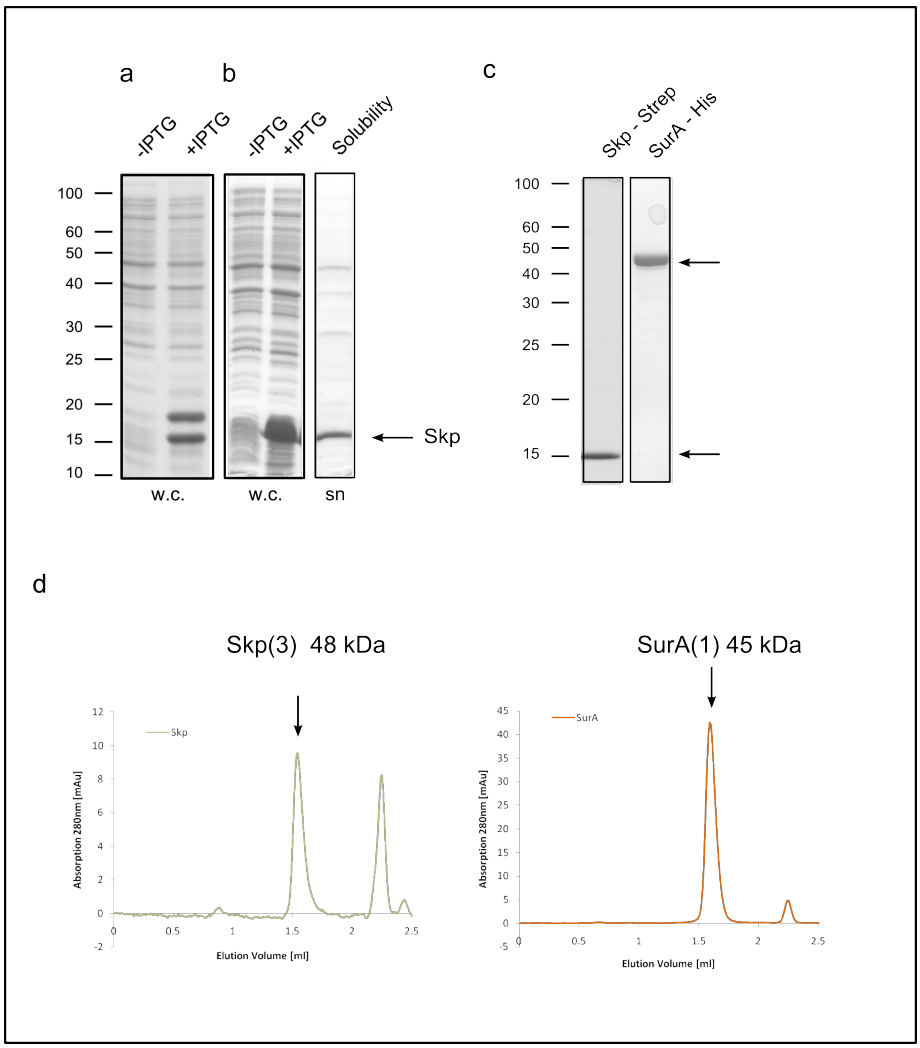

Figure 3.6: Purification of the periplasmic chaperones Skp and SurA. 12% SDS-PAGE gels are stained with Coomassie Blue. For expression trials lanes are marked with -IPTG for uninduced sample and with +IPTG for induced expression (aliquot taken 1 hour after induction) and the gels are labelled with either 'w.c.' (whole cells extraction) or 'sn' (supernatant). **a.** Expression trials of Skp full-length construct. **b.** Expression trials of Skp construct without SP. 'Solubility' shows soluble proteins. **c.** Skp-Strep and SurA-His after Streptactin and NiNTA purification, respectively. Arrows indicate the respective bands. **d.** Purified proteins were applied to analytical SEC to ascertain protein purity. They show Skp in trimeric (Skp(3)) and SurA in monomeric (SurA(1)) state.

SP), and insoluble, unprocessed Skp in the cytoplasm, comparable to purification of OmpC (see section 3.1.1).

Upon removal of the SP, mature and soluble Skp was expressed (in the cytoplasm) and expression trials show only one soluble protein (figure 3.6b). Strep-tagged Skp (Skp-Strep) was then purified. After a Streptactin affinity chromatography, Skp-

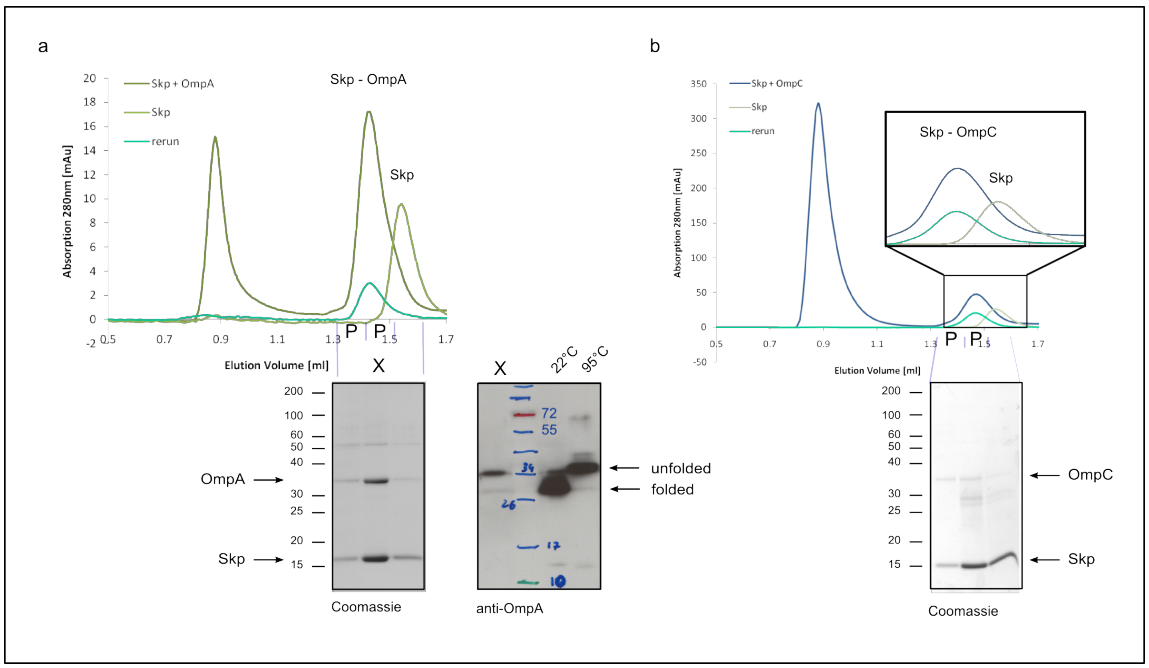

Figure 3.7: Analysis of complex formation between Skp and OmpA/OmpC by analytical SEC. For the individual experiments Skp and ufOmpA/ufOmpC were incubated in a 3:1 ratio and subjected to analytical SEC. Size exclusion chromatograms are labelled accordingly. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to Skp and OmpA are marked. Western blots are stained with anti-OmpA antibodies. **a.** Interaction of Skp and OmpA. Fractions labelled 'P' are combined and reinjected ('rerun'). Fraction X was also used for western blot folding state analysis. **b.** Interaction of Skp and OmpC. The black box shows an enlarged version of the peak area.

Strep was already rather pure (figure 3.6c). Subsequent SEC allowed the purification of a pure functional Skp trimer (Skp(3)). The purity of the final sample was ascertained by analytical SEC (figure 3.6d) and Skp-Strep could be concentrated to 120 mg/ml with a final yield of around 30 mg from 6 l culture.

SurA was expressed with a His-tag (SurA-His). After NiNTA affinity chromatography, SurA was already rather pure (figure 3.6c) and in subsequent SEC monomeric SurA was obtained. General purity was affirmed by analytical SEC (figure 3.6d). SurA yielded around 11 mg protein from 4 ls culture, and was concentrated to 13 mg/ml.

SurA-His and Skp-Strep (noSP) were then used for experiments.

3.2.2 Interaction of Skp and SurA with OmpA and OmpC/F and complex stability

Complexes between Skp, SurA and OmpA, OmpC and OmpF needed to be generated and optimised. Furthermore, the stability of these complexes was investigated.

Complex formation was monitored by analytical SEC. Here, a shift of the respective Skp and SurA elution volume upon addition of unfolded OmpA, OmpC and OmpF indicated complex formation.

Skp and OmpA/OmpC form a stable complex

Skp was incubated with either OmpA or OmpC, and complex formation was analysed by analytical SEC. Interaction conditions of Skp with OmpA were adapted from Walton et al. (2009).

Incubation of Skp with ufOmpA results in a complex with slightly shifted elution volumes of Skp upon addition of OmpA (1.1 ml). SDS - PAGE confirms the coelution of OmpA with Skp, in a ratio that would fit to the expected 3:1 Skp(monomer):OmpA(monomer) ratio. Collection of peak fractions ('P') and reinjection showed the complex to be stable.

Incubation of Skp with ufOmpC leads to a shift of Skp elution volume of 0.06 ml (figure 3.7b). As for the experiments with OmpA, SDS - PAGE confirms the coelution of OmpC with Skp, however less effective compared to OmpA (figure 3.7a). The stability of the Skp-OmpC complex was confirmed by reinjection ('rerun') of the peak fractions 'P', resulting in the same elution profile as the original run.

In summary, it was shown that OmpA and OmpC interact with Skp in a stable manner. The complex formation between Skp and OmpA is more efficient than for Skp-OmpC. Combined with the fact that the OmpA folding state can be monitored, the Skp-OmpA complex was used for further crystallisation experiments and transfer trials to DegP.

SurA prefers binding to OmpA to OmpC/F under the same conditions

The interaction between SurA and OmpA, OmpC/F was tested by analytical SEC (figure 3.8a). A slight peak broadening of SurA upon addition of OmpC and OmpF is observed, while for OmpA a distinct peak appears 0.1 ml prior to the isolated SurA peak. Under the conditions used SurA seems to bind more efficiently to OmpA than to OmpC/F. For this reason, and because the OmpA folding state can be monitored,

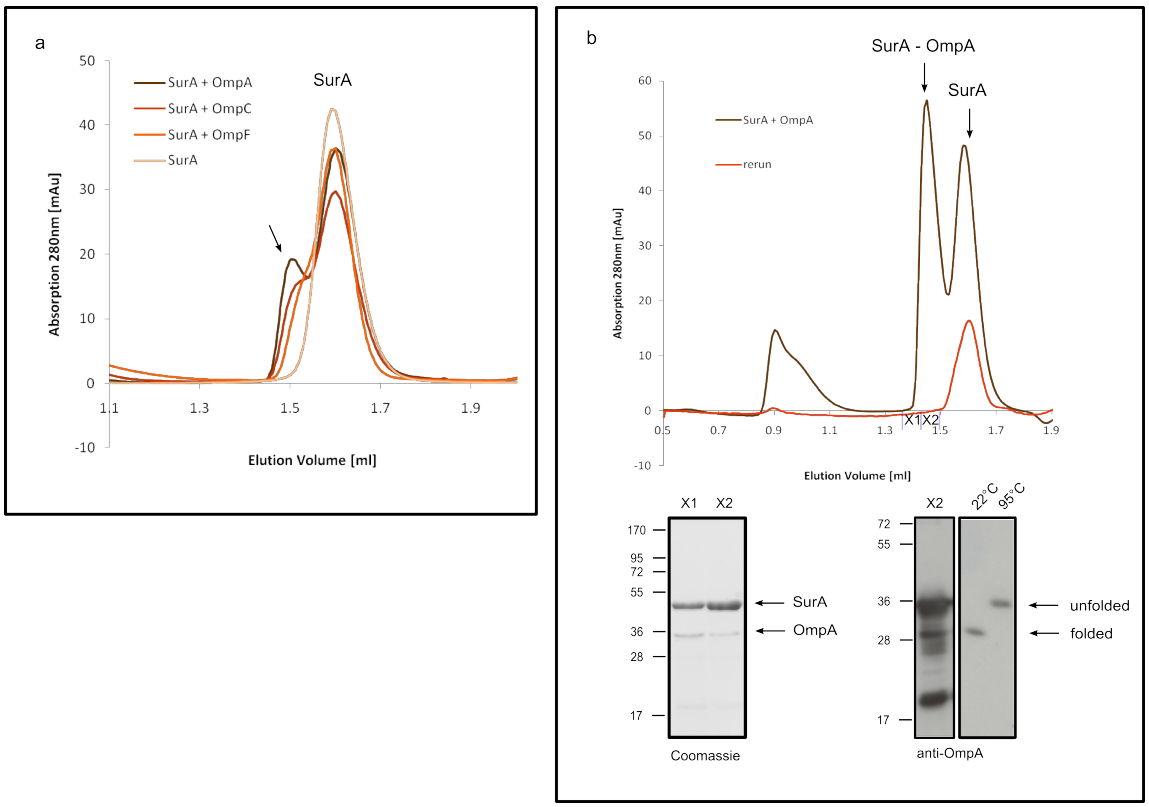

Figure 3.8: Analysis of complex formation between SurA and Omps by analytical SEC. Indicated fractions were analysed on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to SurA and OmpA are marked. Size exclusion chromatograms are labelled accordingly. Western blots are stained with anti-OmpA antibodies. **a.** SurA (20µM) was incubated with equimolar amounts of unfolded Omps (species labelled in graph) and subjected to SEC. SurA-OmpA peak is indicated by a black arrow. **b.** Optimisation of SurA-OmpA. SurA and OmpA were mixed in a 2:1 ratio and incubated at 4°C for 30 minutes.

SurA-OmpA complexes were further optimised, characterised and subsequently used for transfer experiments to DegP.

Optimisation of and stability of SurA-OmpA

In figure 3.8b, the final step in complex optimisation for the SurA-OmpA complex formation is shown. Analysis of corresponding fractions by SDS-PAGE shows an excess of SurA, in X2 more so than in X1. The excess of SurA could be indicative of the SurA-OmpA binding ratio, or it could originate from the a overlap with monomeric SurA.

Testing the stability of the SurA-OmpA complex shows that the complex upon concentration and reinjection dissociates into monomeric SurA and a small raise of aggregated OmpA in the void volume, indicating that the complex is not stable

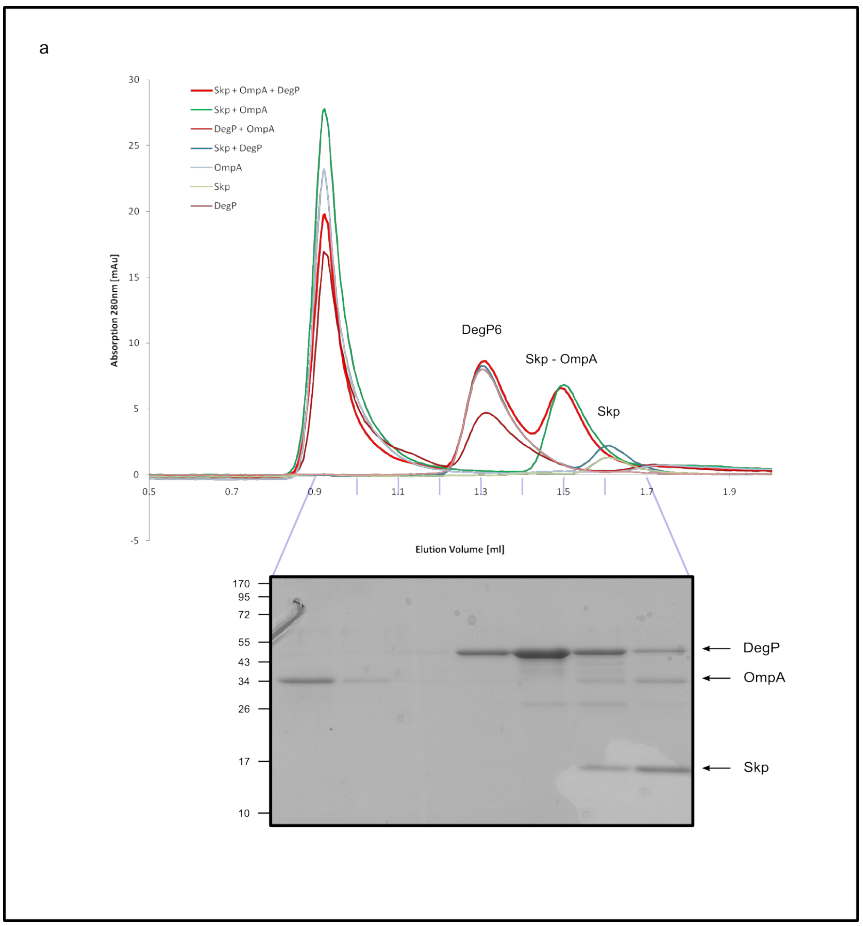

Figure 3.9: Transfer try of OmpA from Skp to DegPSA and DegPwt. All samples were mixed and incubated at 37°C for 10 minutes and subjected to analytical SEC. Size exclusion chromatograms are labelled accordingly. The transfer experiment run is shown as a red line. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to DegP, Skp and OmpA are marked.

(figure 3.8b, 'rerun').

OmpA folding state was determined by semi-native SDS-PAGE followed by western blot (figure 3.3b). Most of the OmpA protein is in an unfolded form, but there is a small fraction corresponding to folded OmpA (figure 3.3b arrow).

3.2.3 Transfer trials of OmpA from Skp/SurA to DegP

For transfer experiments to DegP the respective chaperones, Skp and SurA, were incubated with ufOmpA. Subsequently, DegP 6-mer was added and after additional incubation, analytical SEC was performed.

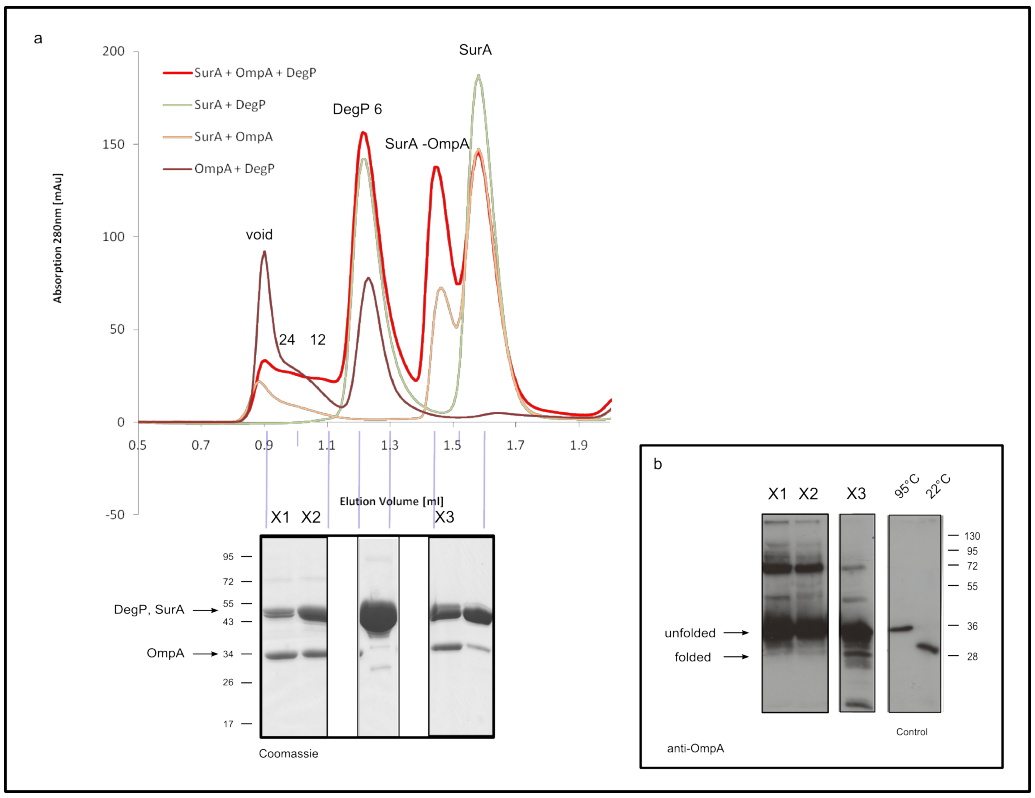

Figure 3.10: Transfer try of OmpA from SurA-OmpA to DegPSA. Analysis was done by analytical SEC. Size exclusion chromatograms are labelled accordingly. Incubation of SurA-OmpA-DegP is shown as a red line. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to DegP, SurA and OmpA are marked. Western blots are stained with anti-OmpA antibodies. **a.** SEC and analysis of complex formation and SDS-PAGE gel of selected fractions. **b.** Western blot of fractions X1, X2 and X3 from (a).

Transfer from Skp does not occur

In figure 3.9 the main experiment is shown in red. Controls of the individual proteins, or mixtures of two of each, are overlaid in the indicated colors in the graph. Here, no formation of DegP 12-mer or 24-mer could be observed as would be expected after sucessful transfer of OmpA to DegP. SDS-PAGE analysis of the indicated fractions shows no elution of DegP and OmpA at the expected fraction for DegP 12/24-mer. Identical results are achieved for DegPSA and DegPwt.

There seems to be no transfer of OmpA from Skp to DegP under these conditions.

Transfer from SurA is improbable

In figure 3.10 the main experiment is shown in red. Minor differences to the controls are visible in the area from around 0.9 ml to 1.1 ml (figure 3.10a). There

are two slight, peak-like raises, which are not present in this way in the control and whose elution volume fits to DegP 12- and 24-mer, respectively. From SDS-PAGE analysis it seems that there are two proteins at the MW of DegP/SurA, indicating that both proteins might be present in this fraction.

To determine whether OmpA in fractions X1 and X2 originates from DegP or SurA, semi-native western blot for these fractions was done (figure 3.10b). Comparing X1 and X2 to fraction X3, which represents OmpA bound to SurA, and to figure 3.3b which shows OmpA bound to DegP, shows that OmpA here resembles OmpA from the DegP-OmpA complex. Furthermore, the folded OmpA in X3 is not present in X1 or X2. This speaks against a transfer of prefolded OmpA from SurA to DegP 6-mer.

These peak-like raises in the red line in figure 3.10 are not always visible peaks, but are generally elevated over base line level.

Summarizing, transfer of OmpA from Skp to DegP is not happening under these conditions. Transfer of OmpA from SurA to DegP seems very unlikely, as there is barely a shift of DegP induced, and no folded OmpA is detected in this fraction.

3.2.4 Crystallization trials of Skp-OmpA

Recent NMR studies of the Skp-OmpA complex show that Skp binds the transmembrane domain of OmpA in an unfolded form inside its cavity, while the folded periplasmic domain sticks into the environment (Walton et al., 2009). A crystal structure of this complex might prove fascinating to acquire deeper insights into how exactly this complex is built up.

Upscaling of complex formation

To generate a sufficient amount of Skp-OmpA complex for crystallisation trials, the purification of the complex was scaled up.

Figure 3.11a shows the Skp-OmpA complex purification on a S200 10/300 column (GE Healthcare). A distinct shoulder corresponding to unbound Skp is visible, which was not detected on analytical SEC (compare figure 3.7a). Analysis by SDS-PAGE confirmed the presence of free Skp, suggesting that free Skp is not separated from the Skp-OmpA complex by SEC, resulting in an inhomogeneous sample.

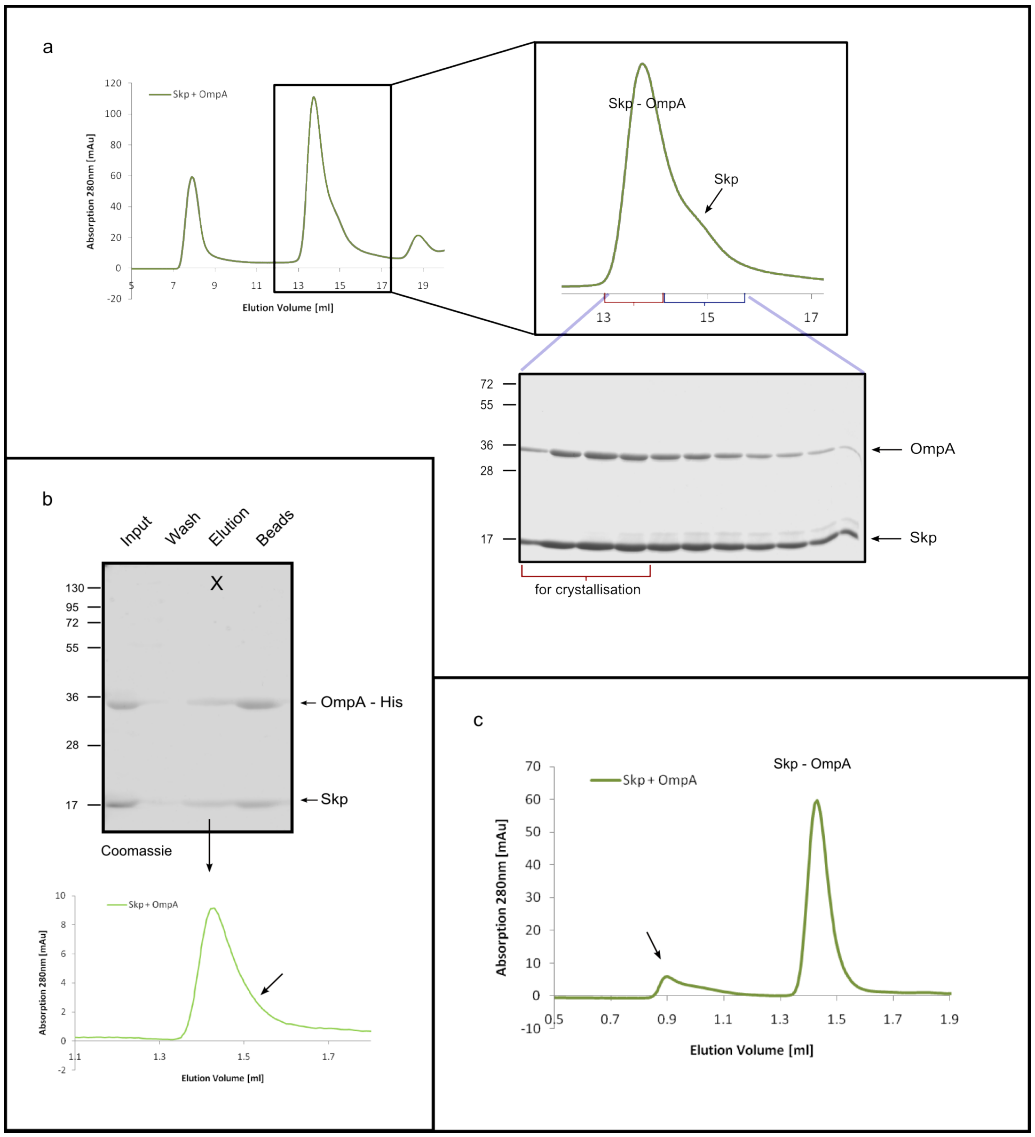

Figure 3.11: Upscaling of Skp-OmpA complex formation. For the individual experiments Skp and ufOmpA were incubated in a 3:1 ratio a and subjected to SEC. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Bands corresponding to Skp and OmpA are marked. Size exclusion chromatograms are labelled accordingly. **a.** Upscaling of the Skp-OmpA complex from analytical SEC to an S200 10/300. The peak fraction is depicted in detail in the black box. The Skp-OmpA peak is labelled. A shoulder in the peak is indicated by a labelled black arrow. Red brackets show the fractions used for crystallisation trials. Blue brackets show the part of the complex reused for further complex formation. **b.** Skp-OmpA-His complex formation. Skp and OmpA-His were incubated and subjected to purification over NiNTA beads. Aliquots were taken at the indicated steps during the purification procedure. Fraction X was subsequently run over analytical SEC. The black arrow indicates a shoulder in the elution profile. **c.** Final purification method for Skp-OmpA complex tested by analytical SEC. Position of the void volume is indicated by a black arrow.

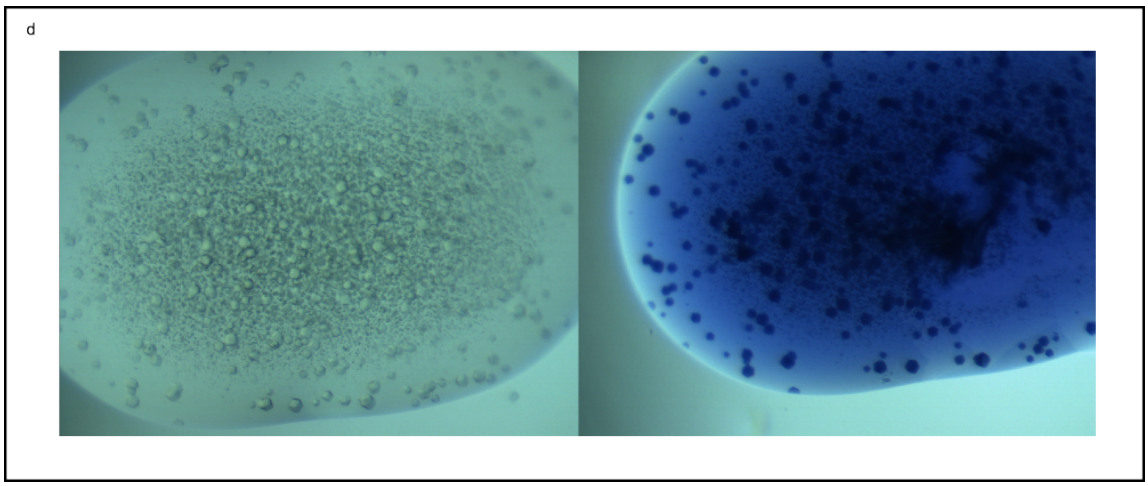

Figure 3.12: Crystallisation of Skp - OmpA. Crystals were grown in condition E5 of Crystal Screen I + II (Hampton): 5% Isopropanol, 2 M Ammmonium Sulfate, grown at 18°C. Protein concentration was 15mg/ml Skp, the ratio of Buffer:Protein was 1:2. Size of the crystals is around 30 µm. Growth time was approximately one month.

OmpA-His NiNTA does not improve sample quality

To separate Skp-OmpA from free Skp, we generated OmpA with an N-terminal His-tag to use it in a subsequent pull-down experiment. Expression and purification of this tagged protein was identical to untagged OmpA.

To separate free Skp from the Skp-OmpA complex, we performed NiNTA affinity chromatography. Therefore, Skp and OmpA-His were mixed, incubated, and subsequently incubated with NiNTA beads (figure 3.11b). Upon elution, we observed that Skp is pulled down with OmpA. Skp is in slight excess, which might approach a Skp:OmpA 3:1 ratio. Purification of the complex seems to work nicely and applying the Skp-OmpA complex after SEC to the NiNTA pull-down showed the same result (not shown).

After elution from the beads, the purified Skp-OmpA-His complex was subjected to analytical SEC (figure 3.11c). Analysis of the elution profile again revealed a trailing of the peak, indicating free Skp. This result indicates that the complex is slightly unstable, and dissociation of Skp-OmpA constantly occurs to a small degree. Therefore, purification by NiNTA did not improve the sample homogeneity of the Skp-OmpA complex.

Optimisation of large-scale complex formation

As the complex is slightly unstable in the course of SEC, complex purification by SEC does not necessarily increase complex homogeneity. Therefore, we tested a second approach for complex purification, by removing chaperone-free, aggregated OmpA from the sample by sedimentation. This kept the sample quality approximately the same, but greatly reduced the time, and highly increased the yield (by minimising losses during SEC) needed for complex purification.

After mixing and incubation of Skp with OmpA, the sample was subjected to centrifugation at 20 000xg for 30 minutes. The aggregated OmpA pelleted while the complex remained in the supernatant. Analytical SEC of the supernatant shows that the Skp-OmpA complex is in the correct position, while almost no aggregated OmpA was detected in the void volume (figure 3.11c). The supernatant was concentrated and used for crystallization trials.

Crystallisation and crystal fine screen

Initial crystal setups were done with the combined peak fractions of several SEC runs (figure 3.11a, highlighted in red brackets). To increase the amount of available protein, the elution fractions containing free Skp (figure 3.11a, highlighted in blue brackets) were incubated with ufOmpA and again purified.

For crystallisation screening, we used sitting-drop, vapour-diffusion experiments with three different commercial screens (Index + Crystal I+II (Hampton), JBS Kinase 1-4 (Rigaku)). In these experiments, we observed crystalline like structures in only one condition (figure 3.12). The protein content (as opposed to salt) of the crystals was affirmed by the deep blue color upon adding Izit Crystal Stain. However, it is not discernible if these crystals contain Skp, OmpA, or a complex of both.

Fine screens of the crystals were designed as described in section 2.11, Materials and Methods. Samples for fine screens (Skp01, Skp02) were prepared collecting SEC fractions. For a second try of fine screen (Skp03) optimised method (figure 3.11c) was used. So far, neither screen produced further crystals.

Summarizing, from a sample containing Skp-OmpA complexes, initial crystal hits were observed. Two methods for complex purification were developed, and both used for crystallisation fine screens. So far, we failed reproducing these crystals.

3.2.5 In vitro refolding trials of OmpC were not successful

Previously, OmpC was purified from inclusion bodies and was subsequently refolded *in vitro* to SDS-stable trimers (Kumar and Krishnaswamy, 2005). Here, the reconstitution of the DegP - Omp complex with *in vitro* refolded Omps might be a valuable tool to generate complexes containing folded Omp species inside the DegP 12/24-mer cavity.

However, when we tried to reproduce published refolding protocols (Kumar and Krishnaswamy, 2005; Robert et al., 2006), we were not able to generate folded OmpC. Figure 3.13a shows a list of the approaches used to reproduce OmpC refolding.

Refolding was monitored as depicted in figure 3.13b. Each sample was analysed by semi-native SDS-PAGE. In case of successful refolding and concurrent trimerisation of OmpC heated fractions would show a migration to around 38 kDa corresponding to the unfolded controls, while the semi-native sample would migrate to around 130 kDa, marked by a labelled black arrow.

When initial refolding attempts failed (see figure 3.13a), we started to vary protein concentration, protein/buffer ratio and the detergent concentration. As this failed we tried different Omp refolding protocols (see section 2.6.7 in Materials and Methods). Neither of these approaches showed any refolding success.

It was so far not possible to reproduce OmpC refolding.

3.3 Complex formation with Omps extracted from the E.coli outer membrane

Besides refolding, another strategy to obtain folded Omps is to extract them directly from the bacterial outer membrane (OM). OmpA and the porins OmpC and OmpF constitute the main bulk of Omps in the OM (see section 1.2). Therefore, in OM extractions, the main protein content consists of these three Omps.

Here, we tested if folded Omps are present in OM preparations (OM preps). Then we incubated these with DegP to see if it is able to form complexes with Omps from OM extractions and subsequently determined the folding state of the Omps.

a

Protocol	Buffer	Detergent	Detergent Concentration [%]	Omp	Ratio Protein : Buffer	Protein Concentration (initial) [mg/ml]	Protein Concentration (final) [mg/ml]
OmpCRF	OmpCRF	C12E9	0,2	C	1:5	1	0,2
OmpCRF	OmpCRF	C12E9	0,2	C	1:5	1	0,2
OmpCRF	OmpCRF	C12E9	0,2	C	1:10	1	0,1
OmpCRF	OmpCRF	C12E9	0,2	C	1:5	0,5	0,1
OmpCRF	OmpCRF	C12E9	0,2	C	1:5	2	0,4
OmpCRF	OmpCRF	C12E9	0,2	C	1:50	10	0,2
OmpCRF	OmpCRF	C12E9	0,2	C	1:50	5	0,1
OmpCRF	OmpCRF	C12E9	0,2	C	1:50	2,5	0,05
OmpCRF	OmpCRF	C12E9	0,2	C	1:100	5	0,05
OmpCRF	OmpCRF	C12E9	0,15	C	1:50	2,5	0,05
OmpCRF	OmpCRF	C12E9	0,18	C	1:50	2,5	0,05
OmpCRF	OmpCRF	C12E9	0,22	C	1:50	2,5	0,05
OmpCRF	OmpCRF	C12E9	0,25	C	1:50	2,5	0,05
OmpCRF(init)	OmpCRF	C12E9	0,2	C	1:5	5	1
OmpCRF (init)	OmpCRF	C12E9	0,2	C	1:10	5	0,5
OmpCRF (init)	OmpCRF	C12E9	0,2	C	1:25	5	0,2
OmpCRF (init)	OmpCRF	C12E9	0,2	C	1:50	5	0,1
X1 BamA	OmpCRF	SB12	0,2	C	1:10	1	0,1
X2 BamA	OmpCRF	SB12	0,2	F	1:10	1	0,1
X3 BamA	Bam RF	SB12	0,5	C	1:10	1	0,1
X4 BamA	BamRF	SB12	0,5	F	1:10	1	0,1
OmpCRF	OmpCRF	C12E9	0,5	C	1:5	1	0,2
OmpCRF	OmpCRF	C12E9	0,5	F	1:5	1	0,2
OmpCRF	OmpCRF	C12E9	0,5	C	1:5	1	0,2

b

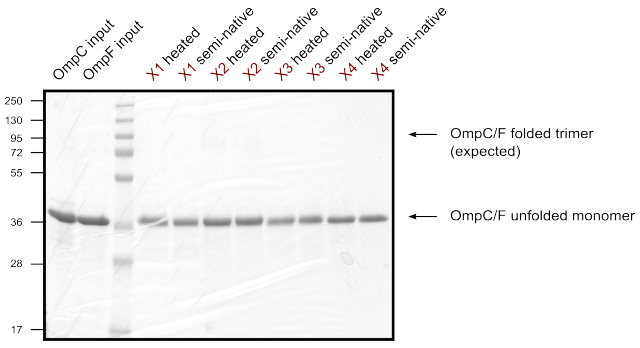

Figure 3.13: OmpC refolding. a. Table of refolding experiments. Details concerning protocols and buffer are found in section 2.6.7 in Materials and Methods. **b.** Evaluation of refolding experiments by mobility-shift assay, exemplified with the setups from (a): X1-X4. Refolding experiments are loaded on a 12% SDS-PAGE gel and stained with Coomassie Blue. Each sample is loaded twice, heated to 95°C, and non-heated (semi-native). In lane 'OmpC input' and 'OmpF input' Urea-denatured OmpC and OmpF, respectively, are loaded as a control for unfolded OmpC and OmpF. The position of the OmpC/F unfolded monomer and of the expected folded trimerised OmpC/F are marked by labelled black arrows.

3.3.1 Purification of outer membrane extracts (OM preps) and determination of folding state by semi-native SDS-PAGE

OM preps were extracted from the *E.coli* strains DH5α and the ΔOmpA strain MR58. The extraction procedure resulted in an OM pellet which was subjected to semi-native SDS-PAGE (described in section 3.1.2) to determine the folding state of the Omps.

In figure 3.14a OM preps from DH5α are shown. Two prominent bands corresponding to unfolded OmpA and OmpC/F are visible (35 and 38 kDa) in the heated fraction whereas in the non-heated fraction these proteins shift to a folded monomeric OmpA at 30kDa, and a higher molecular weight set of bands at around 130 kDa, which corresponds to OmpC/F in folded, trimeric stabilized form.

This indicates that OmpA and OmpC/F are the main protein fraction in the OM preps and that the native folding state of the Omps is retained.

To support the identity of the OmpC/F band, the OM prep from DH5α was subjected to Urea-SDS-PAGE (figure 3.14b). Under denaturing conditions the highly homologous OmpC and OmpF (38 kDa and 37 kDa; 6 amino acids difference in length) can be separated. Controls from OmpC/OmpF purifications show the same migration as the proteins in the OM prep.

In figure 3.14c an OM prep for the ΔOmpA strain MR58 is shown. In the heated fraction unfolded OmpC/F is visible at the same height as the control (OmpC input), while in the non-heated fraction this band is missing and replaced by a higher molecular weight set of bands corresponding to folded trimeric OmpC/F porins.

Determination of Omp folding state by digestion assays with DegPwt

Two methods were used for the determination of Omp folding state. Semi-native SDS-PAGE was already described in section 3.1.2. The second assay utilizes the property of DegP that it digests unfolded proteins, whereas folded proteins remain stable and are protected. By digestion assays with DegP the folding state of the Omp could be determined.

Establishment of digestion assays of DegPwt with OmpC

Before incubation with DegP the Omps had to be extracted from the OM pellet by detergents. For our experiments we use the detergent SB-12 with a concentration of 0.5% for extraction and with a final concentration of 0.25% in the incubation

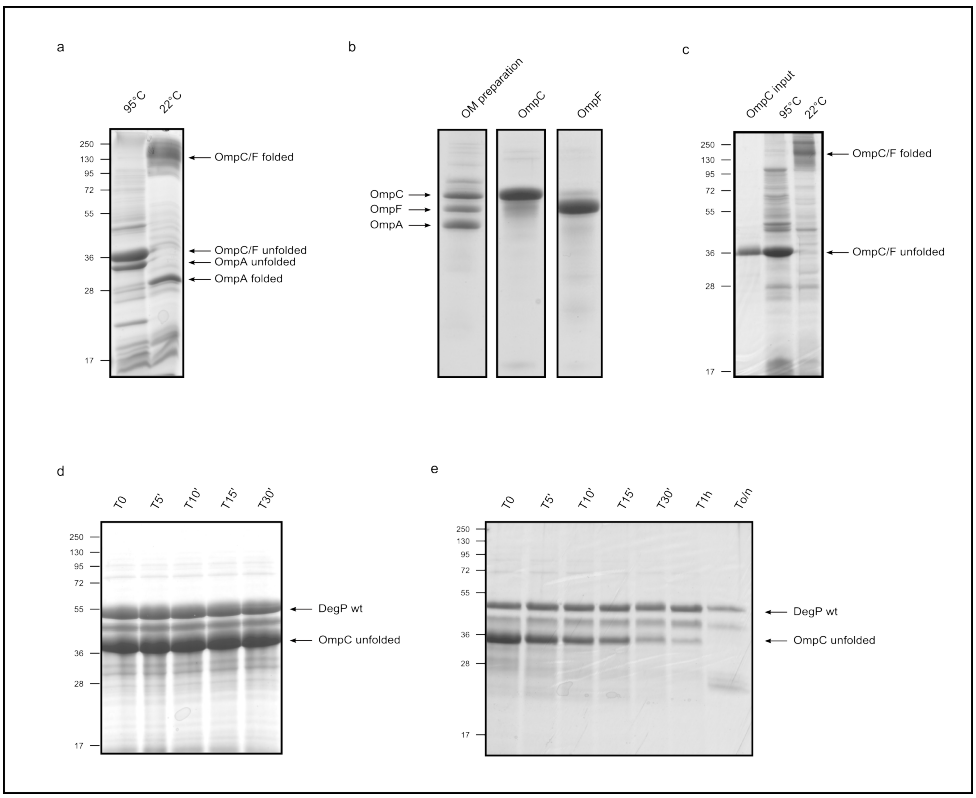

Figure 3.14: Purification of outer membrane extracts and determination of Omp folding state. Samples were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Relevant bands are marked by labelled arrows, naming their presumed folding state. **a.** OM preps from *E.coli* DH5α. **b.** Urea-SDS-PAGE of OM preparations, and IB purified, denatured OmpC and OmpF. **c.** OM preps from *E.coli* ΔOmpA strain MR58. For 'OmpC input' purified Urea-denatured OmpC was loaded as control. **d.** Digestion assay of DegPwt (30µM) against Urea-denatured OmpC (60µM) in the presence of 0.25% SB-12. At the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C. **e.** Digestion assay. DegPwt (40µM) was incubated in buffer containing 0.25% SB-12 for 10 minutes at 37°C before being subjected to analytical SEC. DegPwt peak fractions were then taken, mixed with Urea-denatured OmpC (7.5µM) and incubated at 37°C. At the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C.

mixture.

Inhibition of proteolytic activity of DegP in the presence 0.04% SDS was described previously in the lab (personal communication). We also tested this effect for the detergent SB-12 (0.25%). When DegPwt was incubated with ufOmpC in the presence of 0.25% SB-12 no digestion of ufOmpC was observed (figure 3.14d).

To test the Omp folding state by digestion assays, DegPwt needs to be active. As complex formation analyses for DegP and OM preps are performed on analytical SEC without detergent in the running buffer, we tested whether SEC could dialyse

the detergent, and digestion activity of DegP thereby be reestablished (figure 3.14e). Isolated DegPwt was incubated in buffer containing 0.25% SB-12. After analytical SEC, DegP fractions were collected and ufOmpC was added. After 1 hour most of ufOmpC was digested.

Summarising, it has been shown that OmpA and OmpC/F are present and folded in OM preps. Folding state has been shown by mobility-shift assays. Furthermore, it has been demonstrated that DegPwt activity, which is abolished during incubation in presence of detergent, is reestablished by dialysis over SEC.

3.3.2 Incubation with OM preps induces higher oligomeric species of DegP

Complex formation between DegP and OM preps was tested by analytical SEC. In the following, OM preps from DH5α are used.

In these experiments we faced the problem that the elution profiles varied with the individual OM preps, indicating variation in the extraction efficiency. The gelfiltrations in figure 3.15a and b originate from two different batches of OM preps. Even though the purification and the setup of the experiments are the same, the elution profiles look different. Most notably, in the batch from (a) there is a much clearer aggregate in the void volume and less Omps visible coeluting on SDS-PAGE. Upon incubation of OM prep with DegPwt 6-mer the elution profiles reveal the formation of DegP 12/24-mer (figure 3.15a and b). Although elution profiles vary in the OM preps, we observed batchwise consistency.

It was shown that OM preps induce higher oligomeric DegP particle formation. To exclude the presence of empty DegP particles due to higher oligomer formation induced by membrane lipids Shen et al. (2009), we further analysed the complexes.

DegP and Omps coelute

We analysed the fractions of the SEC runs of figure 3.15a and b on SDS-PAGE and semi-native SDS-PAGE. In figure 3.15a red arrows indicate very little amounts OmpA and OmpC/F.

The control with OM preps alone revealed no protein (not shown), indicating an enrichment of Omps in the fractions containing DegP.

In the results for another OM purification (figure 3.15b), Omps are clearly visible on SDS-PAGE. Here, the strongest Omp signals are in fraction 2 and 3, eluting with the DegP 12/24-meric peak, indicating coelution. Unfortunately, no control of OM

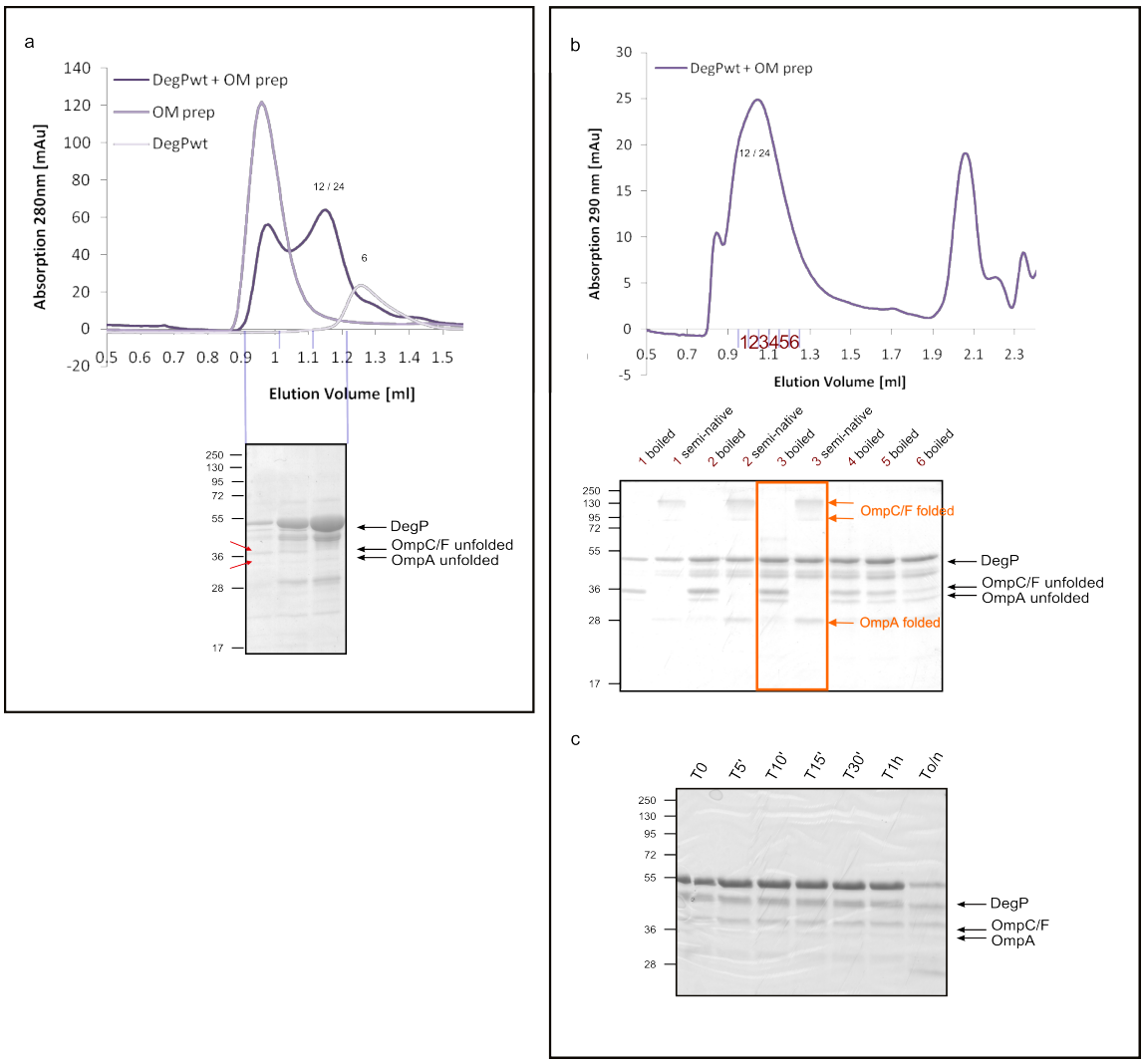

Figure 3.15: Analysis of DegPwt interaction with DH5α OM preps by analytical SEC and SDS-PAGE. OM prep pellet from *E.coli* DH5α was resuspended in buffer 0.5% SB-12 and incubated with DegPwt (40 µM) prior to analytical SEC. DegP oligomeric states are labelled by numbers above the peaks. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. Relevant bands are marked by labelled arrows. **a.** SEC elution profile and analysis of coelution by SDS-PAGE of DegPwt and Omps from OM preps. Red arrows show the position of OmpA and OmpC/F. **b.** Same SEC experiment as in (a), using different OM preparation. The orange box highlights mobility shift-assay by SDS-PAGE (heated) and semi-native SDS-PAGE (non-heated) of fraction 3. Orange arrows and labels label the folded state of the Omps in the semi-native SDS-PAGE which were analysed by mass spectrometry. **c.** Digestion assay of peak fractions of (b). At the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C.

prep was analysed for this purification.

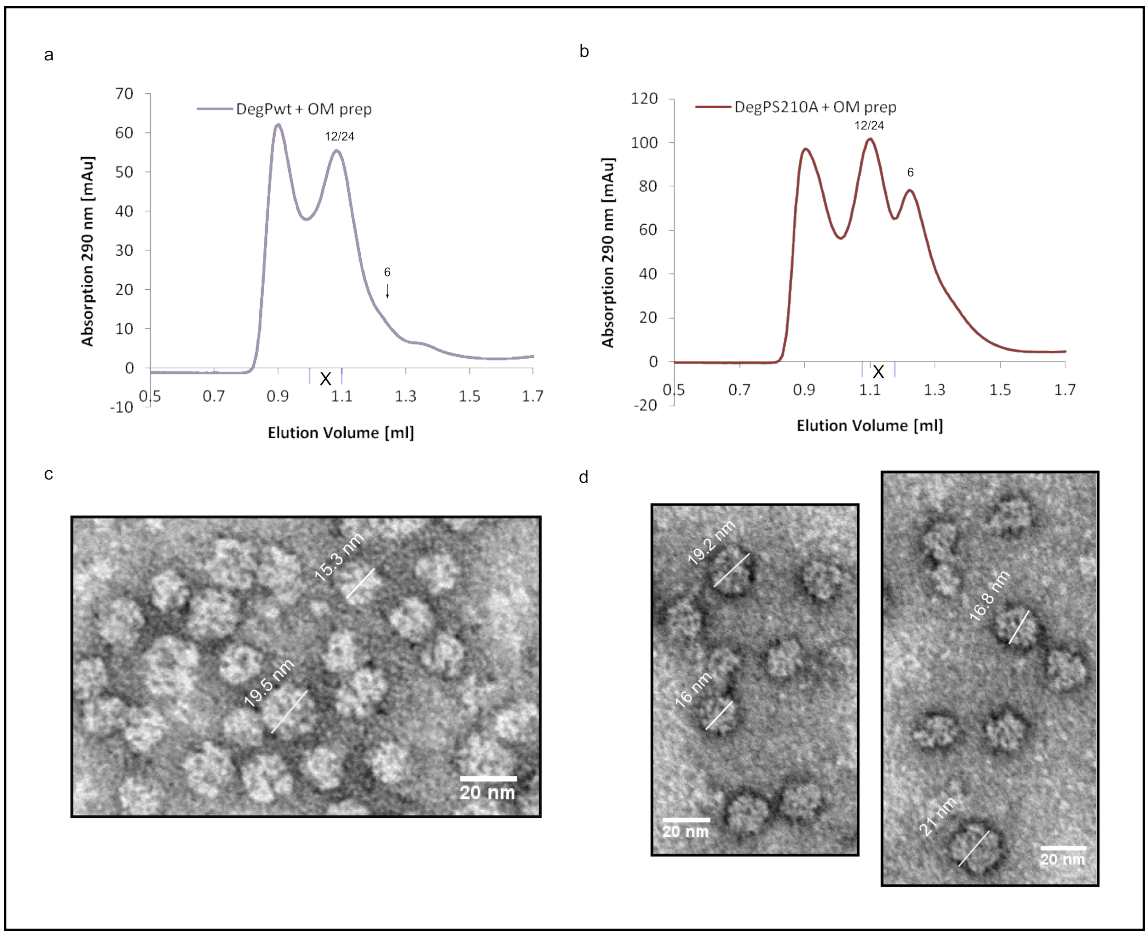

Figure 3.16: Analytical SEC profiles for DegPwt and DegPSA and subsequent negative-stain electron microscopy. Sample preparation and analytical SEC done as in figure 3.15. Negative stain was done with 2% Uranyl Acetate. For the electron micrographs a scale bar is depicted in the left or right lower corner of the picture. The diameter of selected particles is marked and labelled with their length. The expected particle size for DegP 12-mer is 16 nm, for the 24-mer 19.5 nm (Krojer et al., 2008b). **a and b.** DegPwt(a) and DegPSA(b), respectively, incubated with OM preps. **c and d.** Negative-staining electron microscopy image of fractions X from a. (c) and b. (d), respectively.

MS analysis confirms identity of bands corresponding to folded Omps

To ascertain the identity of the Omps in the sample, bands indicated by orange arrows in figure 3.15b were excised and analysed by mass spectrometry. The identity of the respective Omps was thereby confirmed.

3.3.3 Omps that coelute with DegP are stable to digestion and have the folding pattern typical for folded Omps

The folding state of the Omps coeluting with DegPwt was checked by two methods.

Semi-native PAGE is shown for peak fraction 3 in figure 3.15b, highlighted by the orange box. Upon heating, unfolded OmpA and OmpC/F are visible, which are not present in the semi-native sample, where folded OmpA migrates to 30 kDa and folded, trimeric OmpC/F to 130 kDa.

Additionally, the folding state was checked by a DegP digestion assay (figure 3.15c). Peak fractions containing DegPwt and Omps were collected and incubated at 37°C and after indicated timepoints aliquots were taken. The next morning, the Omps could still be detected, while the amount of DegP had decreased, most likely by auto-proteolysis.

Both experiments suggest that folded Omps could be bound in DegP 12/24-mer cavities.

Summarizing, it seems that DegP can take up Omps from OM preps, even though a control for DegP with membrane lipids alone is missing. The coelution seems to indicate complex formation between Omps and DegP and the Omps in these complexes were shown to be folded.

3.3.4 DegPSA and DegPwt show different SEC profiles in response to OM preps

The incubation of DegPwt and DegPSA with OM preps results in different elution profiles upon SEC, indicating a different tendency to encapsulate Omps in DegP 12/24-mer particles (figure 3.16a and b). While DegPwt shifts completely to a 12/24-meric state (figure 3.16a), for DegPS10A a considerable proportion remains in a substrate free 6-meric form, using the same concentrations of DegP and the same amount of OM prep. This result is consistent in both Om prep batches.

3.3.5 Negative-stain electron microscopy (EM) confirms particles of correct size and shape

To confirm the induced formation of DegP 12/24-mer particles upon incubation with OM preps negative-staining EM was performed.

From the OM prep incubations of DegPwt and DegPSA, figure 3.16a and b respectively, indicted peak fractions were taken and subjected to negative-staining EM (figure 3.16c and d). Here, the sample quality was generally very good, showing many particles and very little debris. For the particles consisting of DegPwt (figure 3.16c) and DegPSA (figure 3.16d) both a mixture of bigger and smaller particles

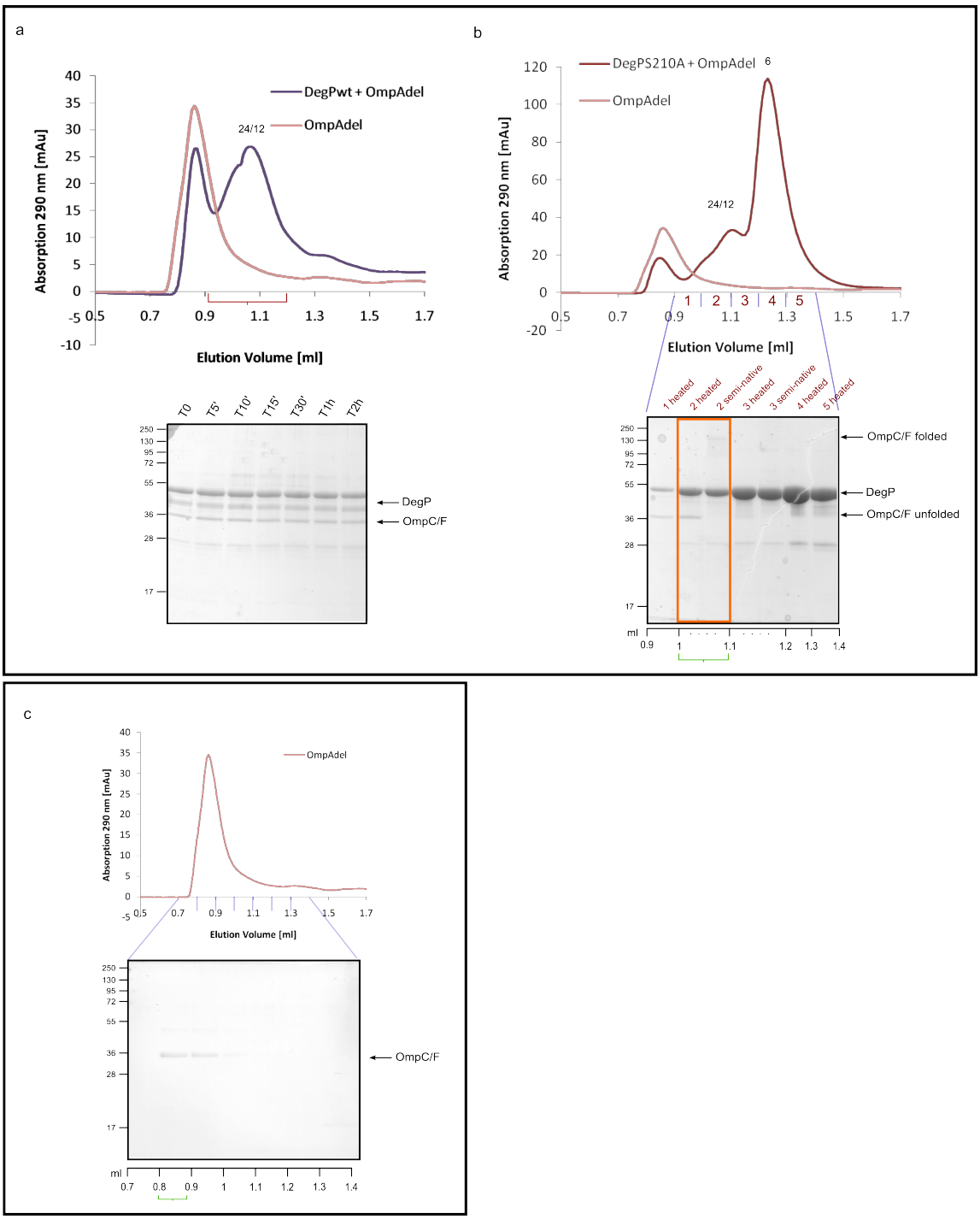

Figure 3.17: Analysis of DegPwt interaction with OM preps from the ΔOmpA strain MR58 by analytical SEC and SDS-PAGE. OM prep pellet from *E.coli* ΔOmpA strain MR58 was resuspended in buffer containing SB-12 and incubated with DegPwt (40 µM) and subsequently subjected to analytical SEC. DegP oligomeric states are labelled with the respective numbers above the peak. Indicated fractions were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. The elution volume corresponding to the fractions is labelled below the gel. Relevant bands are marked by labelled arrows. **a.** Incubation of DegPwt with ΔOmpA OM prep and digestion assay. The red bracket shows the peak fractions which were collected and used for the digestion assay. At the indicated timepoints aliquots were taken and the reaction stopped by adding SDS-sample buffer and boiling the sample for 10 minutes at 95°C. **b.** Incubation and coelution analysis of DegPSA with ΔOmpA OM prep. The orange box highlights SDS-PAGE and semi-native SDS-PAGE for fraction 2. The green brackets show the elution volume where the OmpC/F band is most prominent. **c.** Control run for isolated ΔOmpA OM prep and SDS-PAGE. The green brackets mark the elution volume where OmpC/F band is most prominent.

are visible, which correspond in size to DegP 24-mer (19.5 nm) and DegP 12-mer (16 nm) (selected particles are marked with their diameter). This is in line with the peak elution volume, which indicated a mixture of DegP 12/24-mers. Therefore, EM particles show the expected sizes and phenotype, and there is no obvious difference visible between DegPSA and DegPwt.

3.3.6 Incubation of DegPwt and DegPSA with OM preps from an ΔOmpA strain

For the OmpA deletion strain MR58 the experiments described in section 3.3.2 were repeated. The absence of OmpA in the sample should provide more homogeneous complexes.

In figure 3.17a analytical SEC after incubation of DegPwt with ΔOmpA OM preps is shown. Here, DegPwt shifts to a 12/24-mer form. Peak fractions indicated by a red bracket were collected and the digestion assay was performed as described in section 3.3.2. OmpC/F remains stable over time, indicating a folded state.

In figure 3.17b DegPSA was incubated with OM preps from the ΔOmpA strain. Here, as for DegPSA confronted with DH5α OM prep, a main subfraction of DegP did not bind Omps, and relatively little of DegP shifted to 12/24-meric form. Analysis of fractions of the DegP 12/24-mer peak by SDS-PAGE shows coelution of DegPSA and OmpC/F (figure 3.17b). Semi-native SDS-PAGE of fraction 2 confirmed the presence folded OmpC as a faint band around 130kDa, and the presence of unfolded OmpC in the heated fraction.

To thereafter confirm the complex of DegP and Omps, figure 3.17c shows the result of analytical SEC of the ΔOmpA prep without prior incubation with DegP. In the control we find the main OmpC/F amount eluting between 0.8-0.9 ml (green brackets), whereas the DegP-Omp complexes elute between 1 and 1.1 ml (figure 3.17b). This shows a enrichment of OmpC/F in the DegP containing fraction.

Summarizing, the results of the OmpA deletion strain are in line with the ones achieved for DH5α.

4 Discussion

4.1 Folding activity of DegP

We investigated the capacity of the DegP 12/24-mer cavity to provide an environment suitable for de novo Omp folding. Therefore, we incubated DegP with unfolded OmpA, respectively OmpC/F, and monitored both complex formation and the Omp folding state. As DegPwt immediately degrades unfolded Omps, we used the proteolytically inactive DegPSA mutant to test theoretically the DegP folding activity.

Complex formation of DegPSA with unfolded OmpC/F was very efficient, in contrast to OmpA where complex formation was rare. In both cases, however, we detected no folded Omp species, suggesting that DegP does not promote Omp folding (figure 3.2 and 3.3).

In another approach we aimed to trap unfolded OmpA in peptide-induced DegPwt 12-mer cages and monitored its folding state. In these experiments, we used the signal peptides (SP) of OmpC/F. These peptides are cleaved off the Omp pro-peptides after Sec translocation and might signal nascent Omp arrival to the DegP chaperone. We used DegPwt for better approximation of *in vivo* conditions and since OmpC/F SPs induced DegP 12-mer only in the DegPwt and not in the proteolytically inactive DegPSA mutant (figure 3.4). In this setup, barely any complex formation between DegPwt and OmpA was observed, and OmpA was immediately degraded by DegPwt (figure 3.4). Interestingly, the SP itself induced conformational changes in unfolded OmpA. In all experiments where the SP was present in the SEC buffer an OmpA species with a similar migration pattern in semi-native conditions as the folded species was observed (figure 3.4). These pseudo-folded OmpA species also appeared in the absence of DegP and at inconsistent elution volumes, strongly indicating an experimental artifact introduction by the SPs. We surmise that the highly hydrophobic properties of the OmpC/F SPs allowed interaction with hydrophobic OmpA patches and introduced structural rearrangements, which we observed as a folded-like pattern in our experiments. However, no folded OmpA could be detected in complex with DegP.

These results show that preforming of DegP 12-mer does not promote DegP chaperone and folding activity.

The obtained results and the failure of de novo Omp folding by DegP suggest that the DegP cavity might not provide an environment suitable for de novo Omp folding. As Omps were shown to fold into lipid micelles and membranes spontaneously (Kleinschmidt, 2003; Surrey and Jähnig, 1995), we suggest that a lipid-like environment is necessary for Omp folding while DegP might not provide the appropriate hydrophobic contacts.

4.2 OmpC/F signal peptides show different effects on DegPwt and DegPSA

OmpC/F signal peptides interestingly induced DegP 12-mer formation only for DegPwt, but not for the proteolytically inactive DegPSA mutant (section 4.1). This indicates the relation of the effects of these peptides to DegP protease activity. To further investigate this effect we mutated 3 putative DegP cleavage sites within the OmpF SP (V to E) and indeed observed differences in the behaviour of DegPwt in dependence on the individual mutations (figure 3.5).

Binding the C-terminus of substrates to the DegP PDZ domain of triggers oligomeric rearrangement and also tethers the substrate in a favorable distance of the cleavage site (Krojer et al., 2008a). DegP cleaves C-terminal of amino acids, which in turn favourably bind to the PDZ domain, thereby ensuring efficient processive cleavage of substrates. PDZ domain binding however is not essential for cleavage activity (Krojer et al., 2008a).

The failure of DegPSA to form 12-mers in presence of the SP indicates that the OmpC/F SP C-terminus (-ANA) inefficiently interacts with the DegP PDZ domain (figure 3.5) and therefore no DegP 12-mers are formed. In contrast, while in DegPwt the SPs bind the PDZ domains with equal inefficiency, they can reach the proteolytic site independently. There they are cleaved, creating peptide fragments with a C-terminus capable of PDZ domain binding. These peptide fragments allosterically activate DegP to form 12-mer particles.

The effects of the SP V to E mutations in the SP are, as expected, only observed for DegPwt and not for DegPSA (figure 3.5). The three valines mutated on the SP sequence can be separated into 2 cleavage sites, as two valines are in close proximity in the middle of the sequence (site 1) while a single valine is in the last third of the peptide sequence (site 2) (figure 3.5). For the peptides 2808 and 2809 (figure 3.5),

where one of the two valines at site 1 is mutated, DegP 12-mer formation is reduced by about 50%. For the peptides 2810 and 2308 (figure 3.5) however, where one of the two cleavage sites is fully mutated, DegP 12-mer formation is almost completely abolished. The different effects of the SPs on DegP 12-mer formation indicate that these mutations either impair binding, cleavage, or both. To further investigate the binding of these peptide mutants to DegP we aimed to do ITC studies which unfortunately were not sucessful due to inefficient peptide solubility. So, the detailed workings of this cleavage pattern could not be determined in the course of this work. However, it poses an interesting question for further studies.

4.3 Complex generation of the chaperones DegP, Skp and SurA with OmpA and OmpC/F

Chaperone-Omp complexes between the DegP, Skp and SurA and Omps were generated by incubation of the chaperones with unfolded OmpA and OmpC/F. DegP very efficiently formed complexes with OmpC/F (figure 3.2), while complex formation with OmpA was rare (figure 3.3). In contrast, the chaperones Skp and SurA formed complexes with OmpA more efficiently than with OmpC/F (figure 3.7 and 3.8), suggesting a substrate specificity of chaperones in respect to the three major Omps.

DegP substrate specificity may be caused by substrate binding via the PDZ domains. The DegP PDZ domains coordinate the C-terminal carboxylate group of substrates by providing binding pockets for the three C-terminal amino acids (Krojer et al., 2008a). The C-terminal motif of OmpC/F is -YQF and of OmpA -PQA. The OmpC/F -YQF motif was shown to bind and activate DegP (Krojer et al., 2008a), while for OmpA -PQA no data is available. However, the inefficient complex formation with OmpA (figure 3.3) indicates weak PDZ domain binding. We suspect that the proline in position -3 might disturb binding by β-augmentation to the PDZ domain, while the Q and A in the -PQA motif are in favorable PDZ binding positions (Krojer et al., 2008a). Another reason for inefficient binding of OmpA might be its C-terminal periplasmic domain that folds spontaneously in solution (Smith et al., 2007), probably making the OmpA C-terminus less accessible for PDZ binding.

Skp and SurA both bind more efficiently to OmpA than to OmpC/F (figure 3.7 and 3.8). This may be caused by the different domain morphology of the Omps.

The OmpC/F sequence encodes for one large transmembrane β-barrel domain, while OmpA is separated into a small β-barrel domain and an additional soluble periplasmic domain. The periplasmic domain might increase OmpA solubility and thereby facilitate access for the chaperones. In contrast to DegP, Skp and SurA recognise not only the C-termini of substrates, but may depend on larger motifs for substrate recognition (Bitto and McKay, 2002; Qu et al., 2007; Walton et al., 2009; Xu et al., 2007).

For Skp, OmpA binding results in a rather strong shift on gelfiltration and upon complex formation more OmpA coeluting compared to OmpC (figure 3.7). A reason for the increased shift in SEC may be the Skp binding mode, in which the β-barrel domain is enclosed in the Skp trimer cavity (Qu et al., 2009; Walton et al., 2009). Of OmpA only the β-barrel domain is bound by Skp, but the soluble periplasmic domain protrudes into the environment (Walton et al., 2009). For OmpC the entire protein, solely composed of a β-barrel domain, might need to be protected inside the Skp cavity. This might make the Skp-OmpA complex more bulky than the Skp-OmpC complex and causing a higher apparent mass weight on SEC. The smaller amount of coelution of OmpC with Skp indicates that complex formation might not be as efficient due to substrate accessibility.

Complex formation between SurA and OmpA was more efficient compared to OmpC/F (figure 3.8). Here, the SurA peptidyl-prolyl-isomerases (PPIase) function might explain the discrepancy. OmpA contains 19 prolines in its sequence (while OmpC/F contain only 4) and therefore might have an increased affinity for SurA binding. Even though SurAs PPIase function is decoupled from its chaperone function (Behrens et al., 2001), it might nevertheless influence binding effiencency of Omps to SurA.

Analysis of the coelution of fractions of SurA and OmpA after SEC by SDS-PAGE showed a clear excess of SurA over OmpA (figure 3.8). This might either be explained by inefficient chromatographic separation of SurA and SurA-OmpA complex and/or an indication of multiple SurA molecules binding one Omp substrate. This is in line with results observed by Hagan et al. (2010) who suggested that several SurA molecules per outer membrane protein are required for transport of substrates to the Bam complex. Recently, the crystal structure of a homologous protein to SurA, the Trigger Factor, was solved in complex with substrate showing an equimolar (2:2) ratio (Martinez-Hackert and Hendrickson, 2009). This structure might provide

some hints into a probable SurA-Omp binding mode, which would be interesting to elucidate.

For transfer experiments of Omps from Skp and SurA to DegP, OmpA was chosen as a model Omp, because its monomeric folding state can be monitored.

In our experimental setup no transfer of OmpA from Skp or SurA to DegP was detected (figure 3.9 and 3.10), indicating that transfer of Omps either does not happen or an additional factor is needed. In contrast to the SurA-OmpA complex the Skp-OmpA complex was quite stable under the given conditions (figure 3.7 and 3.8). Previous studies described LPS as a possible release trigger for the Skp-OmpA complex (Qu et al., 2009). In future transfer studies, survey of LPS as a release factor might enable transfer of Omps.

4.4 Interaction of DegP with folded Omps extracted from the outer membrane

To test the interaction of DegP with *in vivo* folded Omps, *E.coli* outer membranes were prepared (OM preps) and Omps extracted with the detergent SB-12. Upon incubation with DegP, DegP formed 12/24-meric particles containing folded Omps (figure 3.15).

Unfortunately, *E.coli* total lipid was previously shown to induce higher oligomeric forms of DegP (Shen et al., 2009) so we set up further experiments to rule out a membrane-induced formation of empty particles.

We showed that folded OmpA and OmpC/F did coelute with DegP and were enriched in DegP 12/24-mer containing fractions (figure 3.15 and 3.17).

Negative-staining electron microscopy (EM) confirmed the presence of DegP 12/24-mers and showed the expected particles (figure 3.16). The mixture of DegP 12- and 24-mer states observed on SEC was consistent with the EM results. To distinguish empty particles from substrate bound particles, our images were compared to negatively stained substrate-bound and substrate-free 12-mers of the close DegP homologue DegQ (personal communication). In substrate-free DegQ 12-mers the pores in the 12-meric structure are clearly visible, while in the substrate-bound DegQ 12-mers no pores can be observed. In our DegP 12/24-mers no pores are visible and particles resemble substrate-bound DegQ, further confirming DegP-Omp complex formation.

We furthermore observed reduced complex formation capabilities of the proteolyt-

ically inactive DegPSA in contrast to DegPwt (figure 3.16). The only difference between DegPwt and DegPSA is the substrate processing proteolytic site and indicates a substrate-dependent, as opposed to membrane-induced, behaviour. The higher complex formation efficiency of DegPwt might indicate that DegPwt not only forms complexes with intact, folded Omps, but also forms protease-active particles degrading misfolded Omps present. These may be present in the sample because they may have lost their secondary structure due to the disruptive, stressful outer membrane extraction process.

It is necessary to state that results from experiments with OM preps were batch-wise consistent, results from different batches varied in respect to the different quantity of complex formation (figure 3.15). Despite this, the conclusion gained from the individual experiments were consistent. However, a more exact reproducibility would advantageous. To achieve this, the detergent content of 0.5% SB-12 could be increased to 1-2 % to ensure complete and reproducable Omp extraction from the OM preps. Also, the choice of detergent might be altered to C12E9. DegP was shown to interact with positively charged lipids, but very weakly with neutral ones (Shen et al., 2009). So a change from the zwitterionic SB-12 to the neutral C12E9 might decrease the background DegP oligomerisation even further. SB-12 as well as C12E9 were used in Omp refolding protocols (figure 3.13) (Kumar and Krishnaswamy, 2005; Robert et al., 2006) and therefore seem a suitable choice for Omp solubilisation.

DegP complexes with folded OmpA and OmpC/F resulted in a mixture of DegP 12/24-mer complexes. From coelution and folding state studies, we determined that folded OmpA, as well as trimeric OmpC/F were bound inside DegP (figure 3.15 and 3.17). For structure solution studies it would be interesting to enrich the DegP 24-mers as they have the possibility to accommodate trimeric OmpC in their cavity. Furthermore, it would be interesting to test whether OmpC/F trimers are always in the DegP 24-mer, and OmpA in the DegP 12-mer. Unfortunately, in experiments with the ΔOmpA strain (figure 3.17), we observe DegP 12- and 24-mers in complex with OmpC/F, indicating that DegP also binds OmpC/F monomers. To generate complexes of DegP solely with folded OmpA, we might overexpress tagged OmpA in the ΔOmpA strain and purify folded OmpA by affinity purification.

4.5 Reconstitution of DegP-Omp complexes

The aim of this study was to generate DegP-Omp complexes *in vitro* for structure determination. First, we achieved complexes between DegP and unfolded OmpC/F very efficiently while with OmpA complex formation was rather rare (section 4.1). These complexes however are not usable for further structure elucidation of the DegP-Omp complex, as for this aim a folded Omp inside DegP is imperative. Complexes with prefolded Omps were not achieved as the transfer of Omps from Skp or SurA to DegP failed (section 4.3).

Finally, complexes of DegP with folded Omps extracted from the OM were achieved (section 4.4). In OM preps from the *E.coli* DH5α strain the main protein fraction were OmpA and OmpC/F. Since this strategy does not provide a sufficiently homogeneous sample, we generated complexes of DegP, which contain mainly the porins OmpC and OmpF by using OM preps from a ΔOmpA strain. To achieve more homogeneous samples by this method, selective expression of either OmpC or OmpF could be induced by growth media composition (Baslé et al., 2006; Batchelor et al., 2005). However, drawbacks of the OM prep usage concern the exact composition and purity of OM preps. Furthermore, for DegP-OM prep interaction, inter-batchwise inconsistencies were observed.

Ultimately, to circumvent the drawbacks of OM extractions the usage of *in vitro* refolded Omps purified from inclusion bodies would be advantageous. *In vitro* refolded Omps have clear advantages in sample purity and reproducibility, and furthermore allow complete control over the reaction conditions, especially concerning the detergent/lipid composition. *In vitro* refolding of OmpC was previously performed by Kumar and Krishnaswamy (2005). Unfortunately, despite a variety of attempts, we were not able to reproduce these experiments so far (figure 3.13). However, a continuation of experiments in this direction would be sensible to provide good samples for subsequent structure elucidation. *In vitro* refolded Omps complexed with preferably DegPwt (to remove any unfolded Omps) would provide an excellent basis for detailed crystallographic or cryo-EM structure solution.

4.6 DegPs role in Omp biogenesis and protein quality control

The data obtained in this study does not support a potential role of DegP as a folding factor in Omp biogenesis (section 4.1 and 4.3). We found no evidence for

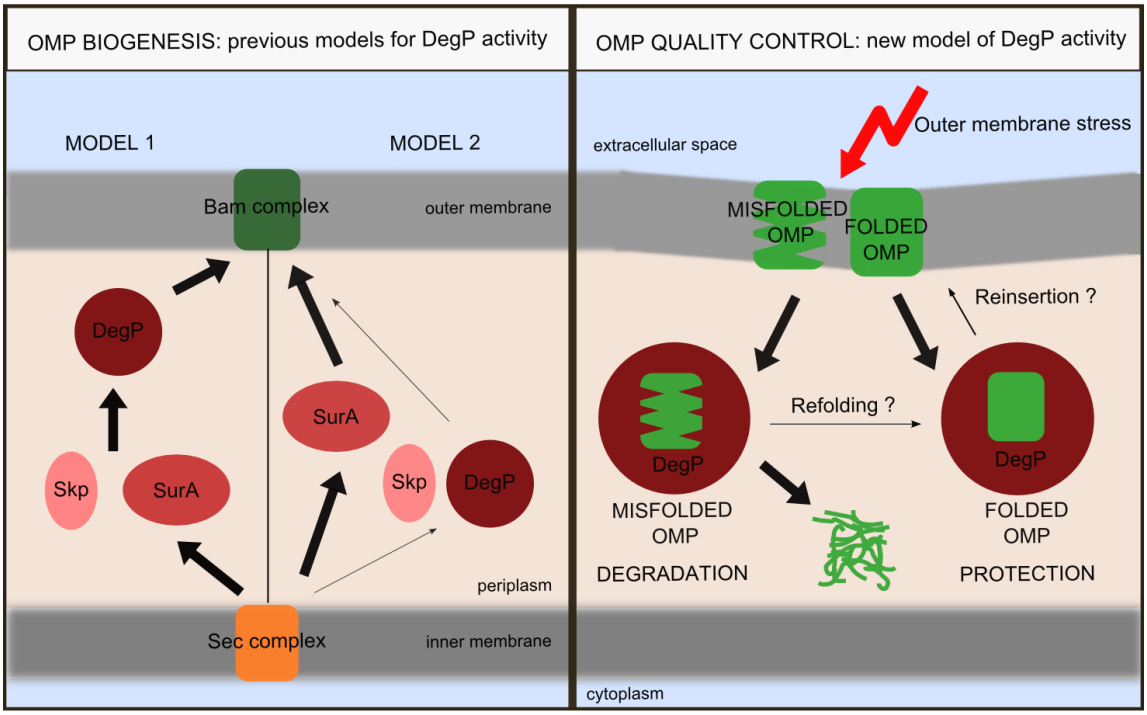

Figure 4.1: DegPs role in Omp biogenesis and Omp quality control. (Left side) Previous models propose a role of DegP in Omp biogenesis. Figure adapted from Introduction figure 1.5 in section 1.2.4. Here, we observed no transfer of Omps from Skp and SurA to DegP (Model 1) and also found no evidence for a role of DegP in Omp biogenesis. (Right side) We propose a new model for DegP activity, with major involvement in Omp quality control. In the event of outer membrane stress, DegP takes up misfolded Omps and degrades them to prevent their aggregation and subsequent damage to the cell, while folded Omps are protected inside the DegP cavity. If DegP is capable of refolding only slighly misfolded Omps and of reinserting folded Omps into the outer membrane is currently not known.

a de novo Omp folding activity of DegPSA. Furthermore, immediate digestion of unfolded Omps by DegPwt, even upon preforming of DegP 12-mer cages (section 4.1), disagrees with a folding activity *in vivo*. We also found no evidence for a second-step quality control factor function of DegP in Omp biogenesis, and we observed no transfer of 'prefolded' Omps from Skp or SurA to DegP (section 4.3). Taken together, these results speak against a DegP folding factor role in Omp biogenesis. Furthermore, a role of DegP as a folding factor is controversial to the models proposed for Skp and SurA, which keep their substrates deliberately in an unfolded state (Allen et al., 2009).

Nevertheless, folded Omps were found inside the DegP cavity from isolated DegP 12/24-mers (Krojer et al., 2008b). The results presented in this work suggest that these Omps did not get folded in the cavity but might have been enclosed by DegP

in an already folded form. For the extremely stable Omp β-barrels the hydrophobic environment provided by the DegP cage might be sufficient to protect their folded form outside the membrane.

Our results support the ability of DegP to enclose folded substrates (section 4.4). We purified folded Omps from detergent-solubilised outer membranes, which DegP subsequently encapsulated in a stable manner. During the harsh process of OM extraction, the Omps contained are exposed to a great amount of stresses due to cell disruption. So they might simulate a stressed DegP substrate population, substantiating a role of DegP as a very important protein quality control factor in the periplasm.

Even though a role of DegP in Omp biogenesis is not strongly supported, deletion of DegP show decrease of major Omps present in the OM (Krojer et al., 2008b). This might hint at a hitherto undiscovered role in Omp biogenesis, but also indicate that DegP might have the ability to reinsert captured, folded Omps into the membrane independently of the Bam complex. However if this happens or how it could work mechanistically is not yet clear.

In the end, many open questions concerning the exact role of DegP remain. The experiments done in this study favour a main role of DegP as a protein quality control factor over a role in Omp biogenesis and are summarised in figure 4.1.

4.7 Summary and outlook

We generated complexes of the *E.coli* protein quality control factor DegP with unfolded and folded Omps with the aim of subsequent structure elucidation. We furthermore investigated DegPs role in Omp biogenesis and its capability of providing a folding chamber for unfolded Omps.

Even though in the course of this study we advanced several steps further in elucidating DegPs versatile roles in the periplasm, there are many questions that remain unresolved. To further delve into DegPs role as a chaperone detailed structural information of DegP complexed to folded substrates would be necessary. An oriented arrangement of Omps inside the DegP cavity could present essential clues as to the binding mode and binding requirements for Omps inside the DegP cavity. This could further lead to insights as to how folded substrates could be released from the DegP cavity and if or what kind of factors could trigger this.

Abbreviations:

ATP: adenosine triphosphate, Da: Dalton DegPSA: DegPS210A, proteolyitcally inactive mutant, DegPwt: DegP wildtype, EC: extracellular space, NiNTA: nickel-nitrilotriacetic acid, IB: inclusion body, IEXC: ion-exchange chromatography, IM: inner membrane, IPTG: Isopropyl β-D-1-thiogalactopyranoside, LPS: lipopolysaccharides, MW: molecular weight, OM: outer membrane, OM prep: OM preparation, Omp: outer membrane protein, PPIase: Peptidyl-Prolyl-Isomerase, PQC: Protein quality control, SDS: sodium dodecyl sulfate, SDS-PAGE: SDS polyacrylamid gel electrophoresis, SEC: size exclusion chromatography, SP: periplasmic localisation signal peptide, TMD: transmembrane domain, ufOmpA/C/F: unfolded OmpA/C/F,

Bibliography

Allen, W. J., Phan, G., and Waksman, G. (2009). Structural biology of periplasmic chaperones. *Adv Protein Chem Struct Biol*, 78:51–97.

Arora, A., Abildgaard, F., Bushweller, J. H., and Tamm, L. K. (2001). Structure of outer membrane protein a transmembrane domain by nmr spectroscopy. *Nat Struct Biol*, 8(4):334–338.

Baslé, A., Rummel, G., Storici, P., Rosenbusch, J. P., and Schirmer, T. (2006). Crystal structure of osmoporin ompc from e. coli at 2.0 a. *J Mol Biol*, 362(5):933–942.

Batchelor, E., Walthers, D., Kenney, L. J., and Goulian, M. (2005). The escherichia coli cpxa-cpxr envelope stress response system regulates expression of the porins ompf and ompc. *J Bacteriol*, 187(16):5723–5731.

Behrens, S., Maier, R., de Cock, H., Schmid, F. X., and Gross, C. A. (2001). The sura periplasmic ppiase lacking its parvulin domains functions in vivo and has chaperone activity. *EMBO J*, 20(1-2):285–294.

Bernstein, H. D. (2000). The biogenesis and assembly of bacterial membrane proteins. *Curr Opin Microbiol*, 3(2):203–209.

Bitto, E. and McKay, D. B. (2002). Crystallographic structure of sura, a molecular chaperone that facilitates folding of outer membrane porins. *Structure*, 10(11):1489–1498.

Bitto, E. and McKay, D. B. (2003). The periplasmic molecular chaperone protein sura binds a peptide motif that is characteristic of integral outer membrane proteins. *J Biol Chem*, 278(49):49316–49322.

Bitto, E. and McKay, D. B. (2004). Binding of phage-display-selected peptides to the periplasmic chaperone protein sura mimics binding of unfolded outer membrane proteins. *FEBS Lett*, 568(1-3):94–98.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72:248–254.

CastilloKeller, M. and Misra, R. (2003). Protease-deficient degp suppresses lethal effects of a mutant ompc protein by its capture. *J Bacteriol*, 185(1):148–154.

Chai, T. J. and Foulds, J. (1977). Purification of protein a, an outer membrane component missing in escherichia coli k-12 ompa mutants. *Biochim Biophys Acta*, 493(1):210–215.

Chen, R. and Henning, U. (1996). A periplasmic protein (skp) of escherichia coli selectively binds a class of outer membrane proteins. *Mol Microbiol*, 19(6):1287–1294.

Clausen, T., Southan, C., and Ehrmann, M. (2002). The htra family of proteases: implications for protein composition and cell fate. *Mol Cell*, 10(3):443–455.

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., and Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two e. coli porins. *Nature*, 358(6389):727–733.

Dalbey, R. E. (1991). Leader peptidase. *Mol Microbiol*, 5(12):2855–2860.

Dartigalongue, C., Missiakas, D., and Raina, S. (2001). Characterization of the escherichia coli sigma e regulon. *J Biol Chem*, 276(24):20866–20875.

den Berg, B. V., Clemons, W. M., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature*, 427(6969):36–44.

Driessen, A. J. (2001). Secb, a molecular chaperone with two faces. *Trends Microbiol*, 9(5):193–196.

Duguay, A. R. and Silhavy, T. J. (2004). Quality control in the bacterial periplasm. *Biochim Biophys Acta*, 1694(1-3):121–134.

Gerken, H., Leiser, O. P., Bennion, D., and Misra, R. (2010). Involvement and necessity of the cpx regulon in the event of aberrant beta-barrel outer membrane protein assembly. *Mol Microbiol*.

Hagan, C. L., Kim, S., and Kahne, D. (2010). Reconstitution of outer membrane protein assembly from purified components. *Science*, 328(5980):890–892.

Hasselblatt, H., Kurzbauer, R., Wilken, C., Krojer, T., Sawa, J., Kurt, J., Kirk, R., Hasenbein, S., Ehrmann, M., and Clausen, T. (2007). Regulation of the sigmae stress response by degs: how the pdz domain keeps the protease inactive in the resting state and allows integration of different omp-derived stress signals upon folding stress. *Genes Dev*, 21(20):2659–2670.

Hennecke, G., Nolte, J., Volkmer-Engert, R., Schneider-Mergener, J., and Behrens, S. (2005). The periplasmic chaperone sura exploits two features characteristic of integral outer membrane proteins for selective substrate recognition. *J Biol Chem*, 280(25):23540–23548.

Hong, H., Szabo, G., and Tamm, L. K. (2006). Electrostatic couplings in ompa ion-channel gating suggest a mechanism for pore opening. *Nat Chem Biol*, 2(11):627–635.

Hung, D. L., Knight, S. D., Woods, R. M., Pinkner, J. S., and Hultgren, S. J. (1996). Molecular basis of two subfamilies of immunoglobulin-like chaperones. *EMBO J*, 15(15):3792–3805.

Jarchow, S., Lück, C., Görg, A., and Skerra, A. (2008). Identification of potential substrate proteins for the periplasmic escherichia coli chaperone skp. *Proteomics*, 8(23-24):4987–4994.

Jiang, J., Zhang, X., Chen, Y., Wu, Y., Zhou, Z. H., Chang, Z., and Sui, S.-F. (2008). Activation of degp chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. *Proc Natl Acad Sci U S A*, 105(33):11939–11944.

Justice, S. S., Hunstad, D. A., Harper, J. R., Duguay, A. R., Pinkner, J. S., Bann, J., Frieden, C., Silhavy, T. J., and Hultgren, S. J. (2005). Periplasmic peptidyl prolyl cis-trans isomerases are not essential for viability, but sura is required for pilus biogenesis in escherichia coli. *J Bacteriol*, 187(22):7680–7686.

Kim, S., Malinverni, J. C., Sliz, P., Silhavy, T. J., Harrison, S. C., and Kahne, D. (2007). Structure and function of an essential component of the outer membrane protein assembly machine. *Science*, 317(5840):961–964.

Kleinschmidt, J. H. (2003). Membrane protein folding on the example of outer membrane protein a of escherichia coli. *Cell Mol Life Sci*, 60(8):1547–1558.

Knowles, T. J., Jeeves, M., Bobat, S., Dancea, F., McClelland, D., Palmer, T., Overduin, M., and Henderson, I. R. (2008). Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. *Mol Microbiol*, 68(5):1216–1227.

Knowles, T. J., Scott-Tucker, A., Overduin, M., and Henderson, I. R. (2009). Membrane protein architects: the role of the bam complex in outer membrane protein assembly. *Nat Rev Microbiol*, 7(3):206–214.

Koebnik, R. (1999). Structural and functional roles of the surface-exposed loops of the beta-barrel membrane protein ompa from escherichia coli. *J Bacteriol*, 181(12):3688–3694.

Korndörfer, I. P., Dommel, M. K., and Skerra, A. (2004). Structure of the periplasmic chaperone skp suggests functional similarity with cytosolic chaperones despite differing architecture. *Nat Struct Mol Biol*, 11(10):1015–1020.

Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002). Crystal structure of degp (htra) reveals a new protease-chaperone machine. *Nature*, 416(6879):455–459.

Krojer, T., Pangerl, K., Kurt, J., Sawa, J., Stingl, C., Mechtler, K., Huber, R., Ehrmann, M., and Clausen, T. (2008a). Interplay of pdz and protease domain of degp ensures efficient elimination of misfolded proteins. *Proc Natl Acad Sci U S A*, 105(22):7702–7707.

Krojer, T., Sawa, J., Huber, R., and Clausen, T. (2010). Htra proteases have a conserved activation mechanism that can be triggered by distinct molecular cues. *Nat Struct Mol Biol*, 17(7):844–852.

Krojer, T., Sawa, J., Schäfer, E., Saibil, H. R., Ehrmann, M., and Clausen, T. (2008b). Structural basis for the regulated protease and chaperone function of degp. *Nature*, 453(7197):885–890.

Kumar, P. D. and Krishnaswamy, S. (2005). Overexpression, refolding, and purification of the major immunodominant outer membrane porin ompc from salmonella typhi: characterization of refolded ompc. *Protein Expr Purif*, 40(1):126–133.

Macario, A. J. L. and de Macario, E. C. (2005). Sick chaperones, cellular stress, and disease. *N Engl J Med*, 353(14):1489–1501.

Martinez-Hackert, E. and Hendrickson, W. A. (2009). Promiscuous substrate recognition in folding and assembly activities of the trigger factor chaperone. *Cell*, 138(5):923–934.

Matsuyama, S., Yokota, N., and Tokuda, H. (1997). A novel outer membrane lipoprotein, lolb (hemm), involved in the lola (p20)-dependent localization of lipoproteins to the outer membrane of escherichia coli. *EMBO J*, 16(23):6947–6955.

Meltzer, M., Hasenbein, S., Mamant, N., Merdanovic, M., Poepsel, S., Hauske, P., Kaiser, M., Huber, R., Krojer, T., Clausen, T., and Ehrmann, M. (2009). Structure, function and regulation of the conserved serine proteases degp and degs of escherichia coli. *Res Microbiol*, 160(9):660–666.

Misra, R., CastilloKeller, M., and Deng, M. (2000). Overexpression of protease-deficient degp(s210a) rescues the lethal phenotype of escherichia coli ompf assembly mutants in a degp background. *J Bacteriol*, 182(17):4882–4888.

Mogensen, J. E. and Otzen, D. E. (2005). Interactions between folding factors and bacterial outer membrane proteins. *Molecular Microbiology*, 57(2):326–346.

Naveed, H., Jackups, R., and Liang, J. (2009). Predicting weakly stable regions, oligomerization state, and protein-protein interfaces in transmembrane domains of outer membrane proteins. *Proc Natl Acad Sci U S A*, 106(31):12735–12740.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev*, 67(4):593–656.

Patel, G. J., Behrens-Kneip, S., Holst, O., and Kleinschmidt, J. H. (2009). The periplasmic chaperone skp facilitates targeting, insertion, and folding of ompa into lipid membranes with a negative membrane surface potential. *Biochemistry*, 48(43):10235–10245.

Pautsch, A. and Schulz, G. E. (2000). High-resolution structure of the ompa membrane domain. *J Mol Biol*, 298(2):273–282.

Phale, P. S., Philippsen, A., Kiefhaber, T., Koebnik, R., Phale, V. P., Schirmer, T., and Rosenbusch, J. P. (1998). Stability of trimeric ompf porin: the contributions of the latching loop l2. *Biochemistry*, 37(45):15663–15670.

Qu, J., Behrens-Kneip, S., Holst, O., and Kleinschmidt, J. H. (2009). Binding regions of outer membrane protein a in complexes with the periplasmic chaperone skp. a site-directed fluorescence study. *Biochemistry*, 48(22):4926–4936.

Qu, J., Mayer, C., Behrens, S., Holst, O., and Kleinschmidt, J. H. (2007). The trimeric periplasmic chaperone skp of escherichia coli forms 1:1 complexes with outer membrane proteins via hydrophobic and electrostatic interactions. *J Mol Biol*, 374(1):91–105.

Raivio, T. L. and Silhavy, T. J. (2001). Periplasmic stress and ecf sigma factors. *Annu Rev Microbiol*, 55:591–624.

Rizzitello, A. E., Harper, J. R., and Silhavy, T. J. (2001). Genetic evidence for parallel pathways of chaperone activity in the periplasm of escherichia coli. *J Bacteriol*, 183(23):6794–6800.

Robert, V., Volokhina, E. B., Senf, F., Bos, M. P., Gelder, P. V., and Tommassen, J. (2006). Assembly factor omp85 recognizes its outer membrane protein substrates by a species-specific c-terminal motif. *PLoS Biol*, 4(11):e377.

Rouvière, P. E. and Gross, C. A. (1996). Sura, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. *Genes Dev*, 10(24):3170–3182.

Ruiz, N., Kahne, D., and Silhavy, T. J. (2006). Advances in understanding bacterial outer-membrane biogenesis. *Nat Rev Microbiol*, 4(1):57–66.

Sawa, J., Heuck, A., Ehrmann, M., and Clausen, T. (2010). Molecular transformers in the cell: lessons learned from the degp protease-chaperone. *Curr Opin Struct Biol*, 20(2):253–258.

Schlapschy, M., Dommel, M. K., Hadian, K., Fogarasi, M., Korndörfer, I. P., and Skerra, A. (2004). The periplasmic e. coli chaperone skp is a trimer in solution: biophysical and preliminary crystallographic characterization. *Biol Chem*, 385(2):137–143.

Schulz, G. E. (2002). The structure of bacterial outer membrane proteins. *Biochim Biophys Acta*, 1565(2):308–317.

Schweizer, M., Hindennach, I., Garten, W., and Henning, U. (1978). Major proteins of the escherichia coli outer cell envelope membrane. interaction of protein ii with lipopolysaccharide. *Eur J Biochem*, 82(1):211–217.

Shen, Q.-T., Bai, X.-C., Chang, L.-F., Wu, Y., Wang, H.-W., and Sui, S.-F. (2009). Bowl-shaped oligomeric structures on membranes as degp's new functional forms in protein quality control. *Proc Natl Acad Sci U S A*, 106(12):4858–4863.

Sklar, J. G., Wu, T., Gronenberg, L. S., Malinverni, J. C., Kahne, D., and Silhavy, T. J. (2007a). Lipoprotein smpa is a component of the yaet complex that assembles outer membrane proteins in escherichia coli. *Proc Natl Acad Sci U S A*, 104(15):6400–6405.

Sklar, J. G., Wu, T., Kahne, D., and Silhavy, T. J. (2007b). Defining the roles of the periplasmic chaperones sura, skp, and degp in escherichia coli. *Genes Dev*, 21(19):2473–2484.

Skorko-Glonek, J., Laskowska, E., Sobiecka-Szkatula, A., and Lipinska, B. (2007). Characterization of the chaperone-like activity of htra (degp) protein from escherichia coli under the conditions of heat shock. *Arch Biochem Biophys*, 464(1):80–89.

Smith, S. G. J., Mahon, V., Lambert, M. A., and Fagan, R. P. (2007). A molecular swiss army knife: Ompa structure, function and expression. *FEMS Microbiol Lett*, 273(1):1–11.

Spiess, C., Beil, A., and Ehrmann, M. (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell*, 97(3):339–347.

Sugawara, E. and Nikaido, H. (1994). Ompa protein of escherichia coli outer membrane occurs in open and closed channel forms. *J Biol Chem*, 269(27):17981–17987.

Surrey, T. and Jähnig, F. (1995). Kinetics of folding and membrane insertion of a beta-barrel membrane protein. *J Biol Chem*, 270(47):28199–28203.

Tamm, L. K., Hong, H., and Liang, B. (2004). Folding and assembly of beta-barrel membrane proteins. *Biochim Biophys Acta*, 1666(1-2):250–263.

Tokuda, H. (2009). Biogenesis of outer membranes in gram-negative bacteria. *Biosci Biotechnol Biochem*, 73(3):465–473.

Tomkiewicz, D., Nouwen, N., and Driessen, A. J. M. (2007). Pushing, pulling and trapping–modes of motor protein supported protein translocation. *FEBS Lett*, 581(15):2820–2828.

Tormo, A., Almirón, M., and Kolter, R. (1990). sura, an escherichia coli gene essential for survival in stationary phase. *J Bacteriol*, 172(8):4339–4347.

Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T. J., and Collet, J.-F. (2009). Characterization of the role of the escherichia coli periplasmic chaperone sura using differential proteomics. *Proteomics*, 9(9):2432–2443.

Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M., and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science*, 299(5604):262–265.

Walton, T. A., Sandoval, C. M., Fowler, C. A., Pardi, A., and Sousa, M. C. (2009). The cavity-chaperone skp protects its substrate from aggregation but allows independent folding of substrate domains. *Proc Natl Acad Sci U S A*, 106(6):1772–1777.

Walton, T. A. and Sousa, M. C. (2004). Crystal structure of skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. *Mol Cell*, 15(3):367–374.

Watts, K. M. and Hunstad, D. A. (2008). Components of sura required for outer membrane biogenesis in uropathogenic escherichia coli. *PLoS One*, 3(10):e3359.

Wickner, S., Maurizi, M. R., and Gottesman, S. (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science*, 286(5446):1888–1893.

Wilken, C., Kitzing, K., Kurzbauer, R., Ehrmann, M., and Clausen, T. (2004). Crystal structure of the degs stress sensor: How a pdz domain recognizes misfolded protein and activates a protease. *Cell*, 117(4):483–494.

Wülfing, C. and Plückthun, A. (1994). Protein folding in the periplasm of escherichia coli. *Mol Microbiol*, 12(5):685–692.

Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005). Identification of a multicomponent complex required for outer membrane biogenesis in escherichia coli. *Cell*, 121(2):235–245.

Xu, X., Wang, S., Hu, Y.-X., and McKay, D. B. (2007). The periplasmic bacterial molecular chaperone sura adapts its structure to bind peptides in different conformations to assert a sequence preference for aromatic residues. *J Mol Biol*, 373(2):367–381.

Zakharian, E. and Reusch, R. N. (2005). Kinetics of folding of escherichia coli ompa from narrow to large pore conformation in a planar bilayer. *Biochemistry*, 44(17):6701–6707.

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