Structural and functional studies on the regulation of the protease-chaperone function of DegP and DegQ from *Escherichia coli*

Dissertation
zur Erlangung des akademischen Grades
Doktorin der Naturwissenschaften (Dr. rer.nat.)

Eingereicht von
Justyna Sawa-Makarska

Wien, März 2008

Matrikel-Nummer: 0448708
Dissertationsgebiet: Molekulare Biologie (A 091 490)
Betreuerin / Betreuer: Dr. Tim Clausen

Table of contents

Acknowledgements

This work would have not been possible without the help and support of many people.

First I would like to express my gratitude to Tim Clausen for the four years of collaboration that we have had and his supportive company in ups and downs of the project. Thank you for your scientific guidance and the trust you had in me.

I am very grateful to Iwona Adamska and Kristina Djinovic for critical reading and the time they spent evaluating my thesis in such a short notice.

Furthermore, I would like to thank my PhD committee, Jan Michael Peters and Renee Schroeder, for their ideas and scientific support in our annual meetings.

I would like to acknowledge our collaborators Michael Ehrmann and Melisa Merdanovic for hosting and helping me with the experiments during my staying in Essen.

My time in the lab would not have been so great if not for the presence of fantastic lab mates! I would like to acknowledge all past and present members of the 'Clausen herd' for sharing the small space and big hearts. Heartfelt thanks to Karina, Corinna and Robert for such a warm welcome when I arrived in Vienna. I would like to send warm thoughts to all people involved in the project in the course of my PhD: Tobias, Johannes, Karen and Robert. Special thanks to Tobias for sharing his experience and knowledge so generously with me, for endless discussions when none of my questions was left without an answer. I am indebted to Becky for proofreading and 'unPolishing' the text of my thesis. Thank you for your helpful comments and suggestions. Special thanks to my 'bench neighbors' Boril and Linn simply for their tolerance and good mood.

Finally, I would like to acknowledge my family. Without their continuous support and love this work would not have been possible. Thank you Jacek for helping me to keep the right balance, your understanding and never-ending support.

Pracę tę dedykuję Rodzicom w podziękowaniu za troskliwą opiekę, wychowanie i bezwarunkową milosc jaką mnie obdarzyli.

Abstract

The failure to eliminate misfolded proteins can cause the formation of potentially toxic aggregates, inactivation of functional proteins and ultimately cell death. In order to sustain proper homeostasis cells have developed a system of protein quality surveillance. It involves dedicated chaperones and proteases to monitor and control the state of cellular proteins and depending on the degree of damage, either refold or digest aberrant proteins. The pool of periplasmic proteins of *E .coli* is quality-controlled by two representatives of the HtrA proteases family, namely DegP and DegQ.

The heat-shock protein DegP combines digestive and remodelling activities and can switch between these antagonistic functions in a tightly regulated manner. In this study the characterization of different DegP/substrate complexes revealed that binding of misfolded proteins transformed hexameric DegP into large, catalytically active 12- and 24-meric multimers dependent on the size and concentration of the substrate. The same mode of regulation, i.e. protease activation by substrate-induced oligomer reassembly, also appears in DegQ indicating that this unique regulatory mechanism is a conserved feature of HtrA proteins. Moreover, structural and biochemical analysis of DegP complexes with outer membrane proteins (OMPs) revealed that DegP represents a protein packaging device whose central compartment serves antagonistic functions. While encapsulation of folded OMP protomers is protective and might allow safe transit through the periplasm, misfolded proteins are eliminated in the molecular reaction chamber.

In parallel to elucidate common HtrA features, this study focused on regulatory and mechanistic differences between the two closely related protease-chaperones DegP and DegQ. Activity assays and size exclusion chromatography analysis demonstrated that low pH (5.5) induces remodeling of the DegQ particle, most likely from hexamer to dodecamer. Remarkably, the conversion of the oligomeric state was accompanied by a change in the protease activity being, in contrast to DegP, the most pronounced at low pH. *In vivo* DegQ was shown to affect the growth of *E. coli* at lower pH values, while the presence of DegP had no effect. Thus the pH dependent activity of DegQ might reflect adaptation of the bacterium to habitats with variable pH values. Furthermore, the growth of *degQ* null mutant strain shows an elongated adaptation phase compared to the wild type, indicating an important house keeping function of DegQ, which is essential in the highly unstable environment of the bacterial envelope.

Zusammenfassung

Die fehlerhafte Entfernung von ungefalteten Proteinen kann zur Bildung von möglicherweise gefährlichen Aggregaten, zur Inaktivierung von funktionellen Proteinen bis hin zum Tod einer Zelle führen. Um eine einwandfreie Homöostase aufrechtzuerhalten hat die Zelle ein System zur Überwachung der Proteinqualität entwickelt. Dieses Kontrollsystem umfasst spezielle Chaperone und Proteasen, die den Zustand von zellulären Proteinen unter physiologischen und unter Stressbedingungen überwachen. Abhängig vom Grad der Beschädigung der nicht-nativen Proteine werden diese entweder rückgefaltet oder aber entfernt. In *Escherichia coli* wird der Zustand von periplasmatischen Proteinen von zwei Repräsentanten der HtrA Proteasefamilie überwacht: DegP und DegQ.

Das Hitzeschockprotein DegP verfügt über eine abbauende und eine rückfaltende Aktivität und es kann zwischen diesen beiden gegensätzlichen Funktionen in regulierter Weise umschalten. In dieser Arbeit wurden verschiedene DegP/Substrat-Komplexe charakterisiert. Es zeigte sich, dass fehlgefaltete Proteine das hexamere DegP in große, katalytisch aktive 12- und 24-mere Partikel umwandeln, abhängig von der Größe und der Konzentration des vorliegenden Substrates. Die gleiche Art der Regulation, d.h. eine Aktivierung der Proteaseaktivität durch eine substratinduzierte Umwandlung des Oligomers, konnte auch für DegQ festgestellt werden. Diese Beobachtung deutet darauf hin, dass dieser einzigartige Regulationsmechanismus ein konserviertes Merkmal der HtrA Familie darstellt. Weiterhin zeigte die strukturelle und biochemische Analyse von DegP im Komplex mit Außenmembranproteinen (outer membrane proteins, OMPs), dass DegP Proteine in einer zentralen Kammer einschließt, die sowohl als Chaperon als auch Protease-Kompartiment dienen kann. Während die Einkapselung von gefalteten OMP Protomeren schützend wirkt und möglicherweise den sicheren Transport durch das Periplasma gewährleistet, werden fehlgefaltete Proteine in der molekularen Reaktionskammer abgebaut.

Um weitere typische Merkmale der HtrA Familie zu bestimmen, konzentrierte sich diese Studie darauf, regulatorische und mechanistische Unterschiede zwischen den beiden eng verwandten Protease-Chaperon Systemen, DegP und DegQ zu ermitteln. Untersuchungen der Proteaseaktivität und des Molekulargewichtes des DegQ Oligomers

mittels Gelpermeationschromatografie ergaben, dass niedrige pH-Werte (5.5) eine Umwandlung von DegQ von einem hexameren zu einem möglicherweise dodekameren Zustand induzieren. Auffälligerweise war die Veränderung des oligomeren Zustandes mit einer Veränderung der Proteaseaktivität verbunden, die im Gegensatz zu DegP, am höchsten bei niedrigen pH-Werten war. Bei der weitergehenden Untersuchung der *in vivo* Relevanz dieser Beobachtung zeigte sich, dass DegQ vor allem für das Wachstum von *Escherichia coli* bei niedrigen pH-Werten wichtig ist. Dies deutet darauf hin, dass die pH-abhängige Regulation von DegQ die Adaption des Bakteriums an eine Umgebung mit veränderbarem pH-Wert wiederspiegelt. Weiterhin zeigte das Wachstum eines *degQ* null Stammes eine verlängerte Adaptionsphase im Vergleich zum Wildtyp, was auf eine grundlegende Rolle von DegQ in der Proteinhomöostase hinweist, welche essentiell in der äußerst instabilen Umgebung der bakteriellen Zellhülle ist.

1. Introduction

1.1 The cell envelope of gram-negative bacteria

The cell envelope is the outer portion of a bacterial cell, which is localized externally to the cytoplasmic membrane. The cell envelope of gram-negative bacteria is composed of two membranes, the inner membrane (IM) and the outer membrane (OM), which are separated by the periplasm (Figure 1.1). The two membranes have different structure and composition reflecting their different functions and neighboring environments. The IM is a phospholipid bilayer, whereas the OM is an asymmetrical bilayer, with an inner leaflet composed of phospholipids and the outer leaflet consisting mainly of lipopolysaccharides (LPS) (Muhlradt and Golecki 1975; Smit *et al.* 1975). The two bilayers contain species of integral proteins and several lipid modified proteins, called lipoproteins. The integral proteins span the membrane, while most lipoproteins are anchored to the membrane through the attached lipids. However, the two membranes differ with respect to the structure of their integral membrane proteins. Whereas integral IM proteins are typically α-helical, integral OM proteins (OMPs) generally consist of amphipathic β-strands that fold into cylindrical β-barrels (Koebnik *et al.* 2000).

The aqueous periplasmic compartment between the inner and outer membranes is occupied by soluble proteins and the peptidoglycan layer. The peptidoglycan, also termed murein sacculus, constitutes an extracytoplasmic cytoskeleton that protects the cell from rupture by the internal osmotic pressure (turgor) and contributes to the cell shape (Vollmer 2007). It is a heteropolymer composed of glycan strands that are cross-linked by short peptides, forming a netlike structure (Vollmer and Holtje 2004). The murein layer binds lipoprotein (Lpp, Braun's lipoprotein), which links it to the outer membrane (Braun 1975).

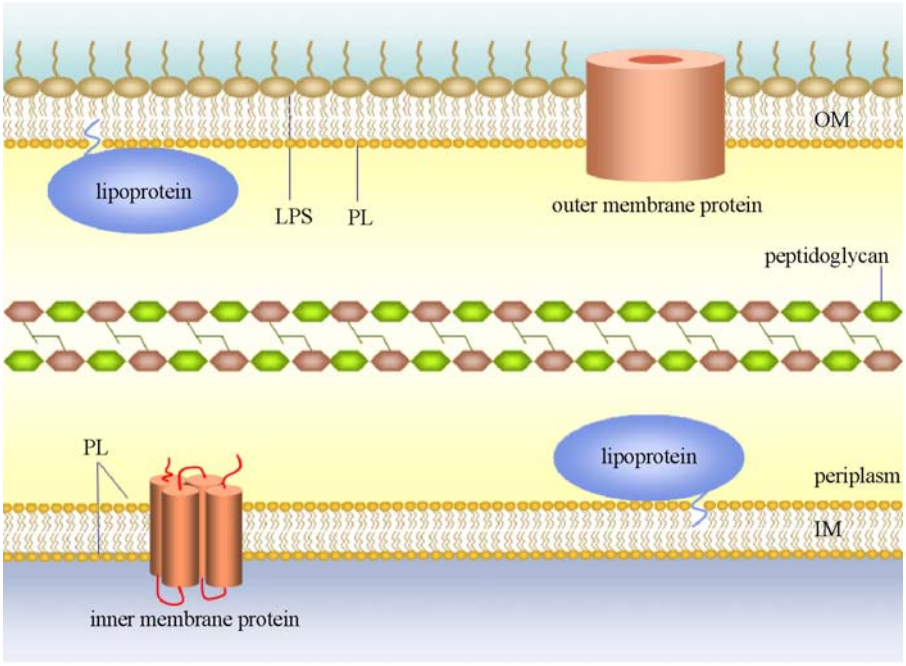


Figure 1.1. The periplasm of *Escherichia coli*. The cell envelope of gram-negative bacteria like *Escherichia coli* is typically built up by an inner (IM) and an outer membrane (OM). Both membranes consist of phospholipid (PL) bilayers and are separated by the periplasmic space. The OM contains lipopolisaccharide (LPS) in its outer leaflet and β-barrel transmembrane proteins (outer membrane protein, OMP). IM integral proteins contain characteristic α-helical transmembrane domains. Both membranes contain lipoproteins that are anchored to their periplasmic faces. The murein sacculus (peptidolycan) is located at the periplasmic side of the OM. Figure reproduced from (Ruiz *et al.* 2006).

1.1.1 Dimensions and physico-chemical properties of the periplasm

The periplasm of *Escherichia coli* comprises ~20% of the total cell volume (Van Wielink and Duine 1990). Electro microscopy analysis and recent structural work on the proteins of the cell envelope have contributed to a more precise estimation of the dimensions of the periplasm (Dubochet *et al.* 1983; Leduc *et al.* 1985; Van Wielink and Duine 1990; Ferguson 2007). Based on these studies the width of the periplasmic compartment has been estimated to be between 17 – 33 nm. The width of the periplasm, however, may vary with both the organism and particular growth conditions.

The environment within the periplasm is very different from the aqueous solutions typically used for *in vitro* experiments. Measurements of lateral diffusion rates of proteins within the periplasm have revealed a 1000-fold lower number than comparable measurements yielded *in vitro* and a 100-fold lower number than expected for cytoplasmic

diffusion rates (Brass *et al.* 1986). This implies that the periplasm has a gel-like consistency which could be caused by the presence of non-polymerised peptidoglycan present in the periplasm and by high protein concentrations. In some growth conditions the effective concentration of periplasmic proteins can reach the milimolar range (Ferguson 2007). This phenomenon is termed macromolecular crowding (Zimmerman and Minton 1993).

The conditions found in the periplasm are different when compared to the cytoplasm. First, the periplasm is a naturally oxidative compartment that favors the formation of disulfide bonds, hence the presence of the crucial Dsb family of enzymes, which are involved in remodeling disulfide bridges (Nakamoto and Bardwell 2004). Moreover, it is a compartment devoid of adenosine 5'-triphosphate (ATP) that is an essential energy source for cytoplasmic molecular processes (Rosen 1987; Wulfing and Pluckthun 1994). Additionally, being separated from the extracellular milieu only by the porous outer membrane, the periplasm is more susceptible to changes in the external environment than the cytoplasm. In fact, the conditions in the periplasm resemble the external environment due to the permeable character of the outer membrane. Recent fluorimetric studies have shown that the pH of the periplasm in *E. coli* is similar to the pH of the medium under all conditions tested. In contrast, cytoplasmic pH (7.2 - 7.8) was recovered within 10 seconds to 5 minutes depending on the analyzed conditions (Wilks and Slonczewski 2007).

All the described features contribute to the unique character of the periplasmic space. Remarkably, in this environment periplasmic proteins are able to fulfil their basic molecular functions similarly to their cytoplasmic counterparts.

1.1.2 Periplasmic proteins

Proteins residing in the periplasmic space fulfill a number of important functions. They are responsible for the detection and processing of essential nutrients and their transport into the cell. They promote the biogenesis of proteins entering this compartment along with compounds destined for incorporation into the peptidoglycan and outer membrane. Furthermore, the sensing domains of most inner membrane receptor proteins read environmental signals from this location (Oliver 1996). Based on the different functions the proteins can be divided into several categories. First the solute or ion binding proteins that

function in conjunction with ABC-transporters or chemotaxis receptors. Second and third the catabolic and the detoxifying enzymes. Finally, the enzymes that promote the biogenesis of major envelope proteins (Oliver 1996):

a) Disulphide bond formation (Dsb oxydoreductases):

The formation of appropriate disulfide bonds is crucial for the proper folding of many proteins in the envelope. Due to the oxidizing nature of the periplasm, spontaneous disulfide bond formation can occur. It is a very slow process and may be incorrect thus there is the requirement for enzymes dedicated to the task of formation and isomerization of disulfide bonds. These enzymes belong to the Dsb oxydoreductase family (Rietsch *et al.* 1996). Interestingly, it has been shown that two of its members, namely DsbG and DsbC, function as molecular chaperones in addition to their role in disulfide bond formation (Chen *et al.* 1999; Shao *et al.* 2000).

b) Peptidyl-Prolyl Isomerases (PPIases):

These proteins catalyze the *cis-trans* isomerization of prolyl peptide bonds. In the absence of catalysts, the isomerization of peptidyl-prolyl bonds is a slow process that is thought to be the rate-limiting step in protein folding (Levitt 1981). At the moment four proteins with this activity are known, namely SurA (Rouviere and Gross 1996), PpiD (Dartigalongue and Raina 1998), FkpA (Ramm and Pluckthun 2000) and PpiA/RotA (Liu and Walsh 1990). Additionally to their ability to isomerize prolyl peptide bonds some of these PPIases have been also demonstrated to facilitate folding of envelope proteins (Figure 1.2) (Lazar and Kolter 1996; Rouviere and Gross 1996; Bothmann and Pluckthun 2000; Arie *et al.* 2001).

c) Chaperones:

As no nucleoside triphosphates are present in the periplasm (Rosen 1987) chaperones that work in this compartment cannot be similar to the extensively studied ATP-dependent chaperones of the Hsp60 and Hsp70 families (Ben-Zvi and Goloubinoff 2001). The family of periplasmic chaperones comprises the following proteins:

– Pili-specific chaperones:

The multiple subunits of pili are secreted across the inner membrane and are complexed with a specific chaperone in the periplasm. These chaperones form a large PapD-like superfamily of specialised periplasmic chaperones that facilitate folding and assembly of over 30 diverse adhesive surface organelles (Holmgren *et al.* 1992; Hung *et al.* 1996).

– LolA/LolB:

The incorporation of lipoproteins into the outer membrane is catalysed by LolA, a periplasmic shuttle protein, and the outer membrane lipoprotein LolB (Matsuyama *et al.* 1995; Matsuyama *et al.* 1997).

– Skp

Skp has been proposed to be a general chaperone for outer membrane proteins (OMPs). It has been demonstrated to bind selectively to outer membrane proteins but not to periplasmic or cytosolic proteins. In agreement with these results, skp null mutants showed a moderate reduction in properly folded OMPs (Chen and Henning 1996). The structure and function of Skp are presented more detailed in section 2.4.

– DegP (HtrA)

Spiess et al. (Spiess *et al.* 1999) could demonstrate that this heat-shock protein exhibits general molecular chaperone activity in addition to its protease activity. The process is controlled in a temperature-dependent manner. DegP is a major focus of this work and it is described thoroughly in the next sections.

To summarize, the main function of these chaperones and folding factors is to stabilize non-native conformations of target proteins thus facilitating their folding (molecular chaperones), and to catalyze the rate limiting steps of isomerization during folding (Dsb, PIPases). It is necessary to note that such classification of folding catalysts is not strict, as some of them display more than one acitvity (Figure 1.2).

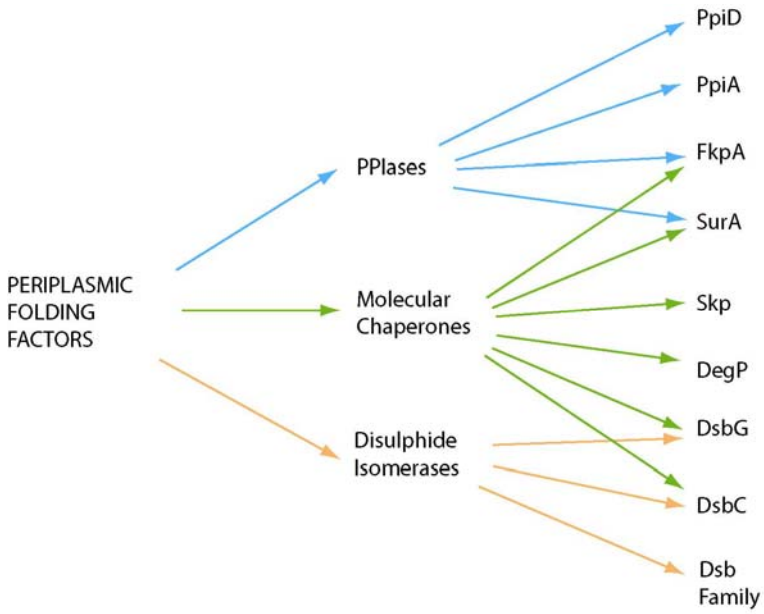


Figure 1.2. Schematic summary of periplasmic folding factors. Their categorization falls into three groups: peptidyl-prolyl isomerases (PPIases), molecular chaperones, and disulphide isomerases. Members of each group are depicted. Some of the proteins display more than one activity. Figure modified from (Mogensen and Otzen 2005).

1.2 Outer membrane biogenesis

The OM of Gram-negative bacteria is an essential organelle designed to shield against the entry of toxic compounds into the cell, while simultaneously allowing for the selective entry of nutrients and other small molecules required for cell survival. The biogenesis of its compounds requires their synthesis, translocation across the IM, transport through the periplasmic space, and finally incorporation into the OM. It is highly intriguing to observe how a cell tackles the multiple problems of a protein crossing a hydrophobic barrier, prevention of misfolding in the aqueous environment, and guaranteeing insertion into the correct membrane with the correct topology. The main focus of this section is to highlight the mechanisms of biogenesis of integral outer membrane proteins (OMPs).

1.2.1 Biogenesis of lipoproteins and lipopolisaccharide

The biogenesis of OM lipoproteins involves the translocation of their precursors across the inner membrane by the Sec machinery, followed by processing of the signal

sequence and remodeling of their N-termini by adding a lipid moiety. Next, a mature lipoprotein is released from the IM, escorted through the periplasm and inserted into the OM by the specialised Lol system (Figure 1.3) (Pugsley 1993; Ruiz, Kahne et al. 2006).

The hallmark of the outer membrane is the presence of lipopolisaccharide (LPS) in its outer leaflet (Muhlradt and Golecki 1975; Smit, Kamio et al. 1975). The typical LPS molecule comprises three structurally and functionally distinct domains: lipid A, core oligosaccharide, and O-antigenic polysaccharide and it is synthesized at the cytoplasmic leaflet of the IM. (Raetz and Whitfield 2002). Upon synthesis LPS is translocated across this bilayer by means of the ABC transporter MsbA which flips LPS from its site of synthesis in the inner leaflet to the outer leaflet of the IM (Doerrler *et al.* 2001; Doerrler *et al.* 2004). Although it has been shown that the outer membrane protein Imp is required for the assembly of LPS to the OM, it still remains unresolved how LPS travels from the IM to the OM, and how it is flipped to the cell surface (Braun and Silhavy 2002) (Figure 1.3).

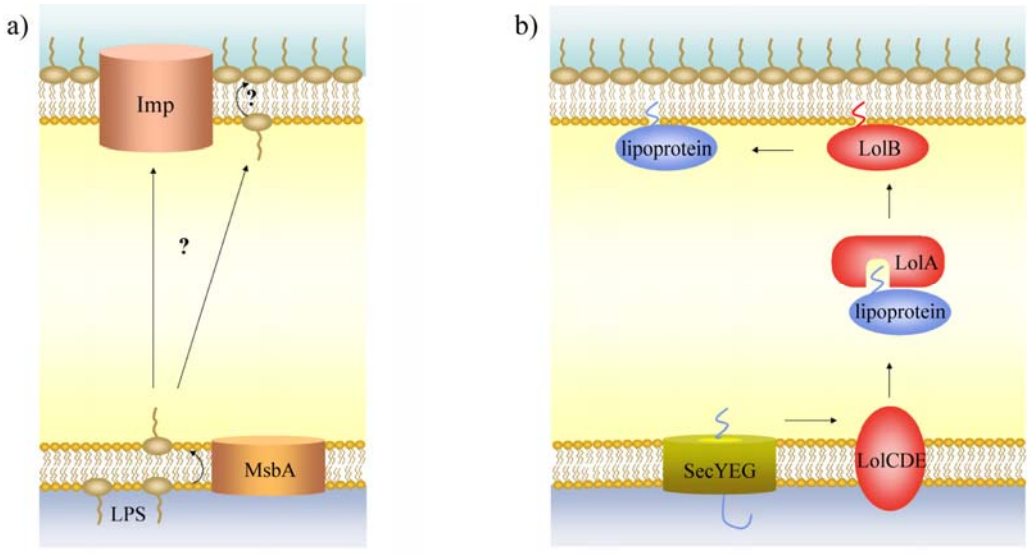


Figure 1.3. A model of lipopolisaccharide (LPS) and lipoproteins biogenesis. a) LPS is synthesized at the inner leaflet of the IM. It is then translocated across the IM by MsbA, transported through the periplasm by an unknown mechanism and inserted into OM by means of Imp. b) OM lipoproteins, after transport via the Sec system and subsequent modification, bind to the ABC-transporter LolCDE. When the LolA-lipoprotein complex interacts with the OM receptor LolB, the lipoprotein is transferred to LolB and then inserted into the OM. Figure adapted from (Ruiz, Kahne et al. 2006)

1.2.2 Integral outer membrane proteins

Integral membrane proteins are embedded in the lipid bilayer (Figure 1.1). Unlike integral inner membrane proteins, integral outer membrane proteins do not consist of transmembrane α-helices. All currently known OMPs from bacteria form a cylindrical β-barrel with even numbers of antiparallel β-strands ranging from 8 to 22 (Schulz 2002) (Figure 1.4). Their hydrophobic residues point outward to create, a single very wide transmembrane segment bearing a hydrophilic interior which often serves as a channel for small molecules (Table 1.1). Even though OMPs share the same architectural principle, they differ regarding their size, oligomeric state, and surface loops enabling them to fulfill various functions (Table 1.1). The smaller transmembrane β-barrels (8 β-strands) have solid cores partially filled with water. They usually they bind to other macromolecules or work as enzymes by means of additional soluble domains attached to the barrel (Table 1.1). The larger barrels of this type (OmpC, OmpF) have channels along their axis that allow the passage of small hydrophilic molecules across the barrier; hence they are often referred to as “porins”. Beside the structural protein OmpA, porins are the most abundant proteins in the OM. The largest known 22-stranded transmembrane β-barrels (e.g. FhuA) are used for the active transport of rare cargos through the bacterial OM. β-barrel proteins were also found in the membrane of mitochondria, peroxysomes and chloroplasts (Nikaido 2003). The functions of OMPs with exemplary proteins and their basic characteristics are summarized in Table 1.1 (Schulz 2002; Kleinschmidt 2007).

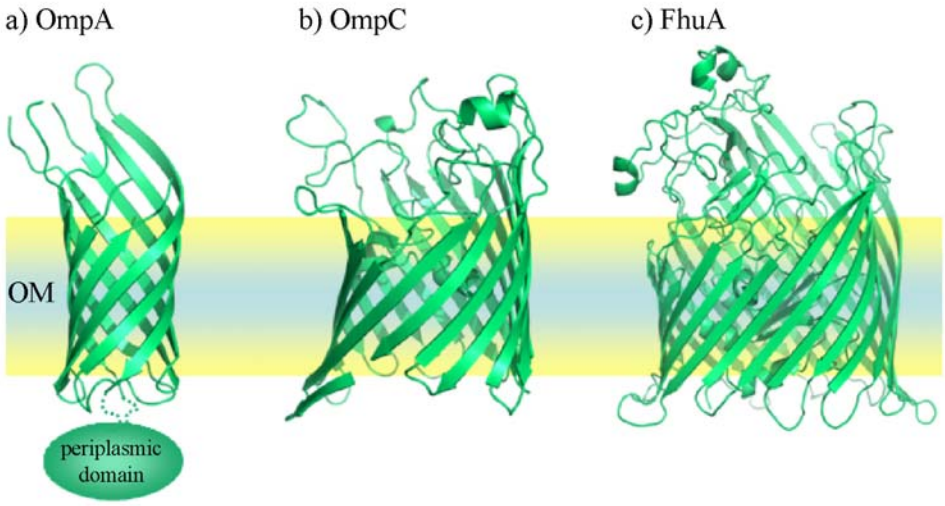


Figure 1.4. Representative outer membrane β-barrels. a) transmembrane domain of OmpA – a structural protein linking peptidoglycan to the OM by a soluble periplasmic domain; 8 β-strands (PDB entry code: 1BXW; (Pautsch and Schulz 1998)) b) monomer of OmpC – non-specific diffusion porin; 16 β-strands (PDB entry code: 2J1N; (Basle *et al.* 2006)) c) FhuA - an active transporter for ferrichrome iron; 22 β-strands (PDB entry code: 2FCP; (Ferguson *et al.* 1998)) . The images of the structures were produced using PyMOL (DeLano 2002).

functional group	OMP	MW [kDa]	β-strands number	oligomeric state
general non specific diffusion pores	OmpC	38.2	16	trimer
	OmpF	37.1	16	trimer
passive specific transporters (e.g. sugars, nucleotides)	LamB	47.4	18	trimer
	Scry	53.2	18	trimer
active transporters for iron complexes or cobalamin	FhuA	78.7	22	monomer
	FepA	79.8	22	monomer
	FecA	81.7	22	monomer
	BtuB	66.3	22	monomer
enzymes:				
protease	OmpT	33.5	10	monomer
acyltranferase	PagP	19.5	8	monomer
lipase	OmP1A	30.8	12	dimer

Table 1.1. Examples of integral outer membrane proteins: their function and basic characteristics (Schulz 2002; Kleinschmidt 2007)

Although OMPs display certain differences, their biogenesis encompasses the same three basic steps, namely i) targeting and transport across the inner membrane ii) shuttling

across the periplasm iii) incorporation into the outer membrane. The next sections will summarize the current state of knowledge on the three processes.

1.2.3 Transport of OMPs across the inner membrane

There are two ways of transporting extracytoplasmic proteins across the inner membrane to the periplasm. The vast majority of periplasmic proteins are exported by the general secretory (Sec) pathway in an unfolded state (Danese and Silhavy 1998; Veenendaal *et al.* 2004). However, it is worth noting that approximately 10% proteins are exported already folded, and sometimes as hetero-oligomers, by the *t*win *a*rginine protein *t*ransport (Tat) pathway (Palmer and Berks 2003). The latter system is mostly involved in transferring proteins that bind cofactor molecules in the cytoplasm, and are thus folded prior to the export (Berks *et al.* 2000; Palmer and Berks 2003; Sargent 2007). Most of the extracellular proteins, including all OMPs studied so far, employ the Sec system for their translocation indicating that they reach the periplasm in an unfolded state (Bernstein 2000).

Integral OMPs are synthesized in the cytoplasm as precursors (preproteins) with N-terminal signal sequences, which are essential for targeting OMPs to the Sec translocon and for transport across the IM (von Heijne 1990). The synthesis and translocation of preproteins are not coupled events (Randall 1983). The targeting of OMP precursors to the IM is mediated by a molecular chaperone called SecB. It recognizes and binds the signal sequence of an emerging newly synthesized polypeptide on the ribosome and delivers the client protein to the cytoplasmic face of the IM in an unfolded state (Figure 1.5) (Driessen 2001). Its crystal structure suggests that polypeptides are wrapped around the tetrameric SecB protein (Xu *et al.* 2000; Driessen and Nouwen 2007).

The next step of the process involves the interaction of SecB with SecA. SecA is a central component of the Sec translocase functioning as an ATP-dependent motor protein which pushes the unfolded preprotein through the SecYEG channel in a step-wise process (Tomkiewicz *et al.* 2007). The protein-conducting channel SecYEG consists of three proteins, termed SecY, SecE, and SecG, that together form a stable complex embedded in the cytoplasmic membrane (Brundage *et al.* 1990). The translocated preproteins are in an unfolded conformation and pass through the membrane via the aqueous pore of the

complex. The crystal structure of the SecYEG channel revealed the presence of the pore of an appropriate size to accommodate an unstructured polypeptide chain (Van den Berg *et al.* 2004).

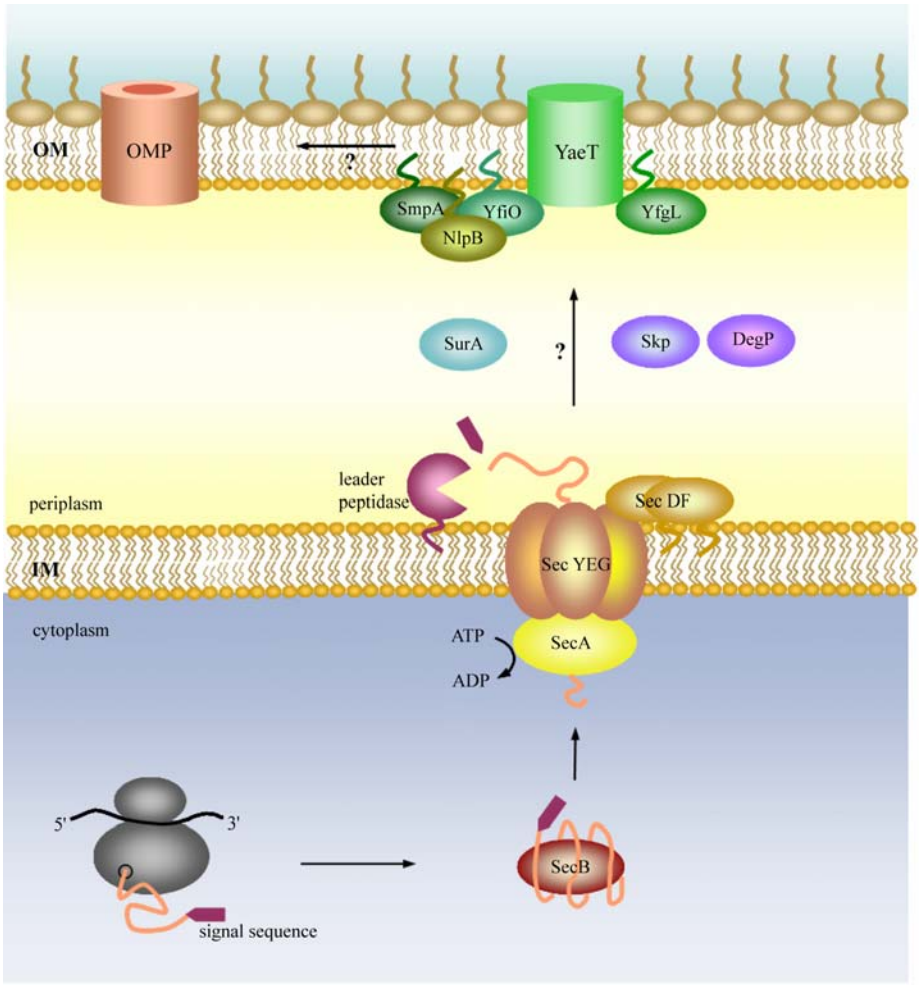


Figure 1.5. Bigenesis of integral OMPs. OMPs are synthesized in the cytoplasm. As soon as they emerge from the IM Sec translocon, their signal sequence is cleaved off. Next they are sequestered by periplasmic chaperones, which prevent premature folding and aggregation. Ultimately, chaperone-assisted OMPs reach the OM where they fold and are inserted to the bilayer by means of the Omp85 complex. (Veenendaal, van der Does et al. 2004; Ruiz, Kahne et al. 2006; Kim *et al.* 2007)

The least understood component of the Sec translocon is the SecDF complex. Both SecD and SecF are membrane proteins with large periplasmic domains (Gardel *et al.* 1990).

They can bind to the SecYEG channel but their exact function in the protein translocation remains unclear (Driessen and Nouwen 2007). After translocation the signal sequence is processed by the membrane anchored leader protease thereby preparing the protein to cross the periplasm prior to its assembly into the OM (Dalbey and Wickner 1985; Dalbey 1991).

1.2.4 Periplasmic trafficking

One of the most intriguing and yet the least described aspect of OMP biogenesis is their transport to the OM across the aqueous periplasm. Two general paths for the transfer have been proposed. The first model postulates that OMPs are ferried to the outer membrane via zones of adhesion between the inner and outer membranes. Alternatively, the second model proposes a chaperone-mediated transit through the periplasm.

The zones of adhesions are also called 'Bayer's Junctions' named after their discoverer Manfred Bayer, who in the 1960s, by using electron microscopy observed contact sites between the IM and the OM of plysmolized *E. coli* cells (Bayer 1968). This model would also satisfactorily explain the mode of transport of other OM components like LPS and phospholipids. Nevertheless, the cryo-fixation technique employed in the sample preparation was greatly disputed raising doubts in the adhesion concept (Ruiz, Kahne et al. 2006). The existence of membrane-adhesion sites has not been completely disproved, however in the light of the latest discoveries the model of chaperone assistance is favored.

This model proposes that after they are translocated to the periplasm by the Sec complex, OMPs are kept soluble in an unfolded form by periplasmic chaperones (Figure 1.5). Among the periplasmic proteins that could either bind unfolded OMPs or affect their assembly are Skp, SurA and DegP (Chen and Henning 1996; Missiakas *et al.* 1996; Rouviere and Gross 1996; Rizzitello *et al.* 2001). As mentioned in section 1.2, SurA and DegP exhibit more than one activity independent of their chaperone function. It is worth noting that the most likely function of periplasmic chaperones is to bind to non-native form of OMPs thus preventing their aggregation and targeting them to the OM. Their role in active OMP folding remains unsolved (Kleinschmidt 2007).

The three mentioned chaperones constitute two overlapping, periplasmic chaperone pathways for delivery of proteins to the outer membrane, the first uses DegP and Skp, and

the other uses SurA, and at least one of these pathways must be functional for viability (Rizzitello, Harper et al. 2001). Recently, it has been suggested that SurA is the key trafficking factor whereas Skp and DegP are involved as a rescue mechanism when OMPs fall off the main pathway (Sklar *et al.* 2007). The full understanding of the interplay among the periplasmic chaperons is still to be achieved.

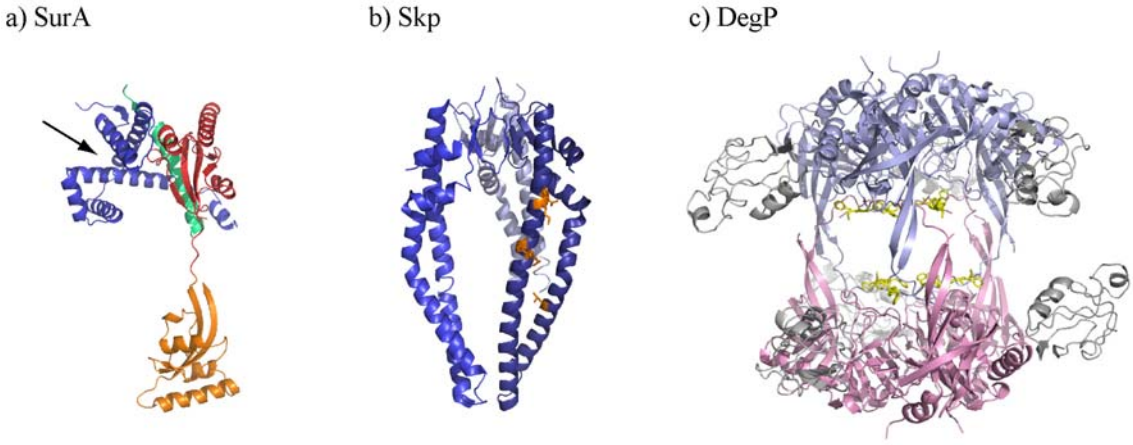


Figure 1.6. Three dimesional structures of periplasmic chaperones involved in OMPs biogenesis. **a)** SurA (PDB entry code: 1m5y, (Bitto and McKay 2002)). The color code for the domains: the N-terminal "core" domain - blue, P1 domain (PPIase I) - red, P2 (PPIase II) – orange, and C-terminal domain – green. The N-terminal domain binds OMP peptides with the consensus motif Ar-X-Ar. The putative binding site is depicted by a black arrow. **b)** Skp (PDB entry code: 1sg2 (Korndorfer *et al.* 2004)). Three protomers are shown in different shades of blue. Residues involved in constituting the putative LPS binding site (E49, K97, Q99, R107, and R108) are shown in orange only for one protomer. **c)** DegP (PDB entry code: 1ky9, (Krojer *et al.* 2002)). The two trimeric rings are depicted in violet and magenta. The PDZ1 domain is shown in grey. The PDZ2 domain is not shown. The hydrophobic Phe-clusters are shown in yellow. The images of the structures were produced using PyMOL (DeLano 2002).

The structure solution of the three proteins was a step forward in understanding the mode of action but not enough to fully explain the mechanisms underlying their chaperone activity. The structure of SurA reveals a monomer built up by four distinct domains: the N-terminal "core" domain, P1 domain (PPIase I) and P2 (PPIase II) domain, which connects P1 with the C-terminal domain (Figure 1.6) (Bitto and McKay 2002). Biochemical analysis revealed the core to bind OMP peptides with the consensus motif Aromatic-X-Aromatic

thus suggesting a binding site for unfolded OMPs during their periplasmic passage (Bitto and McKay 2003; Bitto and McKay 2004). Interestingly, the two PPIase domains are dispensable for both SurA chaperone-like activity and OMPs biogenesis as shown *in vitro* and *in vivo* (Behrens *et al.* 2001).

The structure of the Skp trimer resembles a jelly fish composed of three α-helical tentacles protruding from a central body composed of a β-barrel (Figure 1.6) (Korndorfer, Dommel et al. 2004; Walton and Sousa 2004). The tentacles form a chamber that is proposed to constitute the substrate binding site due to the presence of hydrophobic patches. The molecule displays strong polarity with negative charges at the central body and positive ones at the tips of the tentacles. This characteristic could help to orient Skp relative to cell membranes. Interestingly, Skp resembles prefoldin from *Methanobacterium thermoautotrophicum*, a cytosolic chaperone that prevents aggregation of non-native proteins and delivers them to chaperonins – foldases (Siegert *et al.* 2000). Additionally, a putative LPS binding site found in the structure coincides with biochemical findings on the connection between the function of Skp and LPS binding (De Cock *et al.* 1999; Bulieris *et al.* 2003).

The structure of DegP revealed a hexameric assembly of protomers containing one protease and two PDZ domains (Figure 1.6) (Krojer, Garrido-Franco et al. 2002). The cavity encompassed between two trimeric rings is lined by conserved hydrophobic residues suggesting binding sites for unfolded proteins or proteins exposing hydrophobic character like OMPs. Furthermore, the height of the inner cavity (15Å) excludes folded proteins from entering (Clausen *et al.* 2002). Although the structure revealed an impressive architecture of DegP, it did not sufficiently explain how the employment of the OMP substrate may occur. The structure of DegP is discussed in more detail in section 4.4.

1.2.5 Incorporation of OMPs into the outer membrane

Although OMPs can fold spontaneously in the presence of detergents *in vitro* (Sen and Nikaido 1991; Surrey and Jahnig 1992; Marsh *et al.* 2006) the *in vivo* insertion into the OM is an assisted process (Kleinschmidt 2003). It requires the presence of an assembly machinery, which in *E. coli* consists of at least five interacting components: four

lipoproteins (YfgL, YfiO, NlpB, and SmpA) and the core integral membrane protein, YaeT belonging to the conserved Omp85 family (Wu *et al.* 2005; Sklar *et al.* 2007). According to recent structural studies on bacterial members of this family, Omp85 contains a membrane embedded β-barrel and an N–terminal periplasmic extension encompassing five polypeptide transport–associated (POTRA) domains (Clantin *et al.* 2007; Kim, Malinverni et al. 2007). It interacts with unfolded OMPs by recognizing their C-terminal sequence most probably by POTRA domains (Robert *et al.* 2006). Binding of the substrate protein initiates folding, which results in the release of an assisting periplasmic chaperone (De Cock, Schafer et al. 1999)? There is not much data available on the next steps of the process, however the current model suggests a conformational change in the C-terminal domain of Omp85 upon OMP binding. This alteration allows the OMP to insert into the OM between the Omp85 subunits. Consecutively, the subunits would dissociate and release the assembled OMPs into the OM. The insertion through the channel of Omp85 is not a plausible idea as the lateral opening of the β-barrel is rather unlikely due to the high rigidity of the structure (Bos *et al.* 2007; Tommassen 2007). The precise role of the accessory lipoproteins (YfgL, YfiO, NlpB) still remains to be elucidated.

Interestingly, an impact of LPS on OMP assembly has been proposed. This finding still remains ambiguous though and it is strongly debated in the field. The current position is that *in vitro* folding of most investigated OMPs can be facilitated by LPS, although it is not required (Bos, Robert et al. 2007). The situation *in vivo* is less clear. The *E. coli* rough mutant containing truncated LPS molecules shows that the assembly of certain OMPs was affected (Koplow and Goldfine 1974; Kloser *et al.* 1998). Unexpectedly, the finding of the LPS deficient mutant of *Neisseria meningitis* which is viable and has a correctly assembled OM brought confusion to the field, and yet an excellent tool to investigate OMP assembly in the absence of LPS (Steeghs *et al.* 1998; Steeghs *et al.* 2001). Currently it is suggested that LPS may stabilize assembled porins (Laird *et al.* 1994) or that it can act indirectly by binding to periplasmic shutter chaperones like Skp (De Cock, Schafer et al. 1999; Walton and Sousa 2004).

The mechanism described above explains the OM incorporation process of monomeric barrels. However, as mentioned in section 2.2 porins may also form trimers. *In vitro* folding studies on purified OmpF and OmpF released from spheroplasts could

elegantly show that folding and trimerization events are coupled and that they occur at or in the membrane (Sen and Nikaido 1990; Sen and Nikaido 1991; Surrey *et al.* 1996). Presumably, in the cell the trimer assembly takes place during OM insertion assisted by Omp85. The detailed mechanism of this process *in vivo* remains elusive though.

1.3 Protein quality control in *Escherichia coli*.

The appearance and maintenance of functional proteins within cells does not depend exclusively on the fidelity of transcription and translation. Although all the information necessary for a protein to reach a native structure is contained in its amino acid sequence (Anfinsen 1973), *in vivo* protein folding requires the participation of molecular chaperones, folding catalysts and proteases, that monitor or regulate this process through many cellular functions. Under physiological conditions, these factors survey quality control of protein biosynthesis, thus errors or failures of the protein folding process are rare (Miot and Betton 2004). However, upon exposure to environmental stress incorrectly folded or misfolded proteins can appear rapidly thus jeopardizing cell homeostasis and leading to fatal consequences. For this reason there is a requirement for a protein quality control system which efficiently recognizes such proteins and quickly counteracts the damage. As soon as it is identified, a misfolded protein may either be degraded by proteases, or repaired by chaperones (Figure 1.7). Members of the two protein families recognize previously buried hydrophobic regions that are commonly found in non-native proteins (Wickner *et al.* 1999). The failure to eliminate misfolded proteins can cause the formation of potentially toxic aggregates, inactivation of functional proteins, and ultimately cell death. The number of diseases linked to aberrant protein conformations and lack of proper protein quality control include Huntington's disease, Alzheimer's disease, Parkinson's disease, and more (Dobson 2004; Macario and Conway de Macario 2005). They underline the importance of effective quality control for cell survival.

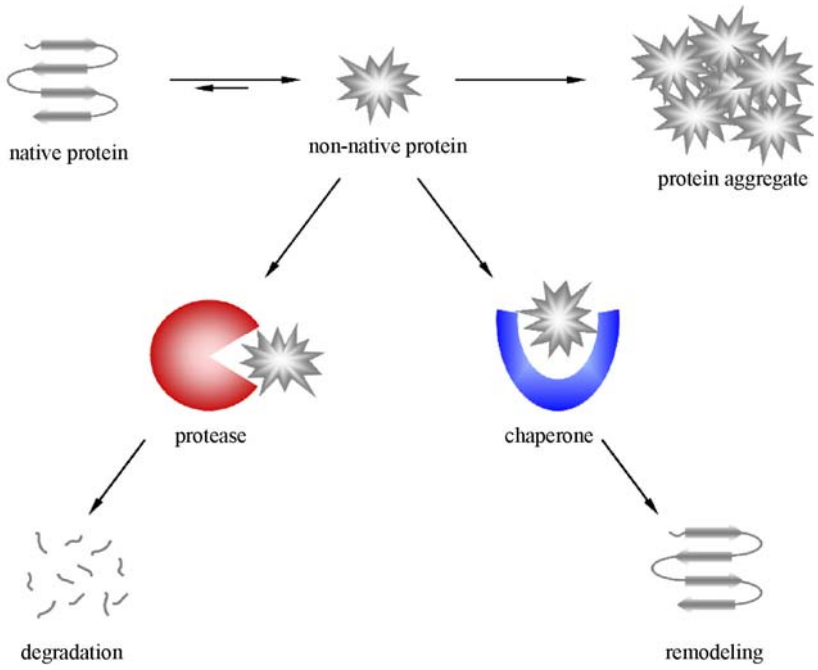


Figure 1.7. Schematic representation of the protein quality control concept. Non-native proteins can either be refolded by molecular chaperones or degraded by proteases. Both principles serve the same goal, namely the avoidance of protein aggregates, which are lethal for the cell. Achieving the correct balance between folding and degradation of misfolded proteins is critical for cell viability.

1.3.1 Heat-shock proteins

The heat-shock response is one of the fundamental responses of living cells. It is characterized by the induction of a set of proteins (heat-shock proteins - Hsps) as a result of rapid change in the environmental temperature. The Hsps protect the cell against environmental stress, and they produce tolerance against high temperature, high salt, and heavy metals (Rosen and Ron 2002). Many Hsps are highly conserved in evolution from bacteria to human and they comprise a group of molecular chaperons (e.g., GroEL, GroES, DnaK, and DnaJ) and a group of ATP-dependent proteases (e.g. ClpAP, ClpXP, HslUV (ClpYQ), Lon and FtsH) that play a critical role in the monitoring of correct protein folding and in protein degradation under normal and stress conditions (Rosen and Ron 2002). The cytoplasmic heat shock response and its factors have been extensively studied and growing evidence brings more and more understanding to the exact functions of Hsps in this cellular compartment and interplay among them (Dougan *et al.* 2002; Liberek *et al.* 2008). Figure

1.8 schematically summarizes the contribution of major cytoplasmic Hsps to the protein quality control in this compartment.

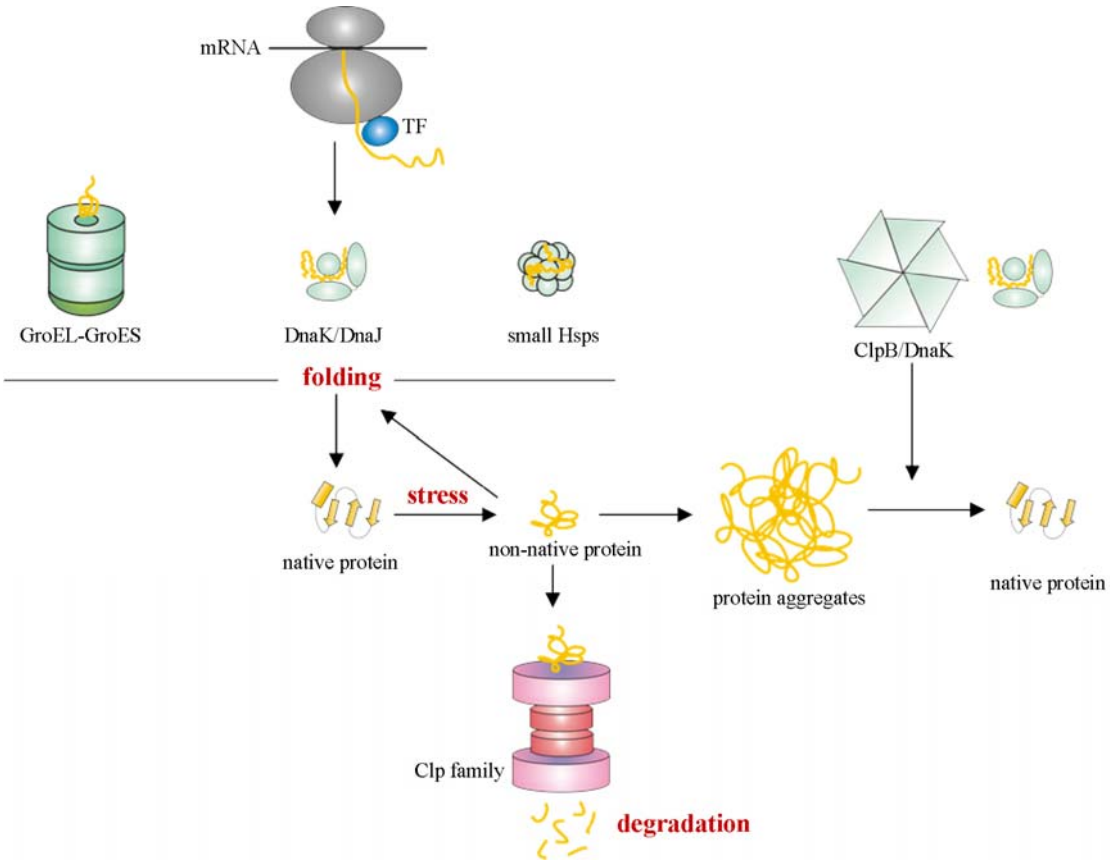


Figure 1.8. The network of chaperones and proteases controlling protein quality in the cytoplasm of *E. coli*. The newly translated proteins are associated with trigger factor (TF), which prevents their aggregation. Most newly synthesized proteins fold to their native state with the assistance of chaperone systems (GroEL, DnaK/J). Upon exposure to stress stimuli some proteins may misfold and consecutively be refolded to the native state by the chaperone systems or alternatively they are degraded by proteases. Under severe stress conditions the misfolded proteins tend to aggregate. The aggregated proteins may either be rescued by the ClpB/DnaK/J bi-chaperone unfolding system or degraded by the ClpAPS proteolytic machine (Houry 2001; Dougan, Mogk et al. 2002; Deuerling and Bukau 2004; Young *et al.* 2004).

1.3.2 Multi subunit proteases and chaperones

Beside other important biological functions, a special class of multi-subunit proteases is almost exclusively responsible for the removal of damaged or denatured proteins and the recycling of their amino acids (Schneider and Hartl 1996). Structural studies of the proteasome (Groll *et al.* 1997), ClpP (Wang *et al.* 1997) and HslV (Bochtler *et al.* 1997) have revealed that the multi-subunit proteases share the common feature of a

relatively large central cavity. The proteolytic subunits associate into multimeric rings that stack upon each other to form a barrel-shaped complex (Figure 1.9). The active sites are physically sequestered in a gated protein chamber to ensure complete degradation of a substrate and to avoid its unintended proteolysis (Pickart and Cohen 2004).

The degradation process is accompanied by regulatory ATPase complexes which deliver substrates to the internal proteolytic chamber. For HslV, the corresponding ATPase is HslU (Figure 1.9) and for ClpP it is ClpA or ClpX. The ATPase components also consist of oligomeric rings, that stack vis-à-vis on the protease complex (Ramachandran *et al.* 2002; Pickart and Cohen 2004; Hanson and Whiteheart 2005). The ATPase complex and the protease chamber function together to degrade a substrate protein. The substrate protein first gets unfolded in the substrate binding chamber of the ATPase complex. Then the unfolded protein is translocated through a narrow axial channel to the protease complex where it gets degraded (Figure 1.9) (Ishikawa *et al.*, 2001).

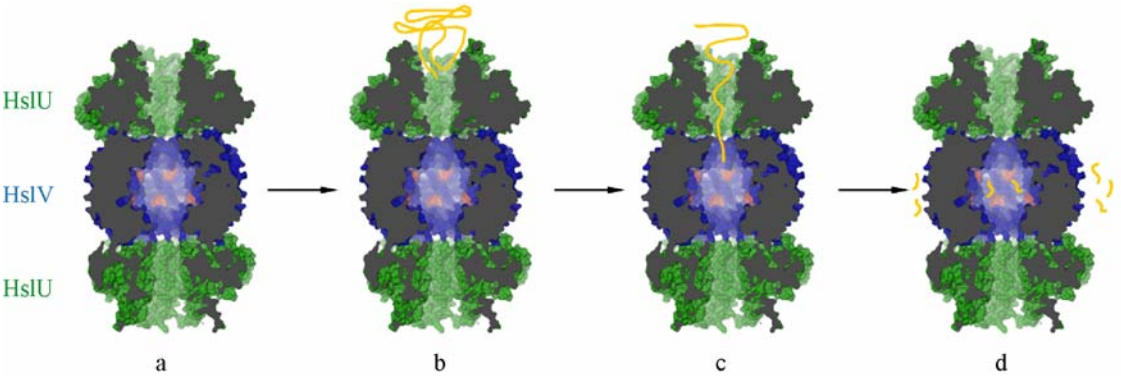


Figure 1.9. Degradation of abnormal proteins by the HslVU protease. a) Cross-section view of the HslUV (PDB entry code: 1G3I (Sousa *et al.* 2000)). Protease with the following colour code: ATPase subunits (HslU): green; protease subunits (HslV): blue; active sites: red. b-d) The non-functional target protein (yellow) is first bound to the ATPase subunit (b). There the protein is unfolded by using ATP and subsequently translocated through the narrow internal channel to the protease subunit (c). In the proteolytic chamber, the protein is degraded to oligopeptides in an ATP-independent fashion (d) (Wickner, Maurizi et al. 1999).

Another important cage forming protein involved in protein quality control in the bacterial cytoplasm is the GroEL/GroES chaperone complex also named chaperonin. It belongs to the Hsp60 family of molecular chaperones and was shown to be the only chaperone that is essential for the growth of *E. coli* (Fayet *et al.* 1989). The barrel shaped

chaperone GroEL is a homo-oligomer of fourteen subunits arranged into two heptameric rings forming two hollow cylinders, which can accommodate a protein of 20-60 kDa molecular weight (Figure 1.10) (Houry *et al.* 1999). The smaller co-chaperone GroES forms a homo-heptameric, dome shaped single ring. The interaction between GroEL and GroES approximately doubles the size of the chamber and allows proteins to fold under otherwise prohibitive conditions (Deuerling and Bukau 2004). The process requires ATP which binds to a substrate-occupied GroEL and initiates a series of conformational changes that bury the substrate-binding sites and lower the affinity for non-native polypeptides thereby alternating the character of GroEL between hydrophobic (binding) and hydrophilic (folding/release) (Ranson *et al.* 1998). ATPase cycles control both GroES cap dissociation and substrate binding and release. About 10–15% of total cytoplasmic *E. coli* proteins use the GroEL/GroES system for *de novo* folding under normal growth conditions and about twice as many under stress conditions (Ewalt *et al.* 1997; Houry, Frishman et al. 1999).

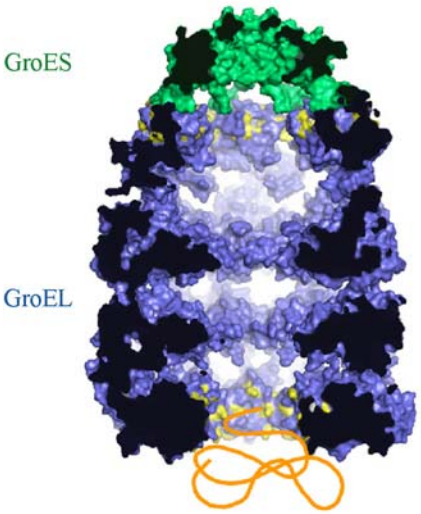


Figure 1.10. Bacterial chaperonin - a cross-section of GroEL-GroES complex structure. Two heptameric rings of GroEL (blue) associate with GroES (green) (PDB entry code: 1aon (Xu *et al.* 1997)) to form a functional chaperonin complex. b) A cross-section view of the GroEL barrel. The substrate protein (orange) binds through the hydrophobic interactions with the substrate binding sites (yellow) of the unoccupied ring of a GroEL-GroES asymmetric complex. Association of GroES with GroEL and the nucleotide binding results in enlargement of the size of the cavity. The images of the structures were produced using PyMOL (DeLano 2002).

All of the chamber-forming factors involved in protein quality control clearly shows common characteristics. They all exhibit a compact homo-oligomeric architecture with either active centers or binding sites encompassed in the interior of the cavity. The self-compartmentalized particles seclude the substrate protein from the cytoplasmic environment and ensure an undisturbed completion of the molecular process. The limited flexibility of the subunits makes them rigid structures with a defined oligomeric state.

Furthermore, these barrel shaped complexes (GroEL, HslU, ClpP) require both cofactors (GroES, HslV, ClpA) and ATP for their proper function.

1.4 Protein quality control in the periplasm

It is worth highlighting the importance of protein quality control in the periplasm for cell survival. Unlike the cytoplasm, the envelope is exposed directly to the external environment owing to the porous character of the outer membrane. Thus the bacterial envelope is a physiologically distinct compartment with proteins that are continually exposed to the changing conditions of the external environment (Raivio and Silhavy 2001).

Owing to the special nature of the envelope proteins that reside there must have evolved unique mechanisms of activity compared to their cytoplasmic counterparts. The discoveries made for the cytoplasmic protein quality control factors are not directly applicable to the extracytoplasmic ones due to the fact that traditional Hsps require ATP for their activity, and the envelope is devoid of nucleotides. Thus it is of great interest to reveal the mechanisms underlying protein quality in the periplasm.

1.4.1 Stress in the periplasm

Optimal cellular growth requires that the cell is able to sense and respond to changes in subcellular compartments. Due to the presence of the envelope, the stress response in Gram-negative bacteria is compartmentalized into cytoplasmic and extracytoplasmic responses. In contrast to cytoplasmic stress, where the sensing of misfolded proteins and the accompanying response take place in the same compartment, extracytoplasmic stress signals must cross the cytoplasmic membrane by a signal transduction system. *E. coli* senses and responds to the extracytoplasmic stress via at least two overlapping, but distinct, transduction pathways: the Cpx two-component system and the σ^E heat shock pathway (Figure 1.11) (Raivio and Silhavy 1999). Both regulatory systems control the expression of several genes whose products are envelope-localized protein folding catalysts (PPIases, disulphide isomerases), chaperones and proteases (DegP), as well as genes involved in lipid and lipopolysaccharide metabolism (Miot and

Betton 2004). Notably, many of these factors also participate in the biogenesis of the cell envelope in the absence of stress as described in section 1.2. (Ruiz and Silhavy 2005)

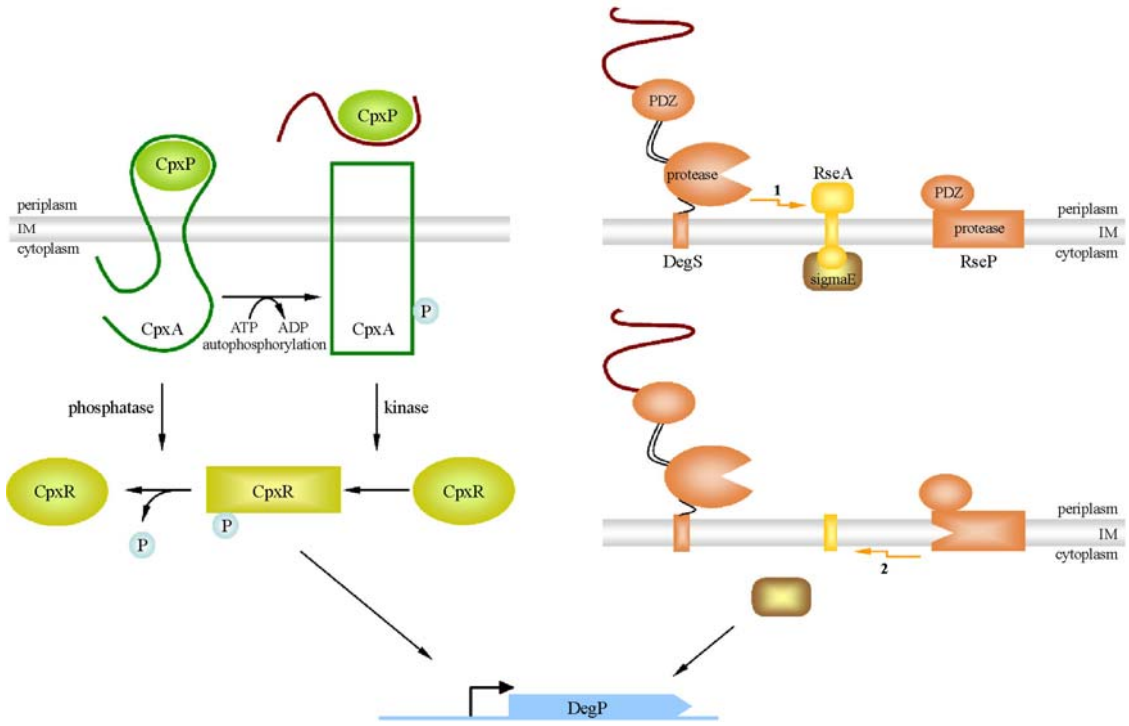


Figure 1.11. Two signaling pathways for the extracytoplamic stress response in *E. coli*. When induced by unfolded proteins (dark red) both pathways trigger a signaling cascade that leads to the regulation of factors needed to combat envelope damage (see text for details). Apart from specific targets, CpxA and σ^E control transription of common factors such as DegP. Figure adapted from (Raivio and Silhavy 2001; Ruiz and Silhavy 2005; Hasselblatt *et al.* 2007).

Cpx signal transduction is mediated by a two-component regulatory system consisting of a sensory histidine kinase CpxA and the response regulator CpxR. Envelope stresses are sensed by the inner membrane localized CpxA (Figure 1.11). In the absence of envelope stress, CpxA functions as a CpxR phosphatase. In the presence of envelope stress, CpxA undergoes a conformational change, which causes it to take on autokinase and CpxR kinases activities. Phosphorylation of CpxR converts it to a transcription factor able to bind to the promoters of target genes. The small periplasmic protein CpxP modulates the actions of CpxA. Under normal conditions it is bound to CpxA and thus prevents autophosphorylation. When unfolded proteins accumulate, CpxP interacts with them and no longer inhibits CpxA, which can then activate CpxR (Raivio and Silhavy 2001; Duguay and Silhavy 2004).

The second pathway leads to the activation of the alternative σ^E factor which is a transcription regulator. Under nonstress conditions, the activity of σ^E is negatively regulated by its antisigma factor, RseA and by the periplasmic protein RseB (De Las Penas *et al.* 1997; Missiakas *et al.* 1997; Collinet *et al.* 2000). When complexed with RseA, σ^E is trancriptionally inactive. In response to extracytoplasmic stress cells degrade RseA via a proteolytic cascade (Figure 1.11). RseA is cleaved first by DegS and then RseP (YaeL) to release the RseA/ σ^E complex from the membrane (Alba *et al.* 2002; Kanehara *et al.* 2002). The cytoplasmic part of RseA is then degraded by ClpXP and active σ^E is released (Flynn *et al.* 2004). This proteolytic cascade is activated by unassembled OMPs which accumulate during appropriate envelope stress. Their C-termini are recognized and bound by the PDZ domain of DegS, which causes a conformational change in DegS resulting in its activation (Walsh *et al.* 2003; Wilken *et al.* 2004; Hasselblatt, Kurzbauer et al. 2007).

Although activation of σ^E-mediated transcription induces the expression of many genes (folding catalysts, proteases), it also down-regulates the expression of a subset of outer membrane proteins (OmpC, OmpF, and OmpA), thereby reducing the accumulation of unassembled OMPs and limiting the duration of the response (Rhodius *et al.* 2006). Taken together, both regulatory systems serve to ensure proper biogenesis of the bacterial envelope by sensing and counteracting any perturbation in periplasmic protein folding.

1.4.2 HtrA family

The role of afore mentioned DegS and DegP proteins in maintaining envelope homeostasis is significant. Interestingly they belong to the same family of proteins, namely High temperature requirement (HtrA). The family exhibits a characteristic domain composition comprising a conserved protease domain and one or two C-terminal PDZ domains (Clausen, Southan et al. 2002) (Figure 1.13). Some members of this family possess additional N-terminal domains, such as a transmembrane region or an insulin growth factor-binding domain (IGFBP) (Kim and Kim 2005). Functionally HtrAs monitor protein homeostasis in the cell. Prokaryotic HtrAs underlie processes involved in tolerance against various folding stresses and pathogenicity (Jones *et al.* 2001; Cortes *et al.* 2002; Mo *et al.* 2006), whereas human homologues are involved in the onset of diseases related to

disturbed protein quality control. The examples include arthritis, Parkinson's and Alzheimer's disease (Gray *et al.* 2000; Grau *et al.* 2006; Plun-Favreau *et al.* 2007). The cellular localization of HtrAs is connected to extracytoplasmic compartments such as periplasm in Gram-negative bacteria or membranes in Gram positive organisms. In eukaryotes Htra proteases were found in endoplasmic reticulum, mitochondria and chloroplasts (Huesgen *et al.* 2005; Zurawa-Janicka *et al.* 2007; Vande Walle *et al.* 2008).

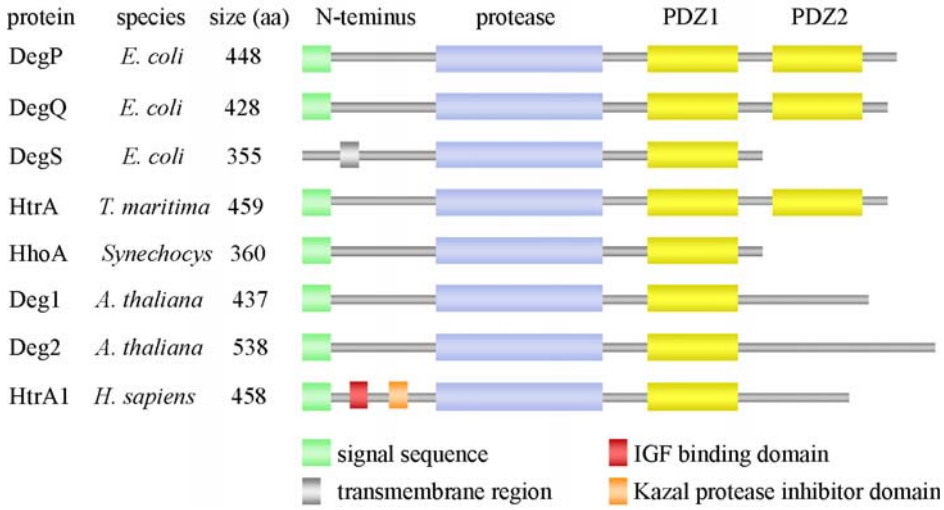


Figure 1.13 Comparison of HtrA family members. Schematic representation of the domain organization of selected HtrAs. The protease domain is colored in blue, PDZ1 and PDZ2 in yellow. The N-terminal segments may contain a signal sequence (green), a transmembrane segment (dark grey), an insulin growth factor (IGF) binding domain (red) or Kazal protease inhibitor domain (orange). The sizes refer to mature proteins. Figure adapted from (Clausen, Southan et al. 2002).

Up to date, four crystal structures of HtrA family members are available, namely DegP (Krojer, Garrido-Franco et al. 2002), DegS (Wilken, Kitzing et al. 2004; Hasselblatt, Kurzbauer et al. 2007), human HtrA2/Omi (Li *et al.* 2002) and the protease domain of HtrA from *Thermotoga maritima* (Kim *et al.* 2003). A trimer constitutes the basic building block which is stabilized by interactions between protease domains while the PDZ domains constitute mobile elements (Figure 1.12 a-b). The protease domain adopts a chymotrypsin-like fold with a catalytic triad composed of histidine, aspartate, and serine residues, an oxyanion whole which stabilizes reaction intermediates and substrate binding sites with defined specificity pockets. It is important to note that the structure of an activated form of

DegS is the only one that exhibits correct architecture and accessibility of all the elements (Wilken, Kitzing et al. 2004).

PDZ domains are conserved protein modules that mediate specific protein-protein interactions (Doyle *et al.* 1996). Most known PDZ-mediated contacts occur through the recognition of short COOH-terminal peptide motifs (Oschkinat 1999). The available crystal structures of HtrAs imply high mobility of the domains where they act as molecular gatekeepers of the proteolytic chamber (DegP) or regulators of proteolytic activity (DegS, hHtrA1). Among HtrAs of *E. coli,* only two proteins posses two C-terminal PDZ domains namely DegP and its close homolog – DegQ.

1.4.3 Function and structure of DegP

DegP (also named HtrA or protease Do) was discovered nearly 25 years ago, when Goldberg and co-workers undertook a systematic study of proteases in *E. coli* (Swamy *et al.* 1983). DegP is synthesized as a precursor protein with an N-terminal signal peptide (26 amino acids long) that targets the protease to the periplasm (Pallen and Wren 1997). The *htrA* gene was identified by two phenotypes of *htrA* null mutants. These mutants were thermosensitive (Lipinska *et al.* 1989) and showed a decreased degradation of abnormal periplasmic proteins (Strauch *et al.* 1989). DegP is a heat-shock protein and transcription of its gene is regulated by both σ^E and Cpx pathways in response to unfolded protein stress in the cell envelope (Erickson and Gross 1989; Danese *et al.* 1995) (section 4.1). Biochemical data confirm that the protease recognizes the nonnative states of proteins and that exhibits strong preference for cleavage after small hydrophobic residues (valine, isoleucine) (Kolmar *et al.* 1996). Based on these results DegP was proposed to degrade misfolded proteins, thus reducing damage in the periplasm, as a primary physiological role. Yet, DegP was shown to display a chaperone activity additionally to its digestive properties (Spiess, Beil et al. 1999). A switch between the two functions is mediated by temperature: the chaperone activity prevails at low temperatures (28˚C), whereas the protease activity is dominant at higher temperatures (42˚C) (Spiess, Beil et al. 1999). Recent studies have shown however, that the proteolytically inactive DegPS210A mutant is able to prevent aggregation of unfolded substrates over a wide range of temperatures (30–45˚C) (Skorko-

Glonek *et al.* 2007). Overall, DegP represents an important protein quality factor in the bacterial periplasm reversibly switching between the two functions, which may be necessary to rapidly respond to environmental changes (Clausen, Southan et al. 2002).

Although the molecular mechanisms of the switch and substrate partitioning between the two activities remain elusive, the crystal structure of DegP was a step forward in an attempt to understand a possible mode of action of DegP. The crystal structure of DegP shows the protein to be arranged as a hexamer composed of two staggered trimeric rings (Figure 1.12a) (Krojer, Garrido-Franco et al. 2002). The proteolytic sites are located in an inner chamber, which is accessible only laterally (Figure 1.12b). Additionally, the chamber is lined with hydrophobic patches that might constitute the substrate binding sites for nonnative polypeptides (Figure 1.6). The highly flexible PDZ domains form sidewalls that restrict the access to the cavity. Remarkably, the same crystal yielded two significantly different conformations of PDZ domains. In one they protrude from the core thus creating a lateral passage with a freely accessible inner cavity ('open state') while in the second they cover the entrances to the chamber ('closed state') (Figure 1.12). This striking feature could imply a possible role of PDZ domains which would couple two events: substrate binding and subsequent translocation into the inner chamber (Clausen, Southan et al. 2002). Although the PDZ domains do not constitute the essential hexameric interactions ('open' state), the PDZ2 domain has been demonstrated to be indispensable for the hexamer formation in solution (Sassoon *et al.* 1999; Iwanczyk *et al.* 2007).

The dimerization of trimeric rings is mediated by the interaction between the loops LA from opposite trimers. They form spacing pillars of the chamber containing an intersubunit β-sheet (Figure 1.12a). It has been proposed that shortening of the loop LA results in the decrease of the volume or the collapse of the cavity. Such mutants can still fulfill their functions suggesting that neither specific dimensions of the cage nor the presence of the enclosed cavity itself is essential for the chaperone or protease activities in DegP (Jomaa *et al.* 2007). Moreover, in the crystal structure, the loop LA from each DegP monomer protrudes into the active site of the opposite monomer, where it interacts with the active-site loops (L1 and L2) and thereby distorting their spatial organization (Figure 1.12c). The resulting twist of the loops impedes proper adjustment of the catalytic triad and formation of the oxyanion hole, as well as the S1 specificity pocket (Krojer, Garrido-

Franco et al. 2002). Thereby the reported structure of DegP shows a proteolytically inactive conformation and could represent a chaperone state.

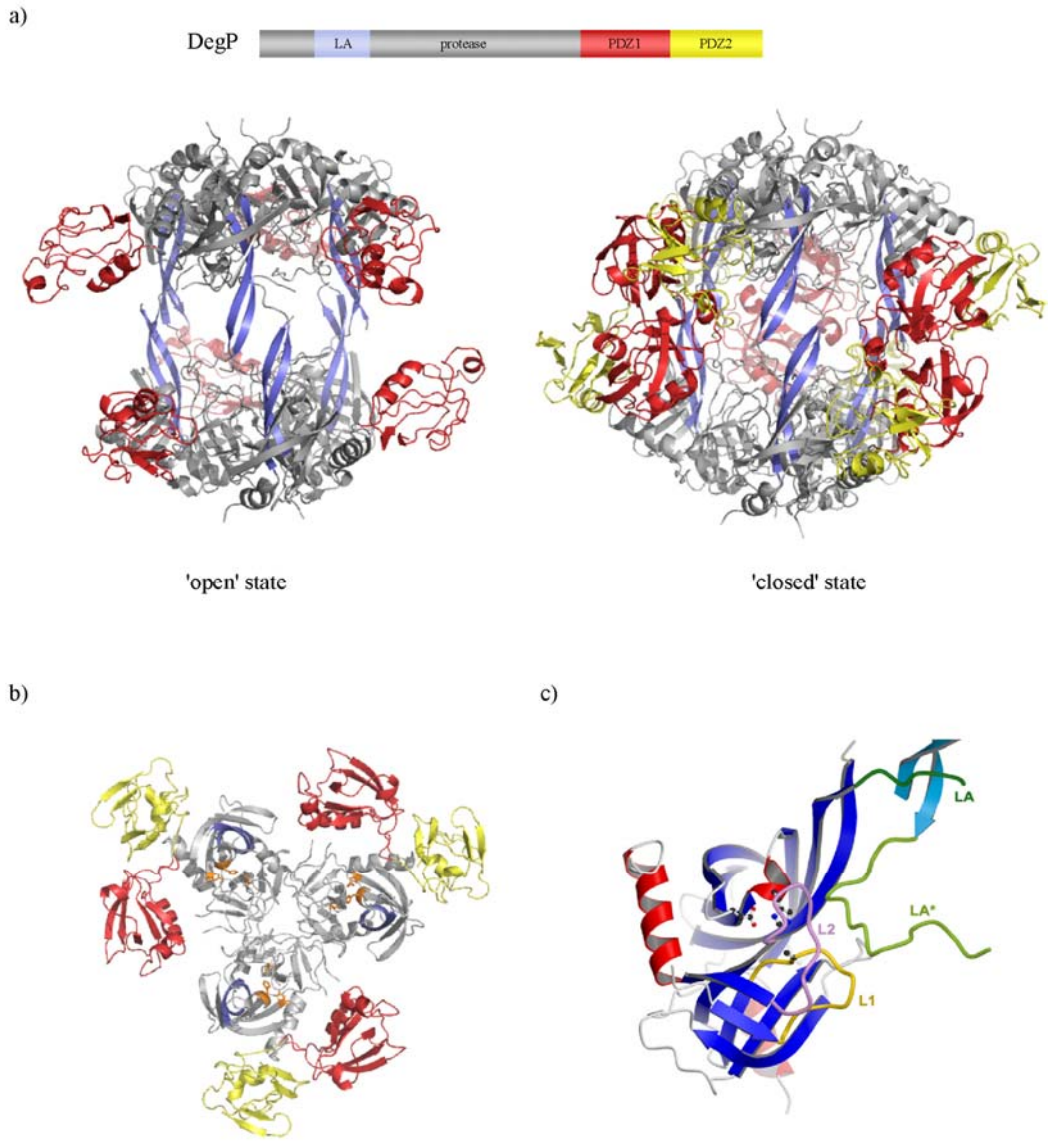


Figure 1.12. The crystal structure of *E. coli* DegP. a) Side view of DegP hexamer in two states: 'open' (left) and 'closed' (right). Trimeric rings of the protease domain (grey) are connected by 'loop LA pillars' by forming an inter-subunit β-sheet (blue). PDZ domains are depicted in red (PDZ1) and yellow (PDZ2). PDZ2 domains of the 'open' state were too flexible to be resolved. b) A trimeric building block of HtrA proteases viewed from the inside of the DegP cavity 'closed' state. Active sites (orange) line the ceiling of the cavity. c) Loop LA (LA*) protrudes into the active site of the opposite monomer and distorts the conformation of active site loops L1 and L2. Active site residues are depicted in ball and stick representation. Note that, an active site mutant Ser210Ala was used for crystallization. PDB entry code for DegP: 1ky9 (Krojer, Garrido-Franco et al. 2002). The images of the structures were produced using PyMOL (DeLano 2002).

Compared to the other HtrA proteases, the loop LA of *E. coli* DegP contains an extraordinarily long stretch of 48 residues (Clausen, Southan et al. 2002) (Figure 1.14). This region is a proline/serine/glutamine rich segment that has the characteristics of a Q-linker (Wootton and Drummond 1989) and it is positioned between the β1 and β2 strand of the protease domain (Kim and Kim 2005). This region constitutes an important difference between DegP and other members of the HtrA family in which Q-linkers are much shorter. The length and structure of the Q-linkers may be considered a major criterion with regard to the classification of DegP, DegQ, and DegS subfamilies, as well as in the determination of their functions (Kim and Kim 2005)

1.4.4 DegQ - a homolog of DegP

E. coli DegQ (also designated HhoA for 'HtrA homology') was first identified and characterized by Bass et al. (Bass *et al.* 1996) and Waller and Sauer (Waller and Sauer 1996). The *degQ* gene is located directly upstream of *degS* at the considerable distance of *htrA*. The two genes appear to be regulated independently and, unlike *degP*, neither of them is heat inducible (Waller and Sauer 1996). Similarly to DegP, DegQ is synthesized with an N-terminal signal sequence (27 amino acids) and it is targeted to the periplasm (Waller and Sauer 1996).

The proteins DegQ and DegP have a similar size, consisting of 455 and 474 residues, respectively. They also share the same domain composition being the only members of the *E. coli* Deg family encompassing two PDZ domains. DegP and DegQ exhibit 60% overall sequence identity and 23% similarity. Despite very high overall sequence homology within the protease domain the afore mentioned Q-linker of DegQ is 20 amino acids shorter and according to secondary structure predictions it harbors a short α-helix (Figure 1.14). The same α-helix was predicted and observed in the crystal structure of the protease domain of *T. maritima* where it was proposed to play a role in the regulation of the protease (Kim, Kim et al. 2003) (Kim *et al.* 2008). In solution DegQ was observed to form hexamers, however when covalently cross-linked, dodecamers could be detected (Kolmar, Waller et al. 1996).

DegQ is not essential for normal growth and *degQ* null mutants do not show an obvious phenotype under a variety of growth conditions (Waller and Sauer 1996).

However, it was demonstrated that overproduction of DegQ rescues the temperature-sensitive growth defect of a *degP* null strain suggesting DegQ to be a functional substitute for DegP. Furthermore, both proteins exhibit similar substrate specificity towards several substrates *in vitro* and are inhibited by diisopropyl fluorophosphate (DFP), a serine protease inhibitor (Kolmar, Waller et al. 1996; Waller and Sauer 1996). It was discovered that several DegP/DegQ homologs are implied in the pathogenic virulence of bacteria where DegP was an essential factor while the role of DegQ was not entirely evident (Johnson *et al.* 1991; Farn and Roberts 2004; Mo, Peters et al. 2006). Taken together DegP and DegQ display both overlapping features and dissimilarities, however the exact physiological function of DegQ remains poorly understood.

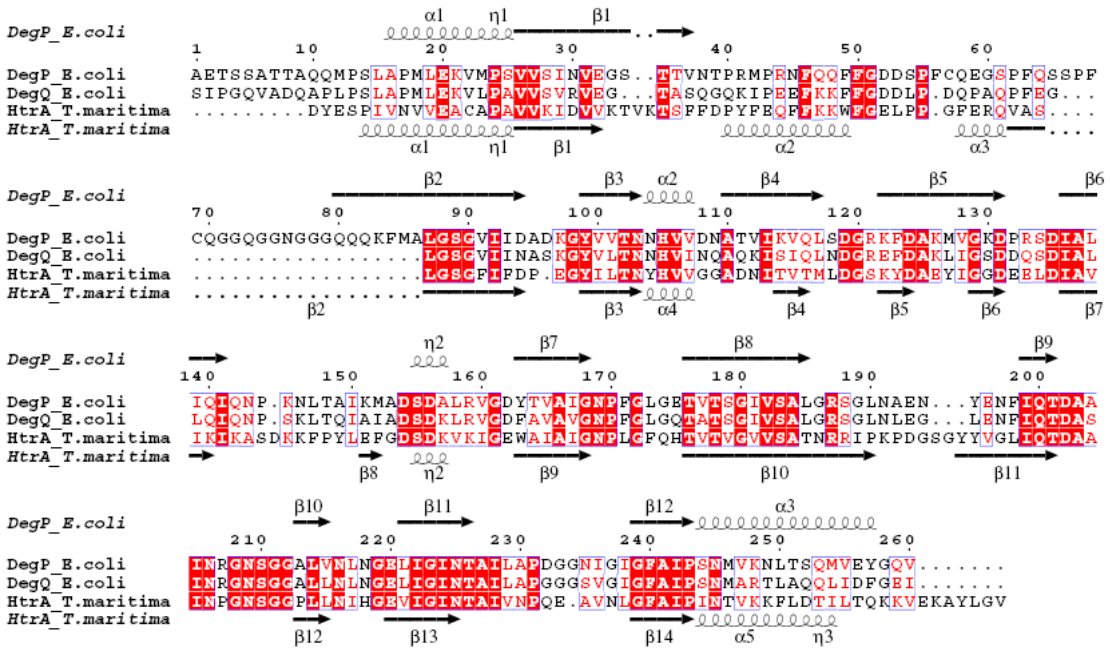


Figure 1.14. Multiple sequence alignment of protease domains of DegP, DegQ from *E.coli* and HtrA from *T. maritima*. The secondary structure information of DegP (PDB entry: 1ky9) and HtrA from *T. maritima* (PDB entry: 1L1J) can be seen above and below the corresponding amino acid sequence, respectively. Identity and similarity in the sequences of the three HtrA proteins is indicated by red boxes and red font, respecitevly. The Q-linker is positioned between β1 and β2 strand. The regulatory α-helix 2 of HtrA2 from *T. maritima* was not observed in the crystal structure of DegP. The alignment was performed using ClustalW (Chenna *et al.* 2003) and the figure was produced using ESPript (Gouet *et al.* 1999).

1.5 Aim of the study

The focus of this study comprises the characterization of the two central factors of periplasmic protein quality control, namely DegP and DegQ. The unique ability of DegP to act as both a chaperone and a protease raises the question how one protein fulfills the two antagonistic functions. A structural and biochemical approach was chosen to reveal how substrates are discriminated between the two activities and to better understand the mechanism how a single quality control factor switches in a tightly regulated manner between refolding and digestive function.

In addition, following the reported ability of DegP to interact with OMPs, an *in vivo* and *in vitro* approach was planned to be employed to investigate the role of DegP in OMP biogenesis and to analyze its function as a potential shuttle chaperone ensuring targeted transport of OMPs through the periplasm.

Moreover, by moving the attention to another protein quality factor of *E. coli* periplasm, namely DegQ, we wanted to compare the mechanistic features of DegQ and DegP in order to pinpoint common features and specialized properties within the HtrA family of protease-chaperones. The planned experiments should provide novel insights how such highly similar proteins complement their abilities to sense and counteract the aggregation-prone misfolding in the envelope of gram-negative bacteria.

2 Results and Discussion

2.1 Manuscript: 'Structural basis for the regulated protease and chaperone function of DegP'

The cryo EM figures (Figure 5, Supplementary Figure 2 and 6) were contributed by Eva Schäfer and Helen R. Saibil from Crystallography Department and Institute of Structural Molecular Biology, Birkbeck College, London.

Structural basis for the regulated protease and chaperone function of DegP

Tobias Krojer[1a], Justyna Sawa[1a], Eva Schäfer[2a], Helen R. Saibil[2], Michael Ehrmann[3] and Tim Clausen[1*]

[1]*Research Institute for Molecular Pathology - IMP, Dr. Bohrgasse 7, A-1030 Vienna, Austria*

[2]*Crystallography Department and Institute of Structural Molecular Biology, Birkbeck College, Malet St, London WC1E 7HX, UK*

[3]*Centre for Medical Biotechnology, FB Biology and Geography, University Duisburg-Essen, Universitätsstrasse, D-45117 Essen, Germany*

[a] *These authors contributed equally to the work.*

[*] *Corresponding author: Tim Clausen, phone: +43-1-797303350, fax: +43-1-7987153 email: clausen@imp.univie.ac.at*

All organisms have to precisely monitor the folding state of cellular proteins. The heat-shock protein DegP is a protein quality control factor in the bacterial envelope that is involved in eliminating misfolded proteins and in the biogenesis of outer membrane proteins (OMPs). To investigate the molecular basis of these dual activities we characterized different DegP/substrate complexes. Binding of misfolded proteins transformed hexameric DegP into large, catalytically active 12- and 24-meric multimers. Structural analysis of these particles revealed that DegP represents a protein packaging device whose central compartment is adaptable to the size and concentration of substrate. Moreover, the inner cavity serves antagonistic functions. While encapsulation of folded OMP protomers is protective and might allow safe transit through the periplasm, misfolded proteins are eliminated in the molecular reaction chamber. Oligomer re-assembly and concomitant activation upon substrate binding may also be critical in regulating other HtrA proteases implicated in protein folding diseases.

All living organisms employ dedicated chaperones and proteases to monitor and control the state of cellular proteins. Failure of this quality control can lead to protein aggregation, a malfunction correlated with fatal protein folding diseases (Selkoe 2003; Macario and Conway de Macario 2005). The protease-chaperone DegP represents a unique model system for uncovering mechanisms that protect cells from misfolded or damaged proteins as it combines digestive and remodelling activities on a single polypeptide and can switch between these dual functions in a tightly regulated manner (Spiess *et al.* 1999; Iwanczyk *et al.* 2007; Meltzer *et al.* 2007). DegP is a member of the widely conserved HtrA family of serine proteases that are crucial to maintain protein homeostasis in extracytoplasmic compartments (Clausen *et al.* 2002). The bacterial representatives DegP and DegS play key roles in the unfolded protein response of the cell envelope, whereas the four human HtrAs are implicated in many severe disorders including Parkinson's and Alzheimer's disease (Grau *et al.* 2005; Plun-Favreau *et al.* 2007). HtrA proteins encompass a catalytic domain with a chymotrypsin-like fold and one or two C-terminal PDZ domains, which are well-characterized protein-protein interaction modules (Harris and Lim 2001). The protease

domains of three protomers closely interact to form a trimer that represents the basic building block of HtrA oligomers. The projecting PDZ domains either participate in protein degradation by presenting substrates to the protease (Iwanczyk, Damjanovic et al. 2007) or offer a binding site for an allosteric activator that stimulates protease function (Wilken *et al.* 2004; Hasselblatt *et al.* 2007). The reversible activation mechanism ensures that the digestive mode of HtrA proteins can be precisely switched on and off depending on the needs of the cell. The available crystal structures suggest that HtrAs differ in their molecular architecture, ranging from trimers with surface accessible active sites to hexamers that belong to the class of self-compartmentalising proteases (Clausen, Southan et al. 2002). For these proteases, trimer association positions a regulatory loop into the active site of a neighbouring molecule, thereby blocking substrate access and deforming the proteolytic site (Krojer *et al.* 2002). In addition to its housekeeping function, DegP is also involved in outer membrane protein (OMP) biogenesis (Misra *et al.* 1991; CastilloKeller and Misra 2003; Ruiz *et al.* 2006; Purdy *et al.* 2007). OMPs are translocated as unfolded polypeptide chains across the cytoplasmic membrane via the general SecYEG secretion complex (Ruiz, Kahne et al. 2006). In the periplasm, OMPs are targeted to a translocation machinery in the outer membrane composed of the integral OMP YaeT and the four lipoproteins NlpB, SmpA, YfgL and YfiO (Wu *et al.* 2005). As partially folded OMPs would be substrates for various periplasmic proteases, cells must ensure a safe transit of OMP precursors between inner and outer membrane. Moreover, unfolded OMPs could be prone to protein aggregation and would continuously stimulate the σE stress response (Alba and Gross 2004). So far, the three chaperones SurA, Skp and DegP have been implicated in guiding OMPs through the periplasm (Chen and Henning 1996; Rouviere and Gross 1996; Rizzitello *et al.* 2001; Sklar *et al.* 2007). However, their exact contributions remain to be fully understood.

To better understand how a single cellular factor selectively binds unstructured proteins and then decides whether a substrate will be degraded, repaired or transported to its ultimate cellular destination, we aimed to characterize *in vivo* substrates of DegP and tested which proteins co-purify with the proteolytically inactive $DegP_{S210A}$ (Supp.Fig.1). Size exclusion chromatography (SEC) led to the identification of three DegP oligomers, the 6-mer ($DegP_6$), 12-mer ($DegP_{12}$), and 24-mer ($DegP_{24}$), of which the two larger particles had

additional proteins bound (Fig.1a). Analysis of solubilized crystals of the $DegP_{24}$ complex revealed that the co-purified and -crystallized proteins were the outer membrane proteins OmpA, OmpC, OmpF and LamB.

Crystal structure of $DegP_{24}$

The crystal structure of the $DegP_{24}$ complex was solved by the single wavelength anomalous dispersion method and refined to an R-factor of 21.2% at 3.0 Å resolution (R_{free} 27.4%, Supporting Table 1). In contrast to the previously solved hexameric structure of DegP, the protease domain as well as the PDZ1 and PDZ2 domains are well-defined by electron density and exhibit good stereochemistry. Only one protease loop (residues 36-81) was too flexible to be traced in the electron density. The co-crystallized OMPs were also not defined by electron density, presumably due to conformational and chemical heterogeneity.

The 24-mer of DegP has a molecular weight of 1.13 MDa and forms a spherical shell with 432 symmetry (Fig.1b). Its diameter of 195 Å is consistent with electron microscopic (EM) images of negatively stained $DegP_{24}$/OMP particles, which were about 190 Å in diameter (Supp.Fig.2c,g). In the crystal structure of $DegP_{24}$, eight trimers are located at the vertices of an octahedron that assembles a protein shell of about 31 Å thickness enclosing a large internal cavity of about 110 Å in diameter. The inside volume of the sphere is about 700,000 $Å^3$, which is approximately eight times larger than an open cavity of GroEL (Fig.1c). Superposition of Deg_{24} with $DegP_6$ illustrates that this remarkable large cavity could, in theory, accommodate a 300 kDa protein (Supp.Fig.3). The protein shell has wide pores allowing access to the inner cavity. The largest of these pores is 35 Å wide and runs along the particle's 4-fold axes, whereas smaller channels that coincide with the 2-fold axes are 14 Å in diameter. The 24 proteolytic sites are only accessible from the interior of the cavity. Thus protein substrates would have to be encapsulated in the central compartment during oligomer assembly or enter the particle through one of the six pores. The size of these pores is large enough to allow small folded proteins (< 25 kDa) or unfolded polypeptides to diffuse in and out of the protein shell. The overall organization of the DegP trimer, in which three protease domains are encircled by six PDZ domains, dictates the

assembly of $DegP_{24}$. The outwards extending PDZ domains, PDZ1 and PDZ2, are in close contact with the PDZ domains of two adjacent trimeric rings. Four DegP trimers are arranged by these interactions around the 4-fold symmetry axis and form the large pores of the particle by constituting a ring of four PDZ1/PDZ2* pairs (the asterisk denotes a neighbouring molecule) (Supp.Fig.4).

Regulation of protease activity by oligomer re-assembly

The crystal structure of the DegP hexamer revealed that regulation of protease activity depends on loop LA (Krojer, Garrido-Franco et al. 2002) (for nomenclature of protease loops see Fig.2a). In the corresponding inactive conformation, loop LA protrudes into the active site of one subunit of the opposite trimeric ring, where it closely interacts with the active site loops L1* and L2*. The resulting loop triad LA-L1*-L2* obtains an entirely twisted conformation that blocks the entrance to the active site and distorts adjustment of the catalytic triad, oxyanion hole and substrate specificity pocket (Fig.2a). Our structural data indicate that transformation of the hexamer into the larger oligomers extracts loop LA from the active site of the molecular neighbour and releases loops L1 and L2 to set up a functional proteolytic site. For example, the stretched conformation of loop L1 observed in the inactive DegP is remodelled into the typical turn structure that is essential to form the oxyanion hole (Fig.2b). Thus conversion of $DegP_6$ into $DegP_{12}$ or $DegP_{24}$ plays a key role in regulating protease activity.

To study determinants of oligomer re-assembly, we incubated unfolded protein substrates with the hexameric form of $DegP_{S210A}$ and followed complex formation by SEC. While the larger substrates bovine serum albumin (BSA) and casein were generally captured in the $DegP_{24}$ complex (Supp.Fig.5a), lysozyme affected oligomerisation in a concentration dependent manner triggering formation of $DegP_{12}$ at lower and $DegP_{24}$ at elevated concentrations. Since the redistribution of oligomers did not depend on the amount of DegP (Fig.2c), we presume that the higher order particles mainly reflect the size and concentration of substrate.

When we tested the ability of proteolytically active DegP to form such complexes, we detected transient formation of $DegP_{24}$ and $DegP_{12}$. Short incubation with substrates

transformed $DegP_6$ into the larger oligomers, whereas after prolonged incubation, when degradation was completed, DegP reverted to its hexameric state (Fig.2d, Supp.Fig.5b). To probe the functionality of $DegP_6$, we assayed protease activity with a previously identified chromogenic peptide substrate (Meltzer, Hasenbein et al. 2007). Although no larger complexes were formed (data not shown), DegP slowly hydrolyzed the model peptide indicating that $DegP_6$ is capable of hydrolyzing oligopeptides. However, when we added denatured lysozyme and stimulated $DegP_{12/24}$ formation, degradation of the chromogenic substrate was accelerated 15-fold (Supp.Fig.5c). Similarly, at elevated temperature, where the protease activity of DegP is markedly upregulated (Spiess, Beil et al. 1999), $DegP_6$ was destabilized and the oligomer equilibrium shifted to $DegP_3$ (Supp.Fig.5d). Together, these data indicate that DegP exists in a dynamic equilibrium of different multimers that have specific functions in protein quality control. While $DegP_6$ appears to represent the resting state with reduced peptidase activity, $DegP_{12}$ and $DegP_{24}$ should function as protease-chaperone complexes acting on misfolded proteins. Because the high molecular weight particles are only stabilized as long as misfolded proteins are bound, the activity of DegP is directly linked to folding stress. Furthermore, the flexible encapsulation mechanism should guarantee quality control of a broad range of client proteins.

DegP functions as a chaperone for folded OMPs

Identification of the co-purified and co-crystallized DegP/OMP complexes suggests that DegP plays an active role in OMP biogenesis. To address the *in vivo* relevance of our findings, we analyzed the OMP composition of wildtype and *degP* null mutant strains (Fig.3a). In the *degP* mutant, the levels of OmpA and OmpF in the outer membrane were reduced, whereas the level of OmpC was also lowered but to a lesser degree. It is known that the expression of OMPs is tightly regulated. For example, the σE stress response that is triggered by folding stress can decrease the synthesis of several OMPs by RNA-regulated transcriptional repression (Rhodius *et al.* 2006; Guisbert *et al.* 2007). To test the consequence of deleting *degP* on OMP expression levels, we determined the amounts of OMPs in whole cell lysates and observed that the total amounts of expressed OMPs were similar in wildtype and *degP* null strains (Fig.3a). Thus the observed depletion of several

OMPs in the outer membrane seems to be due to the lack of DegP activity in OMP biogenesis.

To functionally characterize the observed complexes, we examined the stability of OMPs bound to proteolytically active DegP. In contrast to misfolded model substrates, which were degraded within a few minutes, the co-purified OMPs were remarkably stable (Fig.3b). Even in the presence of externally applied proteases, the bound OMPs were almost entirely resistant to proteolytic degradation (data not shown). They remained stably bound to DegP over a period of 30 minutes, a time frame that should be sufficient for targeted transport to the outer membrane.

As it is known that DegP specifically degrades misfolded proteins, we asked whether the bound OMPs might contain tertiary structures that protect them from degradation. Thermodynamic stability studies of OmpA and other β-barrel membrane proteins indicated that formation of tertiary structure can be conveniently followed by a shift of the apparent mass on SDS-PAGE gels (Schweizer *et al.* 1978). The SDS gel-shift assay revealed that at least 50% of bound OmpA is present in a folded state in the higher order particles of $DegP_{S210A}$ (Fig.3c). Thus DegP seems to stabilize a similar assembly intermediate as the functionally related SurA chaperone that favours formation of a folded LamB protomer (Rouviere and Gross 1996). When we analyzed the folding state of OmpA bound to the proteolytically active DegP, we observed that DegP degrades unfolded OmpA and stabilizes the folded protomers. Thus DegP functions as a bonafide OMP chaperone. Moreover, OmpC trimers could not be detected in the large oligomeric complexes (Fig.3c) suggesting that DegP selectively stabilizes folded OMP protomers, but cannot support subsequent assembly (trimerization) steps, which are known to require additional folding factors such as LPS and YaeT (Sen and Nikaido 1991).

Membrane attachment of $DegP_{24}$

To explore how DegP may interact with other molecules, we calculated the electrostatic potential of $DegP_{24}$ (Fig.4a). Most interestingly, clusters of lysine and arginine residues that originate from both PDZ domains render the electrostatic potential of the outer rim of the

large pores strongly positive (Fig.4a) thereby generating candidate sites for membrane attachment. Consistently, recent reports emphasize the importance of PDZ domains for membrane localization (Zimmermann *et al.* 2002; Mortier *et al.* 2005; Yan *et al.* 2005; Pan *et al.* 2007). To test the binding of $DegP_{24}$ to lipid membranes, we conducted a lipid sedimentation assay using liposomes prepared from bovine brain lipid extracts (Fig.4b). Remarkably, $DegP_{24}$ bound to liposomes with a similar affinity as other membrane-associated PDZ proteins (Wu *et al.* 2007). To directly monitor the influence of the PDZ domains on lipid binding, we generated two mutants, in which the surface exposed lysines 305, 379, 381 and 416 were replaced by either alanine ($DegP_{4A}$) or glutamate ($DegP_{3E}$). Dose-response experiments revealed that the lipid affinity of the $DegP_{4A}$ 24mer is significantly reduced and that lipid binding of $DegP_{3E}$ is almost entirely impaired (Fig.4b). These data indicate that DegP has exploited the PDZ domains to target cellular membranes. Alternatively, as the distance between cytoplasmic and outer membrane is believed to be between 150 and 330 Å (Winkler *et al.* 1977; Leduc *et al.* 1985), the assembled $DegP_{24}$ could become wedged between the two membranes with the positively charged openings directly facing the phospolipid layers. Thus DegP could function as a periplasmic macropore allowing protected diffusion of OMP precursors from the inner to the outer membrane.

EM analysis reveals the encapsulated OMP density

The $DegP_{12}$/OMP and $DegP_{24}$/OMP complexes were analysed by EM. Negative stain EM analysis of $DegP_{24}$/OMP yielded a map with octahedral symmetry that resembles the X-ray data filtered to an equivalent resolution (Supp.Fig.2g-k). However, the $DegP_{12}$/OMP complex was more homogeneous (Supp.Fig.2a vs c). To define the subunit assembly and OMP density, we examined the $DegP_{12}$/OMP complex by cryo EM.

The cryo EM map of $Degp_{12}$/OMP shows a tetrahedral cage with a diameter of ~160 Å (Fig.5a). Each face is made of a triangular density that fits well to a DegP trimer. In contrast to $DegP_{24}$, the inter-trimer contacts of $DegP_{12}$ are made by adjacent PDZ1 domains and do not appear to involve PDZ2 (Fig.5b). Furthermore, the fitted cryo EM map indicates that the catalytic sites open up into the central cavity of the particle, which has a diameter

of about 78 Å. Most interestingly, the central cavity is occupied by a cylindrical density that fits remarkably well to the native β-barrel of OmpC (Fig.5b). Because OmpA, OmpC, OmpF and LamB all form β-barrels with similar dimensions, the density observed in the central compartment could accommodate any of the potential OMP substrates. As it is unlikely that unfolded proteins or the unstructured loop LA give rise to such a defined shape, the extra density most likely represents an OMP monomer in a close to native conformation. Thus, the cryo EM data provides further evidence that DegP sequesters OMP monomers in a substantially folded state and provides a remarkable view of a membrane protein precursor before its insertion into the membrane.

Conclusions

Large protein complexes with octahedral 432 or icosahedral 532 symmetry often form hollow protein shells that are used to store specific molecules. Classical storage devices for iron atoms and nucleic acids are ferritin and virus particles, respectively (Winkler, Schutt et al. 1977; Banyard *et al.* 1978). Our data show that DegP represents another high-symmetry packaging device, whose central compartment is used to sequester unfolded proteins in the periplasm and to partition them between refolding and degradation pathways. In a first step, DegP has to sort out aberrant proteins that exhibit partially folded, aggregation-prone structures from properly folded proteins. $DegP_6$ appears to function as a substrate filter, as only unfolded proteins are capable of entering the cavity and assembling the functional protease-chaperone. Oligomer formation should not depend on the nature of the unfolded substrate and thus OMPs secreted into the periplasm as well as stress-damaged proteins should be equally well encapsulated by DegP. However, in contrast to other protease-chaperone systems, the inner cavity of $DegP_{12/24}$ combines the dual characteristics of a folding and a proteolytic compartment. Since the proteolytic activity of DegP is restricted to unfolded peptide structures, the fate of an encapsulated protein should mainly depend on its propensity to readily adopt its native, folded conformation and to escape the degradative machinery of DegP. Consistently, *in vitro* studies showed that several OMPs including PhoE, LamB and OmpA spontaneously fold into their β-barrel structures (Park *et al.* 1988;

De Cock *et al.* 1990; Klose *et al.* 1993; Rouviere and Gross 1996) and could thus, in contrast to unfolded soluble proteins, remain protected inside the high molecular weight DegP particle.

METHOD SUMMARY

An improved purification procedure allowed separation of the $DegP_6$, $DegP_{12}$ and $DegP_{24}$ multimers. Mass spectrometry and Western blot analysis revealed that specific OMPs were bound to $DegP_{12}$ and $DegP_{24}$. The $DegP_{24}$/OMP complex was crystallized and the structure solved by the single anomalous dispersion method. In parallel, the structure of the $DegP_{12}$/OMP complex was determined by single particle cryo-EM. In vitro complexes of DegP with several model substrates were analyzed by SEC and SDS PAGE illustrating the reassembly of the resting $DegP_6$ into the proteolytically active $DegP_{12}$ and $DegP_{24}$ complexes. The transient nature of the higher order complexes was shown by incubating wildtype DegP with an excess of substrate and immediate separation of the mixture by SEC. Degradation assays revealed the remarkable stability of OmpA and OmpC bound to proteolytically active DegP. The folding state of OMPs in corresponding complexes was analyzed by mobility shift assays taking advantage of the different migration behaviors of folded/unfolded OmpA and monomeric/trimeric OmpC. To explore the *in vivo* relevance of our findings, we isolated the outer membrane fraction from *E. coli* wildtype and *degP* null mutant strains and analyzed OMP levels on SDS PAGE in the presence of 6 M urea to resolve OmpC and OmpF bands. As a control we analyzed the corresponding OMP composition of whole cell extracts. Mutational analysis uncovered PDZ residues critical for membrane attachment of $DegP_{24}$ as shown by liposome binding assays.

Full methods accompany this paper.

FIGURES

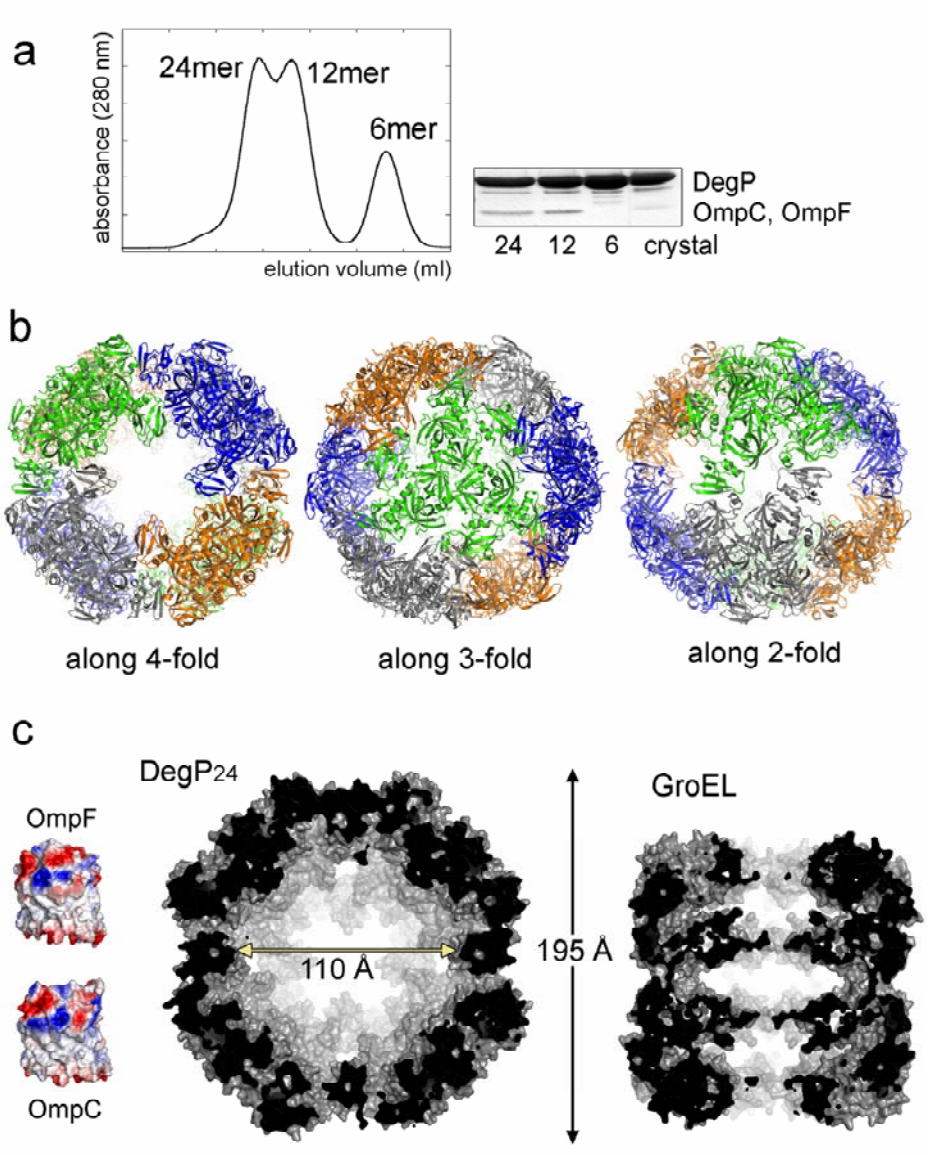


Figure 1: The $DegP_{24}$ particle

(a) Identification of $DegP_6$, $DegP_{12}$ and $DegP_{24}$ by SEC. Only the two larger oligomers had different OMPs bound. Mass spectrometry of dissolved $DegP_{24}$ crystals revealed that the visible additional band corresponds to OmpC and OmpF.

(b) Ribbon plot of $DegP_{24}$ illustrating its overall architecture with the trimeric units coloured differently. The particle is shown in three different orientations along the molecular 4-fold, 3-fold and 2-fold axes.

(c) To illustrate the size of the inner cavity of $DegP_{24}$, the molecular surfaces of OmpF and OmpC (with mapped electrostatic potential) are shown together with the surfaces of the sliced-open $DegP_{24}$ and GroEL chaperones.

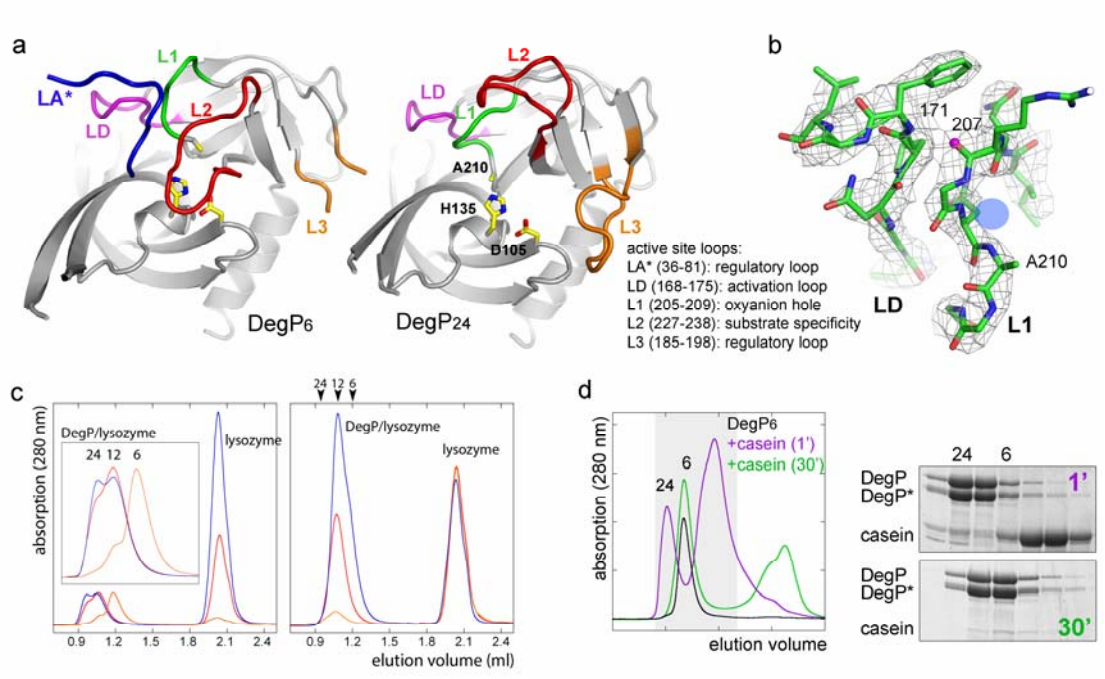


Figure 2: Regulation of protease activity by oligomer reassembly

(a) Ribbon plot of the protease domain of $DegP_6$ and $DegP_{24}$ highlighting the mechanistically important loops LA*, LD, L1, L2 and L3. Residues of the catalytic triad (Asp105, His135, Ala210) are shown in stick mode and the used loop nomenclature (Perona and Craik 1995; Krojer, Garrido-Franco et al. 2002) is indicated.

(b) Electron density of the active site loops L1 and LD. The $2F_o$-F_c simulated annealing omit map was calculated at 3.0 Å resolution (contoured at 1.1 σ.) after omitting loops L1 and LD from the refined model. The oxyanion hole (blue sphere) and the main-chain carbonyl of Arg207 are highlighted. The position of the latter oxygen is a distinctive feature of proteolytically active HtrA proteases.

(c) Denatured lysozyme and $DegP_6$ were incubated in different ratios and the resulting complexes analyzed by SEC. Left: Incubation of different amounts of lysozyme (orange, 30

μM, red, 300 μM, blue, 600 μM) with $DegP_6$ (15 μM). Right: Incubation of different amounts of $DegP_6$ (orange, 3 μM, red, 15 μM, blue, 65 μM) with lysozyme (170 μM).

(d) Short incubation of wildtype DegP with casein (one minute, magenta line) resulted in formation of the $DegP_{24}$/casein complex (the pronounced low molecular weight peak represents unprocessed casein). After completed degradation (30 minutes, green line), DegP recycled into its hexameric state. Composites of individual elution peaks are indicated on the SDS gel with the self cleavage products of DegP labelled as DegP*.

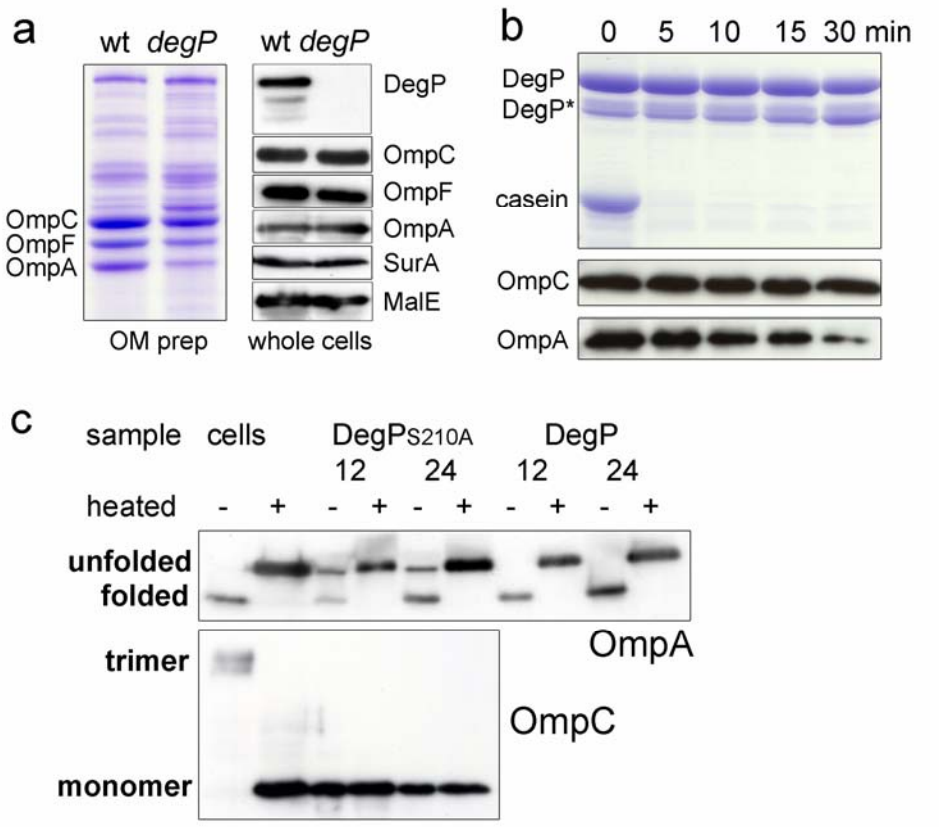


Figure 3: Function of DegP in OMP biogenesis

(a) Steady-state levels of OmpA, OmpC and OmpF in wildtype and *degP* null mutant strains. Outer membranes were prepared from an equivalent number of wildtype and *degP*$^-$ cells. The right panel shows the corresponding steady-state levels of whole cell extracts. The constant SurA levels exclude stimulation of the σE stress response in the mutant, whereas MalE serves as a loading control.

(b) SDS PAGE of the cleavage of different substrates by higher order DegP complexes (40 µM). The cleavage reactions with casein (130 µM), OmpC and OmpA were carried out at 37°C and stopped at various time points. Self cleavage products of DegP are labelled as DegP*.

(c) Folding state of OmpA and OmpC in $DegP_{12}$ and $DegP_{24}$. Upper panel: Heated and unheated samples of whole cells, $DegP_{12}$ and $DegP_{24}$ were analyzed by SDS PAGE to distinguish between folded and unfolded OmpA. Unboiled samples of folded OmpA migrate at 30 kDa, whereas unboiled samples of partially folded or unfolded OmpA migrate at 35 kDa. Lower panel: Without heating, OmpC trimers do not dissociate on SDS gels. Therefore both DegP particles bind OmpC in its monomeric state.

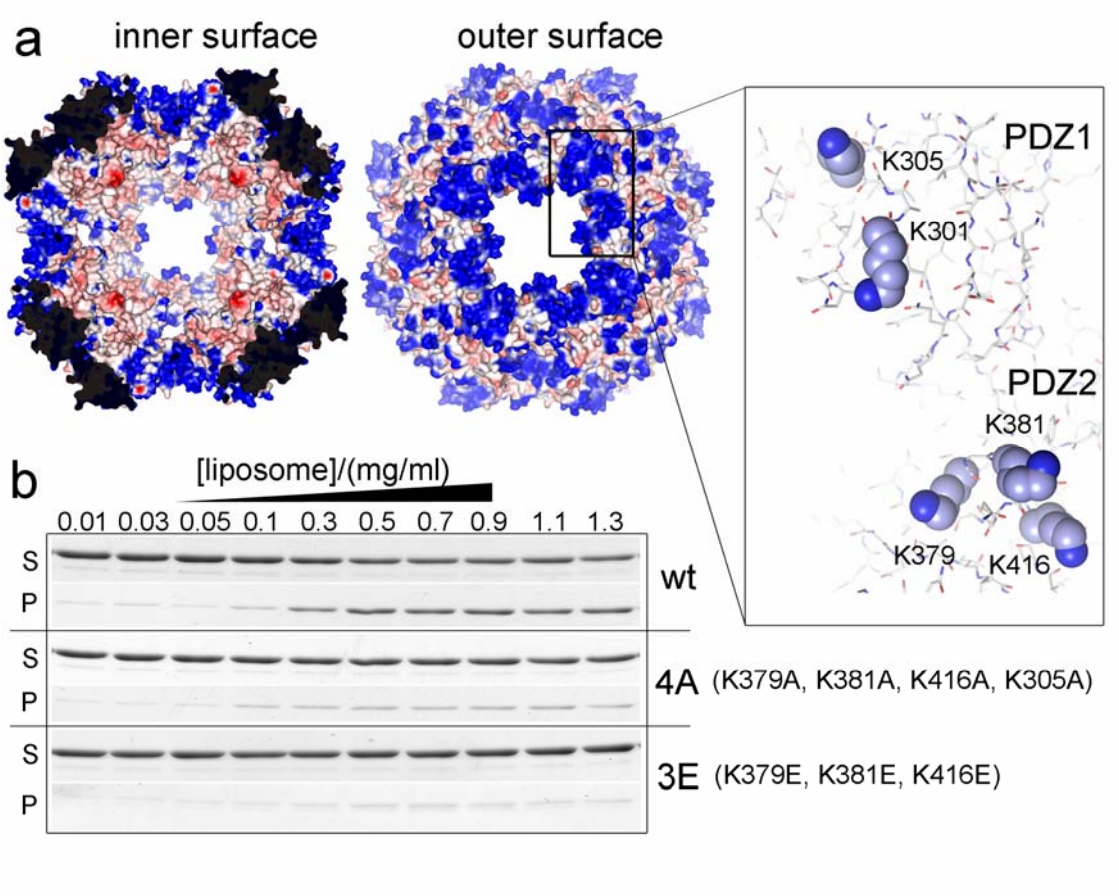


Figure 4: Membrane attachment of $DegP_{24}$

(a) The electrostatic potential of $DegP_{24}$ was calculated with PYMOL (DeLano 2002) and mapped on the molecular surface of the particle. Red indicates negatively charged, blue

positively charged regions. Lysine residues of PDZ1 and PDZ2 that enclose the outer rim of the large pore and contribute to the positively charged patches are shown (right panel).

(b) Sedimentation assay of $DegP_{24}$/OMP binding to bovine brain liposomes. Dose-response experiments were carried out with a fixed amount of $DegP_{24}$/OMP (0.1 mg/ml) and increasing concentrations of liposomes. "S" and "P" refer to proteins present in the supernatant or pellet after centrifugation and mutations of "4A" and "3E" are listed.

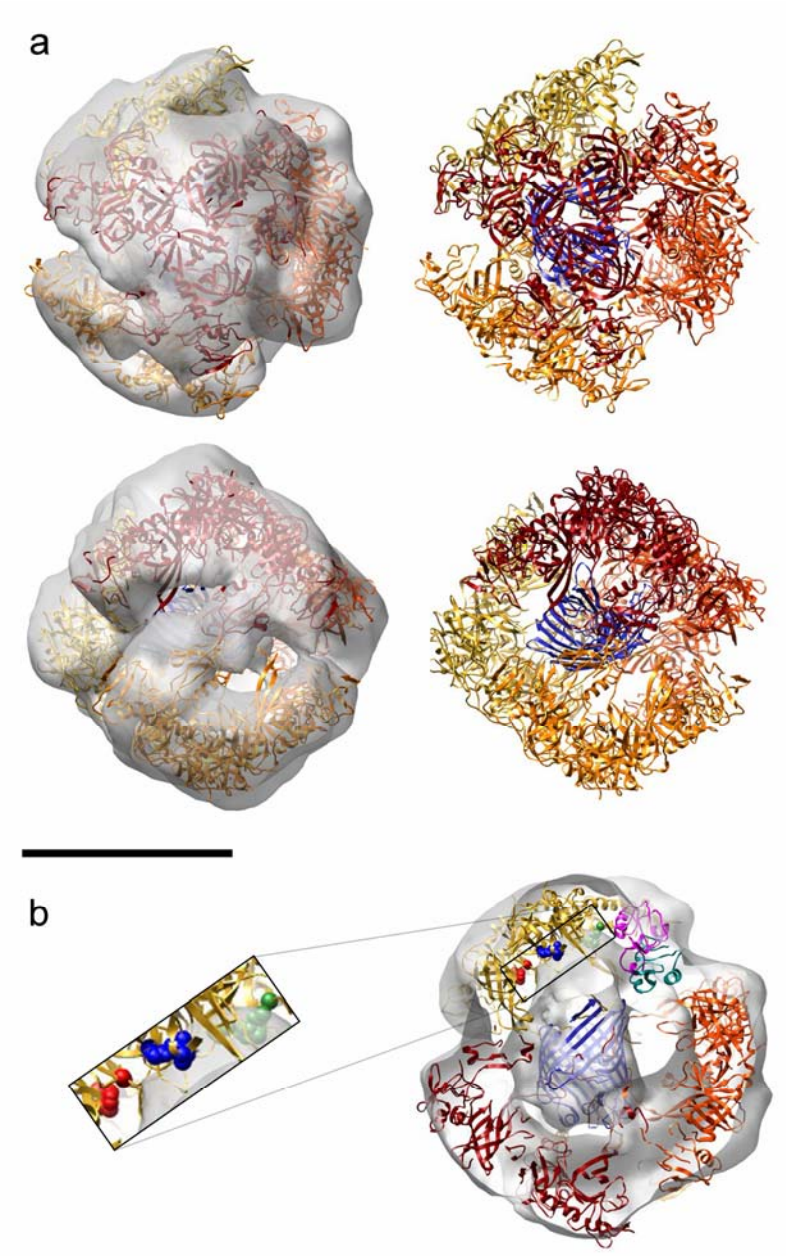


Figure 5: Cryo EM structure of the $DegP_{12}$/OMP complex

(a) The asymmetric $DegP_{12}$/OMP complex viewed along the approximate three-fold (upper panels) and two-fold axes (lower). In the left panels, the ribbon model of the DegP dodecamer is overlaid with the semi-transparent 3D map.
(b) Central section of the $DegP_{12}$/OMP EM map with an OmpC monomer (blue) modelled in the central density. The adjacent PDZ1 domains from neighbouring trimers are coloured in cyan and magenta. Three catalytic triads are coloured in red, green and blue and magnified in the left panel. The scale bar represents 100 Å.

ACKNOWLEDGEMENTS

We thank Karl Mechtler and Ines Steinmacher for assistance with mass spectrometry; the staff at ESRF and SLS for assistance with collecting synchrotron data; N. Boisset and R. Trujillo for providing image processing scripts; Luchun Wang for EM support; David Houldershaw for computer support. The Research Institute of Molecular Pathology (IMP) is funded by Boehringer Ingelheim. E.S. and H.R.S. were supported by the UK Biotechnology and Biological Sciences Research Council, M.E. by Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie, T.C. by the EMBO Young Investigator Program and T.K. and J.S. by the Austrian Science Fund.

AUTHOR INFORMATION

The Protein Data Bank accession number for $DegP_{24}$ is XXX. The EM 3D map will be deposited at the EM database http://www.ebi.ac.uk/msd-srv/emsearch/index.html.

METHODS

Isolation of $DegP_6$, $DegP_{12}$ and $DegP_{24}$

C-terminally His-tagged DegP, $DegP_{S210A}$, $DegP_{4A}$ and $DegP_{3E}$ were expressed as full-length proteins containing the periplasmic signal sequence in a *degP* null strain (CLC198, *degP*::Tn*10*) (Spiess, Beil et al. 1999). Site-directed mutagenesis was done with the QuikChange multi site-directed mutagenesis kit (Stratagene) and all DegP variants were isolated at 4°C via the following purification procedure: Cells were harvested by centrifugation, resuspended in 200 mM NaCl, 100 mM HEPES/NaOH pH 8.0 (buffer A) and disrupted by sonication. The cleared lysate was purified with a Ni-NTA resin (Qiagen) using standard procedures. Samples containing DegP were applied to a hydroxyapatite column (Biorad) and eluted with a linear gradient of 0 to 500 mM potassium phosphate in buffer A (Supp.Fig.1a). Two distinct DegP fractions could be discerned, one of which represented $DegP_6$ that was crystallized previously (Krojer, Garrido-Franco et al. 2002), whereas the second fraction contained DegP together with prominent protein bands of 35 kDa. The latter sample was concentrated using VIVASPIN concentrators (cut-off 50 kDa) and applied to a Superdex 200 column (prep grade, GE Healthcare) equilibrated with 300 mM NaCl, 50mM HEPES/NaOH pH 8.0. During the SEC run, three DegP oligomers were separated representing $DegP_6$, $DegP_{12}$ and $DegP_{24}$. The overall sizes of the particles were determined by dynamic light scattering (DynaPro-801, Protein-Solutions Inc.) at 20ºC using protein concentrations from 0.5 to 2 mg/ml and 10 sec acquisition times.

Crystallization and structure solution of the $DegP_{24}$/OMP complex

The SeMet-containing $DegP_{24}$(S210A)/OMP complex was crystallized at 19ºC using the vapor diffusion method. For crystallization, 1 µl of 10 mg/ml $DegP_{24}$/OMP was mixed with 0.3 µl FOS-choline-10 and 0.5 µl of a reservoir solution containing 23% (v/v) PEG550MME, 0.1 M Tris/HCl pH 8.5 and 0.1 M NaCl. Prior to flash freezing the crystals in liquid nitrogen, the drop was left open for 20 minutes at 19ºC. The protein crystallized in the cubic space group F432 with unit cell parameters of a=b=c=253.9 Å and one DegP protomer in the crystallographic asymmetric unit. A single wavelength anomalous dispersion dataset to 3.0 Å resolution was collected at beamline ID23-1 at the European

Synchrotron Radiation Facility (ESRF). Diffraction data were processed with programs from the XDS package (Kabsch 1993) and 11 out of 14 selenium sites of DegP were instantly located using Shake and Bake (Weeks and Miller 1999). Subsequent phasing was carried out with Sharp (de la Fortrelle and Bricogne 1997). The model was built with O (Jones *et al.* 1991) and refined with CNS (Brunger *et al.* 1998). Data collection, phasing and refinement statistics are summarized in Supplementary Table 1.

Identification of substrate proteins

To identify co-purified protein substrates, we examined crystals of the $DegP_{24}$ complex for potential binding partners. After extensive washing, the $DegP_{24}$ crystals were solubilized and the proteins separated by SDS-PAGE. In addition to the strong band representing DegP, we detected several faint bands, which were analyzed by mass spectrometry. Protein spots were excised from the SDS PAGE gel, washed, reduced, *S*-alkylated, and digested with trypsin. Resulting peptide fragments were analyzed on a hybrid linear ion trap - Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (LTQ-FT Ultra, ThermoFisher, Bremen, Germany). For peptide identification a database search was performed with Mascot (Matrix Science, London, UK). Ultimately, hits were confirmed by Western blot analysis.

Electron microscopy, image processing and atomic structure fitting

Samples of the proteolytically inactive $DegP_{12}$/OMP and $DegP_{24}$/OMP with final concentrations of 0.0064 mg/ml and 0.01 mg/ml were negatively stained with 2% (w/v) uranyl acetate on glow discharged, carbon-coated grids (Agar Scientific). For cryo measurements, samples containing the $DegP_{12}$(S210A)/OMP complex (0.16 mg/mL) were embedded in vitreous ice using C-flat holey carbon grids (CF-2/2-4C-100, Protochip) and a Vitrobot (FEI) at 20°C and 100% relative humidity. Low dose CCD images were recorded on a 4k x 4k Gatan CCD camera using a Tecnai F20 (FEI) at 200 kV and a defocus of ~2 μm, at a magnification of 68,100x corresponding to a pixel size of 2.22 Å. Subsequently, adjacent pixels were 2x2 averaged to yield a pixel size of 4.44 Å. 9890 particles of the $DegP_{12}$(S210A)/OMP complex were selected from 64 CCD images and windowed into

100x100 pixel boxes using the EMAN/BOXER software package (Ludtke *et al.* 1999), corrected for the effects of the contrast transfer function (CTF) by phase flipping and the contrast was inverted. Images were processed using SPIDER version 11.12 (Frank *et al.* 1996) and IMAGIC-5 (van Heel *et al.* 1996). Images were centered against a circular mask. Initial reference free alignment (Penczek *et al.* 1992) was refined by multi-reference alignment in SPIDER (Frank, Radermacher et al. 1996). The first 3D reconstruction was calculated by angular reconstitution (Van Heel 1987) in IMAGIC. Based on the observation of two- and three-fold views and the subunit number of 12, tetrahedral symmetry was applied. Other possible symmetries such as C3, D2 and D3 were not consistent with the data. After initial projection matching with tetrahedral symmetry, the structure was subsequently refined without symmetry. To validate the substrate density, it was masked out of the map, but it fully reappeared in subsequent refinement. The final 3D map was calculated from 6285 particles (Supp.Fig.2c) and had a resolution of 28 Å as determined by Fourier shell correlation at 0.5 correlation (Supp.Fig.6). The 3D map was contoured at threshold of 3σ giving a volume of 600 kDa, corresponding to the expected $DegP_{12}$/OMP mass. The X-ray structures of the DegP trimer (Krojer, Garrido-Franco et al. 2002) and OmpC (Basle *et al.* 2006) were fitted manually and then refined in Chimera (Pettersen *et al.* 2004). For the $DegP_{24}$/OMP complex, 3828 particles from 40 negative stain CCD images were windowed into 130x130 pixel boxes and processed as for the $DegP_{12}$/OMP complex. The starting map was obtained by angular reconstitution and refined by projection matching with octahedral symmetry. The $DegP_{24}$ X-ray structure was filtered to 25 Å resolution for comparison with the EM images (Supp.Fig.2j,k).

Biochemical characterization of DegP/substrate complexes

To follow the degradation of the model substrate casein we incubated 40 □M $DegP_{12/24}$ with 130 μM casein in 25 mM Hepes/NaOH pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$ at 37ºC. In parallel we followed degradation of OmpA and OmpC by incubating the $DegP_{12/24}$/OMP complex in the same degradation buffer at 37ºC. At certain time points the reaction was stopped by adding SDS loading buffer supplemented with 8 M urea to taken aliquots. Subsequently, the aliquots were incubated for 15 minutes at 95ºC and analyzed by

SDS PAGE. DegP and casein were detected by Coomassie stain, whereas degradation of OmpA and OmpC was monitored by Western blot analysis (see below).

To follow complex formation of hexameric $DegP_{S210A}$ with casein, we incubated 20 µl of 80 µM $DegP_{S210A}$ with 20 µL of 170 µM casein in 50 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 10 mM DTT. To survey complex formation with lysozyme (BSA), the protein substrate was initially denatured by preparing a 100 (50) mg/ml protein solution in 4 (8) M urea and 10 mM DTT. Subsequently, 2.5 (1) µl substrate was added to 50 µl degradation buffer containing $DegP_{S210A}$. Both assays were incubated for ten minutes at 37ºC before samples were injected on a Superdex 200 gelfiltration column (PC 3.2/30, GE Healthcare). Hexameric $DegP_{S210A}$ was used as a control.

In order to determine the dynamics of complex formation, we incubated wildtype DegP with casein for different times and explored the size of the resulting complexes. First, we mixed 15 µl of 320 µM DegP with 50 µl of 2 mM casein and analyzed the reaction mixture immediately by SEC. For a second gelfiltration run, we mixed wildtype DegP (320 µM) with a reduced amount of casein (425 µM) and incubated the sample for 30 minutes at 37°C. Analogously, we incubated wildtype $DegP_{12}$/OMP for 3 hours at 37ºC and followed conversion of $DegP_{12}$ to $DegP_6$ upon OMP degradation by SEC and SDS PAGE.

Determination of OMP levels in outer membranes and whole cells

Outer membranes of *E. coli* MC4100 wildtype and *degP* null mutant strain were prepared as described previously (Matsuyama *et al.* 1984). To evaluate protein levels in whole cells, both strains were grown in LB medium until they reached the stationary phase. After measuring the optical density, we took standardized aliquots to obtain pellets of equal cell number. Cell pellets were dissolved in equivalent volumes of SDS loading buffer and boiled for 15 minutes at 95°C. 10 µl of each sample were resolved by SDS-PAGE, transferred to a PVDF membrane (Immobilon-P, Millipore) and probed with antibodies against DegP (1:10,000), OmpA (1:20,000), OmpC (1:20,000), OmpF (1:20,000) and SurA (1:20,000). After incubation with the secondary antibody, protein bands were visualized with ECL Plus Western Blotting detection System (GE Healthcare) and Hyperfilm ECL (Amersham Biosciences).

Mobility shift assay of folded and unfolded OMPs

Cells of wildtype strain MC4100 were harvested in the stationary phase and lysed by incubation with 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% SDS, and 1% β-mercaptoethanol. The protein concentration of the cleared lysate was determined and samples with equal protein amounts were prepared. To ensure the complete unfolding of OMPs, one sample was boiled at 95°C for 15 min in SDS loading buffer supplemented with 4 M urea. In order to maintain the folded states of OMPs, the second sample was incubated at room temperature with a loading buffer lacking SDS (Nakae *et al.* 1979). Analogously, samples with/without SDS and with/without heating were prepared from the isolated $DegP_{12}$/OMP and $DegP_{24}$/OMP complexes. Finally, all samples were characterized by SDS-PAGE and Western blot analysis.

Lipid-binding assays

Lipid binding of $DegP_{wt}$, $DegP_{4A}$ and $DegP_{3E}$ 24-mers was carried out as described (Yan, Wen et al. 2005). Briefly, brain lipid extracts (Folch fraction I, Sigma) were suspended in 140 mM NaCl, 20 mM Hepes/NaOH pH 7.4. The proteins were incubated at a concentration of 0.1 mg/ml with varying lipid concentrations for 15 minutes at 37ºC. Subsequently, samples were centrifuged for 30 minutes at 100,000xg and 4ºC. After the supernatant was removed and the pellet resuspended with an equivalent volume, samples were analyzed by SDS-PAGE.

2.1.2 Supporting information

Structural basis for the regulated protease and chaperone function of DegP

Tobias Krojer[1a], Justyna Sawa[1a], Eva Schäfer[2a], Helen R. Saibil[2], Michael Ehrmann[3] and Tim Clausen[1*]

[1]*Research Institute for Molecular Pathology - IMP, Dr. Bohrgasse 7, A-1030 Vienna, Austria*

[2]*Crystallography Department and Institute of Structural Molecular Biology, Birkbeck College, Malet St, London WC1E 7HX, UK*

[3]*Centre for Medical Biotechnology, FB Biology and Geography, University Duisburg-Essen, Universitätsstrasse, D-45117 Essen, Germany*

[a] *These authors contributed equally to the work.*

[*] *Corresponding author: Tim Clausen, phone: +43-1-797303350, fax: +43-1-7987153 email: clausen@imp.univie.ac.at*

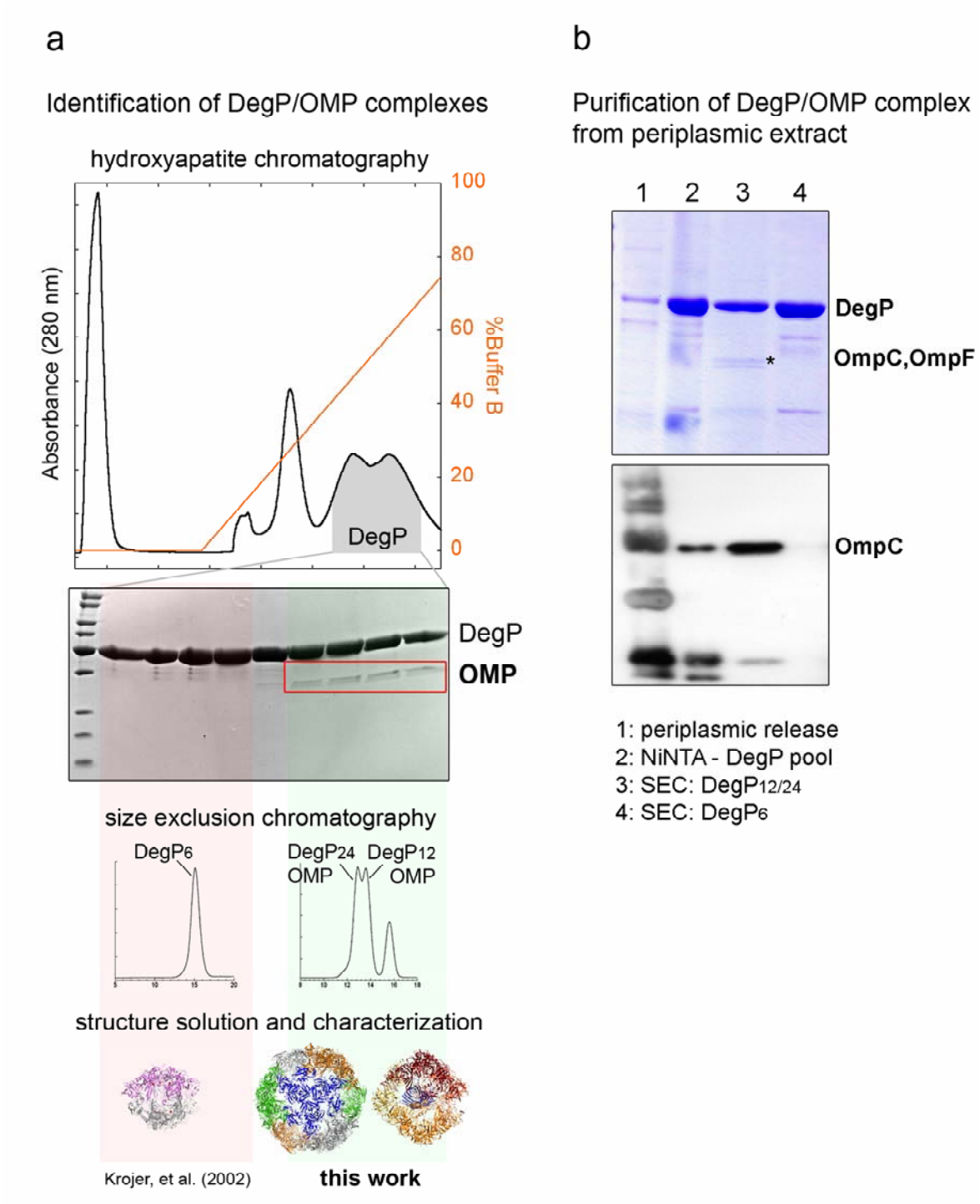
TABLES

Supplementary Table 1: Data collection, phasing and refinement statistics

Data collection	
Space group	F432
Unit cell parameters [Å]	a=253.9, b=253.9, c=253.9
Wavelength [Å]	0.9792
Resolution [Å][1]	30 – 3.0 (3.19-3.00)
Completeness [%]	99.7 (99.9)
R_{sym} [%][2]	9.3 (57.9)
I/σ(I)	13.0 (2.9)
Redundancy	5.1 (5.1)
Phasing	
Phasing Power[3]	1.46
Figure of merit (before/after solvent flattening)	0.31 / 0.85
Refinement	
Resolution [Å]	15 – 3.0
Number of reflections R_{work}/ R_{free}	13,714 / 727
Number of protein atoms	2,893
R_{cryst}/ R_{free}[4]	21.2 / 27.4
Average B-factor [Å^2]	69.0
root mean square deviations of bond length [Å]/ angles [º]/bonded Bs [Å^2]	0.009 / 1.52 / 3.92
Ramachandran statistics (%) most favored, additionally allowed, generously allowed, disallowed region[5]	82.1 , 15.8, 2.1, 0.0

[1]Numbers in parentheses, here and below, refer to the highest resolution shell.
[2]R_{sym} is the unweighted R-value on I between symmetry mates.
[3]Phasing power is the root mean squared value of F_H divided by the root mean squared lack-of-closure.
[4]$R_{cryst} = \Sigma_{hkl} \mid |F_{obs}(hkl)| - k|F_{calc}(hkl)| \mid / \Sigma_{hkl} |F_{obs}(hkl)|$ for the working set of reflections; R_{free} is the R-value for 5% of the reflections excluded from refinement.
[5]The stereochemistry of the model was validated with PROCHECK (Laskowski *et al.* 1993).

SUPPLEMENTARY FIGURES

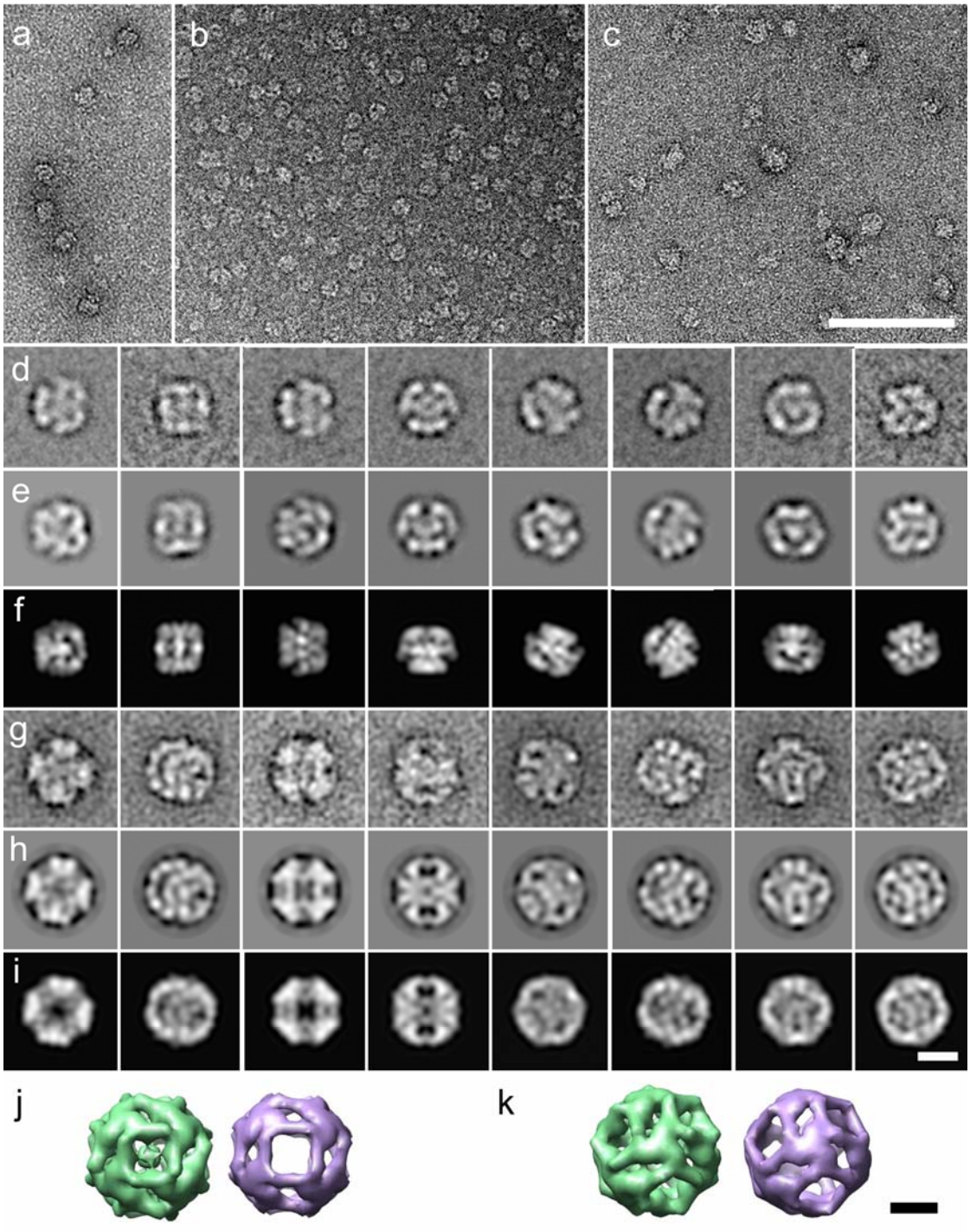


Supp. Fig. 1: Isolation of a DegP/OMP complex

(**a**) Identification of DegP/OMP complexes. DegP was purified via a 3-step purification procedure including NiNTA, hydroxyapatite and size exclusion chromatography. In the second purification step, two distinct DegP fractions could be separated, one of which

represented $DegP_6$ that was crystallized previously (Krojer *et al.* 2002), whereas the second fraction contained DegP together with prominent protein bands of 35 kDa. During the SEC run of the latter sample, three DegP oligomers could be isolated, which correspond to $DegP_6$, $DegP_{12}$ and $DegP_{24}$. The two larger DegP particles captured additional proteins, which were the OMPs OmpA, OmpC, OmpF and LamB. The corresponding higher order particles of DegP were structurally and functionally characterized in this work.

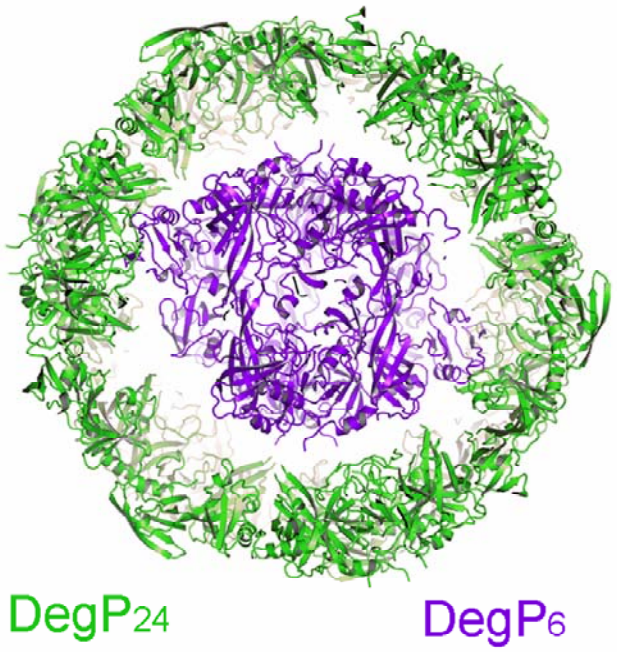
(**b**) To provide evidence that the identified DegP/OMP complex is formed in the periplasm and not by interaction of overexpressed DegP with un-assembled OMPs during purification we prepared periplasmic extracts of *E. coli*. Periplasmic release of overexpressed DegP was achieved by incubating cells with a buffer containing 33 mM Tris pH8, 100 mM KCl, 5 mM EDTA, 40% sucrose and 10 mg/ml lysozyme. The periplasmic fraction was loaded on a NiNTA column and assayed for the presence of DegP by SDS PAGE. Subsequently, samples containing DegP were applied to a SEC column (Superdex 200) and the resolved fractions analyzed by Western blotting using OmpC antibodies. The results clearly indicate that OmpC co-purifies with DegP suggesting that the DegP/OMP complex is formed in the periplasm. (Top): Coomassie stained SDS PAGE gel of the purification with labeled OmpC band. (Bottom): Western blot using OmpC antibodies illustrating its co-purification with $DegP_{12/24}$.



Supp. Fig. 2: EM images of DegP/OMP complexes

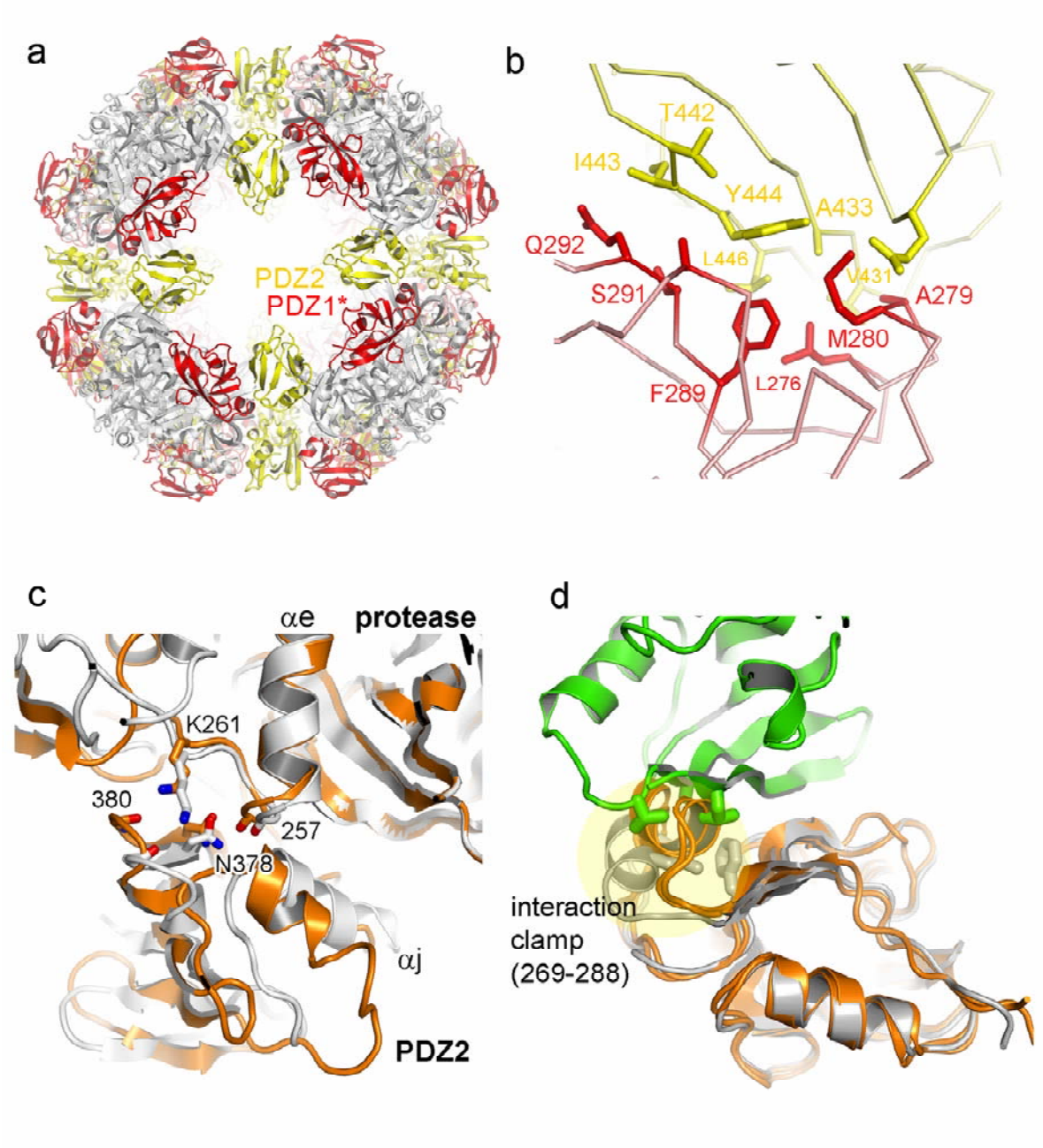
(**a**) Negative stain and (**b**) cryo-EM images of $DegP_{12}$/OMP complexes. (**c**) Negative stain images of $DegP_{24}$/OMP particles. (**d)** Some cryo-EM class averages of $DegP_{12}$/OMP obtained by multi-reference alignment. (**e**) Corresponding re-projections of the cryo-EM 3D map of $DegP_{12}$/OMP. (**f**) Equivalent projections of a density map

derived from the atomic coordinates of the fitted DegP trimers filtered to 25 Å. (**g**) Example negative stain class averages of $DegP_{24}$/OMP obtained by multi-reference alignment. (**h**) Corresponding re-projections of the negative stain map of $DegP_{24}$/OMP. (**i**) Equivalent projections of a model density derived from the $DegP_{24}$/OMP X-ray structure filtered to 25 Å. (**j**) Surface representations of 4-fold and (**k**) 3-fold views of the $DegP_{24}$/OMP EM map (left, green) and the $DegP_{24}$/OMP X-ray structure (right, lilac). The scale bars represent 1000 Å in (**a-c**) and 100 Å in (**d-k**).



Supp. Fig. 3: Alignment of $DegP_6$ and $DegP_{24}$

Superposition of $DegP_{24}$ (green) and $DegP_6$ (lilac) shows that the $DegP_{24}$ cavity can house the entire 300 kDa hexameric particle.



Supp. Fig. 4: Overall architecture of $DegP_{24}$

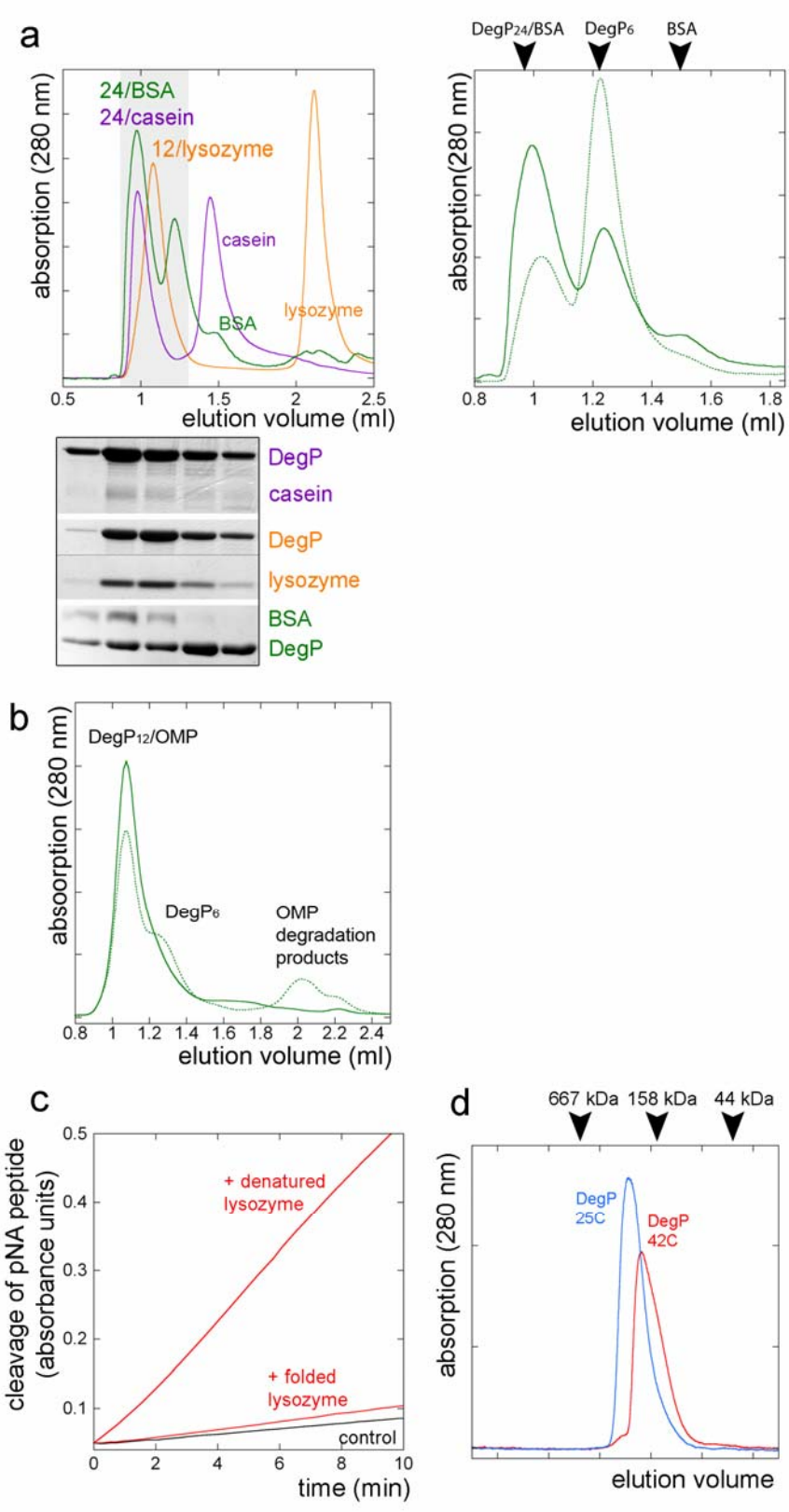
(**a**) Construction of $DegP_{24}$. While the entry/exit pores are formed by four pairs of PDZ1*/PDZ2 domains, which are coloured red and yellow, respectively, the sidewall of the particle is constituted by the protease domains. Moreover, all domains are part of an intricate interaction network that glues trimeric units together and confers rigidity to the entire particle.

(**b**) Interface between PDZ1 (red) and PDZ2* (yellow). The backbones of both PDZ domains as well as residues constituting the hydrophobic core of the interface are

shown. The inter-trimer interactions occur exclusively between residues of PDZ1 and PDZ2* and are different than in $DegP_6$. The PDZ1 domain features an "interaction clamp" (residues 269-288 including helix f), a motif typically found in PDZ domains of HtrA proteins (Clausen *et al.* 2002), that forms most of the contacts. The hydrophobic surface of this molecular "clamp" covers an apolar region of PDZ2* situated above the two C-terminal β-strands (residues 430-448). Here, Leu276, Ala279, Phe289, Val431*, Ala433*, Tyr444*, Thr442*, and Leu446* come together to form the hydrophobic core of the PDZ1-PDZ2* interface. The hydrophobicity of these residues is largely conserved in the DegP protein family suggesting that the observed 24-meric architecture is of general functional importance.

(**c**) We performed a structural comparison of $DegP_6$ and $DegP_{24}$ to pinpoint elements controlling quaternary assembly. In $DegP_{24}$ and the closed form of $DegP_6$, the flexible PDZ2 domain is tethered to the protease by conserved hydrogen bonds formed between the side-chains of Lys261 and Asn378 and the main-chain carbonyls of residues 257 and 380, respectively. Moreover, helices e and j are positioned properly to undergo favorable macrodipole interactions thereby stabilizing the relative domain orientation. These findings imply that the transition between different oligomeric states may not require major structural remodelling of individual protomers, but rather depends on subtle, local changes of components constituting the interface between subunits.

(**d**) Despite their entirely different architecture, the PDZ1 "interaction clamp" is key for the configuration of the inter-PDZ contacts of both $DegP_6$ and $DegP_{24}$. Alignment of the PDZ1 domains indicates that the "interaction clamp" of $DegP_{24}$ is tilted away from the core of the domain by 55° thereby opening a hydrophobic cleft used to interact with a hydrophobic patch on the surface of PDZ2* as described previously. Clearly, the PDZ1 "interaction clamp" attains characteristic orientations that stabilize $DegP_6$ or $DegP_{24}$ by employing either its polar or nonpolar face, respectively.



Supp. Fig. 5: Characterization of the $DegP_6$, $DegP_{12}$ and $DegP_{24}$ multimers

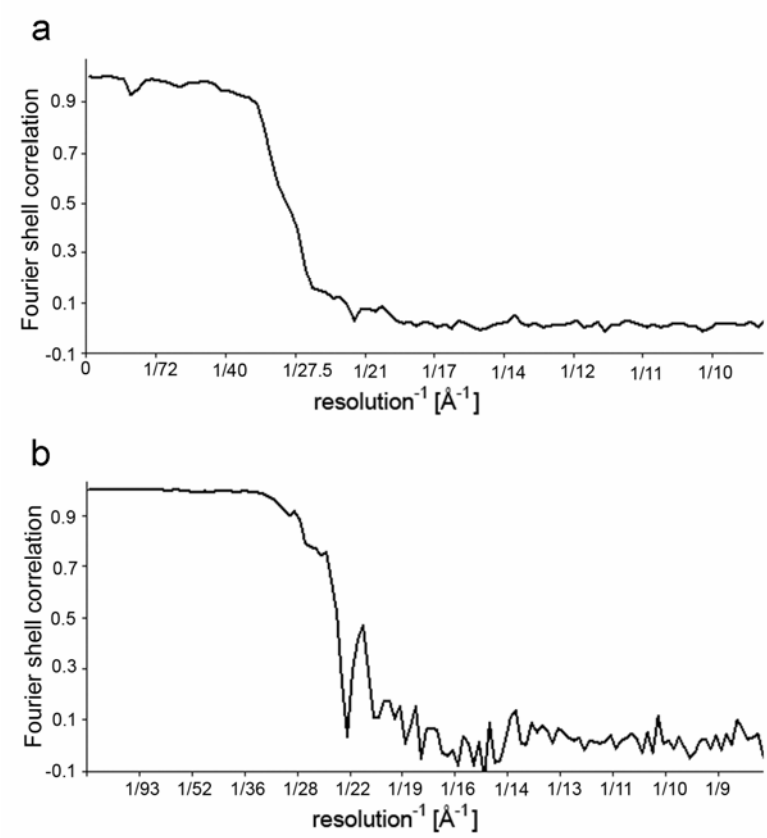
(**a**) Characterization of DegP/substrate complexes. Left: After hexameric $DegP_{S210A}$ was incubated with denatured lysozyme (orange), BSA (green) and casein (magenta), the

resulting complexes were analyzed on a Superdex 200 column. Comparison with marker proteins (not shown) and SDS-PAGE analysis revealed the formation of $DegP_{24}$/casein, $DegP_{24}$/BSA and $DegP_{12}$/lysozyme complexes. Right: Denatured BSA was incubated in two different concentrations (18 μM, solid line; 6 μM, dashed line) with $DegP_6$ and the resulting complexes analyzed by SEC. Notably, BSA triggered even in very little amounts $DegP_{24}$ formation.

(**b**) The $DegP_{12}$/OMP complex comprising proteolytically active DegP (solid line) was incubated for 3 hours at 37°C and applied to a Superdex200 column (dashed line). Elution profiles and SDS PAGE (not shown) illustrate that the decrease of $DegP_{12}$/OMP is directly linked to the appearance of $DegP_6$ and OMP cleavage products.

(**c**) Cleavage of the chromogenic SPMFKGV-pNA substrate (0.5 mM) by DegP (2 μM) in the absence (black) and presence of folded/unfolded lysozyme (red). The reaction was followed by recording the change in absorbance at 405 nm.

(**d**) Conversion of $DegP_6$ into $DegP_3$ at elevated temperatures. Superdex 200 elution profiles of $DegP_6$ recorded at different temperatures. Positions of molecular standards are marked on the top. Notably, $DegP_3$ recycled into $DegP_6$ after incubation at 25°C (data not shown).



Supp. Fig. 6: Fourier shell correlation (FSC) curves

FSC curve of (**a**) the asymmetric cryo EM map of $DegP_{12}$/OMP and (**b**) of the negative stain map of octahedral $DegP_{24}$/OMP.

2.2 Regulation of molecular and cellular function of DegQ

In contrast to DegP, limited information is available on the role and the regulation of DegQ in the periplasm of *E. coli* (introduction section 1.4.4). The two proteins belong to the same family of HtrA serine proteases, which are involved in the maintenance of protein homeostasis in the cell. They exhibit high sequence similarity and the same domain composition, namely one protease domain and two C-terminal PDZ domains (Clausen *et al.* 2002). The protease domain contains a regulatory loop LA, which is significantly longer in DegP compared to DegQ and other HtrAs (Figure 1.13 – introduction). As discussed previously (section 1.4.3) loop LA is involved in spatial rearrangements of active site loops of the DegP hexamer thereby abolishing the proteolytic activity (Krojer *et al.* 2002). DegP occurs as a hexamer in solution and reassembles into higher order oligomers when complexed with a substrate protein, which leads to the extraction of the inhibiting loop LA from the active center (section 2.1). Likewise, DegQ forms hexamers (Kolmar *et al.* 1996), however the regulation of its activity has not been studied so far.

Both proteins reside in the periplasm of *E. coli* (Waller and Sauer 1996; Pallen and Wren 1997). DegP was reported to be up-regulated in response to the presence of non-native proteins and to be indispensable during heat shock (Erickson and Gross 1989; Lipinska *et al.* 1989; Danese *et al.* 1995). Although DegQ is not essential for *E. coli* viability, it can complement a temperature sensitive phenotype in a *degP* null strain implying overlapping functions with DegP (Waller and Sauer 1996). The precise function and regulation of DegQ has not been explored yet, thus *in vitro* and *in vivo* approaches were undertaken to provide some insight into the mechanisms underlying protein quality control in the bacterial envelope.

2.2.1 pH regulates oligomer assembly and activity of DegQ

2.2.1.1 Purification of DegQ

In order to assess its biochemical properties, wild type DegQ and a proteolytically inactive form thereof was overexpressed in *E. coli* with a C-terminal His-tag. In the inactive mutant serine of the catalytic triad was replaced by alanine yielding the

DegQS187A mutant. In the course of the overexpression the recombinant protein was exported to the periplasm due to the presence of an N-terminal signal sequence.

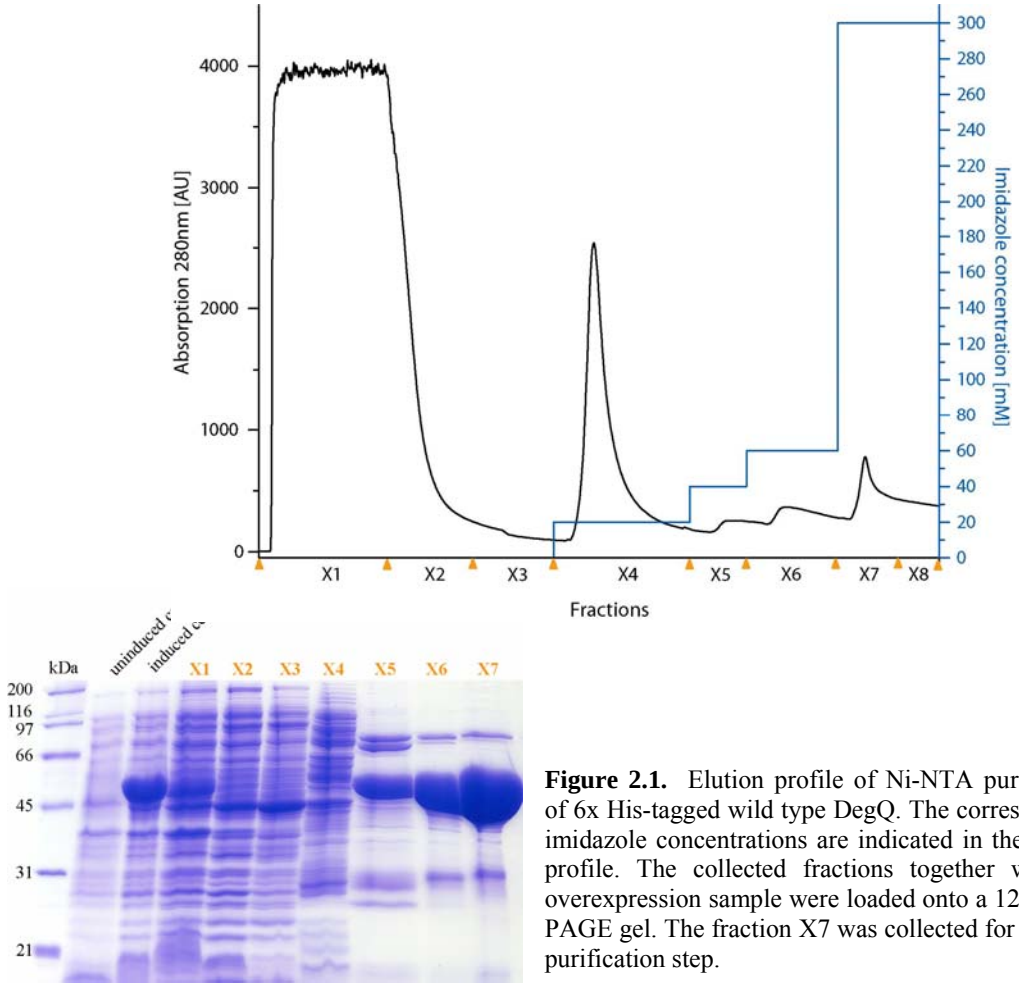


Figure 2.1. Elution profile of Ni-NTA purification of 6x His-tagged wild type DegQ. The corresponding imidazole concentrations are indicated in the elution profile. The collected fractions together with the overexpression sample were loaded onto a 12% SDS-PAGE gel. The fraction X7 was collected for the next purification step.

The purification procedure involved Ni-NTA affinity and size exclusion chromatography (SEC). In the first step, the His tagged DegQ was bound to the resin by applying the whole cell lysate on a Ni-NTA column. The column was washed with buffer containing increasing concentrations of imidazole and finally DegQ was eluted with 300mn imidazole (Figure 2.1). Fraction X7 was collected, concentrated and purified by SEC. The protein yield at this stage was approximately 110 mg from 4 L expression culture.

The size elusion chromatography was performed on a Superdex 200 prep grade gel filtration column (Figure 2.2). The resulting gel filtration profile showed a small

aggregation which peak eluted in the void volume of the column and a single peak corresponding to DegQ. The molecular weight was estimated to be 280 kDa, which would correspond to a hexameric assembly of DegQ, which was later confirmed by analytical SEC (section 2.2.1.3). SDS-PAGE analysis (Figure 2.2) of the peak fractions indicated that the protein preparation was pure except for an additional 80 kDa band which was identified by mass spectroscopy as a cytoplasmic protein, namely polyribonucleotide nucleotidyltransferase. Fractions showing this impurity were not included in the final pool. The collected fractions (6-11) yielded 50 mg of highly pure protein which was subsequently used in further experiments. The overexpression and purification of the proteolytically inactive variant of DegQ was indistinguishable from the wild type.

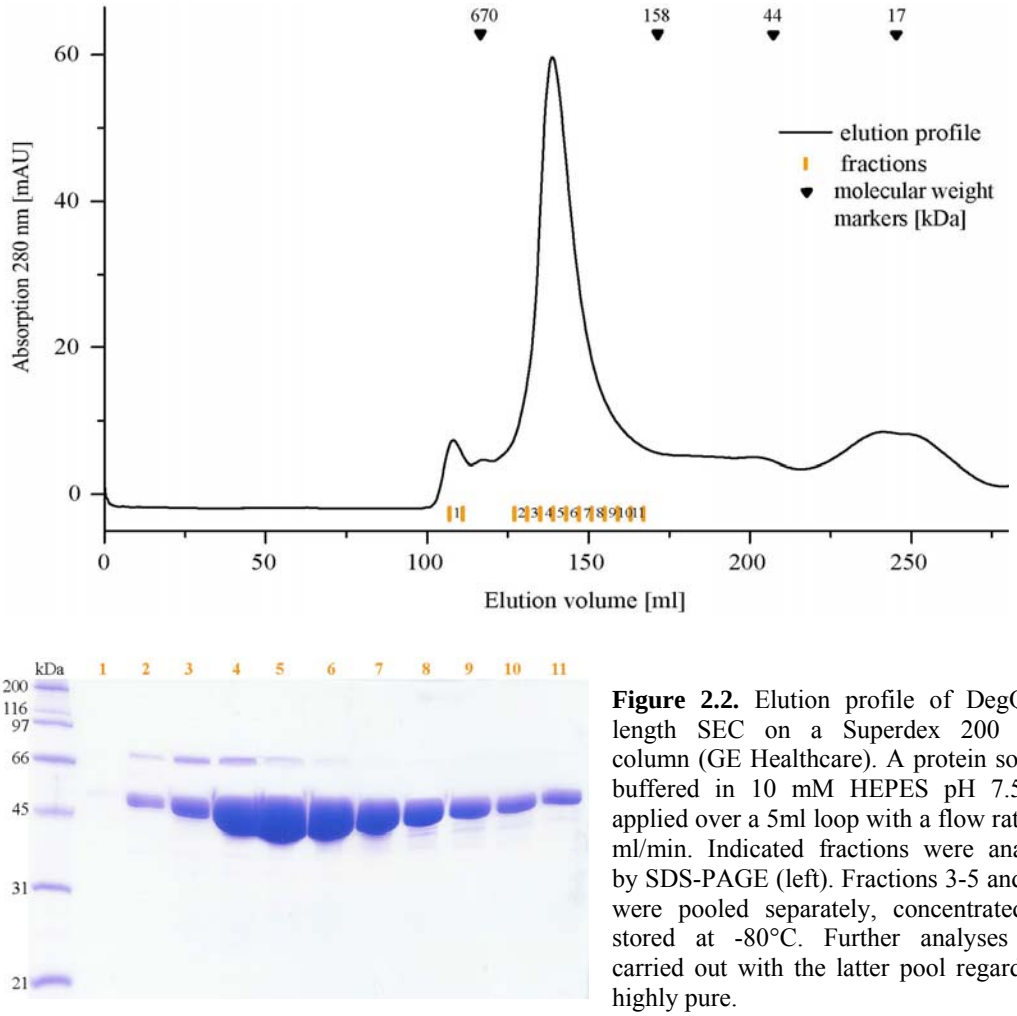


Figure 2.2. Elution profile of DegQ full length SEC on a Superdex 200 26/60 column (GE Healthcare). A protein solution buffered in 10 mM HEPES pH 7.5 was applied over a 5ml loop with a flow rate of 1 ml/min. Indicated fractions were analysed by SDS-PAGE (left). Fractions 3-5 and 6-11 were pooled separately, concentrated and stored at -80°C. Further analyses were carried out with the latter pool regarded as highly pure.

The procedure of His-tagged $DegQ_{delpDZ2}$ expression and purification was the same as for the full length protein. Ni-NTA affinity chromatography resulted in 150 mg relatively pure protein (data not shown) which was concentrated and applied to the Superdex 200 prep grade gel filtration column (Figure 2.3). The protein eluted as a single peak which would correspond to a trimer in solution (105 kDa) as confirmed later by analytical gel filtration (section 2.2.1.3). When analyzed on SDS-PAGE no additional bands were observed (Figure 2.3). The pooled peak fractions yielded 130 mg of highly pure protein.

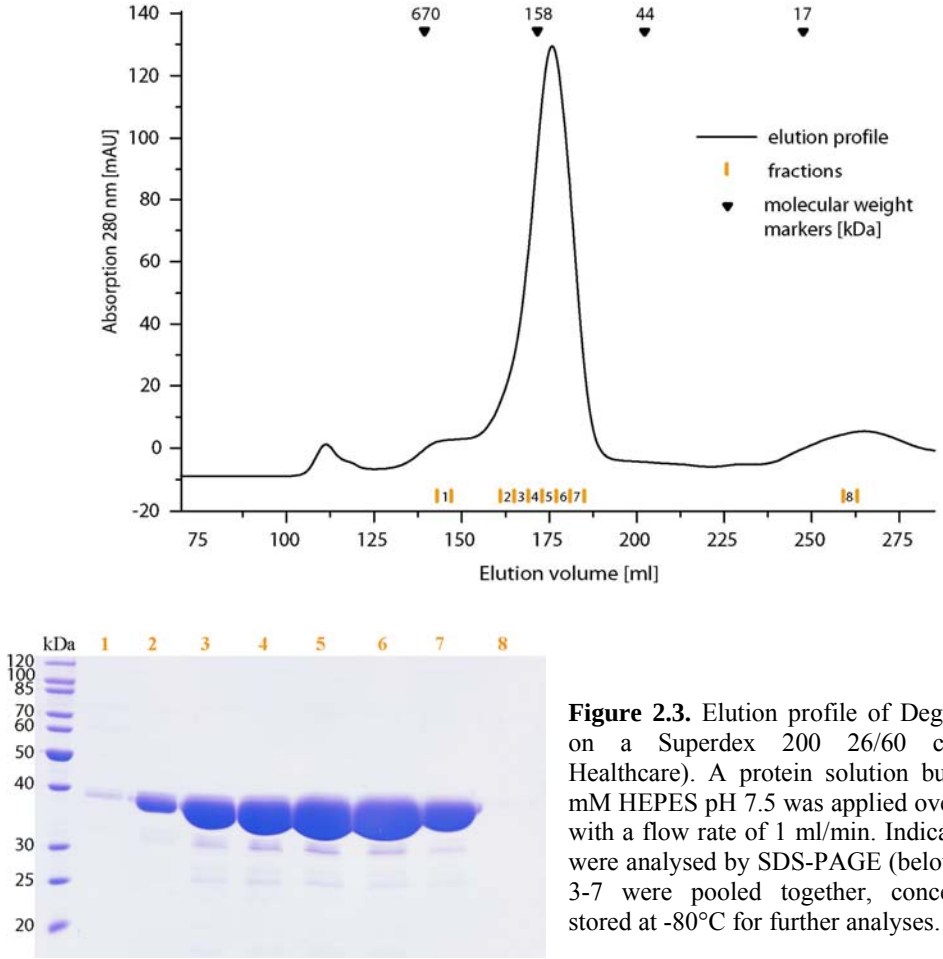


Figure 2.3. Elution profile of $DegQ_{delpDZ2}$ SEC on a Superdex 200 26/60 column (GE Healthcare). A protein solution buffered in 10 mM HEPES pH 7.5 was applied over a 5ml loop with a flow rate of 1 ml/min. Indicated fractions were analysed by SDS-PAGE (below). Fractions 3-7 were pooled together, concentrated and stored at -80°C for further analyses.

2.2.1.2 DegQ proteolytic activity is pH dependent

The digestive function of DegP was reported to be pH independent (Lipinska *et al.* 1990). On the contrary, two HtrA proteases from *Arabidopsis thaliana*, namely Deg1 and Deg2, were demonstrated to cleave substrates (β-casein or gelatine) in a pH dependent manner with the optima at pH 6.0 and 9.5, respectively (Haussuhl *et al.* 2001; Chassin *et al.* 2002). Furthermore, the proteolytic activity of HhoA from *Synechocystis* sp. Strain PCC 6803 was recently reported to increase in basic pH (Huesgen *et al.* 2007). Bearing in mind the fluctuations of pH in the cell envelope due to the permeable character of the bacterial outer membrane, the activity of bacterial DegQ protease could be affected by sudden changes in pH. To test this possibility the proteolytic activity of DegQ, $DegQ_{delPDZ2}$ and DegP against resorufin-labeled β-casein at different pHs was tested. In this assay resorufin-labeled β-casein was incubated with equimolar amounts of DegP, DegQ or $DegQ_{delPDZ2}$. The degradation products could be monitored by the increase of the absorbance at the wavelength 574 nm (Figure 2.4).

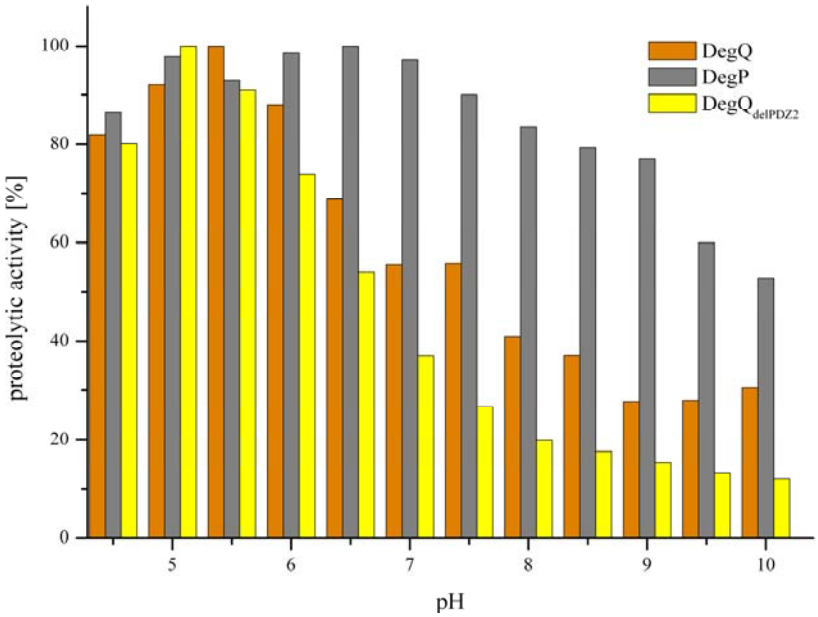


Figure 2.4. Effect of pH on the proteolytic activity of DegQ. DegQ, $DegQ_{delPDZ2}$ or DegP were incubated with resorufin-labeled β-casein substrate in various buffers of pH ranging from 4.5 to 10. The cleavage was stopped after 3 h (DegP) and 12 h (DegQ, $DegQ_{delPDZ2}$). The relative proteolytic activities were calculated by standardization to the highest obtained value, which for each protein is regarded as 100%.

The results demonstrated that, unlike DegP, DegQ digested the substrate in a pH dependent fashion. The degradation was most efficient at pH values between 4.5 and 6 with the highest activity observed at pH 5.5. At slightly basic pHs, the degradation rate dropped, though it was still present. Furthermore, the activity does not depend on the second PDZ domain since $DegQ_{delPDZ2}$ mutant exhibits similar behavior.

The pH dependence of the proteolytic activity of Deg1 and Deg2 from *A. thaliana* chloroplasts has been previously reported to be consistent with the localization of the two proteases (Haussuhl, Andersson et al. 2001; Chassin, Kapri-Pardes et al. 2002). Deg1 was reported to occupy the thylakoid lumen (Itzhaki *et al.* 1998) while Deg2 is associated with the stromal side of the thylakoid membrane (Haussuhl, Andersson et al. 2001). The light induced pH gradient between the two adjacent compartments sustains a low pH (4.5 – 6.0) in the lumen and an alkaline (above 8.0) in the stroma which corresponds to the proteolytic optima for Deg1 and Deg2, respectively (Pfundel *et al.* 1994; Hauser *et al.* 1995).

A similar suggestion could be proposed for DegQ. As observed, DegQ was able to digest resorufin-labeled casein in a pH-dependent manner, which may respond to the changes in the enteric environment where *E. coli* resides. The pH of the human gastrointestinal tract varies between values of 5 to 8 and can change significantly after the food intake or during fasting periods (Evans *et al.* 1988). Not only do bacteria grow and persist in the intestine within a moderate range of external pHs, but they are able to transiently survive in extreme pHs (pH 1 - 4) in the stomach during colonization (Dressman *et al.* 1990; de Jonge *et al.* 2003). It was not possible to test whether the high activity of DegQ was preserved in these extreme pHs since resorufin-labeled casein precipitated in pHs lower than 4.5. It would be interesting, however to find an acid-resistant substrate and investigate a possible function of DegQ in monitoring the state of proteins in the periplasm during the extreme pH stress correlated with the colonization process of *E. coli* and thereby contributing to the survival of the bacteria in highly unfavorable conditions.

2.2.1.3 pH regulates the oligomeric state of DegQ

As shown in section 2.1, the proteolytic activity of DegP is correlated with the reassembly of the hexamer. To test whether the pH dependent digestive function of DegQ is linked to the same phenomenon, analytical SEC analysis was performed in buffers of different pH values. Prior to the injection the samples were dialyzed against the respective buffer for approximately 3 h. The elution profiles are compared in Figure 2.5.

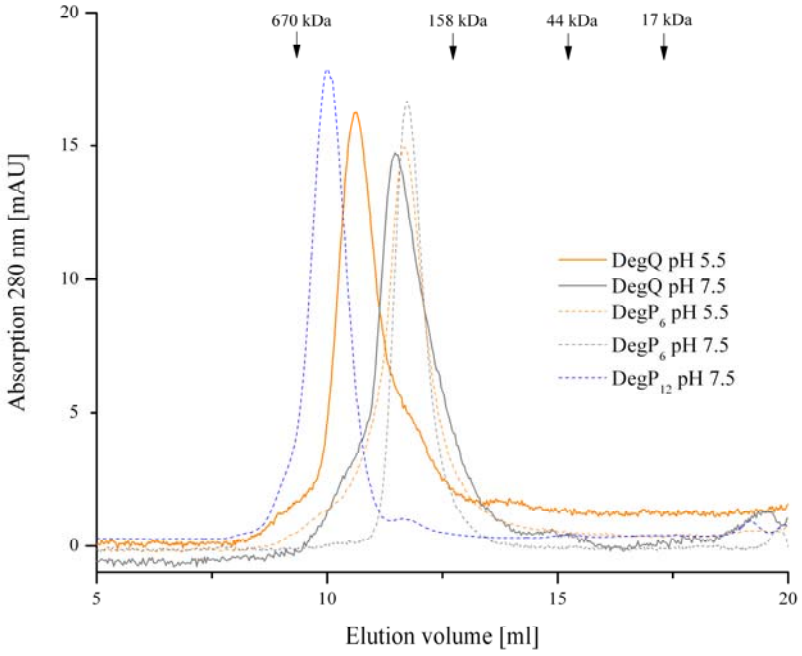


Figure 2.5. **Effect of pH on the size of DegQ**. The 10/300 Superdex 200 column (GE Healthcare) was equilibrated with 50 mM MES pH 5.5 or 10 mM HEPES pH 7.5.buffer. Samples were dialyzed prior to the injection in the respective buffers for 3h. The elution profiles showed a 0.9 ml shift of DegQ in the two different pH values. The elution volumes were 10.6 ml (orange line) and 11.5 ml (grey line) at pH 5.5 and pH 7.5, respectively. The elution profile of $DegP_6$ was not altered (dotted orange and grey lines). In pH 5.5 DegQ elutes between $DegP_6$ (peak at 11.7 ml) and $DegP_{12}$ (peak at 10 ml; dotted blue line). pH had no effect on the elution volumes of marker proteins (data not shown).

The experiment clearly demonstrates the pH dependent shift in elution volume of DegQ, whereas this phenomenon could not be observed for DegP. Consistent with the results of the protease assay the most dramatic change was observed at pH 5.5 while pH 9.0 had no effect on the separation behavior (data not shown). At pH 5.5 DegQ eluted at 10.6 ml as a molecule of an apparent higher weight, while in both pHs 7.5 and 9.0 the elution volume of the peak appeared at 11.5 ml. The behavior of DegP was not affected

by pH. It was eluted at 11.6 ml in both buffers. The estimated molecular weight of DegQ at pH 5.5 and 7.5 was 440 kDa and 256 kDa, respectively. Given the mass of DegQ to be 45 kDa and considering a trimer to be a building block of all HtrAs this calculation suggested that the enzyme was found in two forms, hexameric and in higher oligomeric which could correspond to either a 9-mer or a 12-mer. The assignment of the accurate oligomeric state to the oligomer eluting is more difficult due to limited resolution of such high molecular particles in the gel filtration column. It should be noted, that the calculated molecular weight is critically dependent on the measured retention times or elution volumes of the marker proteins. As there is a logarithmic correlation between the K_{av} value and the molecular weight, even small variations in the measured values may have dramatic effects on the apparent molecular weight.

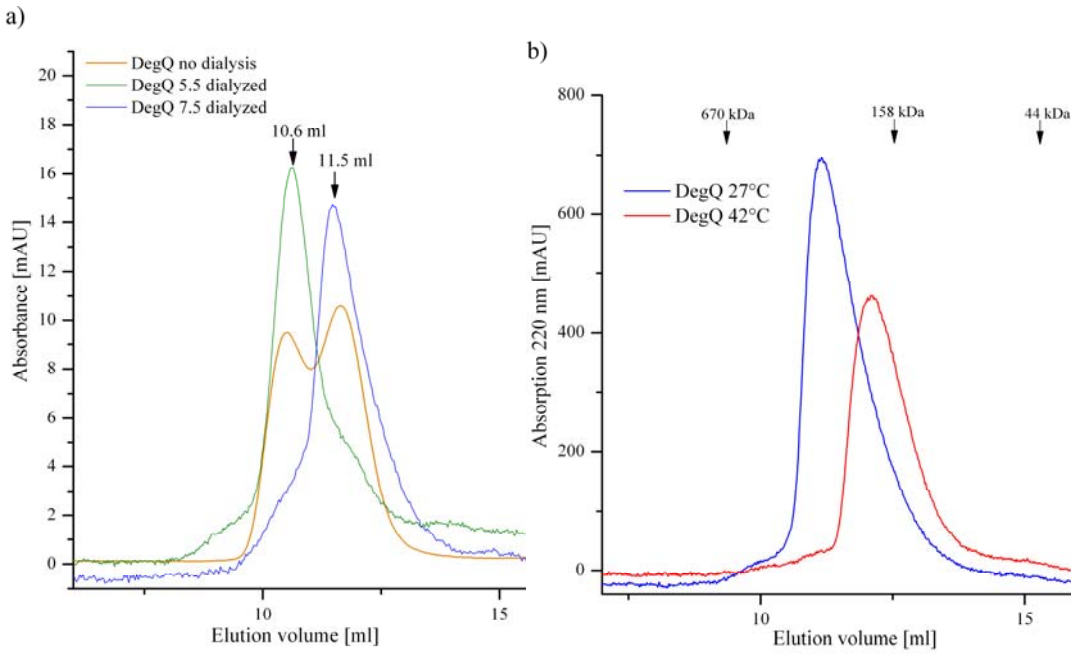


Figure 2.6. a) The dynamic transition between two oligomeric forms of DegQ. Dialized DegQ samples (50 mM MES pH 5.5 - green line and 10 mM HEPES pH 7.5 - blue line) were injected onto a 10/300 Superdex 200 column (GE Healthcare) pre-equilibrated with a respective buffer. An undialyzed sample in 10 mM HEPES pH 7.5 buffer was directly applied on the column pre-equilibrated with a buffer containing 50 mM MES pH 5.5 (orange line) resulting in the shift of the oligomer equilibrium toward the 10.6 ml peak observed for DegQ at pH 5.5 before. b) **The temperature shift in the DegQ oligomer equilibrium**. DegQ was analyzed on the Superdex 200 column in two temperatures: 27°C (blue line) and 42°C (red line). The high temperature moved the oligomer equilibrium to $DegQ_3$.

To test the dynamic equilibrium of the transition process, an undialyzed DegQ sample (HEPES 7.5) was injected directly onto the column, pre-equilibrated with MES pH 5.5 buffer. The elution profile clearly showed two peaks corresponding the 10.6 and 11.5 ml peaks observed before (Figure 2.6 a). The transition of $DegP_6$ to $DegP_{12}$ and $DegP_{24}$ occurs via reassembly of trimers. The temperature triggered hexamer dissociation into trimers was observed when DegP was run in 42˚C (section 2.1). No such intermediates were detected during the DegQ conversion upon sudden pH change (Figure 2.6 a). The temperature dependent behavior of DegQ was also tested. Similarly to DegP the equilibrium was shifted to a trimer due to the high temperature implying that the reassembly happens via a similar mechanism (Figure 2.6 b).

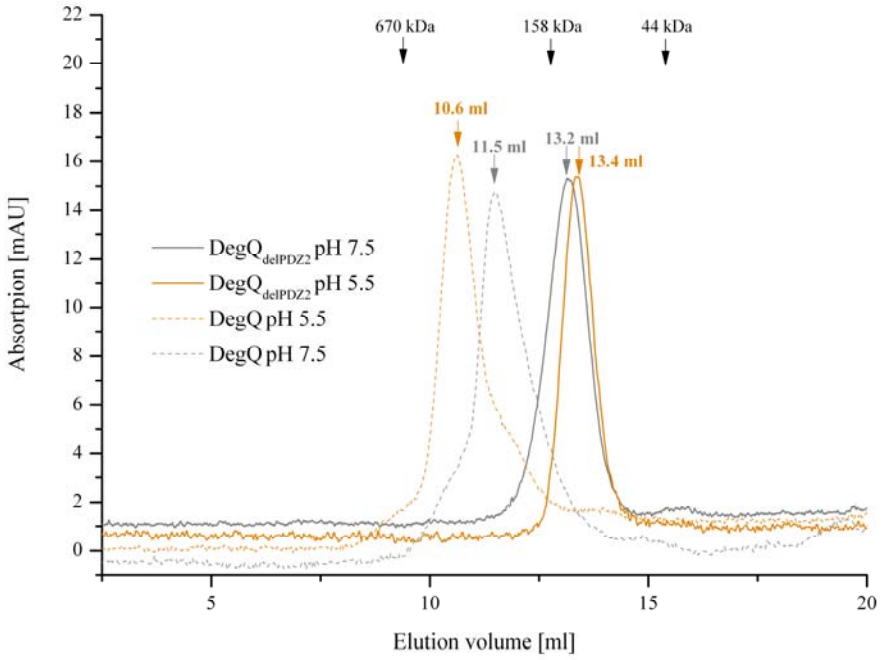


Figure 2.7. Effect of pH on the elution volume of $DegD_{delPDZ2}$. Dialyzed $DegQ_{dePDZ2}$ samples (50 mM MES pH 5.5 - orange line and 10 mM HEPES pH 7.5 - grey line) were injected onto the 10/300 Superdex 200 column (GE Healthcare) pre-equilibrated with a respective buffer. The elution volumes differed insignificantly (0.2 ml) compared to the shift observed for the full length DegQ (dotted orange and grey lines).

The conversion of $DegP_{24}$ complex back to $DegP_6$ was observed when the substrate (casein) digestion was completed (section 2.1). In the case of DegQ the transition between the two oligomeric forms does not require the presence of a substrate

thus it would be worth investigating if the process is pH reversible and what are the conditions for interconvertibility. One of prerequisites is the presence of the PDZ2 domain. The PDZ2 domain of DegP was shown to be required for the hexamer assembly (Sassoon *et al.* 1999; Iwanczyk *et al.* 2007). Upon removal of PDZ2 from DegQ the formation of a hexamer is also impossible and the pH dependent change in the quaternary state can no longer observed (Figure 2.7). The actual oligomeric state of the shortened version of DegQ would correspond to a trimeric assembly of a molecular weight 105 kDa.

The SEC analysis and proteolytic assays of DegQ and $DegQ_{delPDZ2}$ showed a possible regulation of the protease by pH related reorganization of the oligomer. It is still not clear whether this reassembly is substantial for the pH dependent activity though. It was shown that the hexameric form of cross-linked DegP which is no longer able to reassemble into higher oligomeric states, is unable to degrade protein substrates (casein) but it still degrades peptide substrates (Max Roessler personal communication). Therefore it would be interesting to use a suitable peptide substrate for a corresponding DegQ version to test whether the enhancement in proteolytic activity is due to an increased activity of the protease domain or a direct consequence of the change in the oligomeric state.

It has been shown that peptide activators can alter the proteolytic activity of DegS (Wilken *et al.* 2004; Hasselblatt *et al.* 2007). Recent analysis of the interplay between protease and PDZ1 domain of DegP revealed an allosteric mechanism of the protease regulation (unpublished data). Thus it would be interesting to test if such phenomenon applies for DegQ and in addition, if it could be correlated to the pH changes of the environment, thus to the control of the digestive function of DegQ.

2.2.2 DegQ oligomer reassemblies as a result of substrate binding

2.2.2.1 DegQ/substrate complex formation

During proteolysis of substrate proteins, $DegP_6$ undergoes the transformation into a high molecular complex in which it encapsulates target proteins in the newly generated internal cavity. When proteolysis is completed, Deg_{24} or $DegP_{12}$ returns into the

hexameric resting state (section 2.1). The pronounced sequence similarity of DegP and DegQ indicates that this mechanism might be analogous for the two proteins indicating a conserved mechanism of substrate uptake among HtrA family members. To test if the proposed model could be also applied for DegQ, the DegQS187A mutant was incubated with β-casein and subsequently injected on a Superdex 200 gel filtration column. Furthermore, to study a possible effect of pH on the process two different buffers of pH 5.5 and pH 7.5 were used, respectively.

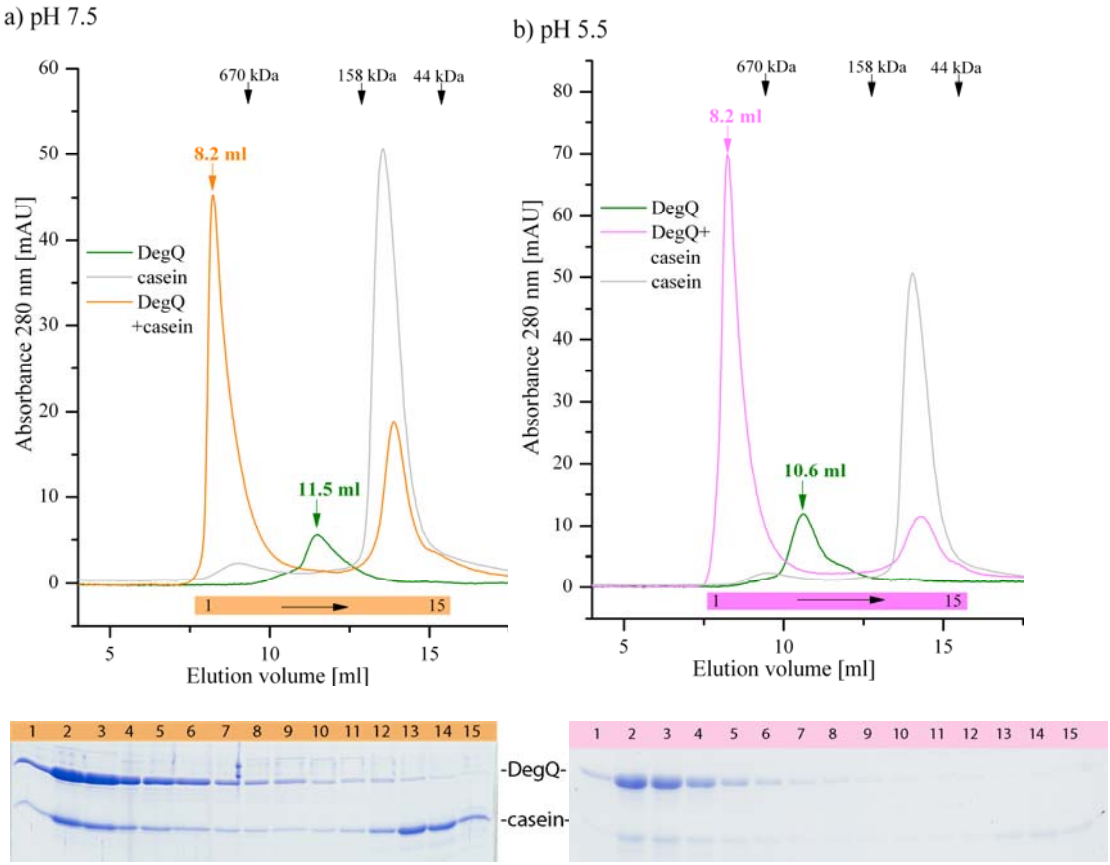


Figure 2.8. Analysis of DegQ/casein complex formation. DegQS187A mutant was incubated with casein in two different buffers: 10 mM HEPES pH 7.5 (a) and 50 mM MES pH 5.5 (b). The resulting complexes (orange and magenta lines) were analyzed by analytical SEC (10/300 Superdex 200 column, GE Healthcare) and SDS-PAGE where consecutive fractions were separated (indicated in orange or magenta). Untreated DegQ (green line) and casein (grey line) served as controls. The arrows indicate the elution volumes.

In both experiments casein binding occurred and lead to a shift in the elution volume of DegQ to 8.2 ml (Figure 2.8 a and b). Since in both buffers substrate binding to DegQ resulted in the same size of the particle it appears that pH has no effect on casein binding and possibly on the quaternary architecture of the resulting complex. It is worth to mention that the resolution of the column is very limited for such large proteins, thus small changes in the molecular weight would not be noticed. Nevertheless, it appears that substrate binding to DegQ leads to the formation of high molecular complexes similarly to DegP. Although, the observed 8.2 ml peaks corresponds to the void volume of the column the dynamic light scattering measurements showed a monodisperse species of molecular weight 2.1 MDa. Overall, it is very likely that in order to fulfill its digestive function DegQ needs to bind a substrate and remodel into a particle of higher oligomeric state as observed for DegP. However, additional experiments with proteolytically active DegQ are required to ultimately prove the relation of the large intermediate oligomer with the degradation of substrates.

The inter-trimeric contacts that stabilize $DegP_{24}$ and $DegP_6$ are different. In the large particle they are mediated by hydrophobic faces of PDZ domains, while the hexamer is stabilized mainly by hydrogen bonds within the β-sheet of the loop LA pillars and additionally polar faces of flexible PDZ domains in the 'closed' form (Krojer, Garrido-Franco et al. 2002). Furthermore, the relative buried surface area of $DegP_6$ is larger than of $DegP_{24}$, indicating higher stability of the hexameric particle. Assuming that in neutral pH the quaternary structure of DegQ resembles $DegP_6$ that can be remodeled into a higher oligomeric state in low pH, it is tempting to speculate that 10.6 ml peak of uncomplexed DegQ at pH 5.5 could represent another form of a resting state whose reassembly would require less activation energy due to weaker inter-trimer interactions compared to the more stable hexameric form (peak 11.5 ml at neutral pH). The activation barrier of such particle would be lower at pH 5.5, thus making it more active. In addition, the observed difference in the elution volume of $DegP_{12}$ (10 ml) and DegQ pH 5.5 (10.6 ml) (Figure 2.5) is significant, thus it cannot be directly assumed that the quaternary structure of DegQ in pH 5.5 resembles exactly $DegP_{12}$. Based on the EM structure of $DegP_{12}$/OMP the pores of the particle are too narrow to allow the substrate access; thus

the particle has to open up in order to degrade the substrate protein. It is completely elusive how the large DegQ particle should be spatially arranged though.

2.2.2.2 $DegQ_{delPDZ2}$/substrate complex formation

The PDZ domains of DegP have been assigned two distinct functions. While the PDZ2 domain is required for the oligomer assembly, the PDZ1 domain mediates substrate and activator binding (Sassoon, Arie et al. 1999; Iwanczyk, Damjanovic et al. 2007; Meltzer *et al.* 2007). Similarly, the role of the PDZ2 domain of DegQ is linked to the initial hexamer formation in neutral pH and subsequent oligomer reassembly in acidic pH (section 2.2.1). It is not essential for pH dependent proteolysis though, presumably due to the dispensability for substrate binding. In order to test if the lack of the PDZ2 domain influences substrate complex formation the $DegQ_{delPDZ2}$S187A mutant was incubated with casein and applied onto a Superdex 200 analytical column.

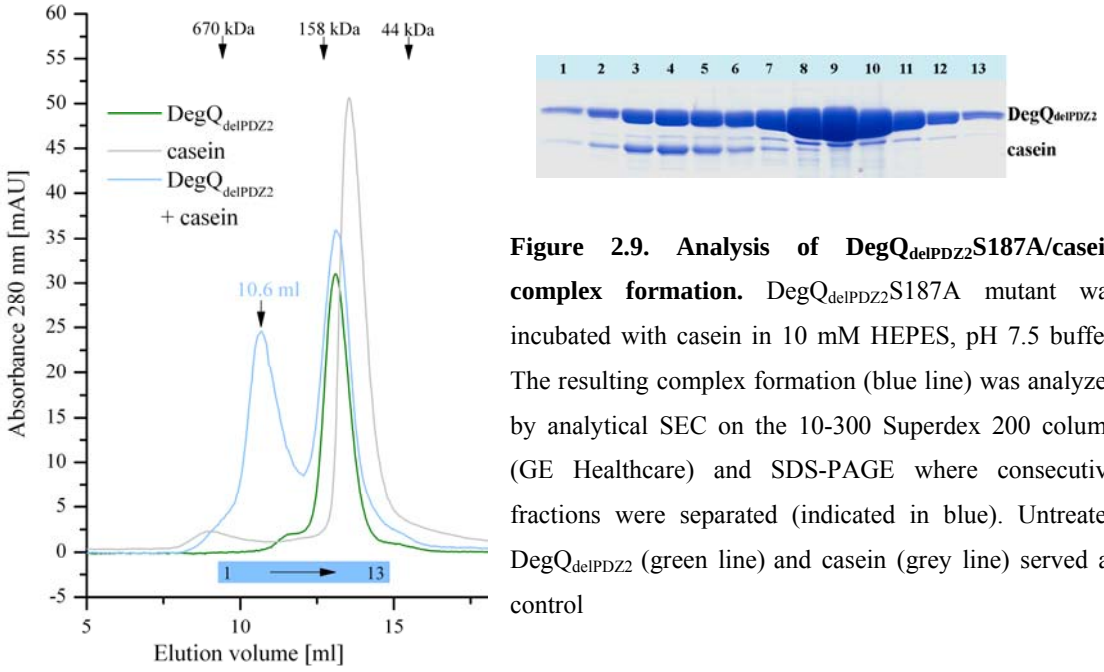


Figure 2.9. Analysis of $DegQ_{delPDZ2}$S187A/casein complex formation. $DegQ_{delPDZ2}$S187A mutant was incubated with casein in 10 mM HEPES, pH 7.5 buffer. The resulting complex formation (blue line) was analyzed by analytical SEC on the 10-300 Superdex 200 column (GE Healthcare) and SDS-PAGE where consecutive fractions were separated (indicated in blue). Untreated $DegQ_{delPDZ2}$ (green line) and casein (grey line) served as control

The experiment clearly showed a complex formation indicated by the appearance of a 10.6 ml peak in the elution profile (Figure 2.9). SDS-PAGE analysis confirmed the

presence of both casein and $DegQ_{delPDZ2}S187A$ in the additional peak (Figure 2.9). The particular nature of DegP and DegQ among other HtrA family members is based on the presence of two consecutive C-terminal PDZ domains (Figure 13 introduction). Human and plant homologs contain only one of such domain and yet are able to sustain their proteolytic function. The removal of the PDZ2 domain from DegQ did not impair its ability to bind and degrade casein (Figure 2.9 and 2.4), thus one might conclude that the reassembly of trimers into higher molecular weight complexes and the associated encapsulation of the substrate are hallmarks of the HtrA family in general. It should refer to the HtrAs involved in the indiscriminate degradation of unfolded proteins thus participating in the general protein quality control, unlike specialized members (e.g. DegS) which plays a single defined step in a signal transduction pathway.

2.2.3 Study on the role of DegQ in the periplasm

Periplasmic proteins are continually exposed to the changing conditions of the external environment due to the porous character of the outer membrane (Raivio and Silhavy 2001). Thus the need of an efficient system of protein homeostasis surveillance is indispensable. DegP was shown to be an important factor of periplasmic protein quality control encompassing two antagonistic activities namely, a protease and a chaperone function (Spiess *et al.* 1999). In the absence of DegP in the periplasm, *E. coli* is unable to remove misfolded proteins and to survive exposure to elevated temperatures (Lipinska, Fayet et al. 1989; Strauch *et al.* 1989). DegQ is another periplasmic serine protease (Bass *et al.* 1996; Waller and Sauer 1996) so its possible function could be to degrade misfolded protein substrates, thereby contributing to the release of the potential danger caused by non-native proteins. The precise role of DegQ in quality control of envelope proteins of *E. coli* has not been clarified though.

2.2.3.1 Analysis of aggregate formation in the presence of DegQ

The mechanism of oligomer reassembly of DegP explains the chaperone-like activity, which occurs by encapsulation of unfolded protein substrates in the chamber and thereby preventing the formation of aggregates in the periplasm (section 2.1). As shown by SEC analysis DegQ undergoes similar rearrangements, thus could possibly display the

chaperone-like activity, too. To test this possibility, DegQS287A variant was incubated with citrate synthase (CS) at 43°C and the kinetics of aggregation were determined by light-scattering measurements (Figure 2.10).

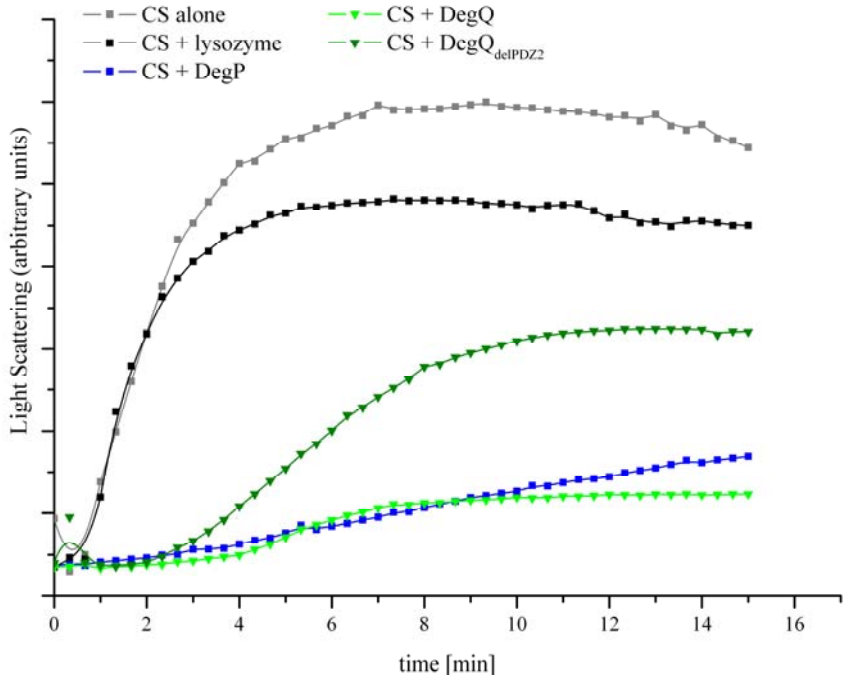


Figure 2.10. Influence of DegQ on the thermal aggregation of citrate synthase. CS was diluted to the final concentration of 1 µM into the thermostated (43°C) buffer (10 mM HEPES pH 7.5) in the presence of 25 µM DegQS187A (light green line, triangles), 100 µM $DegQ_{delPDZ2}$S187A (dark green line, triangles), 25 µM DegPS210A (blue lined; squares), or 25 µM lysozyme (black line, squares) as a control. The concentrations of all proteins are based on monomers. The kinetics of aggregation were determined by light-scattering measurements and plotted together with the CS alone (grey line, squares).

The efficiency of the aggregation suppression by DegP and DegQ is comparable. $DegQ_{delPDZ2}$ could also suppress aggregate formation, however 100:1 (DegQ $_{delPDZ2}$:CS) ratio was required to achieve significant result (Figure 2.10 legend). The reduced degree of aggregate formation is a direct consequence of the encapsulation of the unfolded citrate synthase. In addition to SEC analysis, the aggregation assays could demonstrate that DegQ and DegP function as 'holder' chaperones, similarly to small Hsps (Lee *et al.* 1997). The binding of an unfolded protein by DegP results in high molecular weight assemblies which represent initial protease/substrate complexes (section 2.1). Thus, the

observed 'holding' activity of DegP and DegQ could be interpreted as a byproduct of an impaired degradative cycle, where the captured complex can be regarded as the first intermediate of the proteolytic process. This behavior of the DegPS210A variant might also explain its effective suppression of the temperature-sensitive phenotype of *degP*$^-$ strain (Spiess, Beil et al. 1999).

2.2.3.2 The role of DegQ in OMP biogenesis

The biogenesis of outer membrane proteins is a complex process (introduction section 1.2). It involves the OMP synthesis, translocation across the inner membrane, transport through the periplasmic space, and finally incorporation into the outer membrane. The shuttling between the two membranes requires the assistance of molecular chaperones. They ensure a targeted transit to the OM and prevent the triggering of the stress response by the presence of unfolded OMPs in the periplasm.

There have been three chaperones postulated to participate in the OMP biogenesis, namely SurA, Skp, and DegP. The periplasmic purification of DegP allowed the isolation of DegP/OMP complexes (section 2.1). Further analysis revealed that the bound OMPs were stable and exhibited tertiary structure elements protecting them from degradation. Furthermore, the composition of OM fraction isolated from *degP* null strain demonstrated altered levels of OMPs compared to the wild type strain. To test a possible role of DegQ in OMP biogenesis, purification profiles of periplasmically expressed DegQ were analyzed and OM fractions of the *degQ* null strain were examined (Figure 2.11).

During the production of recombinant proteins, both DegQ and DegP were C-terminally His-tagged and exported to the periplasm where the complex formation with OMPs could occur. When the two SEC elution profiles were compared, no additional peaks corresponding to $DegP_{12}$ and $DegP_{24}$ were observed during purification of DegQ (Figure 2.11). The presence of an active site serine had no impact on the result (data not shown). In addition, SDS-PAGE analysis of the OM isolated from the *degQ* null strain showed no alteration in the OMPs composition compared to the wild type (Figure 2.11).

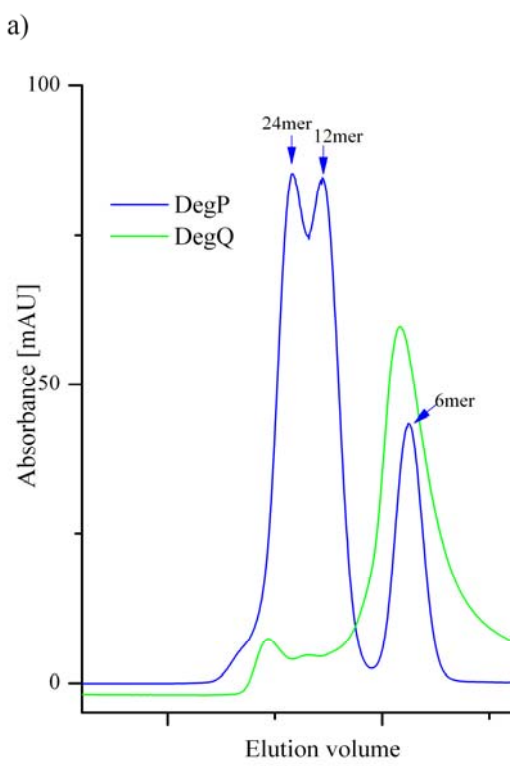


Figure 2.11. Analysis of the relation of DegQ with OMPs. a) Both, recombinant DegQS187A and DegPS210A were overproduced in the periplasm of *E.coli*. SEC elution profiles which were recorded during the final purification step of the two proteins are compared (Superdex 200, prep grade). The DegP preparation resulted in two additional peaks (blue line) corresponding to $DegP_{12}$/OMP and $DegP_{24}$/OMP complexes. No additional peaks were observed during DegQ purification (green line). b) Outer membranes were prepared from an equivalent number of wildtype , *degP*$^-$ and *degQ*$^-$ cells and the steady-state levels of OMPs was analysed by SDS-PAGE.

These observations demonstrate that no obvious interactions between DegQ and OMPs occur. The OM composition remains intact in the absence of DegQ in the periplasm thus suggesting that the role of DegQ in OMP biogenesis does not appear to be essential. Interestingly, it is the heat-shock regulated DegP that plays the major part in the homeostasis of OMPs in the *E. coli* envelope, although the two proteins display high similarity. It is elusive why OMPs interact exclusively with DegP. It cannot be excluded however, that DegQ comes into a transient contact with OMPs and does not form stable complexes, which could be easily detected. This possibility however does not explain the abundance of DegP/OMP assemblies on the contrary to DegQ. As discussed before (Introduction: section 1.4.4) the sequence comparison of the two proteins (Figure 1.14 - Introduction) reveals an extended Q-linker (Loop LA) region in the protease domain of DegP. Taking into account a distinct character of the loop LA within HtrAs and its regulatory function in the DegP molecule, it may be considered as the main interaction

platform essential to mediate contacts of DegP with OMPs. It would be exciting to confirm the hypothesis by exchanging the Q-linkers between DegP and DegQ and examine the properties of the hybrid proteins.

2.2.3.3 Analysis of the growth of *degQ* and *degP* null strains

Following the discovery of the pH dependent behavior of DegQ on the molecular level (section 2.2.1), an *in vivo* approach was employed to study the relevance of this finding on the growth of *E. coli*. The growth rate of *degQ* and *degP* null mutant strains was monitored by measurements of the optical density of liquid bacterial cultures (Figure 2.12). In the first experiment Luria Bertani (LB) medium was buffered to pH 5.5 or 7.5 and then inoculated with either wild type or *degP* null mutant strain. Remarkably, in pH 5.5 the growth of the mutant was the same as that of its parent strain but in pH 7.5 cells stopped dividing and entered stationary phase 3.5 h earlier. In the absence of DegP in the *E. coli* envelope the accumulation of misfolded proteins is pronounced (Strauch, Johnson et al. 1989). The apparent correlation with the pH dependent proteolytic activity of DegQ leads to the conclusion that DegQ takes over the function of DegP in the *degP* null mutant strain. However, in the course of growth and away from the pH optimum, DegQ is not as efficient anymore and the growth of cells is impaired (Figure 2.12). Since DegQ/substrate complex formation is not pH dependent unlike the proteolytic function, it would be interesting to test if the observed phenotype could be rescued by the DegQS187A variant.

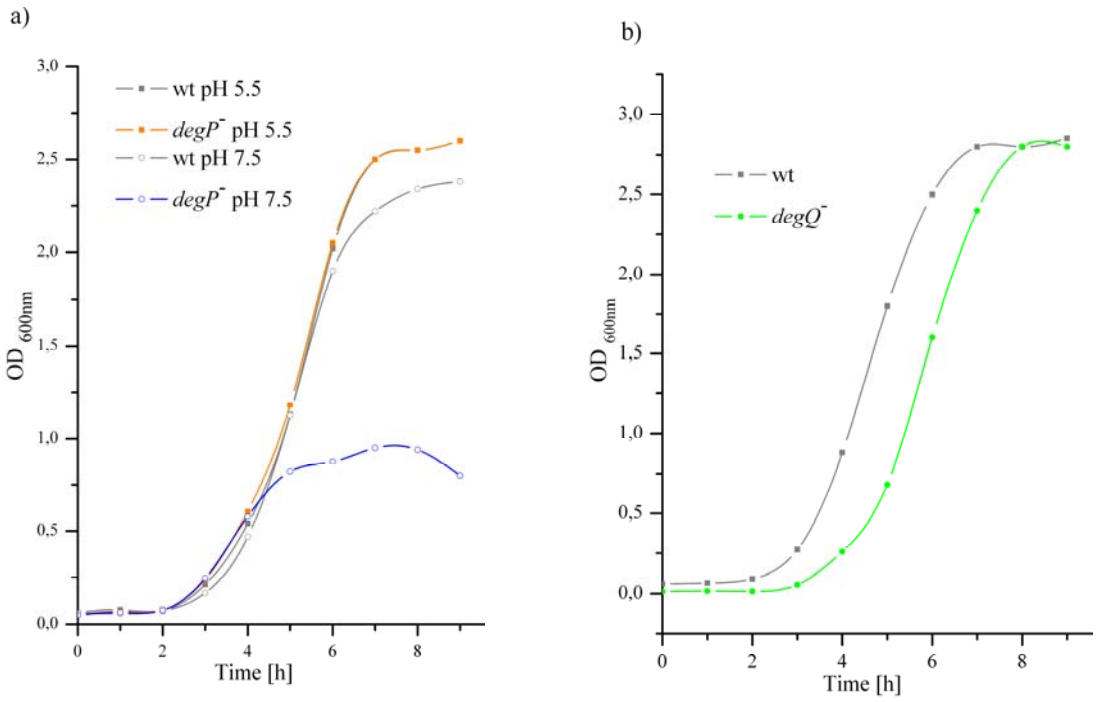


Figure 2.12. Analysis of the growth of *degQ* and *degP* null strains a) Growth of CLC198 (*degP*⁻) and MC4100 (wild type) strains in LB media buffered with either 50 mM MES pH 5.5 or 50 mM HEPES pH 7.5. The growth rate of the wild type strain was not affected by pH (grey line - open and closed symbols), while *degP*⁻ grown in pH 7.5 entered the stationary phase (blue line) 3.5 hours earlier compared to the growth in pH 5.5 (orange line) b) Growth of *degQ*⁻ and MC4100 (wild type) strains in LB media buffered with 50 mM HEPES pH 7.5. The mutant strain (green line) shows longer lag time after inoculation in the medium compared to the wild type (grey line). Growth was monitored by determining the OD at 600 nm in 30 min time points.

The same treatment of the *degQ* null mutant strain did not result in a similar phenotype (data not shown) implying DegP to be independent of pH of the environment of bacterial growth. However, when the initial phase growth of *degQ* null mutant was compared to the wild type strain, the adaptation phase of the cells lacking DegQ was elongated (Figure 2.12), indicating the house keeping function of DegQ whose presence is required for the continuous response to rapid environmental changes. In the absence of DegQ the up-regulation of *degP* has to occur due to the accumulation of unfolded proteins in the envelope during initial steps of the cell culture growth. This finding is consistent with the inducible character of the *degP* promoter (Erickson and Gross 1989; Danese, Snyder et al. 1995). The regulation of *degQ* gene has not been undoubtfully proved, however there are indications of its pH related control (Yohannes *et al.* 2004).

It is important to note that the cytoplasmic adaptation to the pH based changes has been extensively studied and described (Booth 1985; Foster 2004). Recently however, microarray and 2-D electrophoretic analysis demonstrated that remarkable fraction of genes showing pH dependence encoded periplasmic proteins (Blankenhorn *et al.* 1999; Stancik *et al.* 2002; Yohannes, Barnhart et al. 2004; Maurer *et al.* 2005; Hayes *et al.* 2006). The majority of the targeted proteins are involved in the minimization of the pH change by facilitating catabolism-related mechanisms (catabolite binding proteins and transporters). The collection of identified genes additionally included the acid stress chaperones HdeA, HdeB and the folding catalysts DsbA underlying the importance of protein quality control systems during pH stress. Interestingly, Yohannes *et al*. could observe in their 2-D electrophoretic experiments that *degQ* is up-regulated in high pH under anaerobic conditions of growth (Yohannes, Barnhart et al. 2004). However, the same group did not confirm this hit with their microarray assay two years later (Hayes, Wilks et al. 2006). The ambiguous reports cannot exclude the possibility that DegQ facilitates the adaptation to the changes in environmental pH on the molecular level responding to the mild acidic stress by oligomer reassembly and subsequent enhancement of the activity.

2.2.4 Summary

Analogously to DegP, the biochemical analysis of DegQ revealed an oligomer reassembly upon binding of an unfolded substrate, indicating a common basis of the regulation of the activity. The substrate binding and the consecutive transformation into the larger particle was demonstrated to be independent of the presence of the PDZ2 domain. Taking into consideration the fact that the majority of HtrAs encompass only one PDZ domain, the reassembly of trimers into higher molecular weight complexes can be considered as a conserved mechanism regulating the activity of HtrA family members in general.

The observed ability to suppress the formation of aggregates by DegQ points into the chaperone-like function by which the captured unfolded substrates can no longer interact with each other to cause severe aggregation-linked damage. However, the

dynamics and turn over of the interactions would have to be investigated in detail in order to exclude or confirm the possible refolding events within the chamber.

Although DegQ and DegP exhibit very a pronounced sequence identity (60%), the study revealed striking functional differences between the two proteins. The activity assays and size exclusion chromatography demonstrated a pH related remodeling of the DegQ particle in addition to the observed pH dependence of its digestive function. These findings might reflect an additional regulatory mechanism employed by DegQ, which could be associated with the changes of the external pH in the habitat of *E. coli*. Consistently, *in vivo* studies highlighted an important, pH-related house keeping function of DegQ. The highly unstable environment of the periplasm induces very rapid changes of pH, thus the damage has to be reacted on immediately. The danger of misfolding in the periplasm is constant, hence DegQ may represent an important protease-chaperone that ensures continuous protein quality control under slightly/rapidly changing environmental conditions, which might not trigger stress response pathways. Only when the system is overloaded as a result of stress DegP is up-regulated and takes over. These results indicate that DegP and DegQ closely collaborate to ensure the overall robustness and fidelity of the proper protein homeostasis in the cell envelope.

However, when outer membranes of the *degQ* null mutant strain were analyzed, no alterations to the wild type strain were observed, indicating the essential role of DegP, but not DegQ, in OMP biogenesis. It would be of a great interest to investigate the differences and parallels between them further to unveil in more detail the interplay between the two factors in the periplasm.

3 Materials and methods

The following chapter contains experimental methods that were used in section 2.2 ('Regulation of molecular and cellular function of DegQ') and were not described in section 2.1 ('Structural basis for the regulated protease and chaperone function of DegP')

3.1 Reagents and enzymes

Antibiotics were purchased from Sigma and all other chemicals were purchased from Merck, Sigma or Fluka unless otherwise stated and were of the highest grade available. Enzymes used in molecular biology protocols were purchased from Roche or New England Biolabs (NEB), unless specifically stated. Materials for protein purification and chromatography and Ni-NTA Superflow were purchased from Qiagen and columns for self-packing were ordered from GE Healthcare (formerly Amersham Biosciences) as well as pre-packed columns and other FPLC materials.

3.2 Buffers, solutions media and antibiotics

The common buffers and media used are described here; when a more specialized one was used it is described in the appropriate section.

Name	Composition
SDS sample buffer	2% (w/v) SDS, 80 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 50 µl/ml 2-ME
1x SDS-PAGE running buffer	25 mM Tris/HCl pH 8.3, 200 mM glycine, 0.1% (w/v) SDS
10x TAE buffer	0.4 M Tris/HCl, pH 8.0, 10 mM EDTA-Na_2-salt, 0.2 M acetic acid
DNA loading buffer :	30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol
LB medium :	10 g tryptone, 5 g yeast extract, 10 g NaCl, adjusted to pH 7.0 with NaOH; filled up with H_2O to 1000 ml and autoclaved

Ampicillin : 50 mg/ml in H_2O stock solution, stored at -20°C

Kanamycin : 50 mg/ml in H_2O stock solution, stored at -20°C

Lysozyme : 50 mg/ml in H_2O stock solution, stored at -20°C

DNAse : 1 mg/ml in H_2O stock solution, stored at -20°C

3.3 Bacterial strains and vector system

Host strain	Genotype
DH5α chemically competent cells	F-, j 80D lacZDM15 D(lacZYA-argF)U169 deoR recA1 endA1 hsdR17($rk^- mk^+$) phoA supE44 thi-1 gyrA96 relA1
BL21 Star (DE3) One shot chemically competent cells (Invitrogen)	F- ompT hsdSb ($r_B^- m_B^-$) gal dcm rne 131 (DE3)α
K-12 MC4100	F^- *ΔlacU169 araD136 rbsR relA rpsL thi*.
CLC198 (*degP*$^-$)	MC4100 *degP::Tn10*
K-12 MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1
degQ$^-$ *	K-12 MG1655 *degQ::Tn5 Kan*^{R}

* strain ordered from *E. coli* Genome Project (http://www.genome.wisc.edu)

Table 3.1 *E. coli* strains used for cloning, expression and growth analysis.

The used vector pET26b(+) (Novagen) contains the T7lac promoter, which consists of a lac operator sequence downstream of the promoter. BL21 (DE3) *E. coli* cells contain a chromosomal copy of the gene for T7 RNA polymerase. IPTG can be used to induce the expression of T7 RNA polymerase. This provides a tightly controlled expression system where concentrations of IPTG can be adjusted to optimize expression of soluble protein.

3.4 Molecular cloning techniques

3.4.1 Construct design

The *degQ* gene was amplified from the genomic DNA of DH5α strain. The constructs lacks the endogenous signal sequence. It was subcloned to a pET26b(+) (Novagen) vector encompassing N-terminal *pelB* signal sequence for periplasmic localization of the recombinant protein and additional C-terminal His-tag for affinity purification. The active site serine of the DegQS187A variant was replaced with the alanine residue by site directed mutagenesis. The same vector system was used for $DegQ_{delPDZ2}$ mutant.

3.4.2 Cloning

The *degQ* gene was amplified from whole bacterial cells by PCR reaction, in which the first denaturation step was elongated to 10 min in order to disrupt the cells. The blunt end product was subcloned to a shuttle vector by means of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) according to the manufacturer's instructions. The restriction enzymes (NcoI and XhoI) were used to extract the *degQ* gene and to reclone it to the pET26b(+) vector (Novagen).

3.4.3 Oligonucleotides

All used oligonucleotides were synthesized by Life Technologies:

Name	**Sequence 5' → 3'**
DegQ Forward (NcoI)	CCATGGCCCCTCTCCCCAGTCTG
DegQ Reverse (XhoI)	CTCGAGACGCATCAGCAGATAGATGC
$DegQ_{delPDZ2}$ Reverse (XhoI)	GCGCTCGAGCGAAGAGGTGCTGGTATCG
DegQS187A Forward	ATTAACCGCGGTAACGCCGGCGGTGCACTAT
DegQS187A Reverse	ATAGTGCACCGCCGGCGTTACCGCGGTTAAT

3.4.4 Plasmid purification

Plasmid DNA was purified using a miniprep kit (Quiagen) according the manufacturer's instructions. Plasmid concentration was calculated by measuring the absorbance at 280nm in the NanoDrop® (ND-1000) spectrophotometer compared to a blank. Plasmid DNA was sequenced by the in-house sequencing facility.

3.4.5 Polymerase Chain Reaction (PCR)

PCR amplification was carried out using an initial cycle of denaturation at 95°C for 5 min, annealing at 57°C for 1 min and extension at 72°C for 2 min. This was followed by 25-30 cycles of 1 min denaturation, 30 seconds annealing and 2 min extension. Reactions typically contained 1 µg template, 10 pmole of each primer and 2 units of Herculase polymerase (Stratagene) in a total volume of 1 x Herculase buffer supplemented with 2 µl

of 10 mM dNTPs mix. Site-directed mutagenesis was performed using the Quickchange kit (Stratagene).

3.4.6 Restriction enzyme digestion, dephosphorylation and ligation

Restriction digests were performed using the supplied buffers at recommended temperatures (NEB). Typically, 1-2 units of enzyme were used per 1 µg of DNA.

To prepare the vector pET26b for cloning, it was digested as described above. To avoid self-ligation and increase ligation efficiency, 15 U calf intestinal phosphatase (CIP) was added for 30 minutes at 37°C to remove 5' phosphates of cut vector.

After successful restriction digestion of the insert and the vector, both were purified by gel extraction (Gel Extraction Kit, Qiagen). To perform ligation insert and vector were mixed in a 5:1 ratio, respectively. The reaction mix was incubated with 1 U T4 DNA ligase in the appropriate 10x buffer at 20°C for 2 hours or left overnight at 4°C. DH5α cells were transformed and plated out for selection on LB plates containing 25µl kanamycin. Uncut vector was transformed in parallel to monitor transformation efficiency.

3.4.7 Transformation of chemically competent *E. coli*

The plasmids were cloned into DH5α cells during the cloning and selection procedures, whereas supercompetent BL21 (DE3) cells were transformed for overexpression. Aliquots of cells (100 µl DH5α; 50 µl BL21 Star (DE3)) were transformed with 1 µl of DNA and incubated on ice for 30 minutes before exposure to 42°C. After 90 seconds, 1 ml LB medium was added to the heat shocked cells on ice and incubated at 37°C for 1 h on a shaker (1200 rpm). Afterwards, they were either transferred into liquid LB media or plated out on LB selective media containing the appropriate antibiotic and grown overnight at 37°C.

3.4.8 Agarose gel electrophoresis

Agarose gel electrophoresis was employed to separate double-stranded DNA molecules according to their molecular weight after PCR, DNA isolation and restriction enzyme digestion and to quickly determine the yield and purity of a DNA fragment. Therefore a 1% (w/v) solution of agarose gel was produced, by dissolving the agarose in

1x TAE. To visualize the DNA bands ethidium bromide was added. The DNA samples (1–20 µl) were mixed with DNA loading buffer and placed on the gels in the wells formed by the comb. The electrophoresis was performed for 45 min at 85 V. The DNA intercalating character of ethidium bromide allowed the detection of the DNA fragments under UV light at 302 nm as separated bands. The intensity of a stained band reflected the amount of DNA in the sample.

3.5 Protein expression and purification

All variants of DegQ were overexpressed as C-terminal His-tagged protein and purified according to the same procedure.

3.5.1 Expression of recombinant protein in *E.coli*

Freshly transformed chemically competent BL21(DE3) cells were inoculated into 100 ml of LB medium containing kanamycin of final concentration 25 µg/ml and grown overnight in a shaking incubator at 37°C and 220 rpm. 10 ml of saturated overnight culture were used to inoculate 1 L of fresh LB medium. The 1 L flasks were incubated at 37°C and 200 rpm until an OD_{600} of 0.8 – 1.0 was reached and IPTG was added to a final concentration of 1 mM. After 4 hours cells were harvested by centrifugation (30 minutes, 4°C, 3500 rpm in Sorvall RC 3B Plus), resuspended in 5–10 ml Buffer A per L of culture and frozen at -20°C.

3.5.2 Protein purification

Buffers used:

Buffer A	50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0 adjusted with NaOH
Buffer B	50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8.0 adjusted with NaOH
Buffer C	10 mM HEPES/NaOH pH 7.5, 150 mM NaCl

Cells were harvested and resuspended as described before and thawed on ice. One tablet “Complete” protease inhibitor mix (Roche) per 2 L culture, lysozyme (1 mg/ml), DNAse (5 µg/ml) and PMSF dissolved in DMSO (0.1 mM) were added for 15 minutes

incubation on ice. Subsequently, cells were disrupted by sonification (Branson Sonifier 250) with a macrotip applying 50% pulse and 50% output for twice 1 minute. The samples were always kept on ice. The lysate was centrifuged (30 minutes, 4°C, 20000 rpm in Sorvall RC 3B Plus, Rotor: SS-34) and the supernatant was loaded over a sample pump (flow 1 ml/min) on a Ni-NTA Superflow resin (column volume, CV = 15 ml; Ø = 16 mm), pre-washed and equilibrated with Buffer A (without imidazole). Afterwards, the loaded column was washed with Buffer A and subsequently a stepwise gradient of Buffer B was used to remove initially unspecific bound proteins and for the elution of the His-tagged proteins (flow 4 ml/min). DegQ was eluted with 100% Buffer B (300 mM imidazole). All fractions were analyzed by SDS-PAGE and the corresponding fraction to His-tagged protein was concentrated down to 5 ml (Vivaspins; MWCO 30000; Vivascience; 4000 rpm in Heraeus Multifuge 4 KR) and directly applied to a Superdex-200 prep grade column (GE Healthcare) equilibrated with Buffer C. The fractions corresponding to the peak were analyzed by SDS-PAGE. Next, appropriate fractions were collected and concentrated. The final concentration of the full length protein was 10 mg/ml while $DegQ_{delPDZ2}$ reached 30 mg/ml measured by Bradford method. The protein was aliquoted, flash frozen with liquid nitrogen and stored in -80˚C for further analysis.

3.6 Protein analysis

3.6.1 Bradford protein assay

For the determination of the protein concentration according to the method of Bradford (Bradford 1976) 795 µl H_2O were mixed with 5 µl of protein solution and 200 µl of Bradford-solution (BioRad, Germany) in a plastic-cuvette. The Absorption of this mixture was measured in a photometer at a wavelength of 595 nm against a blank solution containing 800 µl H_2O and 200 µl of Bradford-solution. The corresponding protein concentration was calculated from a calibration curve using BSA as a standard.

3.6.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for the electrophoretic separation of proteins according to the method of Laemmli (Laemmli 1970). The stacking and the separating gel were prepared

as described below (Table 3.2). Eight minigels could be poured at once in the apparatus used (MPI Martinsried, Germany). The protein samples were mixed in a 1:1 ratio with 2x SDS loading buffer and boiled for 5 minutes at 94°C before loading onto the gel. The electrophoresis was performed at 25 mA per gel, usually until the dye-front eluted from the gel. Afterwards, gels were stained with Coomassie brilliant blue to visualize protein bands.

component	**separating gel**			**stacking gel**
	10%	12%	15%	5%
1.5 M Tris/HCl pH 8.8	20 ml	20 ml	20 ml	-
0.5 M Tris/HCl pH 6.8	-	-	-	7.5 ml
acrylamide stock*	26.4 ml	32 ml	40 ml	4.5 ml
H_2O	32.8 ml	28.4 m	20 ml	17.8 ml
10% (w/v) SDS	800 µl	800 µl	800 µl	300 µl
10% (w/v) APS	400 µl	400 µl	400 µl	300 µl
TEMED	40 µl	40 µl	40 µl	30 µl

***** 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (30% Protogel)

Table 3.2 Composition of polyacrylamide gels.

The OMP preparations were resolved by means of gels containing additionally 6 M urea to resolve OmpC and OmpF bands. All components of gels except for water were mixed, next 28.8 g (resolving gel) or 10.8 g (stacking gel) solid urea was added and dissolved by stirring. Finally, 5 ml H_2O was added and gels were poured according to the standard procedure.

3.6.3 Coomassie blue staining

The polyacrylamide gels were stained by soaking in a staining solution and boiling in a microwave oven. Afterwards, the gels were gently shaken for 15 – 30 minutes at room temperature. For destaining, the gels were transferred to a destaining solution and again boiled for several seconds and subsequently shaken for approximately half an hour at room temperature. The destaining procedure was repeated until the gel was completely free of background stain.

Staining solution: 2.5 g Coomassie Brilliant Blue R-250, 450 ml ethanol, 100 ml acetic acid, filled up with H_2O to 1000 ml

Destaining solution: 250 ml ethanol, 80 ml acetic acid, filled up with H_2O to 1000 ml

3.6.4 Dynamic light scattering (DLS)

DLS was carried out using a DynaPro-801 molecular sizing instrument (Protein-Solutions Inc.). A 50 µl sample of protein in the buffer used for size-exclusion chromatography was centrifuged for 10 minutes at 4°C and 20000xg to spin down dust particles and large aggregates of denatured protein and transferred into a 45 µl sample cell. The DLS measurements were performed at 19°C. The data were analyzed using the Dynamics V6 software (Protein-Solutions Inc.).

3.6.5 Analytical gel filtration

The analytical gel filtration analyses were performed on a Superdex 200 10/300 column (GE Healthcare). The column was equilibrated with 10 mM HEPES (pH 7.5), 50 mM MES (pH 5.5) or 50 mM Tris-HCl (pH 9.5) buffer at room temperature. All buffers additionally contained 150 mM NaCl. Prior to injection, protein samples were dialyzed against the respective buffer for 3 h in 4°C with slow stirring. The samples (120 µM DegQ, 600 µM $DegQ_{delPDZ2}$, 180 µM $DegP_6$, 60 µM $DegP_{12}$) were applied over 100 µl loop at the 0.5 ml/min flow rate. Fractions were collected and further analyzed by SDS-PAGE. To test how dynamic the formation of the higher oligomeric peak of DegQ at pH 5.5 is, an undialyzed sample (in 10 mM HEPES 7.5, 150 mM NaCl buffer) was directly applied on the column pre-equilibrated with 50 mM MES pH 5.5, 150 NaCl buffer.

To test the *in vitro* complex formation of DegQ with casein, 120 µM DegQS187A or 600 µM $DegQ_{delPDZ2}$S187A was mixed with 160 µM casein. Two buffers were used: 50 mM HEPES, 150 mM NaCl, 10 mM DTT, pH 7.5 or 50 mM MES, 150 mM NaCl, 10 mM DTT, pH 5.5. The sample was incubated for ten minutes at 37ºC before samples were injected to a Superdex 200 10/300 gel filtration column (GE Healthcare) pre-equilibrated with a respective buffer. Comparison with marker proteins and SDS-PAGE analysis revealed the size and composites of the individual complexes.

To calibrate the size-exclusion chromatography column, blue dextran was initially applied to determine its void volume (V_0). Afterwards the elution volumes (V_E) of the following molecular weight standards (BioRad) were determined: thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and Vitamin B_{12} (1.35 kDa). The total volume of the column (V_T) was calculated according to its length and diameter. The K_{AV} value for every molecular weight standard was calculated by the following equation:

$$K_{av} = \frac{V_E - V_0}{V_T - V_0}$$

The K_{AV} values for the molecular weight standards were then plotted against the logarithm of their molecular weights and the points were fitted to a linear equation. For proteins with unknown molecular weight, the K_{AV} value was determined and the corresponding molecular weight was calculated from the calibration curve.

3.7 Biochemical and microbiological techniques

3.7.1 Thermal aggregation of citrate synthase

Aggregation kinetics of citrate synthase were measured with a spectrofluorometer (FluoroMax®-4, Horiba Jobin Yvon) in a stirred and thermostatted quartz cuvette at 43ºC. The excitation and emission wavelength of the instrument were set to 360 nm with a spectral bandwidth of 2 nm. The 25 µM DegP, 25 µM DegQ, 100 µM $DegQ_{delPDZ2}$ and 25 µM lysozyme were adjusted in a buffer containing 50mM HEPES/NaOH pH 7.5. The samples were incubated for 20 minutes in the sample cell until a stable baseline reading was obtained. Then citrate synthase was added up to a final concentration of 1 µM and the light scattering signal was monitored for 15 minutes.

3.7.2 Degradation of resorufin-labeled casein

The proteolytic activity of DegP, DegQ and $DegQ_{delPDZ2}$ was determined with resorufin-labeled casein (Roche, Germany). 15 µl of 0.4% (w/v) resorufin-labeled casein was added to 100 µl incubation buffer containing approximately 10 µg of the respective

protein and incubated at 37°C for 3 h (DegP) and 12 h (DegQ, $DegQ_{delPDZ2}$) The reaction was stopped by precipitation of casein with 480 µl 10% (w/v) TCA. Samples were again incubated for 10 min at 37°C and subsequently centrifuged (10 min, 10000 x g, RT). 400 µl of the supernatant was mixed with 600 µl 1M Tris/HCl, pH 8.8 to determine the absorbance at 574 nm. A sample without the proteases was used as a blank. The following buffers were used:

50mM	Acetic acid	pH 4.0	50mM	Tris-Cl	pH 7.5
50mM	Acetic acid	pH 4.5	50mM	Tris-Cl	pH 8.0
50mM	Acetic acid	pH 5.0	50mM	Tris-Cl	pH 8.5
50mM	Propionic acid	pH 4.0	50mM	Tris-Cl	pH 9.0
50mM	Propionic acid	pH 4.5	50mM	Bicine	pH 8.0
50mM	Propionic acid	pH 5.0	50mM	Bicine	pH 8.5
50mM	Propionic acid	pH 5.5	50mM	Bicine	pH 9.0
50mM	MES	pH 5.5	50mM	Ethanolamina	pH 9.0
50mM	MES	pH 6.0	50mM	Ethanolamina	pH 95
50mM	MES	pH 6.5	50mM	Ethanolamina	pH 10.0
50mM	Bis-Tris	pH 6.0	50mM	Ethanolamina	pH 10.5
50mM	Bis-Tris	pH 6.5	50mM	Carbonate	pH 9.5
50mM	Bis-Tris	pH 7.0	50mM	CAPS	pH 10.0
50mM	HEPES	pH 7.0	50mM	CAPS	pH 10.5
50mM	HEPES	pH 7.5	50mM	CAPS	pH 11.0

The pH was adjusted at 37°C. All buffers were supplied with 150 mM NaCl.

3.7.3 Measurements of bacterial growth

5 ml of an unbuffered LB medium was inoculated with 50 µl of a glycerol stock of the *degP* null, *degQ* null and their parental strains. The cell density of the overnight cultures was determined by measurement of the optical density (OD) in a photometer (Ultrospec 3300 pro, Amersham Biosciences) at a wavelength of 600 nm. LB medium was used as a blank. Next, the overnight cultures were standardized to the same OD and the equal volumes were used to inoculate 100 ml of LB. In the case of *degQ* null strain kanamycin was added to a final concentration of 25 µg/ml. After 30 min of growth (37°C, 220 rpm) LB media were buffered by a direct addition of sterile filtered 10 ml 1 M HEPES pH 7.5 or 10 ml 0.5 MES pH 5.5. Growth was monitored by the OD measurement at 30 min time points until the stationary phase was reached.

4 Bibliography

Alba, B. M. and C. A. Gross (2004). "Regulation of the Escherichia coli sigma-dependent envelope stress response." Mol Microbiol **52**(3): 613-9.

Alba, B. M., J. A. Leeds, C. Onufryk, C. Z. Lu and C. A. Gross (2002). "DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response." Genes Dev **16**(16): 2156-68.

Anfinsen, C. B. (1973). "Principles That Govern Folding of Protein Chains." Science **181**(4096): 223-230.

Arie, J. P., N. Sassoon and J. M. Betton (2001). "Chaperone function of FkpA, a heat shock prolyl isomerase, in the periplasm of Escherichia coli." Mol Microbiol **39**(1): 199-210.

Banyard, S. H., D. K. Stammers and P. M. Harrison (1978). "Electron density map of apoferritin at 2.8-A resolution." Nature **271**(5642): 282-4.

Basle, A., G. Rummel, P. Storici, J. P. Rosenbusch and T. Schirmer (2006). "Crystal structure of osmoporin OmpC from E. coli at 2.0 A." J Mol Biol **362**(5): 933-42.

Bass, S., Q. M. Gu and A. Christen (1996). "Multicopy suppressors of prc mutant Escherichia coli include two HtrA (DegP) protease homologs (HhoAB), DksA, and a truncated RlpA." Journal of Bacteriology **178**(4): 1154-1161.

Bayer, M. E. (1968). "Areas of adhesion between wall and membrane of Escherichia coli." J Gen Microbiol **53**(3): 395-404.

Behrens, S., R. Maier, H. de Cock, F. X. Schmid and C. A. Gross (2001). "The SurA periplasmic PPIase lacking its parvulin domains functions in vivo and has chaperone activity." Embo J **20**(1-2): 285-94.

Ben-Zvi, A. P. and P. Goloubinoff (2001). "Review: Mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones." Journal of Structural Biology **135**(2): 84-93.

Berks, B. C., F. Sargent and T. Palmer (2000). "The Tat protein export pathway." Mol Microbiol **35**(2): 260-74.

Bernstein, H. D. (2000). "The biogenesis and assembly of bacterial membrane proteins." Curr Opin Microbiol **3**(2): 203-9.

Bitto, E. and D. B. McKay (2002). "Crystallographic structure of SurA, a molecular chaperone that facilitates folding of outer membrane porins." Structure **10**(11): 1489-98.

Bitto, E. and D. B. McKay (2003). "The periplasmic molecular chaperone protein SurA binds a peptide motif that is characteristic of integral outer membrane proteins." J Biol Chem **278**(49): 49316-22.

Bitto, E. and D. B. McKay (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): 94-8.

Blankenhorn, D., J. Phillips and J. L. Slonczewski (1999). "Acid- and base-induced proteins during aerobic and anaerobic growth of Escherichia coli revealed by two-dimensional gel electrophoresis." J Bacteriol **181**(7): 2209-16.

Bochtler, M., L. Ditzel, M. Groll and R. Huber (1997). "Crystal structure of heat shock locus V (HslV) from Escherichia coli." Proceedings of the National Academy of Sciences of the United States of America **94**(12): 6070-6074.

Booth, I. R. (1985). "Regulation of cytoplasmic pH in bacteria." Microbiol Rev **49**(4): 359-78.

Bos, M. P., V. Robert and J. Tommassen (2007). "Biogenesis of the gram-negative bacterial outer membrane." Annu Rev Microbiol **61**: 191-214.

Bothmann, H. and A. Pluckthun (2000). "The periplasmic Escherichia coli peptidylprolyl cis,trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines." J Biol Chem **275**(22): 17100-5.

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

Brass, J. M., C. F. Higgins, M. Foley, P. A. Rugman, J. Birmingham and P. B. Garland (1986). "Lateral Diffusion of Proteins in the Periplasm of Escherichia- Coli." Journal of Bacteriology **165**(3): 787-795.

Braun, M. and T. J. Silhavy (2002). "Imp/OstA is required for cell envelope biogenesis in Escherichia coli." Mol Microbiol **45**(5): 1289-302.

Braun, V. (1975). "Covalent lipoprotein from the outer membrane of Escherichia coli." Biochim Biophys Acta **415**(3): 335-77.

Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen and W. Wickner (1990). "The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation." Cell **62**(4): 649-57.

Brunger, A. T., P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson and G. L. Warren (1998). "Crystallography & NMR system: A new software suite for macromolecular structure determination." Acta Crystallographica Section D-Biological Crystallography **54**: 905-921.

Bulieris, P. V., S. Behrens, O. Holst and J. H. Kleinschmidt (2003). "Folding and insertion of the outer membrane protein OmpA is assisted by the chaperone Skp and by lipopolysaccharide." J Biol Chem **278**(11): 9092-9.

CastilloKeller, M. and R. Misra (2003). "Protease-deficient DegP suppresses lethal effects of a mutant OmpC protein by its capture." J Bacteriol **185**(1): 148-54.

Chassin, Y., E. Kapri-Pardes, G. Sinvany, T. Arad and Z. Adam (2002). "Expression and characterization of the thylakoid lumen protease DegP1 from Arabidopsis." Plant Physiol **130**(2): 857-64.

Chen, J., J. L. Song, S. Zhang, Y. Wang, D. F. Cui and C. C. Wang (1999). "Chaperone activity of DsbC." J Biol Chem **274**(28): 19601-5.

Chen, R. and U. Henning (1996). "A periplasmic protein (Skp) of Escherichia coli selectively binds a class of outer membrane proteins." Molecular Microbiology **19**(6): 1287-1294.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins and J. D. Thompson (2003). "Multiple sequence alignment with the Clustal series of programs." Nucleic Acids Research **31(13)**: 3497–3500.

Clantin, B., A. S. Delattre, P. Rucktooa, N. Saint, A. C. Meli, C. Locht, F. Jacob-Dubuisson and V. Villeret (2007). "Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily." Science **317**(5840): 957-61.

Clausen, T., C. Southan and M. Ehrmann (2002). "The HtrA family of proteases: implications for protein composition and cell fate." Mol. Cell **10**(3): 443-55.

Collinet, B., H. Yuzawa, T. Chen, C. Herrera and D. Missiakas (2000). "RseB binding to the periplasmic domain of RseA modulates the RseA:sigmaE interaction in the cytoplasm and the availability of sigmaE.RNA polymerase." J Biol Chem **275**(43): 33898-904.

Cortes, G., B. de Astorza, V. J. Benedi and S. Alberti (2002). "Role of the htrA gene in Klebsiella pneumoniae virulence." Infect Immun **70**(9): 4772-6.

Dalbey, R. E. (1991). "Leader peptidase." Mol Microbiol **5**(12): 2855-60.

Dalbey, R. E. and W. Wickner (1985). "Leader peptidase catalyzes the release of exported proteins from the outer surface of the Escherichia coli plasma membrane." J Biol Chem **260**(29): 15925-31.

Danese, P. N. and T. J. Silhavy (1998). "Targeting and assembly of periplasmic and outer-membrane proteins in Escherichia coli." Annu Rev Genet **32**: 59-94.

Danese, P. N., W. B. Snyder, C. L. Cosma, L. J. B. Davis and T. J. Silhavy (1995). "The Cpx 2-Component Signal-Transduction Pathway of Escherichia- Coli Regulates Transcription of the Gene Specifying the Stress- Inducible Periplasmic Protease, Degp." Genes & Development **9**(4): 387-398.

Dartigalongue, C. and S. Raina (1998). "A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in Escherichia coli." Embo Journal **17**(14): 3968-3980.

de Cock, H., R. Hendriks, T. Devrije and J. Tommassen (1990). "Assembly of an Invitro Synthesized Escherichia-Coli Outer-Membrane Porin into Its Stable Trimeric Configuration." Journal of Biological Chemistry **265**(8): 4646-4651.

de Cock, H., U. Schafer, M. Potgeter, R. Demel, M. Muller and J. Tommassen (1999). "Affinity of the periplasmic chaperone Skp of Escherichia coli for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Skp in the biogenesis of outer membrane protein." Eur J Biochem **259**(1-2): 96-103.

de Jonge, R., K. Takumi, W. S. Ritmeester and F. M. van Leusden (2003). "The adaptive response of Escherichia coli O157 in an environment with changing pH." J Appl Microbiol **94**(4): 555-60.

de Las Penas, A., L. Connolly and C. A. Gross (1997). "The sigmaE-mediated response to extracytoplasmic stress in Escherichia coli is transduced by RseA and RseB, two negative regulators of sigmaE." Mol Microbiol **24**(2): 373-85.

de la Fortelle, E. and G. Bricogne (1997). Maximum-likelihood heavy-atom parameter refinement for multiple isomorphous replacement and multiwavelength anomalous diffraction methods. Macromolecular Crystallography, Pt a. **276:** 472-494.

DeLano, W. L. (2002). The PyMol Molecular Graphics System. Palo Alto, CA, DeLano Scientific.

Deuerling, E. and B. Bukau (2004). "Chaperone-assisted folding of newly synthesized proteins in the cytosol." Crit Rev Biochem Mol Biol **39**(5-6): 261-77.

Dobson, C. M. (2004). "Principles of protein folding, misfolding and aggregation." Semin Cell Dev Biol **15**(1): 3-16.

Doerrler, W. T., H. S. Gibbons and C. R. Raetz (2004). "MsbA-dependent translocation of lipids across the inner membrane of Escherichia coli." J Biol Chem **279**(43): 45102-9.

Doerrler, W. T., M. C. Reedy and C. R. Raetz (2001). "An Escherichia coli mutant defective in lipid export." J Biol Chem **276**(15): 11461-4.

Dougan, D. A., A. Mogk and B. Bukau (2002). "Protein folding and degradation in bacteria: to degrade or not to degrade? That is the question." Cell Mol Life Sci **59**(10): 1607-16.

Doyle, D. A., A. Lee, J. Lewis, E. Kim, M. Sheng and R. MacKinnon (1996). "Crystal structures of a complexed and peptide-free membrane protein-binding domain: Molecular basis of peptide recognition by PDZ." Cell **85**(7): 1067-1076.

Dressman, J. B., R. R. Berardi, L. C. Dermentzoglou, T. L. Russell, S. P. Schmaltz, J. L. Barnett and K. M. Jarvenpaa (1990). "Upper gastrointestinal (GI) pH in young, healthy men and women." Pharm Res **7**(7): 756-61.

Driessen, A. J. (2001). "SecB, a molecular chaperone with two faces." Trends Microbiol **9**(5): 193-6.

Driessen, A. J. and N. Nouwen (2007). "Protein Translocation Across the Bacterial Cytoplasmic Membrane." Annu Rev Biochem.

Dubochet, J., A. W. McDowall, B. Menge, E. N. Schmid and K. G. Lickfeld (1983). "Electron microscopy of frozen-hydrated bacteria." J Bacteriol **155**(1): 381-90.

Duguay, A. R. and T. J. Silhavy (2004). "Quality control in the bacterial periplasm." Biochim Biophys Acta **1694**(1-3): 121-34.

Erickson, J. W. and C. A. Gross (1989). "Identification of the sigma E subunit of Escherichia coli RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression." Genes Dev **3**(9): 1462-71.

Evans, D. F., G. Pye, R. Bramley, A. G. Clark, T. J. Dyson and J. D. Hardcastle (1988). "Measurement of gastrointestinal pH profiles in normal ambulant human subjects." Gut **29**(8): 1035-41.

Ewalt, K. L., J. P. Hendrick, W. A. Houry and F. U. Hartl (1997). "In vivo observation of polypeptide flux through the bacterial chaperonin system." Cell **90**(3): 491-500.

Farn, J. and M. Roberts (2004). "Effect of inactivation of the HtrA-like serine protease DegQ on the virulence of Salmonella enterica serovar Typhimurium in mice." Infect Immun **72**(12): 7357-9.

Fayet, O., T. Ziegelhoffer and C. Georgopoulos (1989). "The groES and groEL heat shock gene products of Escherichia coli are essential for bacterial growth at all temperatures." J Bacteriol **171**(3): 1379-85.

Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs and W. Welte (1998). "Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide." Science **282**(5397): 2215-20.

Ferguson, J. S. (2007). Electron transport activities in the periplasm. The periplasm. M. Ehrmann. Washington DC, ASM Press**:** 235-246.

Flynn, J. M., I. Levchenko, R. T. Sauer and T. A. Baker (2004). "Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation." Genes Dev **18**(18): 2292-301.

Foster, J. W. (2004). "Escherichia coli acid resistance: tales of an amateur acidophile." Nat Rev Microbiol **2**(11): 898-907.

Frank, J., M. Radermacher, P. Penczek, J. Zhu, Y. Li, M. Ladjadj and A. Leith (1996). "SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields." J Struct Biol **116**(1): 190-9.

Gardel, C., K. Johnson, A. Jacq and J. Beckwith (1990). "The secD locus of E. coli codes for two membrane proteins required for protein export." Embo J **9**(12): 4205-6.

Gouet, P., E. Courcelle, D. I. Stuart and F. Metoz (1999). "ESPript: analysis of multiple sequence alignments in PostScript." Bioinformatics **15(4)**: 305-308.

Grau, S., P. J. Richards, B. Kerr, C. Hughes, B. Caterson, A. S. Williams, U. Junker, S. A. Jones, T. Clausen and M. Ehrmann (2006). "The role of human HtrA1 in arthritic disease." J Biol Chem **281**(10): 6124-9.

Gray, C. W., R. V. Ward, E. Karran, S. Turconi, A. Rowles, D. Viglienghi, C. Southan, A. Barton, K. G. Fantom, A. West, J. Savopoulos, N. J. Hassan, H. Clinkenbeard, C. Hanning, B. Amegadzie, J. B. Davis, C. Dingwall, G. P. Livi and C. L. Creasy (2000). "Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response." European Journal of Biochemistry **267**(18): 5699-5710.

Groll, M., L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H. D. Bartunik and R. Huber (1997). "Structure of 20S proteasome from yeast at 2.4 angstrom resolution." Nature **386**(6624): 463-471.

Guisbert, E., V. A. Rhodius, N. Ahuja, E. Witkin and C. A. Gross (2007). "Hfq modulates the sigmaE-mediated envelope stress response and the sigma32-mediated cytoplasmic stress response in Escherichia coli." J Bacteriol **189**(5): 1963-73.

Hanson, P. I. and S. W. Whiteheart (2005). "AAA+ proteins: have engine, will work." Nat Rev Mol Cell Biol **6**(7): 519-29.

Harris, B. Z. and W. A. Lim (2001). "Mechanism and role of PDZ domains in signaling complex assembly." Journal of Cell Science **114**(18): 3219-3231.

Hasselblatt, H., R. Kurzbauer, C. Wilken, T. Krojer, J. Sawa, J. Kurt, R. Kirk, S. Hasenbein, M. Ehrmann and T. Clausen (2007). "Regulation of the {sigma}E 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): 2659-70.

Hauser, M., H. Eichelmann, V. Oja, U. Heber and A. Laisk (1995). "Stimulation by Light of Rapid pH Regulation in the Chloroplast Stroma in Vivo as Indicated by CO2 Solubilization in Leaves." Plant Physiol **108**(3): 1059-1066.

Haussuhl, K., B. Andersson and I. Adamska (2001). "A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II." Embo Journal **20**(4): 713-722.

Hayes, E. T., J. C. Wilks, P. Sanfilippo, E. Yohannes, D. P. Tate, B. D. Jones, M. D. Radmacher, S. S. BonDurant and J. L. Slonczewski (2006). "Oxygen limitation modulates pH regulation of catabolism and hydrogenases, multidrug transporters, and envelope composition in Escherichia coli K-12." BMC Microbiol **6**: 89.

Holmgren, A., M. J. Kuehn, C. I. Branden and S. J. Hultgren (1992). "Conserved Immunoglobulin-Like Features in a Family of Periplasmic Pilus Chaperones in Bacteria." Embo Journal **11**(4): 1617-1622.

Houry, W. A. (2001). "Chaperone-assisted protein folding in the cell cytoplasm." Curr Protein Pept Sci **2**(3): 227-44.

Houry, W. A., D. Frishman, C. Eckerskorn, F. Lottspeich and F. U. Hartl (1999). "Identification of in vivo substrates of the chaperonin GroEL." Nature **402**(6758): 147-54.

Huesgen, P. F., P. Scholz and I. Adamska (2007). "The serine protease HhoA from Synechocystis sp. strain PCC 6803: substrate specificity and formation of a hexameric complex are regulated by the PDZ domain." J Bacteriol **189**(18): 6611-8.

Huesgen, P. F., H. Schumann and I. Adamska (2005). "The family of Deg proteases in cyanobacteria and chloroplasts of higher plants." Physiologia Plantarum **123**: 413-420.

Hung, D. L., S. D. Knight, R. M. Woods, J. S. Pinkner and S. J. Hultgren (1996). "Molecular basis of two subfamilies of immunoglobulin-like chaperones." Embo J **15**(15): 3792-805.

Itzhaki, H., L. Naveh, M. Lindahl, M. Cook and Z. Adam (1998). "Identification and characterization of DegP, a serine protease associated with the luminal side of the thylakoid membrane." Journal of Biological Chemistry **273**(12): 7094-7098.

Iwanczyk, J., D. Damjanovic, J. Kooistra, V. Leong, A. Jomaa, R. Ghirlando and J. Ortega (2007). "Role of the PDZ domains in Escherichia coli DegP protein." J Bacteriol **189**(8): 3176-86.

Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller and C. Hormaeche (1991). "The role of a stress-response protein in Salmonella typhimurium virulence." Mol Microbiol **5**(2): 401-7.

Jomaa, A., D. Damjanovic, V. Leong, R. Ghirlando, J. Iwanczyk and J. Ortega (2007). "The inner cavity of Escherichia coli DegP protein is not essential for molecular chaperone and proteolytic activity." J Bacteriol **189**(3): 706-16.

Jones, C. H., T. C. Bolken, K. F. Jones, G. O. Zeller and D. E. Hruby (2001). "Conserved DegP protease in gram-positive bacteria is essential for thermal and oxidative tolerance and full virulence in Streptococcus pyogenes." Infect Immun **69**(9): 5538-45.

Jones, T. A., J. Y. Zou, S. W. Cowan and M. Kjeldgaard (1991). "Improved Methods For Building Protein Models in Electron- Density Maps and the Location of Errors in These Models." Acta Crystallographica Section a **47**: 110-119.

Kabsch, W. (1993). "Automatic Processing of Rotation Diffraction Data from Crystals of Initially Unknown Symmetry and Cell Constants." Journal of Applied Crystallography **26**: 795-800.

Kanehara, K., K. Ito and Y. Akiyama (2002). "YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA." Genes Dev **16**(16): 2147-55.

Kim, D. Y., D. R. Kim, S. C. Ha, N. K. Lokanath, C. J. Lee, H. Y. Hwang and K. K. Kim (2003). "Crystal structure of the protease domain of a heat-shock protein HtrA from Thermotoga maritima." Journal of Biological Chemistry **278**(8): 6543-6551.

Kim, D. Y. and K. K. Kim (2005). "Structure and function of HtrA family proteins, the key players in protein quality control." J Biochem Mol Biol **38**(3): 266-74.

Kim, D. Y., E. Kwon, Y. K. Shin, D. H. Kweon and K. K. Kim (2008). "The mechanism of temperature-induced bacterial HtrA activation." J Mol Biol **377**(2): 410-20.

Kim, S., J. C. Malinverni, P. Sliz, T. J. Silhavy, S. C. Harrison and D. Kahne (2007). "Structure and function of an essential component of the outer membrane protein assembly machine." Science **317**(5840): 961-4.

Kleinschmidt, J. H. (2003). "Membrane protein folding on the example of outer membrane protein A of Escherichia coli." Cell Mol Life Sci **60**(8): 1547-58.

Kleinschmidt, J. H. (2007). Assembly of integral membrane proteins from the periplasm into the outer membrane. The periplasm. M. Ehrmann. Washington DC, ASM Press**:** 30-66.

Klose, M., A. Storiko, Y. D. Stierhof, I. Hindennach, B. Mutschler and U. Henning (1993). "Membrane Assembly of the Outer-Membrane Protein Ompa of Escherichia-Coli." Journal of Biological Chemistry **268**(34): 25664-25670.

Kloser, A., M. Laird, M. Deng and R. Misra (1998). "Modulations in lipid A and phospholipid biosynthesis pathways influence outer membrane protein assembly in Escherichia coli K-12." Mol Microbiol **27**(5): 1003-8.

Koebnik, R., K. P. Locher and P. Van Gelder (2000). "Structure and function of bacterial outer membrane proteins: barrels in a nutshell." Mol Microbiol **37**(2): 239-53.

Kolmar, H., P. R. Waller and R. T. Sauer (1996). "The DegP and DegQ periplasmic endoproteases of Escherichia coli: specificity for cleavage sites and substrate conformation." J Bacteriol **178**(20): 5925-9.

Koplow, J. and H. Goldfine (1974). "Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of Escherichia coli." J Bacteriol **117**(2): 527-43.

Korndorfer, I. P., M. K. Dommel and A. Skerra (2004). "Structure of the periplasmic chaperone Skp suggests functional similarity with cytosolic chaperones despite differing architecture." Nat Struct Mol Biol **11**(10): 1015-20.

Krojer, T., M. Garrido-Franco, R. Huber, M. Ehrmann and T. Clausen (2002). "Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine." Nature **416**(6879): 455-9.

Laemmli, U. K. (1970). "Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4." Nature **227**(5259): 680-&.

Laird, M. W., A. W. Kloser and R. Misra (1994). "Assembly of LamB and OmpF in deep rough lipopolysaccharide mutants of Escherichia coli K-12." J Bacteriol **176**(8): 2259-64.

Laskowski, R. A., M. W. Macarthur, D. S. Moss and J. M. Thornton (1993). "Procheck - a Program to Check the Stereochemical Quality of Protein Structures." Journal of Applied Crystallography **26**: 283-291.

Lazar, S. W. and R. Kolter (1996). "SurA assists the folding of Escherichia coli outer membrane proteins." J Bacteriol **178**(6): 1770-3.

Leduc, M., C. Frehel and J. van Heijenoort (1985). "Correlation between degradation and ultrastructure of peptidoglycan during autolysis of Escherichia coli." J Bacteriol **161**(2): 627-35.

Lee, G. J., A. M. Roseman, H. R. Saibil and E. Vierling (1997). "A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state." Embo J **16**(3): 659-71.

Levitt, M. (1981). "Effect of proline residues on protein folding." J Mol Biol **145**(1): 251-63.

Li, W., S. M. Srinivasula, J. Chai, P. Li, J. W. Wu, Z. Zhang, E. S. Alnemri and Y. Shi (2002). "Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi." Nat Struct Biol **9**(6): 436-41.

Liberek, K., A. Lewandowska and S. Zietkiewicz (2008). "Chaperones in control of protein disaggregation." Embo J **27**(2): 328-35.

Lipinska, B., O. Fayet, L. Baird and C. Georgopoulos (1989). "Identification, characterization, and mapping of the Escherichia coli Htra gene, whose product is essential for bacterial growth only at elevated temperatures." Journal of Bacteriology **171**(3): 1574-1584.

Lipinska, B., M. Zylicz and C. Georgopoulos (1990). "The Htra (Degp) Protein, Essential For Escherichia-Coli Survival At High-Temperatures, Is an Endopeptidase." Journal of Bacteriology **172**(4): 1791-1797.

Liu, J. and C. T. Walsh (1990). "Peptidyl Prolyl Cis-Trans-Isomerase From Escherichia-Coli - a Periplasmic Homolog of Cyclophilin That Is Not Inhibited By Cyclosporine-a." Proceedings of the National Academy of Sciences of the United States of America **87**(11): 4028-4032.

Ludtke, S. J., P. R. Baldwin and W. Chiu (1999). "EMAN: semiautomated software for high-resolution single-particle reconstructions." J Struct Biol **128**(1): 82-97.

Macario, A. J. and E. Conway de Macario (2005). "Sick chaperones, cellular stress, and disease." N Engl J Med **353**(14): 1489-501.

Marsh, D., B. Shanmugavadivu and J. H. Kleinschmidt (2006). "Membrane elastic fluctuations and the insertion and tilt of beta-barrel proteins." Biophys J **91**(1): 227-32.

Matsuyama, S., K. Inokuchi and S. Mizushima (1984). "Promoter Exchange between Ompf and Ompc, Genes for Osmoregulated Major Outer-Membrane Proteins of Escherichia-Coli K-12." Journal of Bacteriology **158**(3): 1041-1047.

Matsuyama, S., T. Tajima and H. Tokuda (1995). "A Novel Periplasmic Carrier Protein Involved in the Sorting and Transport of Escherichia-Coli Lipoproteins Destined For the Outer-Membrane." Embo Journal **14**(14): 3365-3372.

Matsuyama, S., N. Yokota and H. Tokuda (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 Journal **16**(23): 6947-6955.

Maurer, L. M., E. Yohannes, S. S. Bondurant, M. Radmacher and J. L. Slonczewski (2005). "pH regulates genes for flagellar motility, catabolism, and oxidative stress in Escherichia coli K-12." J Bacteriol **187**(1): 304-19.

Meltzer, M., S. Hasenbein, P. Hauske, N. Kucz, M. Merdanovic, S. Grau, D. Jones, T. Krojer, T. Clausen, M. Ehrmann and M. Kaiser (2007). "Allosteric activation of HtrA protease DegP by stress signals in bacterial protein quality control." Angew. Chem. **in press**.

Miot, M. and J. M. Betton (2004). "Protein quality control in the bacterial periplasm." Microb Cell Fact **3**(1): 4.

Misra, R., M. CastilloKeller and M. Deng (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): 4882-8.

Misra, R., A. Peterson, T. Ferenci and T. J. Silhavy (1991). "A genetic approach for analyzing the pathway of LamB assembly into the outer membrane of Escherichia coli." J Biol Chem **266**(21): 13592-7.

Missiakas, D., J. M. Betton and S. Raina (1996). "New components of protein folding in extracytoplasmic compartments of Escherichia coli SurA, FkpA and Skp/OmpH." Mol Microbiol **21**(4): 871-84.

Missiakas, D., M. P. Mayer, M. Lemaire, C. Georgopoulos and S. Raina (1997). "Modulation of the Escherichia coli sigma(E) (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins." Molecular Microbiology **24**(2): 355-371.

Mo, E., S. E. Peters, C. Willers, D. J. Maskell and I. G. Charles (2006). "Single, double and triple mutants of Salmonella enterica serovar Typhimurium degP (htrA), degQ (hhoA) and degS (hhoB) have diverse phenotypes on exposure to elevated temperature and their growth in vivo is attenuated to different extents." Microb Pathog **41**(4-5): 174-82.

Mogensen, J. E. and D. E. Otzen (2005). "Interactions between folding factors and bacterial outer membrane proteins." Mol Microbiol **57**(2): 326-46.

Mortier, E., G. Wuytens, I. Leenaerts, F. Hannes, M. Y. Heung, G. Degeest, G. David and P. Zimmermann (2005). "Nuclear speckles and nucleoli targeting by PIP2-PDZ domain interactions." Embo J **24**(14): 2556-65.

Muhlradt, P. F. and J. R. Golecki (1975). "Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium." Eur J Biochem **51**(2): 343-52.

Nakae, T., J. Ishii and M. Tokunaga (1979). "Subunit Structure of Functional Porin Oligomers That Form Permeability Channels in the Outer Membrane of Escherichia-Coli." Journal of Biological Chemistry **254**(5): 1457-1461.

Nakamoto, H. and J. C. Bardwell (2004). "Catalysis of disulfide bond formation and isomerization in the Escherichia coli periplasm." Biochim Biophys Acta **1694**(1-3): 111-9.

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

Oliver, D. (1996). Periplasm. Escherichia and Salmonella Cellular and Molecular Biology. F. C. Neidhardt, Washington, D.C., ASM Press.

Oschkinat, H. (1999). "A new type of PDZ domain recognition." Nature Structural Biology **6**(5): 408-410.

Pallen, M. J. and B. W. Wren (1997). "The HtrA family of serine proteases." Molecular Microbiology **26**(2): 209-221.

Palmer, T. and B. C. Berks (2003). "Moving folded proteins across the bacterial cell membrane." Microbiology **149**(Pt 3): 547-56.

Pan, L., H. Wu, C. Shen, Y. Shi, W. Jin, J. Xia and M. Zhang (2007). "Clustering and synaptic targeting of PICK1 requires direct interaction between the PDZ domain and lipid membranes." Embo J **26**(21): 4576-87.

Park, S. H., G. P. Liu, T. B. Topping, W. H. Cover and L. L. Randall (1988). "Modulation of Folding Pathways of Exported Proteins by the Leader Sequence." Science **239**(4843): 1033-1035.

Pautsch, A. and G. E. Schulz (1998). "Structure of the outer membrane protein A transmembrane domain." Nat Struct Biol **5**(11): 1013-7.

Penczek, P., M. Radermacher and J. Frank (1992). "Three-dimensional reconstruction of single particles embedded in ice." Ultramicroscopy **40**(1): 33-53.

Perona, J. J. and C. S. Craik (1995). "Structural basis of substrate specificity in the serine proteases." Protein Sci **4**(3): 337-60.

Pettersen, E. F., T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng and T. E. Ferrin (2004). "UCSF Chimera--a visualization system for exploratory research and analysis." J Comput Chem **25**(13): 1605-12.

Pfundel, E. E., M. Renganathan, A. M. Gilmore, H. Y. Yamamoto and R. A. Dilley (1994). "Intrathylakoid pH in Isolated Pea Chloroplasts as Probed by Violaxanthin Deepoxidation." Plant Physiol **106**(4): 1647-1658.

Pickart, C. M. and R. E. Cohen (2004). "Proteasomes and their kin: proteases in the machine age." Nat Rev Mol Cell Biol **5**(3): 177-87.

Plun-Favreau, H., K. Klupsch, N. Moisoi, S. Gandhi, S. Kjaer, D. Frith, K. Harvey, E. Deas, R. J. Harvey, N. McDonald, N. W. Wood, L. M. Martins and J. Downward (2007). "The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1." Nat Cell Biol **9**(11): 1243-52.

Pugsley, A. P. (1993). "The complete general secretory pathway in gram-negative bacteria." Microbiol Rev **57**(1): 50-108.

Purdy, G. E., C. R. Fisher and S. M. Payne (2007). "IcsA surface presentation in Shigella flexneri requires the periplasmic chaperones DegP, Skp, and SurA." J Bacteriol **189**(15): 5566-73.

Raetz, C. R. and C. Whitfield (2002). "Lipopolysaccharide endotoxins." Annu Rev Biochem **71**: 635-700.

Raivio, T. L. and T. J. Silhavy (1999). "The sigma(E) and Cpx regulatory pathways: overlapping but distinct envelope stress responses." Current Opinion in Microbiology **2**(2): 159-165.

Raivio, T. L. and T. J. Silhavy (2001). "Periplasmic stress and ECF sigma factors." Annual Review of Microbiology **55**: 591-624.

Ramachandran, R., C. Hartmann, H. K. Song, R. Huber and M. Bochtler (2002). "Functional interactions of HslV (ClpQ) with the ATPase HslU (ClpY)." Proceedings of the National Academy of Sciences of the United States of America **99**(11): 7396-7401.

Ramm, K. and A. Pluckthun (2000). "The periplasmic Escherichia coli peptidylprolyl cis,trans- isomerase FkpA - II. Isomerase-independent chaperone activity in vitro." Journal of Biological Chemistry **275**(22): 17106-17113.

Randall, L. L. (1983). "Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is independent of elongation." Cell **33**(1): 231-40.

Ranson, N. A., H. E. White and H. R. Saibil (1998). "Chaperonins." Biochem J **333 (Pt 2)**: 233-42.

Rhodius, V. A., W. C. Suh, G. Nonaka, J. West and C. A. Gross (2006). "Conserved and variable functions of the sigmaE stress response in related genomes." PLoS Biol **4**(1): e2.

Rietsch, A., D. Belin, N. Martin and J. Beckwith (1996). "An in vivo pathway for disulfide bond isomerization in Escherichia coli." Proc Natl Acad Sci U S A **93**(23): 13048-53.

Rizzitello, A. E., J. R. Harper and T. J. Silhavy (2001). "Genetic evidence for parallel pathways of chaperone activity in the periplasm of Escherichia coli." J Bacteriol **183**(23): 6794-800.

Robert, V., E. B. Volokhina, F. Senf, M. P. Bos, P. Van Gelder and J. Tommassen (2006). "Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif." PLoS Biol **4**(11): e377.

Rosen, B. P. (1987). Washington D.C., American Society for Microbiology.

Rosen, R. and E. Z. Ron (2002). "Proteome analysis in the study of the bacterial heat-shock response." Mass Spectrom Rev **21**(4): 244-65.

Rouviere, P. E. and C. A. Gross (1996). "SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins." Genes & Development **10**(24): 3170-3182.

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

Ruiz, N. and T. J. Silhavy (2005). "Sensing external stress: watchdogs of the Escherichia coli cell envelope." Curr Opin Microbiol **8**(2): 122-6.

Sargent, F. (2007). "The twin-arginine transport system: moving folded proteins across membranes." Biochem Soc Trans **35**(Pt 5): 835-47.

Sassoon, N., J. P. Arie and J. M. Betton (1999). "PDZ domains determine the native oligomeric structure of the DegP (HtrA) protease." Molecular Microbiology **33**(3): 583-589.

Schneider, C. and F. U. Hartl (1996). "Cell biology - Hats off to the tricorn protease." Science **274**(5291): 1323-1324.

Schulz, G. E. (2002). "The structure of bacterial outer membrane proteins." Biochim Biophys Acta **1565**(2): 308-17.

Schweizer, M., I. Hindennach, W. Garten and U. Henning (1978). "Major proteins of the Escherichia coli outer cell envelope membrane. Interaction of protein II with lipopolysaccharide." Eur J Biochem **82**(1): 211-7.

Selkoe, D. J. (2003). "Folding proteins in fatal ways." Nature **426**(6968): 900-4.

Sen, K. and H. Nikaido (1990). "In vitro trimerization of OmpF porin secreted by spheroplasts of Escherichia coli." Proc Natl Acad Sci U S A **87**(2): 743-7.

Sen, K. and H. Nikaido (1991). "Lipopolysaccharide structure required for in vitro trimerization of Escherichia coli OmpF porin." J Bacteriol **173**(2): 926-8.

Sen, K. and H. Nikaido (1991). "Trimerization of an in vitro synthesized OmpF porin of Escherichia coli outer membrane." J Biol Chem **266**(17): 11295-300.

Shao, F., M. W. Bader, U. Jakob and J. C. Bardwell (2000). "DsbG, a protein disulfide isomerase with chaperone activity." J Biol Chem **275**(18): 13349-52.

Siegert, R., M. R. Leroux, C. Scheufler, F. U. Hartl and I. Moarefi (2000). "Structure of the molecular chaperone prefoldin: unique interaction of multiple coiled coil tentacles with unfolded proteins." Cell **103**(4): 621-32.

Sklar, J. G., T. Wu, L. S. Gronenberg, J. C. Malinverni, D. Kahne and T. J. Silhavy (2007). "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): 6400-5.

Sklar, J. G., T. Wu, D. Kahne and T. J. Silhavy (2007). "Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli." Genes Dev **21**(19): 2473-84.

Skorko-Glonek, J., E. Laskowska, A. Sobiecka-Szkatula and B. Lipinska (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): 80-9.

Smit, J., Y. Kamio and H. Nikaido (1975). "Outer membrane of Salmonella typhimurium: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants." J Bacteriol **124**(2): 942-58.

Sousa, M. C., C. B. Trame, H. Tsuruta, S. M. Wilbanks, V. S. Reddy and D. B. McKay (2000). "Crystal and solution structures of an HslUV protease-chaperone complex." Cell **103**(4): 633-43.

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

Stancik, L. M., D. M. Stancik, B. Schmidt, D. M. Barnhart, Y. N. Yoncheva and J. L. Slonczewski (2002). "pH-dependent expression of periplasmic proteins and amino acid catabolism in Escherichia coli." J Bacteriol **184**(15): 4246-58.

Steeghs, L., H. de Cock, E. Evers, B. Zomer, J. Tommassen and P. van der Ley (2001). "Outer membrane composition of a lipopolysaccharide-deficient Neisseria meningitidis mutant." Embo J **20**(24): 6937-45.

Steeghs, L., R. den Hartog, A. den Boer, B. Zomer, P. Roholl and P. van der Ley (1998). "Meningitis bacterium is viable without endotoxin." Nature **392**(6675): 449-50.

Strauch, K. L., K. Johnson and J. Beckwith (1989). "Characterization of Degp, a Gene Required For Proteolysis in the Cell-Envelope and Essential For Growth of Escherichia-Coli At High-Temperature." Journal of Bacteriology **171**(5): 2689-2696.

Surrey, T. and F. Jahnig (1992). "Refolding and oriented insertion of a membrane protein into a lipid bilayer." Proc Natl Acad Sci U S A **89**(16): 7457-61.

Surrey, T., A. Schmid and F. Jahnig (1996). "Folding and membrane insertion of the trimeric beta-barrel protein OmpF." Biochemistry **35**(7): 2283-8.

Swamy, K. H. S., H. C. Chin and A. L. Goldberg (1983). "Isolation and Characterization of Protease Do From Escherichia- Coli, a Large Serine Protease Containing Multiple Subunits." Archives of Biochemistry and Biophysics **224**(2): 543-554.

Tomkiewicz, D., N. Nouwen and A. J. Driessen (2007). "Pushing, pulling and trapping--modes of motor protein supported protein translocation." FEBS Lett **581**(15): 2820-8.

Tommassen, J. (2007). "Biochemistry. Getting into and through the outer membrane." Science **317**(5840): 903-4.

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

van Heel, M. (1987). "Angular reconstitution: a posteriori assignment of projection directions for 3D reconstruction." Ultramicroscopy **21**(2): 111-23.

van Heel, M., G. Harauz, E. V. Orlova, R. Schmidt and M. Schatz (1996). "A new generation of the IMAGIC image processing system." J Struct Biol **116**(1): 17-24.

van Wielink, J. E. and J. A. Duine (1990). "How Big Is the Periplasmic Space." Trends in Biochemical Sciences **15**(4): 136-137.

Vande Walle, L., M. Lamkanfi and P. Vandenabeele (2008). "The mitochondrial serine protease HtrA2/Omi: an overview." Cell Death Differ **15**(3): 453-60.

Veenendaal, A. K., C. van der Does and A. J. Driessen (2004). "The protein-conducting channel SecYEG." Biochim Biophys Acta **1694**(1-3): 81-95.

Vollmer, W. (2007). Structure and biosythesis of the murein (peptidoglycan) sacculus. Periplasm. M. Ehrmann. Washington DC, ASM Press**:** 198-213.

Vollmer, W. and J. V. Holtje (2004). "The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer(s)?" J Bacteriol **186**(18): 5978-87.

von Heijne, G. (1990). "Imports, exports, and quality control. Intracellular Routing of Protein Molecules, sponsored by the Karolinska Institute, Center for Biotechnology, Huddinge, Sweden, May 18-19, 1990." New Biol **2**(7): 609-11.

Waller, P. R. and R. T. Sauer (1996). "Characterization of degQ and degS, Escherichia coli genes encoding homologs of the DegP protease." J Bacteriol **178**(4): 1146-53.

Walsh, N. P., B. M. Alba, B. Bose, C. A. Gross and R. T. Sauer (2003). "OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain." Cell **113**(1): 61-71.

Walton, T. A. and M. C. Sousa (2004). "Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation." Mol Cell **15**(3): 367-74.

Wang, J., J. A. Hartling and J. M. Flanagan (1997). "The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis." Cell **91**(4): 447-56.

Weeks, C. M. and R. Miller (1999). "The design and implementation of SnB version 2.0." Journal of Applied Crystallography **32**: 120-124.

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

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

Wilks, J. C. and J. L. Slonczewski (2007). "pH of the cytoplasm and periplasm of Escherichia coli: rapid measurement by green fluorescent protein fluorimetry." J Bacteriol **189**(15): 5601-7.

Winkler, F. K., C. E. Schutt, S. C. Harrison and G. Bricogne (1977). "Tomato bushy stunt virus at 5.5-A resolution." Nature **265**(5594): 509-13.

Wootton, J. C. and M. H. Drummond (1989). "The Q-Linker - a Class of Interdomain Sequences Found in Bacterial Multidomain Regulatory Proteins." Protein Engineering **2**(7): 535-543.

Wu, H., W. Feng, J. Chen, L. N. Chan, S. Y. Huang and M. J. Zhang (2007). "PDZ domains of Par-3 as potential phosphoinositide signaling integrators." Molecular Cell **28**(5): 886-898.

Wu, T., J. Malinverni, N. Ruiz, S. Kim, T. J. Silhavy and D. Kahne (2005). "Identification of a multicomponent complex required for outer membrane biogenesis in Escherichia coli." Cell **121**(2): 235-45.

Wulfing, C. and A. Pluckthun (1994). "Protein folding in the periplasm of Escherichia coli." Mol Microbiol **12**(5): 685-92.

Xu, Z., A. L. Horwich and P. B. Sigler (1997). "The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex." Nature **388**(6644): 741-50.

Xu, Z., J. D. Knafels and K. Yoshino (2000). "Crystal structure of the bacterial protein export chaperone secB." Nat Struct Biol **7**(12): 1172-7.

Yan, J., W. Wen, W. Xu, J. F. Long, M. E. Adams, S. C. Froehner and M. Zhang (2005). "Structure of the split PH domain and distinct lipid-binding properties of the PH-PDZ supramodule of alpha-syntrophin." Embo J **24**(23): 3985-95.

Yohannes, E., D. M. Barnhart and J. L. Slonczewski (2004). "pH-dependent catabolic protein expression during anaerobic growth of Escherichia coli K-12." J Bacteriol **186**(1): 192-9.

Young, J. C., V. R. Agashe, K. Siegers and F. U. Hartl (2004). "Pathways of chaperone-mediated protein folding in the cytosol." Nat Rev Mol Cell Biol **5**(10): 781-91.

Zimmerman, S. B. and A. P. Minton (1993). "Macromolecular crowding: biochemical, biophysical, and physiological consequences." Annu Rev Biophys Biomol Struct **22**: 27-65.

Zimmermann, P., K. Meerschaert, G. Reekmans, I. Leenaerts, J. V. Small, J. Vandekerckhove, G. David and J. Gettemans (2002). "PIP(2)-PDZ domain binding controls the association of syntenin with the plasma membrane." Mol Cell **9**(6): 1215-25.

Zurawa-Janicka, D., J. Narkiewicz and B. Lipinska (2007). "[Characterization of the HtrA family of proteins]." Postepy Biochem **53**(1): 27-36.

Curriculum Vitae

Personal data

Full name:	Justyna Sawa-Makarska
Date of birth:	17th April 1977
Place of birth:	Lublin, Poland
Nationality:	Polish

Academic career

2004 to present	Ph.D. studies in the group of Dr. Tim Clausen, Research Institute of Molecular Pathology (IMP), Vienna, Austria
2001 – 2004	Full-time research-teacher assistant, Department of Molecular Biology, Maria Curie-Skłodowska University (UMCS), Lublin, Poland; Research topic: Structure-function analysis of the acidic ribosomal P proteins.
1996 – 2001	Graduate studies in the Institute of Microbiology and Biotechnology, UMCS Lublin, Poland; M. Sc. Diploma with First Class Honors in Biotechnology; Diploma thesis: 'Proteins and polypeptides of *Galleria mellonella* humoral response. Purification and characterization of apolipophorin III.'
I - V. 2000	One semester studies in Bluffton College, Ohio, U.S.A (Major: Microbiology/ Biochemistry)
VII – VIII.1999	Internship in the School of Biosciences, University of Birmingham, U.K

Publications

"Collaboration of DegQ and DegP in maintaining protein homeostasis in the periplasm of *Eschericha coli*." manuscript in preparation

Krojer, T.*, **Sawa, J**.*, Schäfer, E.*, Saibil, H. R., Ehrmann, M. and T. Clausen (2008). "Structural basis for the regulated protease and chaperone function of DegP" Nature, under revision

*These authors contributed equally to the work.

Grela, P., **Sawa-Makarska, J**., Gordiyenko, Y., Robinson, C. V., Grankowski, N. and M. Tchorzewski (2008). "Structural properties of the human acidic ribosomal P proteins forming the P1-P2 heterocomplex." J Biochem **143**(2): 169-77.

Hasselblatt, H., R. Kurzbauer, C. Wilken, T. Krojer, J. **Sawa, J**. Kurt, R. Kirk, S. Hasenbein, M. Ehrmann and T. Clausen (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): 2659-70.

Kitzing, K., T. B. Fitzpatrick, C. Wilken, **J. Sawa,** G. P. Bourenkov, P. Macheroux and T. Clausen (2005). "The 1.3 A crystal structure of the flavoprotein YqjM reveals a novel class of Old Yellow Enzymes." J Biol Chem **280**(30): 27904-13.