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Titel der Dissertation

**„Analysis of the ATP- independent chaperone activity using
DegQ from *E. coli*“**

Verfasserin

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To Antonio

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“Eu sei e você sabe, já que a vida quis assim que nada nesse mundo levará você de mim.”

Abbreviations

amp	ampicillin
APS	ammonium persulfate
bis-ANS	4,4'-Bis (1-anilinonaphthalene 8-sulfonate)
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DLS	dynamic light scattering
dNTP	deoxynucleotides
DTT	dithiothreitol
Fig.	figure
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IPTG	isopropyl- β -D-thiogalactopyranoside
kDa	kilo dalton
LB	Luria Bertani
2-ME	2-Mercaptoethanol or β -mercaptoethanol
min	minutes
NiNTA	nickel-Nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDZ	<u>P</u> ost synaptic density protein (PSD95), <u>D</u> rosophila disc large tumor suppressor (Dlg1), and <u>Z</u> onula occludens-1 protein (zo-1)- protein-protein interaction domain
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	tris[hydroxymethyl]aminomethane
wt	wild-type
v/v	volume per volume
w/v	weight per volume

1 Abstract

All cells employ proteases and chaperones to setup a competent quality control system, preventing the accumulation of misfolded proteins. In *Escherichia coli*, the quality of the proteins in the cell envelope is controlled by the HtrA proteases DegP, DegS and DegQ. The aim of the project is to determine the molecular features of DegQ, which represents a protease and concomitantly an ATP-independent chaperone residing in the bacterial periplasm. Our studies showed that substrate binding triggers the conversion of the resting DegQ hexamer into catalytically active 12- and 24-mers. Interestingly, substrate-induced oligomer reassembly and protease activation depends on the first PDZ domain, but not on the second. This result implies that the mentioned regulatory mechanism may be a common feature of HtrA proteases that typically encompass a single PDZ domain. Furthermore, our *in vitro* and *in vivo* data point to a pH-related function of DegQ in the bacterial cell envelope. In addition, a DegQ mutant lacking the second PDZ domain was used for structural studies and the high-resolution crystal structure of a dodecameric HtrA complex was determined. The obtained structural data revealed a conserved signaling cascade in which substrate binding and protease activation are coupled. Further structural studies of full length DegQ, using Electron Microscopy, suggested that in addition to protease activity, DegQ has also chaperone activity. In order to characterize the molecular aspects of the chaperone function of DegQ, a mutational analysis was performed. DegQ mutants were designed based on the functional analogy to chaperonins which expose their hydrophobic binding sites to interact with substrates and upon a conformational rearrangement provide a hydrophilic chamber to promote protein folding. DegQ in turn exposes hydrophobic binding sites within its PDZ 1 domain in the hexameric state and provides an enclosed hydrophilic environment upon dodecamer formation. A detailed biochemical characterization of DegQ mutants revealed that mutations of residues F257 and F266 in the PDZ 1 domain indeed affected the chaperone activity of DegQ. However, further experiments are required to elucidate the

underlying causes of the impairment in detail. Additionally, the study showed that the protease activity is affected by the mutations in a substrate-specific manner leading to either lower degradation rates or different degradation products. Taken together, the performed studies provide important insights in the DegQ structure and function leading to a better understanding of general mechanisms underlying the protease and chaperone activities of HtrA proteins.

2 Zusammenfassung

Alle Zellen nutzen Proteasen und Chaperone um ein kompetentes Qualitätskontrolle-System aufzubauen, das die Akkumulierung von ungefalteten Proteinen verhindert. In der Zellhülle von *Escherichia coli* wird die Qualität von Proteinen durch die HtrA-Proteasen DegP, DegS und DegQ kontrolliert. Das Ziel dieses Projekts ist es, die molekularen Funktionen von DegQ zu bestimmen, das gleichzeitig eine Protease und ein ATP-unabhängiges Chaperon im bakteriellen Periplasma darstellt. Es zeigte sich, dass die Umwandlung von ruhenden DegQ Hexameren in katalytisch aktive 12- und 24-mere Partikel durch die Bindung von Substraten ausgelöst wird. Interessanterweise hängt diese Substrat-induzierte Umwandlung des Oligomers und die Aktivierung der Proteaseaktivität von der Anwesenheit der ersten PDZ Domäne ab, jedoch nicht von der zweiten. Diese Beobachtung impliziert, dass der beschriebene Regulationsmechanismus ein allgemeines Merkmal der HtrA-Proteasen ist, die üblicherweise nur eine PDZ Domäne besitzen. Unsere *in vitro* und *in vivo* Daten zeigen des Weiteren, dass die Funktion von DegQ in der bakteriellen Zellhülle vom pH-Wert bestimmt wird. Eine DegQ Mutante, der die zweite PDZ Domäne fehlt wurde für strukturelle Studien benutzt und die hoch-auflösende Kristallstruktur eines dodekameren HtrA-Komplexes wurde bestimmt. Die Struktur offenbarte eine konservierte Signalkaskade in der Substratbindung und Proteaseaktivierung gekoppelt sind. Weitere elektronenmikroskopische Studien von Vollängen-DegQ deuteten darauf hin, dass DegQ zusätzlich zur Proteaseaktivität auch Chaperonaktivität besitzt. Um die molekularen Aspekte der Chaperonaktivität zu charakterisieren wurde eine Mutationsanalyse durchgeführt. Die DegQ Mutanten wurden basierend auf der funktionellen Analogie zu Chaperoninen konstruiert, welche ihre exponierten hydrophoben Bindungsstellen zur Substratbindung nutzen und nach einer Konformationsänderung eine hydrophile Kammer zur Proteinfaltung ausbilden. DegQ hingegen bietet hydrophobe Bindungsstellen in der ersten PDZ Domäne in seinem hexameren Zustand und formt eine geschlossene hydrophile Umgebung in seiner dodekameren Form. Eine detaillierte biochemische Charakterisierung der DegQ Mutanten zeigte, dass die Mutation der Reste F257 und F266 in der ersten PDZ Domäne in der Tat die Chaperonaktivität beeinflusst. Es sind jedoch noch weitere Experimente nötig, um die zugrundeliegenden Ursachen dieser Beeinträchtigung herauszufinden. Des Weiteren zeigte diese Studie, dass die Mutationen die Proteaseaktivität in einer

Substrat-spezifischen Weise beeinflusst haben und entweder zu niedrigeren Abbauraten oder unterschiedlichen Abbauprodukten geführt haben. Insgesamt bieten die durchgeführten Studien wichtige Einblicke in die Struktur und Funktion von DegQ und tragen zu einem besseren Verständnis der zugrundeliegenden allgemeinen Mechanismen der Protease- und Chaperonaktivität von HtrA-Proteinen bei.

3 Introduction

3.1 The Gram-negative bacteria and the periplasm

Bacteria are unicellular organisms which are anucleated and lack membrane enclosed organelles. They are traditionally classified into two main groups, the Gram-positive and the Gram-negative bacteria. This classification is based on their ability to retain the Gram-stain in their peptidoglycan layer. The Gram-negative bacteria are not able to retain the Gram-stain due to their outer cell membrane composition or the lack of an exposed peptidoglycan layer (Gupta, 1998).

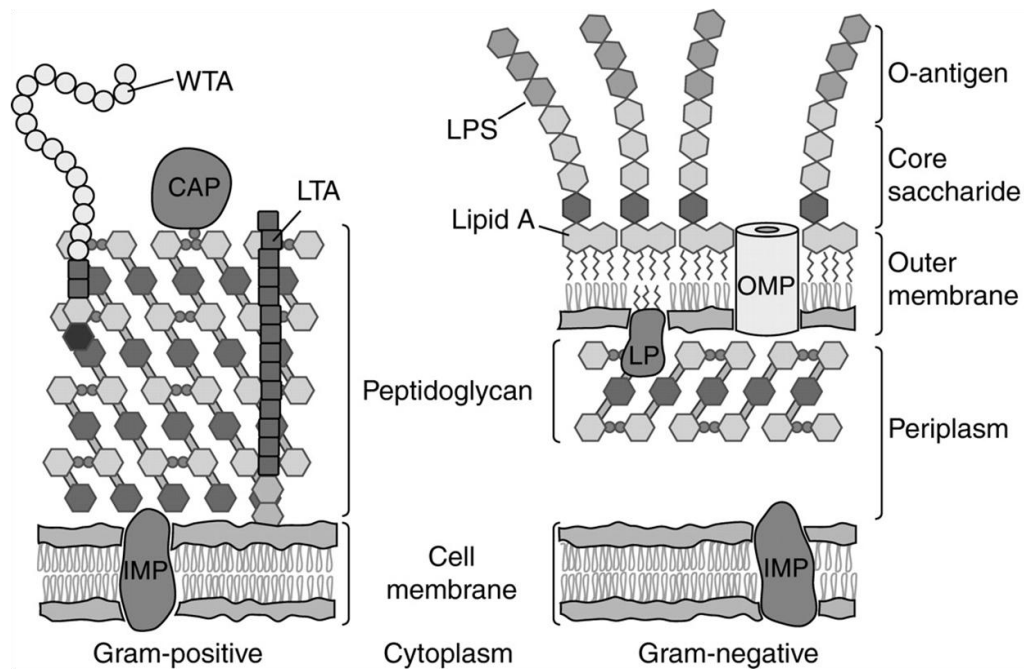


Fig. 1.1- Gram-positive and Gram-negative cell envelopes: CAP = covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid (figure adapted from Silhavy *et al.*, 2010).

In Gram-negative bacteria, the outer cell membrane and the plasma membrane define an intermediate area which is called periplasm (figure 1.1). The periplasm of *Escherichia coli* corresponds to approximately 20 % of the total cell volume (Van Wielink and Duine, 1990). The plasma or inner membrane is composed of a phospholipid bilayer, the outer membrane is an asymmetric bilayer composed of phospholipids and lipopolysaccharides (LPS) (Muhlradt and Golecki, 1975; Smit *et al.*, 1975; Kamio and Nikaido, 1976). While the integral inner membrane proteins are typically α -helical, the outer membrane contains mainly outer membrane proteins (OMPs) which are shaped as cylindrical β -barrels. The inner membrane is impermeable to most solutes and the transport of proteins and small molecules is highly regulated by specific inner membrane proteins. The outer membrane contains pores which allow the free traffic of water and small hydrophilic molecules. In addition, the outer membrane works as an efficient barrier to the transport of hydrophobic molecules bigger than 600 Da (Bos *et al.*, 2007; Nikaido, 2003).

The conditions encountered in the periplasm differ from the cytoplasmic ones. Due to its particular isolation from the external environment and the cytoplasm, the periplasm is a separate cellular compartment with extraordinary and exclusive chaperones and stress-sensing mechanisms (Ehrmann, 2006). Furthermore, the periplasm lacks readily available energy sources as ATP (adenosine 5' triphosphate) to supply the reaction cycles. Additionally, the periplasm is an oxidative compartment which favors formation of disulfide bridges (Nakamoto and Bardwell, 2004). Therefore, the periplasmic proteins had to evolve specialized mechanisms to exert their functions. Since all bacterial proteins are produced in the cytoplasm, their transport to the periplasm, to the outer membrane or to the exterior requires specialized export mechanisms (Durand *et al.*, 2009).

The translocation across the cytoplasmic membrane is mediated by two secretory pathways, the classical secretion (Sec) and the twin arginine translocation (TAT) systems. Whereas substrates of the Sec system are transported in an unfolded conformation and posttranslocationally folded, the TAT substrates fold in the cytoplasm, assuming their functional conformation just after translation (Merdanovic *et al.*, 2011).

However, after folding the periplasmic proteins can be subjected to external environmental changes. Due to the outer membrane porosity, the periplasm is subjected for example to pH variation, which can lead to protein impairment. Unfolded proteins can face different fates. The proteins can be either repaired or degraded, and upon inefficient degradation, harmed proteins can also aggregate.

In order to sense and respond to protein-folding stresses, Gram-negative bacteria employ different signal transduction pathways. The main ones are the sigma E (σ^E) and the Cpx systems that sense misfolded or mislocalized proteins in the periplasm and transmit signals for regulation of the transcription machinery in the cytoplasm.

In this way, not only the cytoplasm but also the periplasm developed effective mechanisms to provide protein quality control in the cell.

3.2 Protein quality control in the cytoplasm – proteases and molecular chaperones

As already mentioned, the Gram-negative bacteria have the periplasm and the cytoplasm as cell compartments. Although the cytoplasm seems to be extra protected from external stresses by the plasma membrane, it is also subjected to environmental changes. In order to deal with all the potential stresses, the cytoplasm evolved an effective and tightly controlled protein quality control system.

The protein quality control in the bacterial cytoplasm comprises many proteases and molecular chaperones. The proteases are in charge of the removal of misfolded proteins and the recycling of their amino-acids (Schneider and Hartl, 1996). As examples for important cytoplasmic proteases, the proteasome and ClpP can be cited. Biochemical and structural studies on these proteases have shown that they belong to multi-subunit ATPase complexes

that are able to unfold and degrade protein substrates (Groll *et al.*, 1997; Wang *et al.*, 1997). Furthermore, these complexes encompass a large central cavity which can be considered as a common feature.

The degradation process inside these ATPase-protease complexes consists of the unfolding of the substrate and its translocation through a narrow channel into the protease chamber where it is finally degraded (Ishikawa *et al.*, 2001). The active proteolytic sites of these proteases are retained in a gated chamber of the protein. The degradation of fully unfolded substrates and avoiding accidental degradation of properly folded proteins which cannot enter the narrow channel are thereby guaranteed (Pickart and Cohen, 2004).

3.2.1 The Chaperonin complex GroEL/GroES

Besides proteases the protein quality control system in the bacterial cytoplasm also relies on molecular chaperones. One example of an important and well-studied cytoplasmic molecular chaperone is the chaperonin GroEL. Chaperonins are a major class of molecular machines that can assist protein folding in cells. GroEL assists the folding of nascent and misfolded polypeptides by alternating cycles of binding and encapsulation (Thirumalai and Lorimer, 2001; Hartl and Hayer-Hartl, 2002). GroEL is a barrel-shaped complex and consists of two seven subunit rings. Each subunit is formed by an apical domain, an intermediate domain and an equatorial domain (figure 1.2). The intermediate domain connects the apical domain with an equatorial ATPase domain which mediates most intersubunit contacts within and between GroEL rings. The apical domains of the subunits expose hydrophobic binding surfaces toward the center of the ring. Additionally, the hydrophobic surfaces engage with non-native substrate protein in multiple contacts (Fenton *et al.*, 1994; Farr *et al.*, 2000). Following substrate binding, combined actions of ATP and GroES trigger a huge conformational change in the complex which results in the formation of a hydrophilic chamber capped by GroES (Mayhew *et al.*, 1996). GroES is a ring structure with approximately seven 10 kDa subunits (Chandrasekhar *et al.*, 1986). The GroEL ring capped by GroES is called the *cis* ring and the non-capped ring is the *trans* ring.

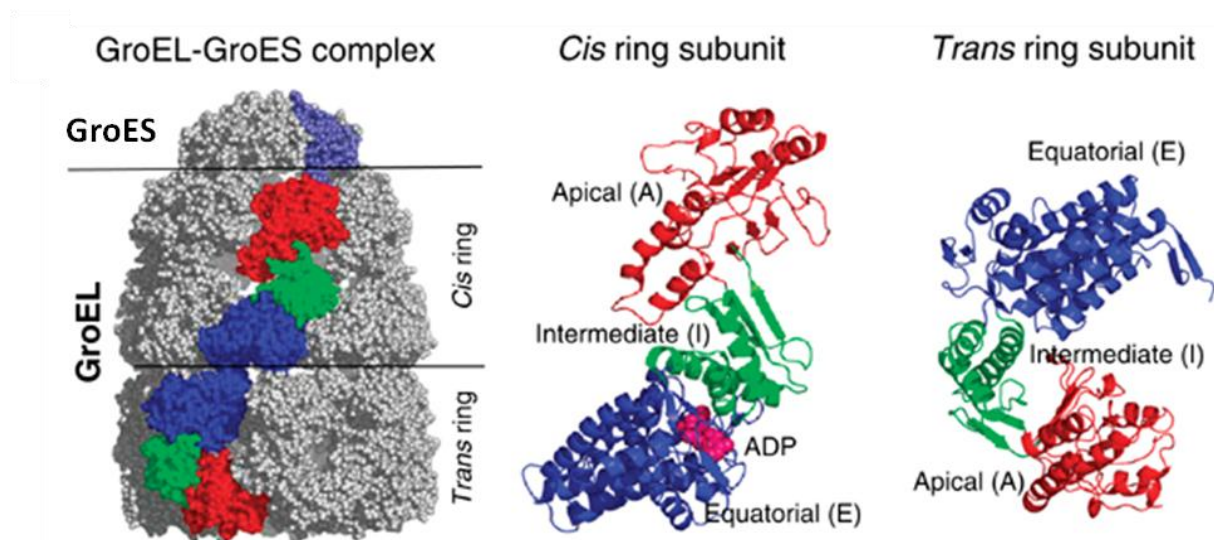


Fig. 1.2- Structure of the GroEL/GroES complex. The figure shows the cylindrical structure of GroEL which is composed of two rings, termed the *cis* and *trans* rings, depending on the position of the GroES lid. Each ring in GroEL is composed of seven subunits. A single subunit in each ring is highlighted. Each subunit consists of three domains, the apical, intermediate and equatorial domains shown in red, green and blue, respectively. In addition, the panel shows an ADP molecule bound to the equatorial domain of the *cis* ring subunit in pink (figure adapted from Chennubhotla and Bahar, 2006).

The process initiates with ATP binding to one GroEL ring followed by the initial contact with GroES which results in the relocation of the GroEL apical domain to form a closed chamber with GroES. ATP binding is followed by substrate binding to the exposed hydrophobic sites (Tyagi *et al.*, 2009). After substrate encapsulation in the closed chamber, ATP is slowly hydrolyzed, providing enough time for substrate folding to happen. Additionally it is important to note that ATP hydrolysis is not needed for substrate folding. Furthermore, ATP hydrolysis in the GroES-bound ring is required for ATP binding in the opposite ring. It triggers the release of GroES, ADP and the substrate.

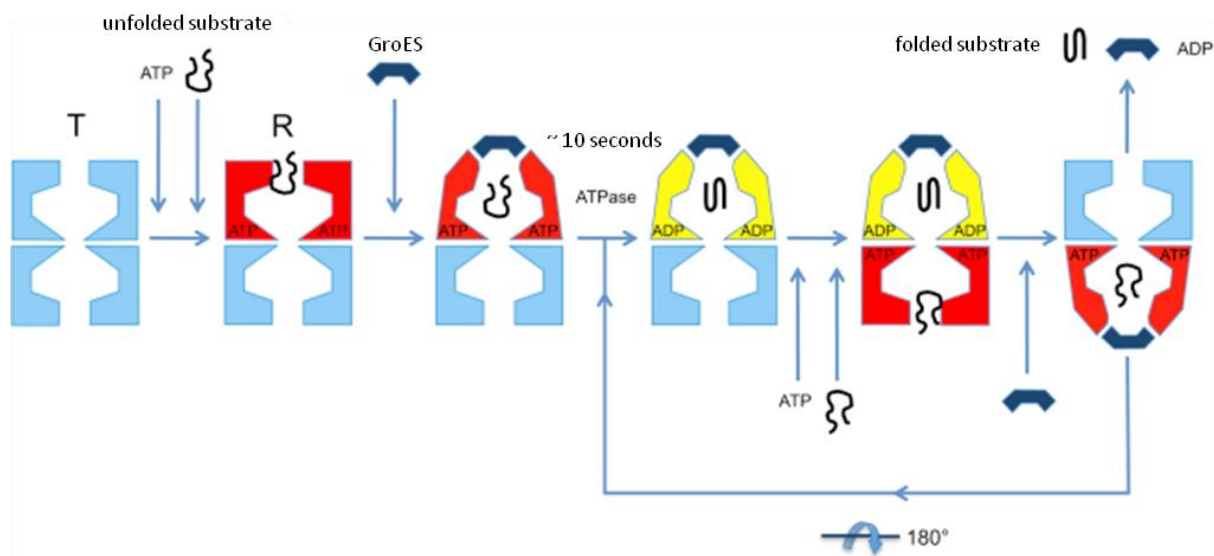


Fig. 1.3- The GroEL/GroES ATPase cycle. The figure shows the steps of substrate binding, encapsulation, folding and substrate release. The ATP hydrolysis in one ring is required to enable subsequent ATP binding in the opposite ring. However, ATP hydrolysis is not required for the folding process within the chamber (figure adapted from Clare *et al.*, 2012).

The fate of the substrate depends on its folding state. If the substrate is fully folded, it is completely released from the chamber. However, if the substrate is still unfolded, it can rebind to the chaperonin complex and undergo further folding cycles (Rye *et al.*, 1999).

3.3 Protein quality control in the cell envelope – HtrA (high temperature requirement A) proteins

In order to deal with unfolding stress the σ^E and Cpx pathways result in upregulation of proteases and chaperones. In Gram-negative bacteria, an exceptional group of proteins provides protein quality control in the cell envelope. The cell envelope of Gram-negative bacteria consists of the periplasm and the outer membrane. Bacterial HtrA proteins, which play key roles in protein quality control, reside in this particular cellular space. DegS, DegP

and DegQ are HtrA proteins which monitor the cell envelope of *E. coli* (Clausen *et al.*, 2002). This group of proteins share a common architecture: all HtrA proteins contain a protease domain and one or more PDZ domains, involved in protein-protein interactions.

A detailed description of these proteins will be presented in the following sections.

3.3.1 DegS

DegS is a regulatory protease which is anchored in the cytoplasmic membrane through an N-terminal transmembrane segment. Additionally, DegS comprises a serine protease and a C-terminal PDZ domain (figure 1.4). Under non-stress conditions, DegS is autoinhibited and therefore inactive. When DegS is inhibited, the PDZ domain interacts with the loop L3 of the protease domain. The active-site of DegS is composed of the loops LD, L1 and L2 and these loops are unable to assume their active conformation when the loop L3 interacts with the PDZ domain, preventing interaction with the loop LD of the neighboring protomer. Thus, in the inactive form, DegS has a disordered catalytic site. However, under stress conditions DegS senses and binds the C-termini of mislocalized outer membrane proteins in the periplasm (Walsh *et al.*, 2003). Upon OMP/substrate binding, the substrate can directly interact with the loop L3 of the protease domain. The reorientation of the loop L3 is the signal that indicates folding stress. Thus, DegS switches from the homotrimeric inactive to the active form. In this way, an ordered catalytic site, in which substrate-specific pockets and an oxyanion hole can be formed, is provided. Consequently, active DegS is able to degrade the anti- σ factor RseA which acts as an inhibitor of the σ^E pathway (Chaba *et al.*, 2007). This activation mechanism is considered as a common feature for serine proteases.

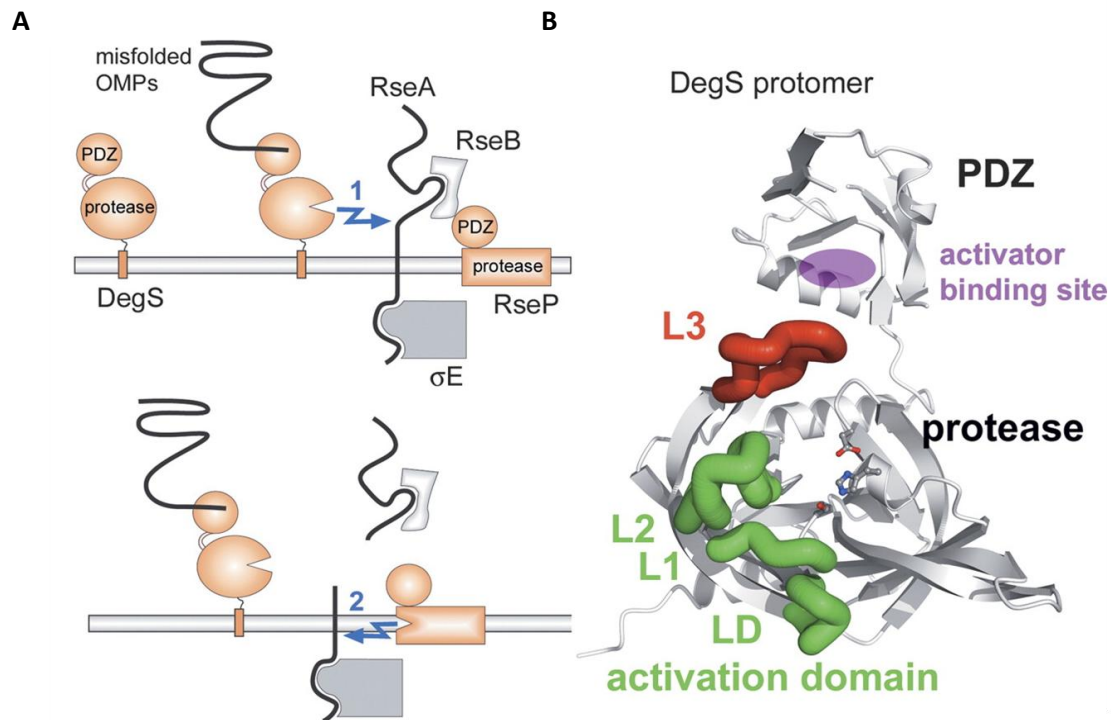


Fig. 1.4- Function of DegS in the σ^E stress response. (A) A schematic presentation of the σ^E stress response is illustrated. (B) The ribbon presentation shows one subunit of the DegS trimer highlighting loop L3, in red. The loop L3 mediates communication between the PDZ and protease domains. The activation domain which contains the loops L1, L2, and LD (green) allows the formation of a functional catalytic triad - shown in ball-and-stick mode (figure adapted from Hasselblat *et al.*, 2007).

3.3.2 DegP

In contrast to DegS which acts exclusively as a regulatory protease, DegP is able to switch between protease and chaperone activities under stress conditions (Spiess, Beil and Ehrmann, 1999). The switch between these functions is triggered by temperature. At lower temperatures, DegP acts rather as a chaperone. However, under high-temperature stress, DegP works mainly as a serine protease (Spiess, Beil and Ehrmann, 1999). The DegP protomer is composed of a protease and two PDZ domains. In the inactive state, DegP is a homohexamer which upon activation by substrate binding is able to disassemble into trimers

and reassemble into higher order oligomeric particles (Krojer *et al.*, 2002; Krojer *et al.*, 2008; Krojer *et al.*, 2010).

The inactive state of DegP is characterized by a distorted catalytic site as seen for DegS (Fig 1.5). DegP has a long loop LA in each protomer which projects into the active site of the opposite protomer (opposite trimer) keeping DegP inactive. The size of the cavity between the two trimers furthermore ensures that folded proteins cannot access the proteolytic sites (Krojer *et al.*, 2002; Clausen *et al.*, 2002). However, upon substrate binding, DegP oligomerizes into proteolytically active higher order oligomers which can encapsulate large substrates within their oligomerized chambers (Merdanovic *et al.*, 2011). Additionally, in the active state, the protease domains of three protomers interact and form the basic building block of the higher oligomers. Furthermore, as in DegS, the PDZ domains contribute to the protease activity of DegP either by presenting unfolded substrates or exposing a binding site for an allosteric activator that triggers the protease function (Wilken *et al.*, 2004; Hasselblatt *et al.*, 2007). Once the substrate is completely degraded, the proteolytic chamber disassembles and DegP returns to its hexameric resting state. This mechanism guarantees a precise activation/inactivation control of the DegP protease activity according to cellular conditions.

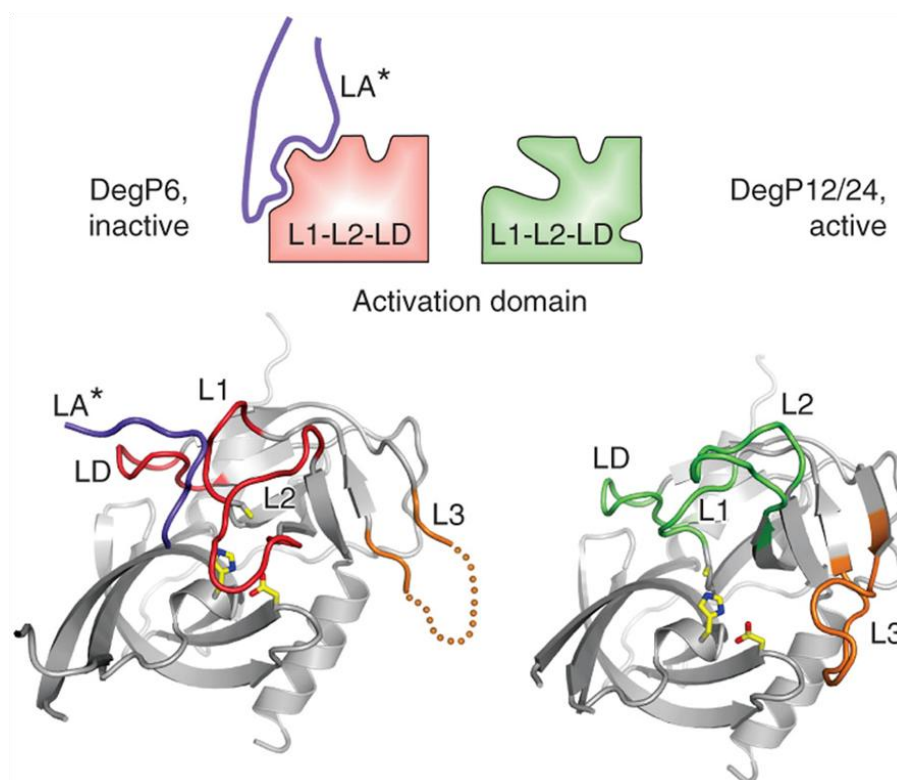


Fig. 1.5- Activation domains of DegP. The loops L1, L2 and LD of DegP6 are shown. In the left panel, the blocked and distorted proteolytic site is shown in red. The functional proteolytic site of the DegP24-mer is depicted in the right panel in green. The inactive state is stabilized by loop LA* originated from a partner subunit in the hexamer, whereas the active state is stabilized by loop L3, which senses the activation signal. The ribbon plots show the protease fold of both states; the catalytic triad is shown in stick mode (figure adapted from Krojer *et al.*, 2010).

As mentioned before, DegP is also able to perform chaperone activity. In order to identify a potential substrate, chaperones recognize hydrophobic features of unfolded polypeptides and thereby distinguish them from native proteins (Clausen *et al.*, 2002). First, MalS which is a periplasmic α -amylase protein was identified as a natural substrate for DegP. Additionally to MalS, DegP chaperone activity was shown *in vitro* for model substrates as citrate synthase (Spiess, Beil and Ehrmann, 1999). Thus, it was suggested that DegP works as a general chaperone in the bacterial cell envelope. DegP is able to encapsulate misfolded substrates protecting them from degradation and aggregation (Clausen *et al.*, 2002). Furthermore, DegP was also shown to be a chaperone for folding of OMPs (Krojer *et al.*, 2008). Krojer and

co-workers were able to co-purify and co-crystallize DegP-OMP complexes and also provided *in vivo* evidence for their results. They were able to show that DegP plays a role in OMP biogenesis. The results suggested that DegP is able to degrade unfolded OMP-A and to stabilize the folded protomers (Krojer *et al.*, 2008).

3.3.3 DegQ

Unlike DegP and DegS which were already extensively studied, the structure and function of DegQ remained poorly understood. *E. coli* DegQ was first identified by Bass and Christen; and Waller and Sauer (Bass and Christen, 1996; Waller and Sauer, 1996). DegQ is a homolog of DegP. As DegP and other periplasmic proteins, DegQ contains a signal peptide sequence targeting it to the periplasm when synthesized (Bass and Christen, 1996; Waller and Sauer, 1996).

DegQ, similarly to DegP is formed by a protease domain and two PDZ domains. DegQ and DegP share approximately 60 % similarity and 23 % amino acid sequence identity. On the protein level, one of the main features which distinguishes DegQ from DegP is the size of the so-called loop LA. In DegP, the long loop reaches the opposite trimer blocking the protease catalytic site, whereas the loop LA is much shorter in DegQ (Kim and Kim, 2005). However, DegQ was also purified as a hexamer although lately other groups also purified *E. coli* and *Legionella fallonii* DegQ as trimers (Wrase *et al.*, 2011; Bai *et al.*, 2011).

In many bacterial genomes only two HtrA proteins are encoded. It is suggested that one is a DegS homologue and the other is rather a DegQ-like than a DegP-like protein, especially when considering the size of the loop LA (Kim and Kim, 2005). It has been shown that DegQ is able to oligomerize into higher order particles even in the absence of a substrate. Later, it was shown by Sawa and co-workers that DegQ does so upon a pH shift to acidic conditions. Thus, in contrast to DegP, DegQ is not a heat-stress activated protein but rather pH sensitive (Sawa *et al.*, 2011).

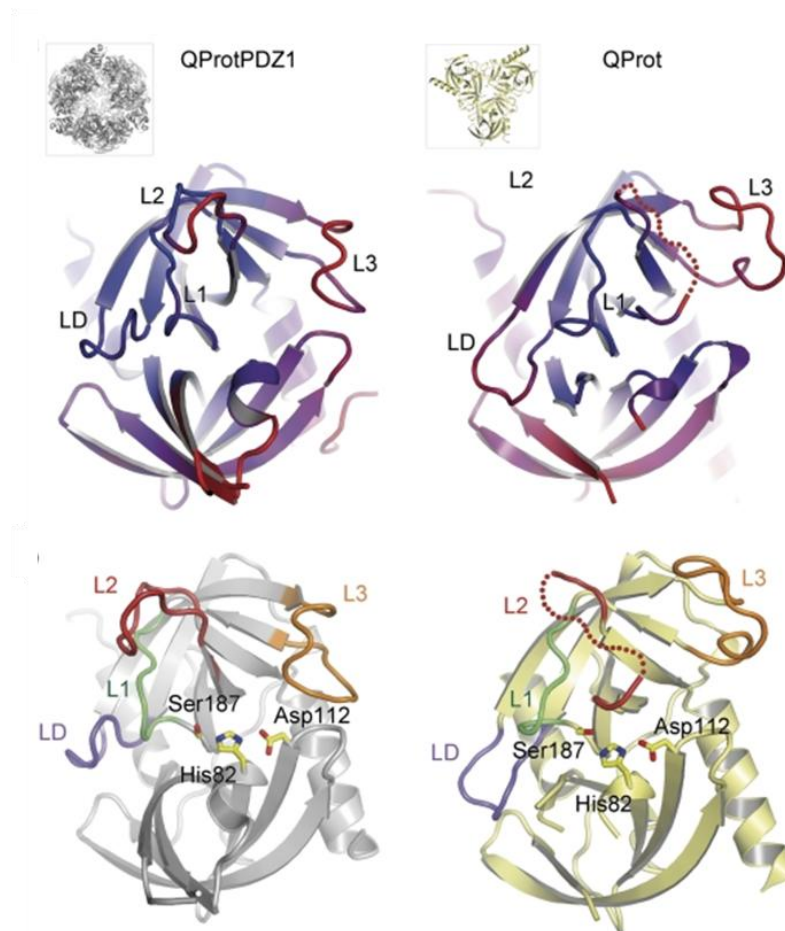


Fig. 1.6- Activation domains of DegQ. The DegQ variants QProtPDZ1 and QProt are shown in the left and right panels, respectively. In the upper panels, the structures are color-coded by the crystallographic temperature factors (rigid portions, blue; disordered regions, red) with the active site loops being labeled. In the lower panels, the active site loops are highlighted. They adopt strikingly different conformations in the two DegQ variants representing the active (QProtPDZ1) and the inactive state (QProt). The catalytic triad is shown in stick mode (figure adapted from Sawa *et al.*, 2011).

Similarly to DegP, it was later shown that DegQ is able to switch between protease and chaperone functions. The activation mechanism of DegQ is very similar to DegP considering that the catalytic site is also distorted in the resting state of DegQ (Fig. 1.6). However, upon substrate binding DegQ oligomerizes into higher order oligomers - such as dodecamers, for example - and encapsulates the unfolded substrates. As DegP, DegQ is able to discriminate folded proteins from unfolded substrates to finally degrade or fold them. Therefore it was

suggested that DegQ and DegP have overlapping functions. It has been shown that DegQ is able to rescue the growth of a temperature-sensitive mutant in a DegP null strain. Even sharing similarities with DegP, DegQ is a fascinating HtrA protein *per se*. DegQ is a pH sensitive protein which exerts protease and chaperone functions. Furthermore, DegQ shows high sequence identity with many HtrA proteins (Kim and Kim, 2005). For these reasons, DegQ was chosen as the subject of this thesis. Within this study, striking features of DegQ concerning its structure and function have been investigated and will be described in the following sections.

4 Aim of the work

The focus of this work is the structural and functional characterization of the bacterial protein DegQ, carrying out protein quality control in the periplasm. The unique ability of this protein to exert protease and chaperone functions in an ATP-independent manner motivated this work. Structural and biochemical approaches were combined to address this point and better understand the driving force of HtrA protease-chaperones.

The obtained data should give insights in the working, activation and regulatory mechanisms of DegQ. Additionally, since DegQ is the central housekeeping protease in the periplasm of most Gram-negative bacteria, DegQ is considered a better model system than the related DegP to better understand the general principle of HtrA protease regulation.

4.1 Experimental approach

As experimental strategy for the molecular characterization of the ATP-independent chaperone activity of DegQ, mutational analysis and biochemical approaches were combined.

Based on the structural analysis of DegQ and in analogy to the chaperonin GroEL/GroES, a working model was put forward and mutants were generated. Subsequently, the effects of the designed DegQ mutations on its chaperone activity were evaluated by biochemical studies. The working model was based on what is believed to characterize the DegQ chaperone activity. While the DegQ protease activity is characterized by a distorted catalytic site in the resting state, the regulated accessibility of hydrophobic substrate binding sites

may define the chaperone activity. In the resting state, DegQ exposes its hydrophobic patches to interact with the unfolded substrates. However, once DegQ oligomerizes into higher order oligomers, the hydrophobic sites are buried within the wall of the DegQ chamber and no longer available to interact with substrate proteins.

In order to carefully select the potential candidate residues to be mutated, DegP was used for comparison. The proteins DegQ and DegP are homologous having 23% overall sequence identity and 60% similarity (Kim *et al.*, 2003). Therefore, mutant design concerning the resting and active states of DegQ was based on the crystal structures of the hexameric DegP and the dodecameric DegQ (figure 2.1).

Additionally, the mutations were based on the working mechanism of GroEL/GroES. The surface of the apical domain in GroEL - facing the central cavity - is enriched by hydrophobic residues which have been implicated in polypeptide binding (Fenton *et al.*, 1994). The apical domain individually preserves the ability of the oligomeric GroEL to capture specific unfolded polypeptides. However, the apical domain is not able to exert chaperone activity; the formation of a hydrophilic folding chamber is required for the substrate protein to fold. The encapsulation by the GroEL/GroES folding chamber is a critical mechanistic step for chaperonin-assisted protein folding (Weber *et al.*, 1998). The flexible hydrophobic surface of the apical domain that is essential for polypeptide binding is now occupied by GroES. Residues important for substrate binding in the first place are excluded from the hydrophilic folding chamber upon GroES binding and the concomitant conformational rearrangement (Fenton *et al.*, 1994; Farr *et al.*, 2000).

This particular switch between the state of capturing substrates by interaction with hydrophobic residues and providing a hydrophilic environment for substrate folding is reminiscent of the oligomerization process of DegQ. In particular, hydrophobic residues within the PDZ 1 domain of DegQ are exposed on the surface within the hexameric state but are buried within the wall of the dodecameric cage.

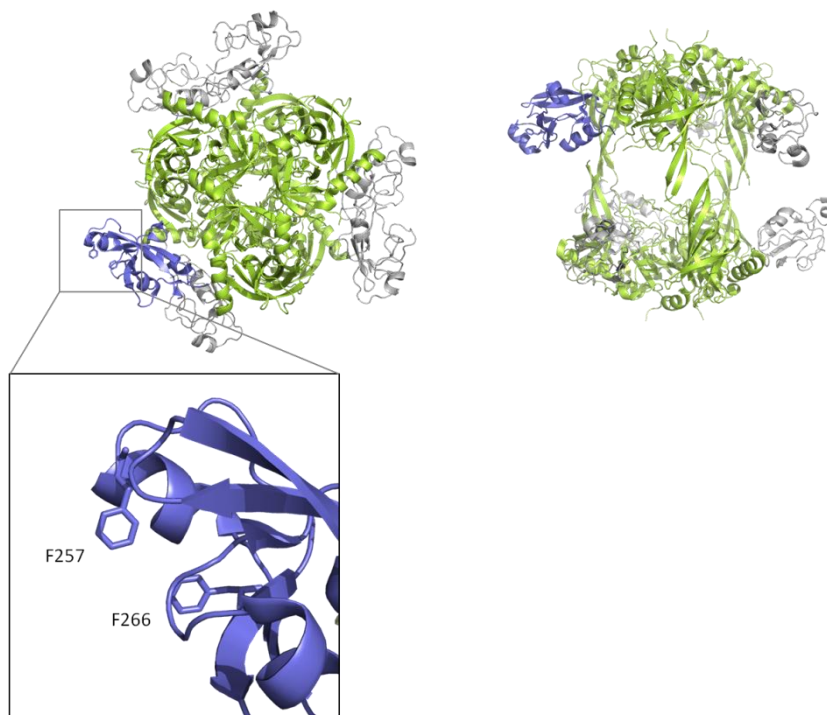


Fig. 2.1- Top and side view of the DegP hexamer with the protease domains shown in green and the PDZ 1 domains shown in grey (1KY9). The PDZ 1 domain of one DegP protomer has been replaced by the PDZ 1 domain of DegQ (shown in blue). The zoom-in view shows the hydrophobic patch in the PDZ 1 domain that is exposed on the surface of the domain in the hexameric state.

Therefore the focus of the mutational analysis of DegQ was the PDZ 1 domain, which would correspond to the apical domain of GroEL. The chosen mutated residues in DegQ, based on the DegP hexameric structure, are assumed to be exposed for interactions with unfolded substrates in the resting state of DegQ. However, upon substrate binding, the oligomerization to dodecamers (or higher order oligomers) occurs and the corresponding residues are supposed to be buried within the interface of the DegQ dodecamer. Based on these assumptions, the chosen mutated hydrophobic residues were I253, F257 and F266. These three residues belong to the interaction clamp of PDZ 1 domain (see figure 2, Sawa *et al.*, 2011). The clamp is formed by interactions between PDZ 1 domains from neighboring molecules (adjacent trimers) as shown in figure 2.2.

As a final point, it is important to mention that ideally the mutations should impair the hydrophobic patch in the PDZ 1 domain of DegQ but shouldn't affect the oligomerization

properties of the protein. In other words, the DegQ mutants should still be able to oligomerize but at the same time they should have impaired interaction with unfolded substrates and impaired chaperone activity.

4.1.1 The selected mutants and the rationale behind the mutations

4.1.1.1 The DegQ S187A I253A F257A F266A mutant

This mutant was designed in order not to impair the hydrophobic patch in the PDZ 1 domain of DegQ completely. Therefore, the original amino acid residues, I253, F257 and F266 were replaced by alanine residues. Since alanine has a residual hydrophobic character, the interaction with unfolded substrates would not be completely abrogated. Hence, the interaction of the DegQ S187A I253A F257A F266A mutant with unfolded substrates and its chaperone activity should be reduced, but not entirely abolished when compared to DegQ.

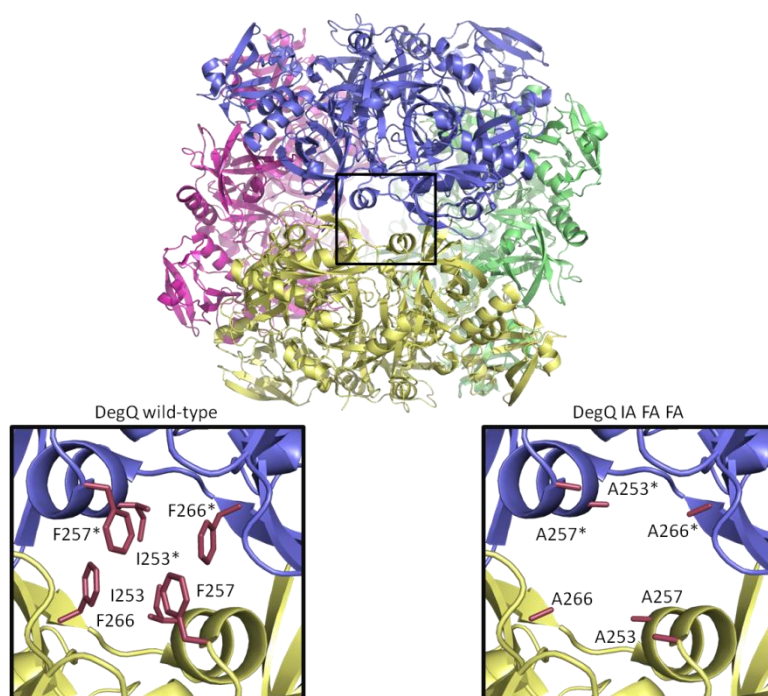


Fig. 2.2- The structure of the DegQ dodecamer is shown with the four trimer building blocks shown in yellow, blue, magenta and green respectively (3STJ). The zoomed areas correspond to the mutated region in DegQ and DegQ I253A F257A F266A.

4.1.1.2 The DegQ S187A F257E F266E mutant

These mutations were chosen in order to completely impair the hydrophobic binding site of the DegQ PDZ 1 domain. The original amino acids were replaced by the negatively charged amino acid glutamate. These specific mutations would confer an additional effect on DegQ, the inability to oligomerize. Since the mutated residues belong to the inter-trimer contacts in the DegQ dodecamer, the glutamate residues would cause repulsion of the trimers, impeding higher order oligomer formation. Thus, the DegQ S187A F257E F266E mutant should not be able to interact with unfolded substrates. Furthermore, this DegQ variant should show impaired chaperone activity due to its inability to promote substrate encapsulation.

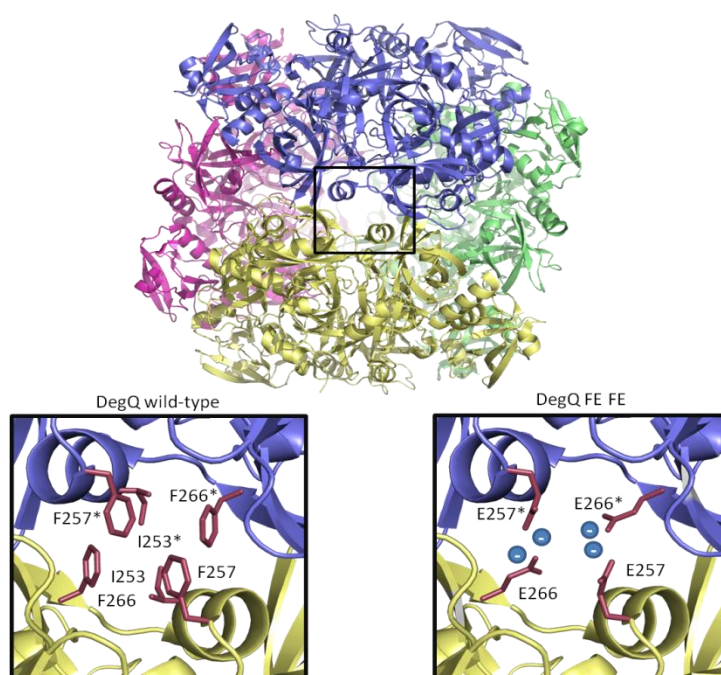


Fig. 2.3- The structure of the DegQ dodecamer is shown with the four trimer building blocks shown in yellow, blue, magenta and green respectively (3STJ). The zoomed areas correspond to the mutated region in DegQ and DegQ F257E F266E

4.1.1.3 The DegQ S187A F257E F266R

The mutant DegQ S187A F257E F266R was designed to provide complete impairment of the hydrophobic patch in the PDZ 1 domain. The original amino acids were replaced by the oppositely charged amino acids glutamate and arginine. This rearrangement of arginine and glutamate residues should preserve the ability to assemble into higher order oligomeric particles. However, the same mutations would confer impairment in substrate interaction and chaperone activity. It is important to note that the chaperone activity of this mutant would be abrogated exclusively due to the mutations. Since the mutant would conserve the ability to encapsulate the substrate, only the substrate binding would be affected causing the impairment in the chaperone activity.

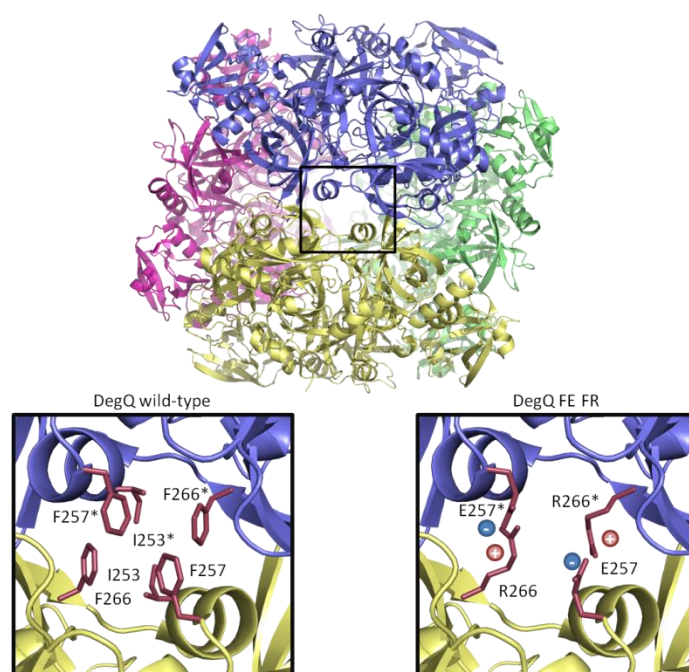


Fig. 2.4- The structure of the DegQ dodecamer is shown with the four trimer building blocks shown in yellow, blue, magenta and green respectively (3STJ). The zoomed areas correspond to the mutated region in DegQ and DegQ F257E F266R.

5 Materials and Methods

In this section the experimental approaches that were not mentioned in the attached publications Sawa *et al.*, 2011 and Malet *et al.*, 2012 are described.

5.1 Buffers, media, enzymes and antibiotics

The common buffers, solutions and media are described in the table below. In case a different one is mentioned, the proper description will follow.

Buffer/ Media/ solutions	Composition	Storage conditions
SDS 2x loading sample buffer	2% (w/v) SDS, 80mM Tris,-HCl pH 6.8, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 50µl/ml 2-ME	Stored at -20°C
1x SDS-PAGE running buffer	25mM Tris- HCl, pH 8.3, 200mM glycine, 0.1% (w/v) SDS	Stored at room temperature
DNA loading buffer	30% (v/v)glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol	Stored at -20°C
LB medium	10g tryptone, 5g yeast extract, 10g NaCl, adjusted pH 7.0 with NaOH, filled up with H ₂ O to 1000ml and autoclaved	Stored at 4°C
Ampicillin	50mg/ml in H ₂ O stock solution	Stored at -20°C
Kanamycin	50mg/ml in H ₂ O stock solution	Stored at -20°C
Lysozyme	50mg/ml in H ₂ O stock solution	Stored at -20°C
DNAse	1mg/ml in H ₂ O stock solution	Stored at -20°C
IPTG	1M isopropyl-beta-D-thiogalatopyranoside in H ₂ O stock solution	Stored at -20°C

Table 3.1 – List of common buffers, media and solutions used for the experimental methods.

5.2 Reagents and enzymes

All used antibiotics were purchased from Sigma and all the additional chemical compounds were purchased from Merck, Fluka or Sigma unless otherwise stated. The used enzymes in molecular biology protocols were purchased from Fermentas, unless particularly stated. Materials used for protein purification and chromatography were purchased from GE Healthcare as well as pre-packed columns and other FPLC materials.

5.3 Bacterial strains and vector system

Host strain	Genotype
DH5 α chemically competent cells	F ⁻ , j 80D lacZDM15 D(lacZY A-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

Table 3.2- *E. coli* strains used for cloning, expression.

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

5.4 Molecular cloning techniques

5.4.1 Construct design

DegQ from *Escherichia coli* was cloned into pET26b(+) (Novagen) by Justyna Sawa. This vector encompasses a N-terminally *pelB* signal sequence for periplasmic localization of the recombinant protein and an additional C-terminal His-tag for affinity purification. The active site of DegQ S187A was replaced by an alanine residue by site directed mutagenesis performed with or without the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene). The same vector system was used for all the mentioned DegQ mutants.

DegP from *Escherichia coli* was cloned into pQE60 (Qiagen) by Tobias Krojer.

5.4.2 Cloning

The mutations in the *degQ* and *degP* gene were performed with Site directed mutagenesis. The QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene) was used with only one primer containing the desired mutation and according to the instructions from the manufacturer.

Used materials:

PfuTurbo® DNA polymerase (2.5 U/ µl)

10× reaction buffer

Dpn I restriction enzyme (10 U/µl)

Oligonucleotide (100 ng/µl)

dNTP mix

XL1-Blue supercompetent cells (blue tubes)

In order to obtain proteolytic inactive mutants, the residue serine 187 was replaced by alanine. For this, a standard PCR (Polymerase Chain Reaction) reaction contained 50 ng template DNA, 10-100 pmol of each primer, 5 µl of a 2.5 mM dNTP solution, 1.5 units of Phusion (Phu, Finnzymes) polymerase and 5 µl of the corresponding 10x buffer in a total volume of 50 µl. The PCR reaction was incubated in a thermo cycler (Peltier Thermal Cycler PTC-200, MJ Research) with the following conditions:

- 1- initial denaturation step for 3 min at 98 °C
- 2- denaturation at 98 °C for 30 sec
- 3- annealing at 65°C for 10-30 sec (30 cycles)
- 4- extension at 72 °C for 90 sec
- 5- final extension at 72 °C for 5 min
- 6- samples were stored at 4 °C

5.4.2.1 Mutant strand synthesis reaction (Thermal cycling)

According to the instructions from the manufacturer, in order to synthesize the DNA mutant strand, the following reaction was performed for each *degQ* gene mutant.

Sample preparation:

5 µl of 10× reaction buffer

X µl (5–50 ng) of dsDNA template

X µl (250 ng) of oligonucleotide primer #1

1 µl of dNTP mix

ddH₂O to a final volume of 50 µl

Then add 1 µl of PfuTurbo DNA polymerase (2.5 U/µl)

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length*

Table 3.3- Thermal cycling parameters used for the Quick Change Site-Directed Mutagenesis Method (table adapted from Quick Change® Site Directed Mutagenesis manual).

5.4.2.2 Oligonucleotides

All used nucleotides were synthesized by Life Technologies and are listed in the table below. The oligonucleotides DegQ S187A forward and DegQ S187A reverse were used to obtain proteolytically inactive mutants.

Name	Sequence 5' → 3'
DegQ forward	CCATGGCCCCCTCTCCCCAGTCTG
DegQ reverse	CTCGAGACGCATCAGCAGATAGATGC
DegQ S187A forward	ATTAACCGCGGTAACGCCGGCGGTGCACTAT
DegQ S187A reverse	ATAGTGCACCGCCGGCGTTACCGCGGTTAAT
FWD MUT Ile253 Ala	CACCGAGATGAGTGCCGATGCCGCCAAAGCCTTCAACCTTGACG
FWD MUT Phe257 Ala	GCCGATATCGCCAAAGCCGCCAACCTTGACGTGCAGCGTGCC
FWD MUT Phe266 Ala	GACGTGCAGCGTGCGCGGCCGTCAGCGAAGTGTTGCC
FWD MUT Phe257Glu	GCCGATATCGCCAAAGCCGAGAACCTTGACGTGCAGCGTGCC
FWD MUT Phe266Arg	GACGTGCAGCGTGCGCGCGTCAGCGAAGTGTTGCC
FWD DegQ A187S	CATTAACCGCGGTAACCTCCGGCGGTGCACTATTAAACC
REV DegQ A187S	GGTTTAATAGTGCACCGCCGGAGTTACCGCGGTTAATG

Table 3.4- List of used oligonucleotides.

5.5 Protein analysis and biochemical methods

5.5.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

In order to perform electrophoretic separation of proteins SDS-PAGE was used according to the method of Laemmli (Laemmli, 1970). The stacking and the separating gel were prepared as described below. Gels were poured in an apparatus for 8 minigels. The protein samples were mixed with 5 μ l of 2x sample buffer, boiled for 5 min and loaded onto the gel. The electrophoresis was performed at 0.25 A for about 60 min. In the end, the gel could be blotted or stained with Coomassie brilliant blue.

Component	Separating gel (15%) (80 ml)	Stacking gel (5%) (30 ml)
1.5 M Tris/HCl pH 8.8	20 ml	-
0.5 M Tris/HCl pH 6.8	-	7.5 ml
10% (w/v) SDS	800 μ l	300 μ l
30 % acrylamide stock	40 ml	4.5 ml
H ₂ O	20 ml	17.4 ml
10 % (w/v) APS	400 μ l	300 μ l
TEMED	40 μ l	30 μ l

Table 3.5- Composition of polyacrylamid gels.

5.5.2 Coomassie blue staining

The polyacrylamide gels were stained by soaking in a staining solution and boiling for 20 sec in a microwave oven. Afterwards, the gels were gently shaken for 20min at RT. In order to destain the gels, they were transferred to a destaining solution and again boiled for 45 seconds and subsequently shaken for approximately one hour at RT. The destaining process was repeated until the gel was free of background stain.

Staining solution:	Destaining solution:
2.5 g Coomassie brilliant blue R-250	450 ml Ethanol
450 ml Ethanol	100 ml acetic acid
100 ml acetic acid	filled with H ₂ O to 1 l
filled with H ₂ O to 1 l	

Table 3.6- Composition of staining and destaining solutions.

5.5.3 Protein quantification

The concentrations of protein samples were determined by measuring the absorption at 280 nm using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific) and calculated according to the Beer-Lambert relation based on the molecular weights (MW) and molar extinction coefficients (ϵ) of the individual proteins.

Protein	Molecular Weight (MW) in kDa	Extinction Coefficients (ϵ) in M ⁻¹ cm ⁻¹
DegQ	45.500	2980
DegQ I253A F257A F266A	45.500	2980
DegQ F257E F266E	45.500	2980
DegQ F257E F266R	45.500	2980
DegP	47.000	7575

Table 3.7- List of molecular weight and extinction coefficients of the proteins.

5.5.4 Western blot

Subsequent to SDS polyacrylamide gel electrophoresis proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Amersham Biosciences) for subsequent Western blots. The transfer was performed at 100 mA till 25 V were reached for approx. 1.5 h using a semi-dry blotting apparatus at room temperature in transfer buffer (50 mM Tris, 380 mM

glycine, 0.1% SDS, 20% methanol). After transferring, the PVDF membrane was blocked with blocking solution for 1 h at RT. The primary antibodies were diluted as following:

OmpA 1:50 000 in TBST + 3% BSA

OmpC 1:10 000 in TBST + 3% BSA

OmpF 1:10 000 in TBST + 3% BSA

Next, the blots were incubated with shaking for 1 hour at RT and then washed. The washing was carried out three times with TBST for 1 h. Afterwards, the secondary antibody (anti-rabbit) was diluted to 1:20 000 in TBTS + 3% BSA (in all cases) and was incubated with the membrane with shaking for 2 hrs at 4°C. Subsequently the blots were washed three times with TBST and finally developed for horseradish peroxidase (HRPO) activity: The blot membrane was incubated for 1 min in a 1:1 solution of stable peroxide and Luminol/Enhancer (Super Signal West Pico Chemiluminescent substrate, Pierce). The excess reagent was drained off and the blot was transferred into a film cassette wrapped in plastic foil. The blot was overlaid with an autoradiographic film (Hyperfilm ECL, Amersham Biosciences) in a dark chamber and exposed for 2 sec up to 5 min. The film was later developed in a developer.

Blocking buffer	TBS(T) solution
3% BSA, 1 mM EDTA, 0.05% Tween 20, 1:1000 20% NaN ₃	10 mM Tris pH 7.5, 150 mM NaCl, (0.05% Tween 20)

Table 3.8- Composition of the blocking buffer and TBS(T) solution used for western blot.

5.5.5 Dynamic Light Scattering (DLS)

DLS was carried out using a DynaPro-801 (Protein-Solutions Inc.) molecular sizing instrument. A 50 µl sample of DegQ S187A F257E F266R dodecamer and trimer at a

concentration of about 1mg/ml in 50 mM HEPES/NaOH, pH 7.5 was given into a 12 μ l chamber quartz cuvette. The measurements were performed at 23°C. To analyze the data the software Dynamics 4.0 (Protein-Solutions Inc.) was used as described by Moradian-Oldak *et al.* (Moradian-Oldak *et al.*, 1998).

5.5.6 Fluorescence titration of bis-ANS binding to DegQ in different oligomeric states

The fluorescence measurements were made on a Horiba Jobin Yvon Spectrofluorimeter model FluoroMax-4. The proteins were used at a fixed concentration of 2 μ M and bis-ANS was used at concentrations ranging from 0 to 6 μ M. The bis-ANS probe was diluted in ethanol and the samples had a final volume of 1 ml. The reactions were performed in buffer containing 50mM HEPES, 150mM NaCl, pH 7.5. For measurements the excitation and emission slit width was set to 2 nm. Samples with bis-ANS were excited at 390 nm, and the emission was measured at 490 nm in a cuvette (Hellma analytics) with a 1-cm path length. Briefly, bis-ANS at several fixed concentrations was mixed with DegQ, and the fluorescence was measured. All the measurements were made at 23 °C. Blanks without protein were prepared in the same buffer.

5.5.7 Software

All structural figures were generated using Pymol (DeLano, 2002).

6 Results

6.1 Publication 1 – “Molecular adaptation of the DegQ protease to exert protein quality control in the bacterial cell envelope.”

6.2 Publication 2 – “Newly folded substrates inside the molecular cage of the HtrA chaperone DegQ.”

6.3 The DegQ mutants: purification

DegQ mutants were generated in order to further characterize the DegQ ATP-independent chaperone activity on a molecular level. The selected mutants were designed based on previous structural studies on DegQ (Sawa *et al.*, 2011). Additionally, they were based on the current working hypothesis that in DegQ, the accessibility to hydrophobic binding sites determines the molecular mechanism underlying the ATP-independent chaperone activity. For the experimental work, the mutants were cloned, expressed and purified. After this the mutants were submitted to biochemical characterization. The corresponding oligomeric state of each mutant and the performed biochemical assays are described in this section.

6.3.1 DegQ S187A mutant

The proteolytically inactive form of DegQ was expressed and purified in *E. coli* with a C-terminal His₆-tag. In this mutant the serine of the catalytic triad was replaced by alanine, resulting in DegQ S187A mutant (hereafter referred to as DegQ SA). During protein overexpression the recombinant protein was exported to the periplasm due to the presence of an N-terminal signal peptide sequence.

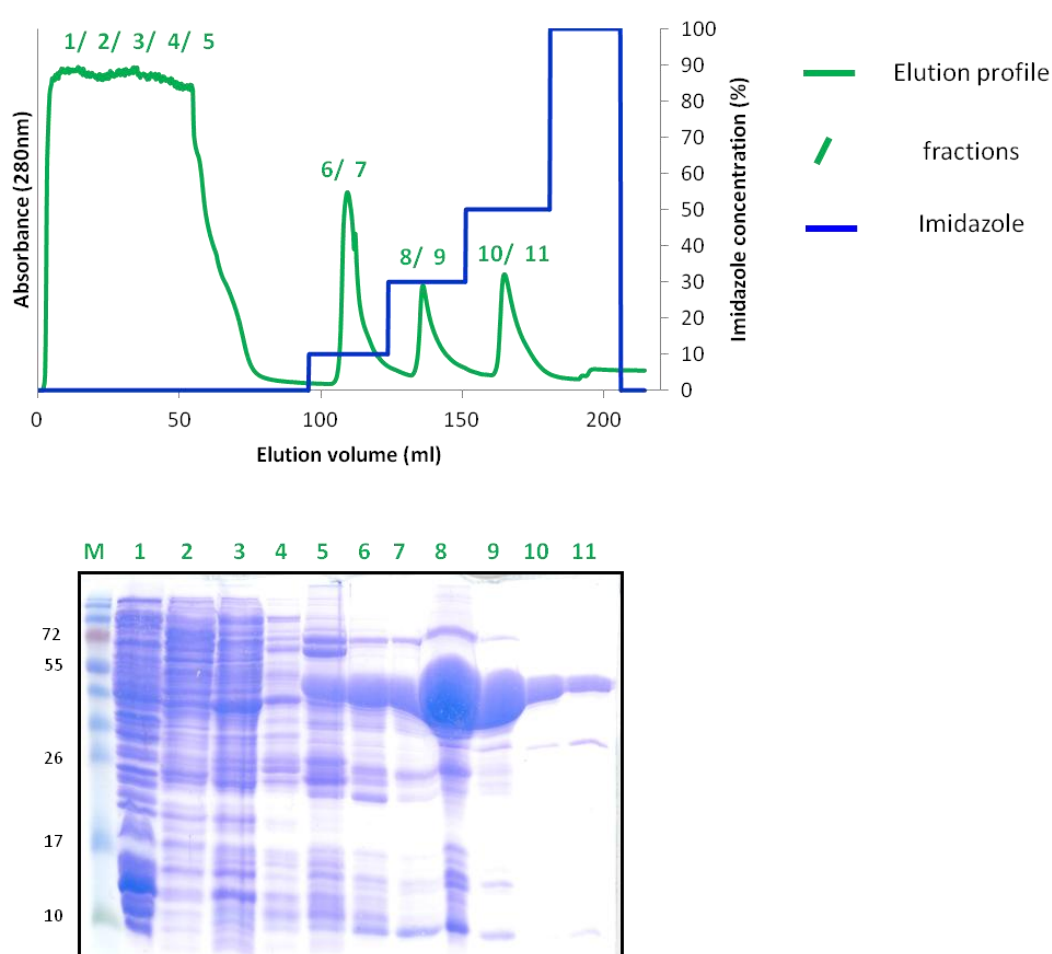


Fig. 4.1- NiNTA affinity purification of DegQ S187A. Elution profile of His-tagged full length DegQ S187A and SDS-PAGE gel corresponding to the eluted fractions.

Finally, the DegQ SA protein was purified as described in the attached publication “Molecular adaptation of the DegQ protease to exert protein quality control in the bacterial

cell envelope” (Sawa *et al.*, 2011). The fractions 9, 10 and 11 from NiNTA affinity chromatography (figure 4.1) were collected, concentrated and further purified by size exclusion chromatography (SEC) in a S200 26/60 column. After SEC, the fractions 9, 10 and 11 were collected, concentrated and stored at -80°C (figure 4.2). The yield was approximately 60 mg of DegQ SA hexamer from 5 L expression culture.

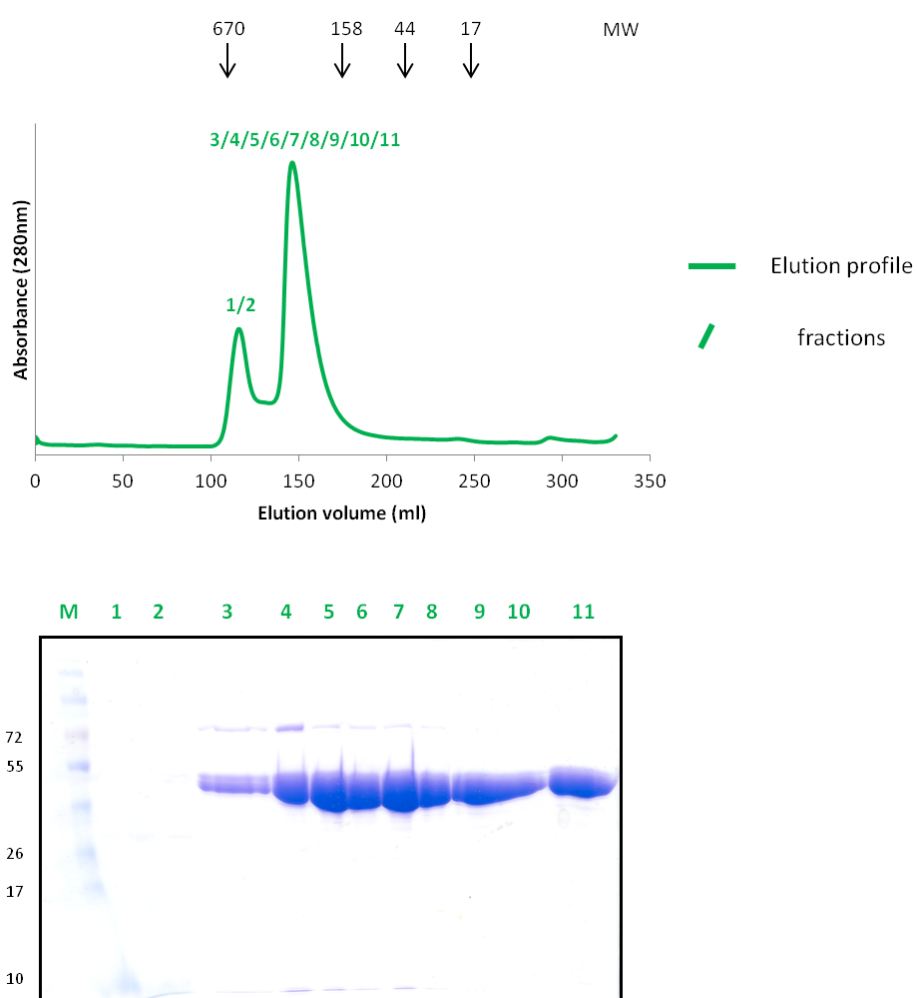


Fig. 4.2- Size exclusion chromatography of DegQ S187A. Elution profile of His-tagged full length DegQ S187A and SDS-PAGE gel corresponding to the eluted fractions.

The proteolytically inactive form of DegP (DegP S210A, hereafter referred to as DegP SA) was purified in exactly the same way as DegQ SA (data not shown).

6.3.2 DegQ S187A F257E F266R mutant

The generated mutants were purified by Ni-NTA affinity and SEC following the same procedures used for DegQ SA purification. The affinity chromatography was performed by binding C-terminally His₆-tagged DegQ SA F257E F266R (hereafter referred to as DegQ SA FE FR) to the resin by applying the cleared whole cell lysate on a Ni-NTA column (figure 4.3). The fractions containing the purest protein were collected, concentrated and further purified by SEC.

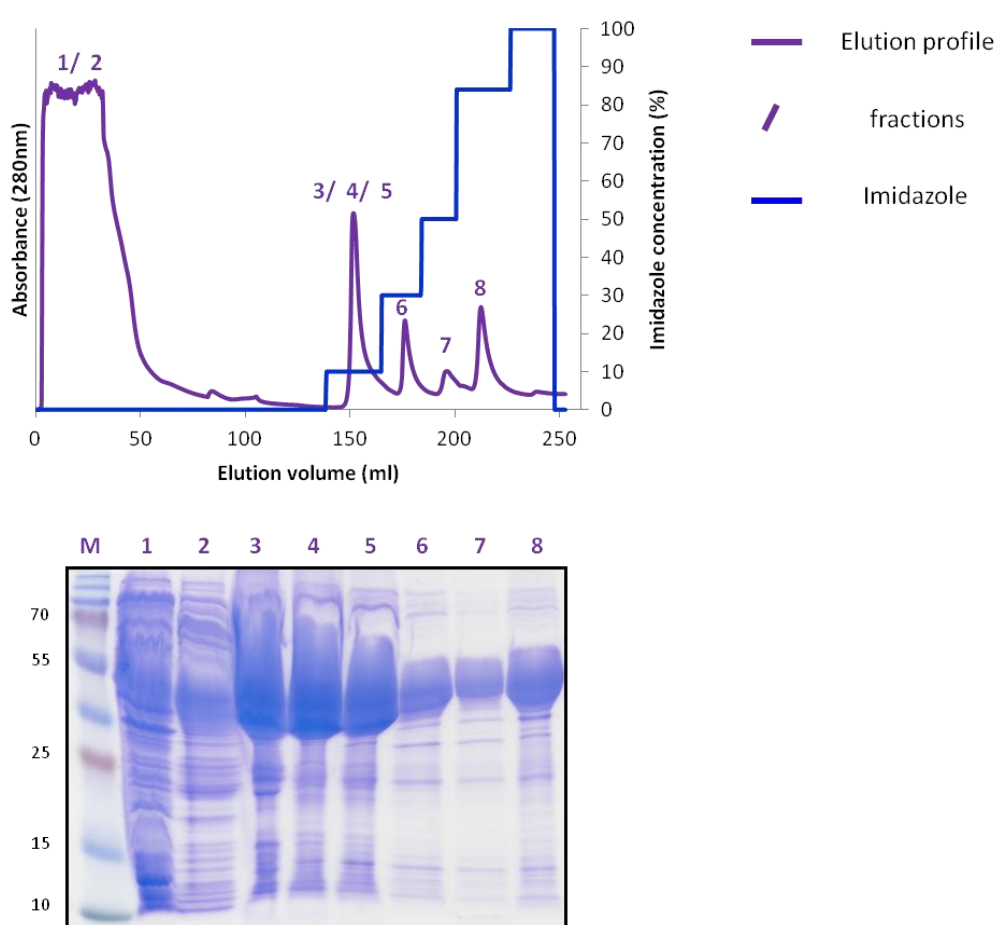


Fig. 4.3- NiNTA affinity purification of DegQ S187A F257E F266R. Elution profile of His-tagged DegQ S187A F257E F266R and SDS-PAGE gel corresponding to the eluted fractions.

The SEC was performed on a Superdex 200 prep grade size exclusion column. The obtained elution profile from SEC showed that this mutant was eluted in two different oligomeric states: trimeric and dodecameric. The estimated molecular weight was approximately

560kDa for the dodecamer and 150kDa for the trimer. SDS-PAGE analysis of the collected fractions was also performed and only the purest fractions were included in the final protein pool. The fractions 6, 7, 8 and 9 corresponding to the dodecamer and fractions 10 and 11 corresponding to the trimers were collected. Finally, the corresponding fractions were concentrated and stored at -80°C. The protein purification yielded approximately 35mg and 10mg of dodecamer and trimer from 6L expression culture, respectively.

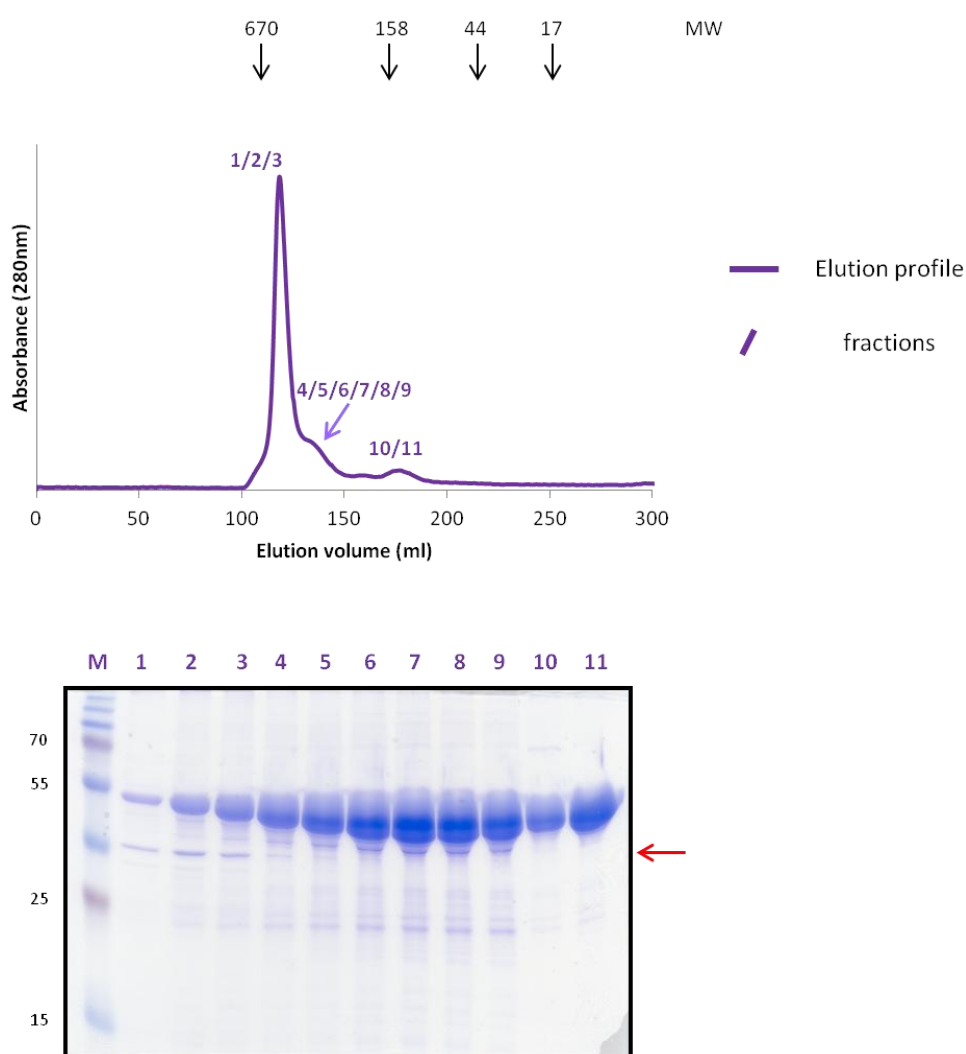


Fig. 4.4- Size exclusion chromatography of DegQ S187A F257E F266R. Elution profile of the DegQ S187A F257E F266R mutant. The lilac arrow indicates the peak corresponding to the DegQ S187A F257E F266R dodecamer. In the SDS-PAGE gel, the eluted fractions are shown. The red arrow indicates the 36kDa band mentioned in the following section.

The obtained protein was used for the subsequent experiments. The overexpression and purification procedures for all active and inactive mutants used in this study were carried out in exactly the same way.

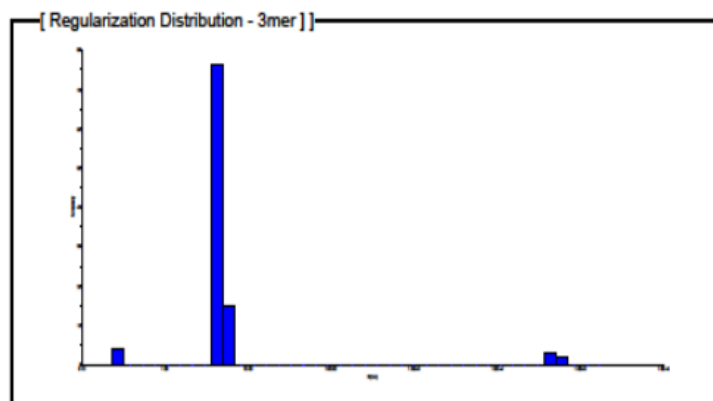
6.4 Detailed characterization of the DegQ SA FE FR mutant

6.4.1 Dynamic Light Scattering (DLS) of the DegQ SA FE FR mutant

Since the DegQ SA FE FR mutant was purified as two different molecular weight species, further characterization of the mutant was required. First, DLS was performed to confirm the molecular weights of the obtained oligomeric states (figure 4.5). DLS is a technique which measures time-dependent fluctuations in the scattering intensity arising from particles undergoing random Brownian motion. Diffusion coefficient and particle size information can be obtained from the analysis of these fluctuations. More specifically, the method permits measurement of size characteristics of proteins in a liquid medium (Moradian-Oldak *et al.*, 1998).

The DLS analysis revealed that the estimated molecular weight of the trimer was approximately 117 kDa and that of the dodecamer 690 kDa. The estimated molecular weight for the DegQ SA FE FR trimer was in agreement with our previous SEC results. However, the molecular weight corresponding to the mutant dodecamer was higher than the molecular weight predicted for the DegQ SA dodecameric form (approximately 560 kDa). SDS-PAGE analysis from the mutant purification revealed an additional protein band in the fractions corresponding to the dodecamer (figure 4.4). The protein band detected on the gel corresponded to approximately 36 kDa.

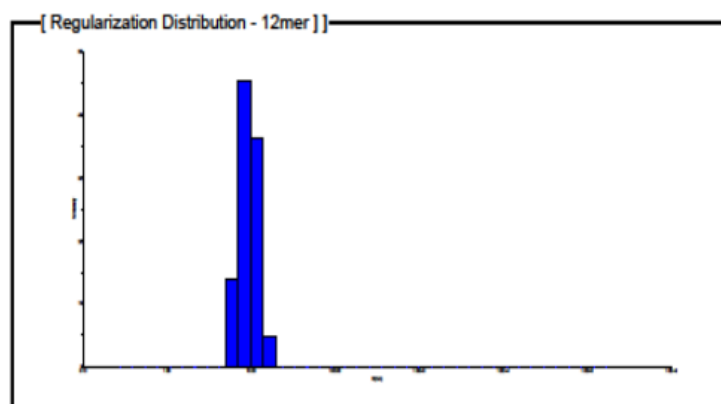
A



[Results Summary - 3mer]

Item	R	%Pd	MW-R	%Int	%Mass
	(nm)		(kDa)		
» Peak 1	0.277702	0.0	0.167983	4.0	99.4
» Peak 2	4.55399	14.1	116.885	91.3	0.6
» Peak 3	49841.5	17.1	3.30087e+011	4.6	0.0

B



[Results Summary - 12mer]

Item	R	%Pd	MW-R	%Int	%Mass
	(nm)		(kDa)		
» Peak 1	9.75056	26.5	693.921	100.0	100.0

Fig. 4.5– DLS analysis of the DegQ S187A F257E F266R mutant. (A) Results summary of the sample corresponding to DegQ S187A F257E F266R trimer. (B) Results summary of the sample corresponding to DegQ S187A F257E F266R dodecamer.

6.4.2 Identification of the purified DegQ SA FE FR dodecameric complex components

The 36kDa band was analyzed by Western blot with antibodies against OMPs - proteins that roughly share the same molecular weight and are known to be co-purified with DegP from *E. coli* (Krojer *et al.*, 2008). The Western blot demonstrated that OMP-C and OMP-F were not present in the dodecameric or in the trimeric forms (figure 4.6). In contrast, the western blot showed the presence of OMP-A co-purified with the mutant dodecamer, but not with the trimeric form (figure 4.6).

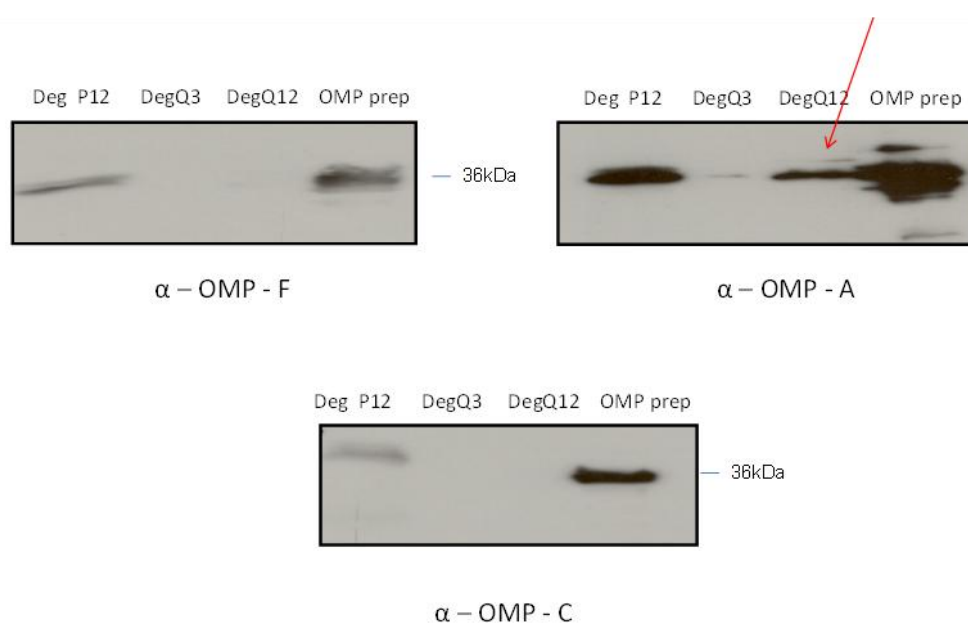


Fig. 4.6– Western blot of DegQ S187A F257E F266R mutant using antibodies against OMPs. The red arrow indicates the presence of OMP-A in the sample corresponding to DegQ S187A F257E F266R dodecamer. DegP 12= DegP dodecamer, DegQ 3= DegQ SA FE FR trimer, DegQ 12= DegQ SA FE FR dodecamer. OMP prep= preparation of outer membrane proteins from *E.coli*.

6.5 Evaluation of the accessibility of hydrophobic binding sites within different DegQ oligomeric states

Subsequently, aiming to show the importance of the accessibility of substrate hydrophobic binding sites for the DegQ chaperone activity, a bis-ANS (bis-1-anilinonaphthalene-8-sulfonate) binding assay was performed (figure 4.7). Bis-ANS is a dimeric analogue of ANS which is a fluorescent probe. Both probes have the ability to bind to hydrophobic parts of a protein and upon binding, their fluorescence increases several fold. To bind to proteins these molecules require ionic interaction of sulfonate with positively charged amino acids and hydrophobic interaction of aromatic rings with hydrophobic residues in an oriented manner (Kundu and Guptasarma, 2002).

The bis-ANS binding assay was performed with DegQ SA in different oligomeric states. According to previous structural analysis of the DegP hexamer, hydrophobic patches of each trimer are exposed to the central cavity of the hexamer (Krojer *et al.*, 2002). Thus, based on the amino acid sequence homology between and same oligomerization behavior of DegP and DegQ, it was suggested that the same occurs within DegQ. Therefore, the hexameric form of DegQ SA was used in this assay. On the other hand, structural studies of DegQ showed that upon substrate binding and oligomerization the hydrophobic patches are buried and are no longer exposed to interact with the substrate. Therefore, the dodecameric state of DegQ SA was also tested. The used dodecamer was oligomerized by peptide binding – as described in the attached publication (Sawa *et al.*, 2011).

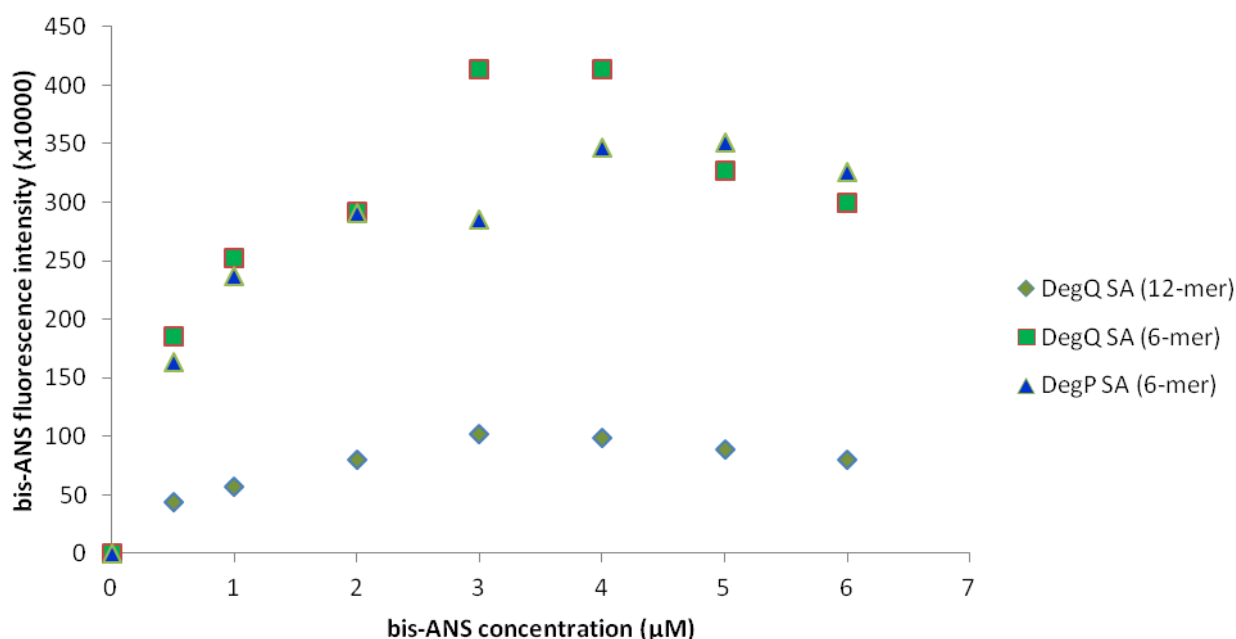


Fig. 4.11- bis-ANS titration experiment. Bis-ANS binding assay using different DegQ oligomers. The DegQ variants were used at 2μM while bis-ANS was used at different concentrations.

The assay consisted of measuring the fluorescence resulting from interaction of DegQ SA with different amounts of the bis-ANS probe. The titration experiment showed that the DegQ SA hexamer was able to bind three times more bis-ANS probe than the DegQ SA dodecamer, indicating that the DegQ hexamer has more hydrophobic patches exposed to interact with the probe than the DegQ dodecamer. In addition, the data obtained suggested the saturation of bis-ANS binding sites in DegQ at a concentration of 4 μM probe, since the maximum level of fluorescence is reached at this concentration. Furthermore, an approximate ratio of two molecules bis-ANS bound per molecule DegQ could be estimated from this data. The DegP SA hexamer was used for comparison and had a similar binding profile to bis-ANS as the DegQ SA hexamer as expected from their homologous nature.

6.6 Analysis of DegQ mutants oligomerization upon substrate binding

The preceding results showed that more hydrophobic binding sites are exposed in the hexameric resting state of DegQ than in the dodecamer. These data are in agreement with our hypothesis concerning the mechanism of the ATP-independent chaperone activity on a molecular level. In an attempt to further investigate the mechanisms underlying DegQ chaperone activity, interaction assays with model substrates were performed. In these assays the ability of the mutants to interact with different unfolded substrates was tested. Since the described DegQ mutants have mutations within a hydrophobic patch in the PDZ1 domain, their capacity to interact with unfolded substrate was tested.

The interaction assays consisted of pre-incubation of a selected DegQ SA mutant and an unfolded substrate - as described in the attached publication (Sawa *et al.*, 2011). The chosen unfolded model substrates were lysozyme and α -lactalbumin - chemically unfolded and β -casein - intrinsically unfolded. After pre-incubation the proteins were applied to analytical SEC. In this way it was possible to monitor a potential complex formation between the DegQ mutants and unfolded substrate proteins. Following the SEC, SDS-PAGE analysis was carried out. Additionally, each DegQ SA variant and each unfolded substrate was separately analyzed by SEC.

6.6.1 Interaction studies with unfolded lysozyme

For the assays, the previously described mutants and chemically unfolded lysozyme were used.

6.6.1.1 Interaction studies with unfolded lysozyme and DegQ SA mutant

For the assays, lysozyme was previously chemically unfolded by 4 M urea and 10 mM DTT. DegQ SA, the proteolytically inactive form of DegQ, is able to interact with unfolded lysozyme forming higher order lysozyme-DegQ SA 12-mer complexes. In the presence of higher concentrations of lysozyme DegQ SA can also oligomerize to 24-mers – as shown in the attached publication (Sawa *et al.*, 2011) and in figure 4.8.

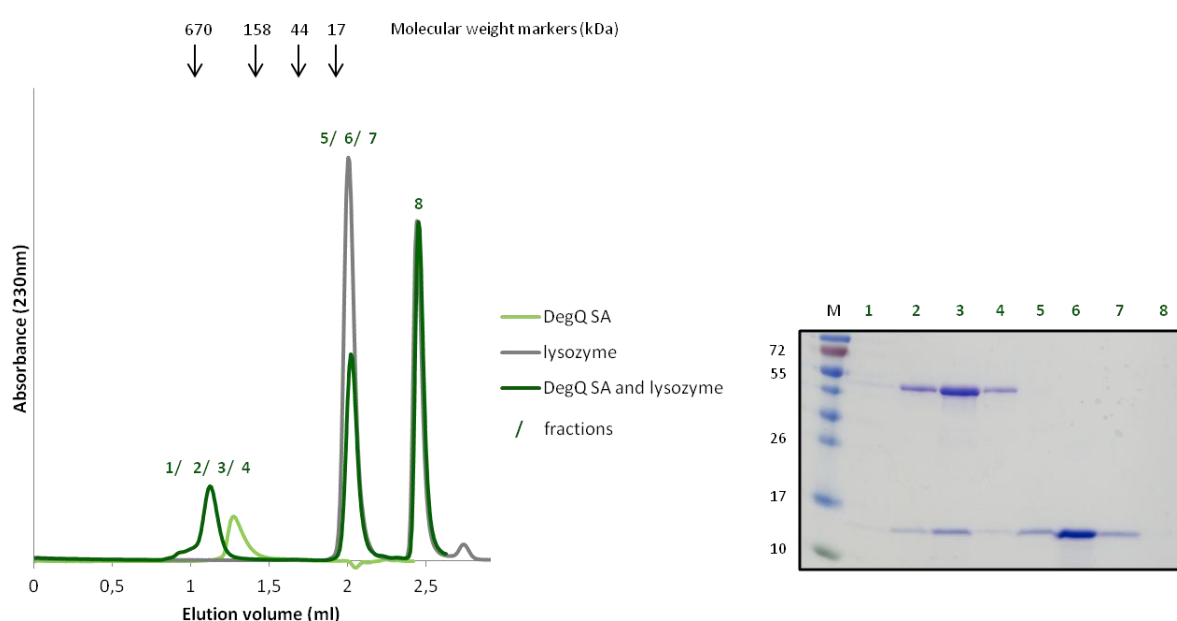


Fig. 4.8- Interaction studies: SEC with DegQ SA and lysozyme.

DegQ SA, when incubated with lysozyme, formed a complex, which applied to analytical SEC (in a S200 PC 3.2/30 column), revealed a shift of the curve towards higher molecular weight particles. Both DegQ SA and lysozyme were eluted as one single peak. The peak's elution volume was approximately 1.15ml and its estimated molecular weight was equivalent to DegQ SA 12mers. The other two observed peaks corresponded to free lysozyme and DTT, respectively. In addition, SDS-PAGE analysis confirmed complex formation between DegQ SA and lysozyme. Both proteins were collected in the same fraction numbers 2, 3 and 4.

6.6.1.2 Interaction studies with unfolded lysozyme and DegQ SA IA FA FA mutant

The ability of the mutant DegQ SA IA FA FA to interact with unfolded lysozyme was tested. The mutant was pre-incubated with unfolded lysozyme and the complex formation was monitored by analytical SEC and SDS-PAGE analysis (figure 4.9).

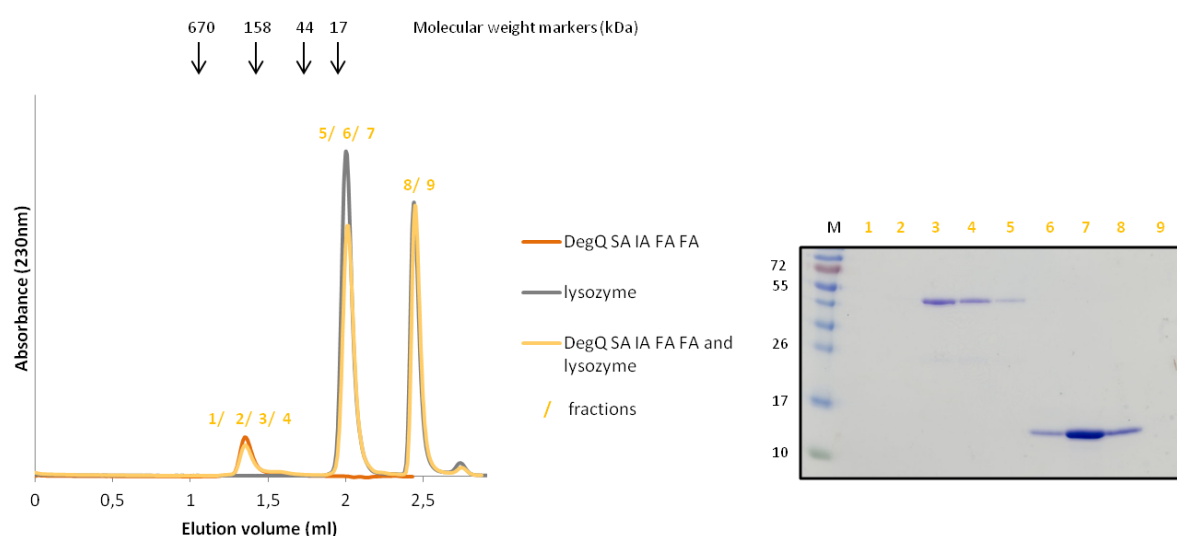


Fig. 4.9- Interaction studies: SEC with DegQ SA IA FA FA and lysozyme.

The results showed that the mutant DegQ SA IA FA FA was not able to interact with unfolded lysozyme under the same conditions as DegQ SA. In the SEC profile no shift to higher molecular weight particles was observed, consequently DegQ SA IA FA FA remained as a trimer. Both proteins were eluted as individual peaks; lysozyme corresponding to 2 ml and mutant to 1.35 ml. In addition the SDS-PAGE analysis showed that the DegQ SA IA FA FA mutant and lysozyme appeared separated in fractions 3, 4, 5 and 6, 7, 8, respectively. Thus, no complex between the mutant and lysozyme was formed.

6.6.1.3 Interaction studies with unfolded lysozyme and DegQ SA FE FE mutant

To test if the mutant DegQ SA FE FE was able to interact with unfolded lysozyme, interaction assays were performed. The mutant was pre-incubated with unfolded lysozyme and complex formation was analyzed by analytical SEC and SDS-PAGE (figure 4.10).

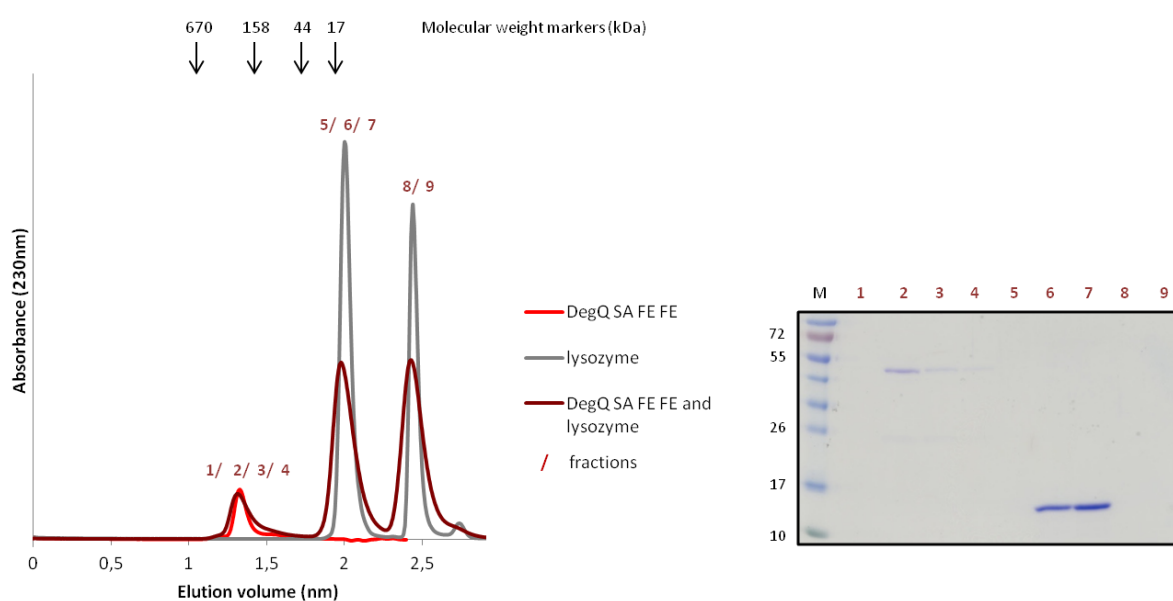


Fig. 4.10- Interaction studies: SEC with DegQ SA FE FE and lysozyme.

The data obtained showed that the mutant DegQ SA FE FE did not interact with unfolded lysozyme. On the SEC no oligomerization of DegQ SA FE FE was observed and both proteins were eluted as individual peaks. DegQ SA FE FE was eluted at 1.35 ml, which corresponds to the trimer and lysozyme was eluted at 2 ml. In addition the SDS-PAGE analysis confirmed that no interaction occurred. Lysozyme was collected in fractions number 6, 7 and the mutant was eluted in fractions 2 and 3.

6.6.1.4 Interaction studies with unfolded lysozyme and DegQ SA FE FR mutant

In order to analyze whether the mutant DegQ SA FE FR sustained the ability to interact with unfolded lysozyme, the same interaction assays as previously described were performed. Thus, analytical SEC and SDS-PAGE analysis were carried out, as demonstrated in figure 4.11.

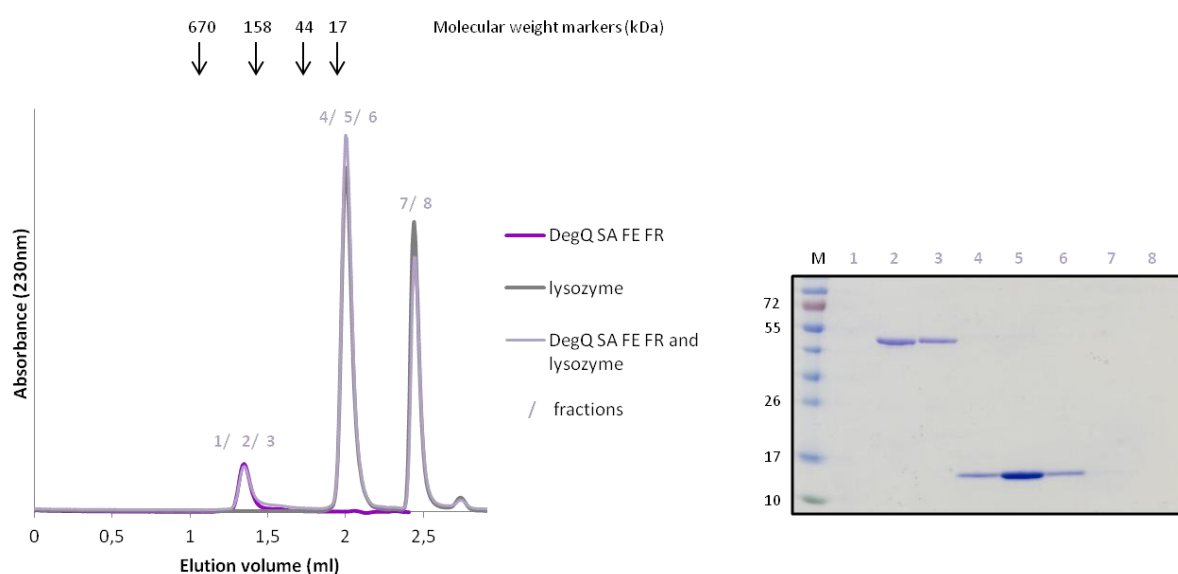


Fig. 4.11- Interaction studies: SEC with DegQ SA FE FR and lysozyme.

The analytical SEC showed that no interaction between the DegQ SA FE FR mutant and unfolded lysozyme occurred. No oligomerization of the DegQ SA FE FR mutant was observed. The mutant was eluted at 1.35 ml, remaining as a trimer, and lysozyme was eluted at 2 ml. The SDS-PAGE analysis showed that DegQ SA FE FR mutant and lysozyme were not collected in the same fraction. The mutant was collected in fractions numbers 2, 3 and lysozyme was collected in fractions 4, 5 and 6, corroborating the SEC data where no complex formation occurred.

6.6.2 Interaction studies with unfolded α -lactalbumin

For the assays the previously described mutants and chemically unfolded α -lactalbumin were used.

6.6.2.1 Interaction studies with unfolded α -lactalbumin and DegQ SA mutant

For the interaction studies with α -lactalbumin the same methods used for interaction studies with lysozyme were applied. The α -lactalbumin was chemically unfolded by 8 M urea and 40 mM DTT buffer. To monitor the interaction the unfolded substrate was pre-incubated with DegQ SA and the reaction was submitted to analytical SEC. After this the sample was also analyzed by SDS-PAGE.

The results obtained from analytical SEC indicated that DegQ SA oligomerized to 24-mers upon interaction with α -lactalbumin. However, the resolution of the obtained curves was not optimal (data not shown). Therefore, the following experiments with unfolded α -lactalbumin were performed with higher concentrations of DegQ SA and DegQ SA mutants, as shown in figure 4.12.

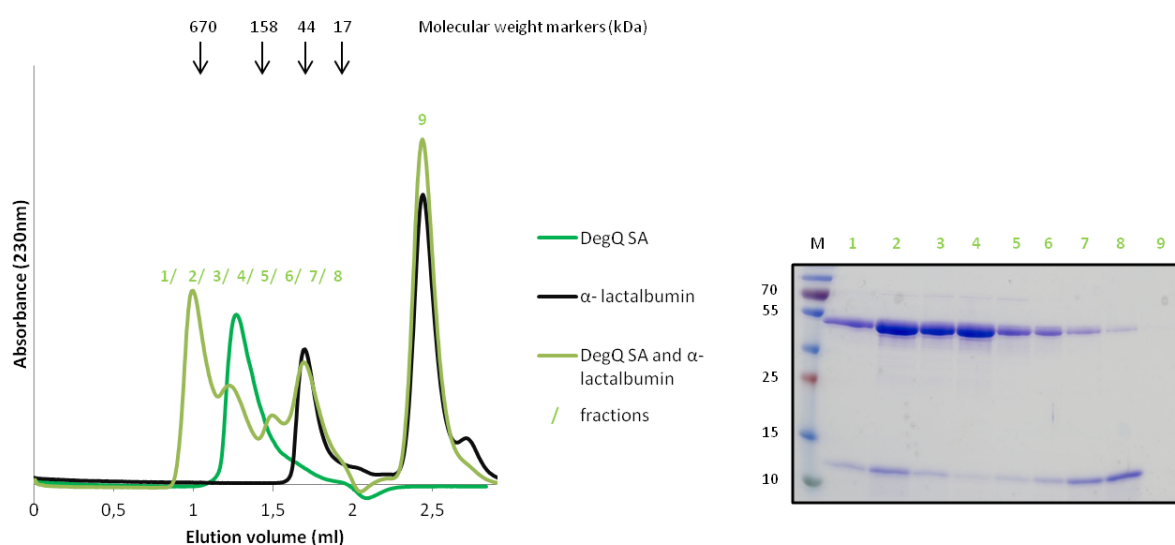


Fig. 4.12- Interaction studies: SEC with DegQ SA and α -lactalbumin.

The analytical SEC performed with higher concentrations of DegQ SA led to curves with improved resolution. The acquired results showed that DegQ SA interacted with α -lactalbumin. The complex of higher order, composed of substrate- engaged DegQ 24-mers, was eluted as a single peak at 1 ml. The sample was also analyzed by SDS-PAGE and the formation of the complex was confirmed. Both proteins were collected in the same fractions, corresponding to fractions 1, 2 and 3.

6.6.2.2 Interaction studies with unfolded α -lactalbumin and DegQ SA IA FA FA mutant

The interaction between the DegQ SA IA FA FA mutant with unfolded α -lactalbumin was also evaluated. The same experimental procedures as previously described were used. The mutant was pre-incubated with unfolded α -lactalbumin and the interaction was monitored by analytical SEC and SDS-PAGE analysis (figure 4.13).

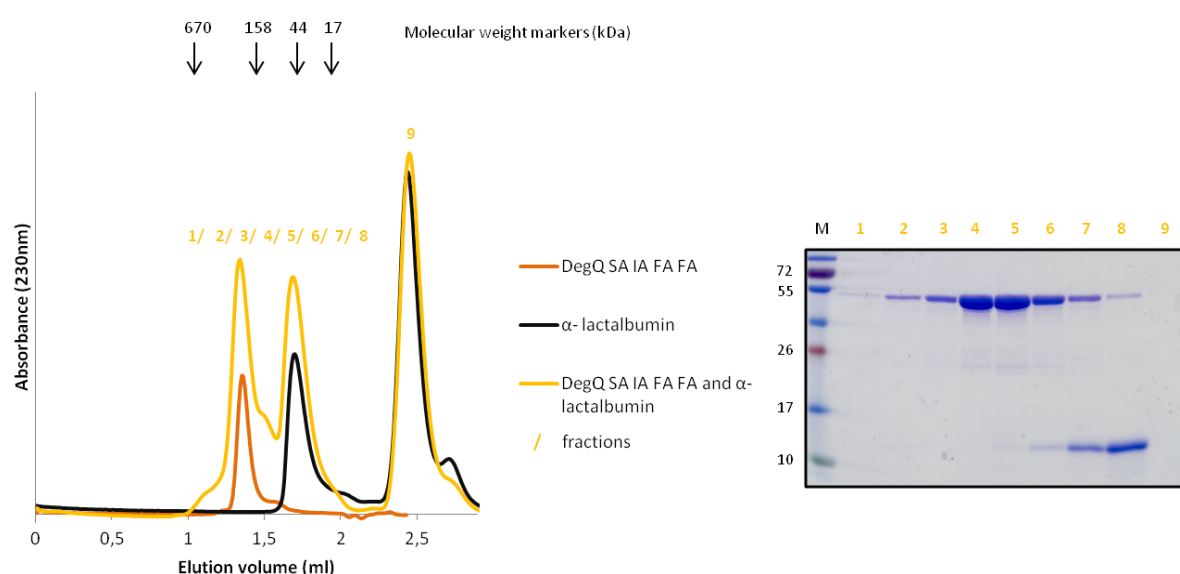


Fig. 4.13- Interaction studies: SEC with DegQ SA IA FA FA and α -lactalbumin.

The SEC and SDS-PAGE analysis revealed that the unfolded substrate α -lactalbumin did not interact with the DegQ SA IA FA FA mutant. In the SEC no shift towards the higher molecular particles was observed. Both proteins were eluted as individual peaks: α -lactalbumin at 1.70

ml, and the mutant as a trimer at 1.35 ml. In addition, the SDS-PAGE analysis showed that the mutant was eluted in fractions 2 to 7, separately from the α -lactalbumin. Thus, no complex had been formed. However, a small “shoulder” could be observed indicating a small shift towards higher order oligomeric particles.

6.6.2.3 Interaction studies with unfolded α -lactalbumin and DegQ SA FE FE mutant

To test the ability of the mutant DegQ SA FE FE to interact with unfolded α -lactalbumin the same experimental methods were used. Analytical SEC and SDS-PAGE analysis were performed, as demonstrated in figure 4.14.

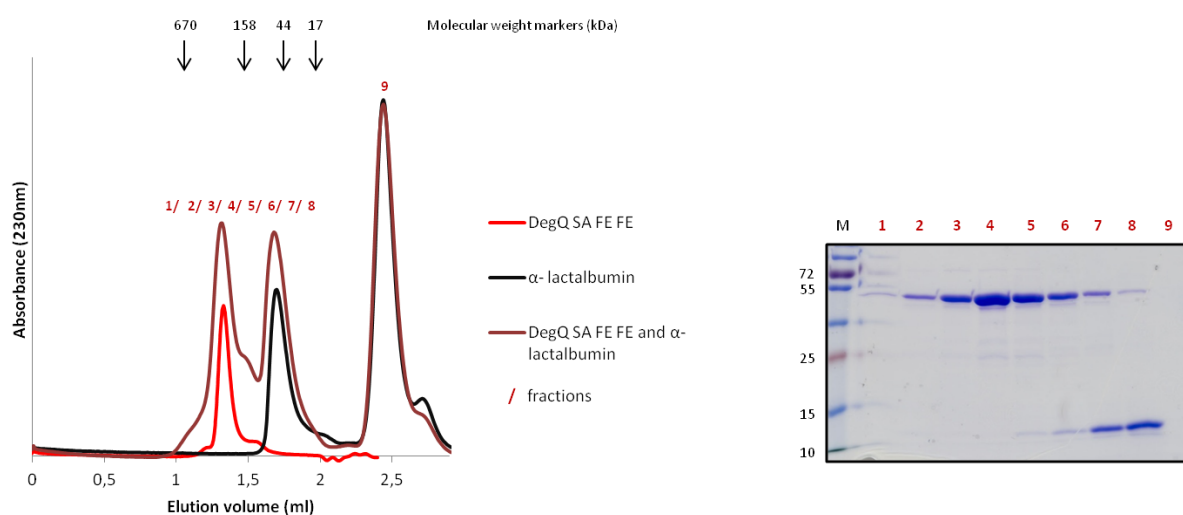


Fig. 4.14- Interaction studies: SEC with DegQ SA FE FE and α -lactalbumin.

The results showed that the DegQ SA FE FE did not interact with α -lactalbumin. No oligomerization of the mutant was observed on the analytical SEC. The mutant, eluted at 1.35 ml, remained as a trimer and the unfolded substrate was eluted in a different peak. Additionally, the SDS-PAGE revealed that both proteins were collected in different fractions. The unfolded substrate α -lactalbumin was collected in fractions 6, 7, 8 and the mutant in fractions 2 to 7. In agreement with the SEC, the SDS-PAGE analysis showed that no complex

formation occurred. However, a small “shoulder” could be observed indicating a small shift towards higher order oligomeric particles.

6.6.2.4 Interaction studies with unfolded α -lactalbumin and DegQ SA FE FR mutant

The capacity of the mutant DegQ SA FE FR to interact with α -lactalbumin was analyzed. As shown in figure 4.15, analytical SEC and SDS-PAGE analysis were carried out under conditions identical to those previously described for the other DegQ mutants.

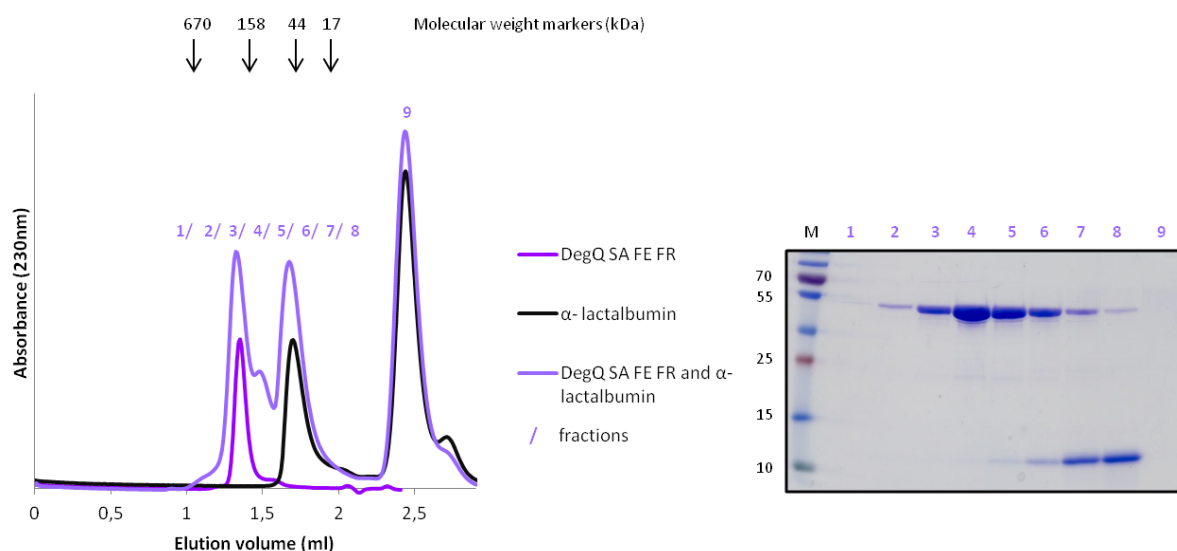


Fig. 4.15- Interaction studies: SEC with DegQ SA FE FR and α -lactalbumin.

The analytical SEC showed that no interaction between the DegQ SA FE FR mutant and α -lactalbumin occurred. The mutant was eluted at 1.35 ml, keeping the trimeric state, and the substrate was eluted as a separate peak. Furthermore, the SDS-PAGE analysis showed that the DegQ SA FE FR mutant was eluted in fractions 2 to 7 and α -lactalbumin in fractions 6, 7 and 8 confirming that no complex between the two proteins had been formed. However, a small “shoulder” could be observed indicating a small shift towards higher order oligomeric particles.

6.6.3 Interaction studies with β -casein

For the interaction studies the previously described DegQ mutants and β -casein were used.

6.6.3.1 Interaction studies with β -casein and DegQ SA mutant

Testing the ability of the mutants to interact with chemically unfolded model substrates was followed by testing with an intrinsically unfolded substrate. In general, intrinsically unfolded proteins have low overall hydrophobicity (Uversky *et al.*, 2000). Therefore, they cannot bury sufficient hydrophobic core to fold as stable globular proteins (Dyson and Wright, 2005). Intrinsically unfolded protein β -casein was used for interaction studies with the selected DegQ mutants.

The experimental methods applied for the interaction studies with β -casein were indistinguishable from those described in the attached publication (Sawa *et al.*, 2011). The assay consisted of pre-incubating β -casein with DegQ SA and monitoring complex formation by analytical SEC and SDS-PAGE analysis, as shown in figure 4.16.

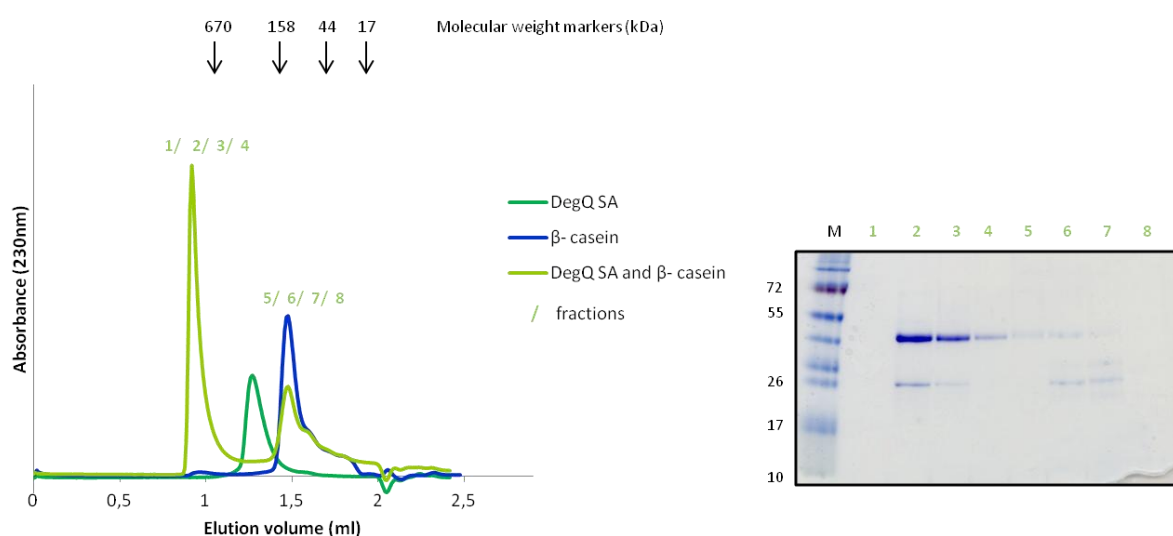


Fig. 4.16- Interaction studies: SEC with DegQ SA and β -casein.

The analytical SEC demonstrated that interaction between DegQ SA and β -casein occurred, leading to the formation of a higher order complex formed of β -casein and DegQ SA 24-mers. The complex was eluted at 0.97 ml. Furthermore, the SDS-PAGE analysis revealed that both proteins were collected in the same fractions number 2 and 3. These data confirmed the formation of a complex between DegQ SA and β -casein. This is in agreement with the interaction studies previously performed, reported in the attached publication (Sawa *et al.*, 2011).

6.6.3.2 Interaction studies with β -casein and DegQ SA IA FA FA mutant

Once it was known that DegQ SA could interact with β -casein, the ability of the DegQ SA IA FA FA mutant to interact with the same substrate was investigated. As previously mentioned, the mutant was pre-incubated with β -casein and the interaction was followed by analytical SEC and SDS-PAGE analysis (figure 4.17).

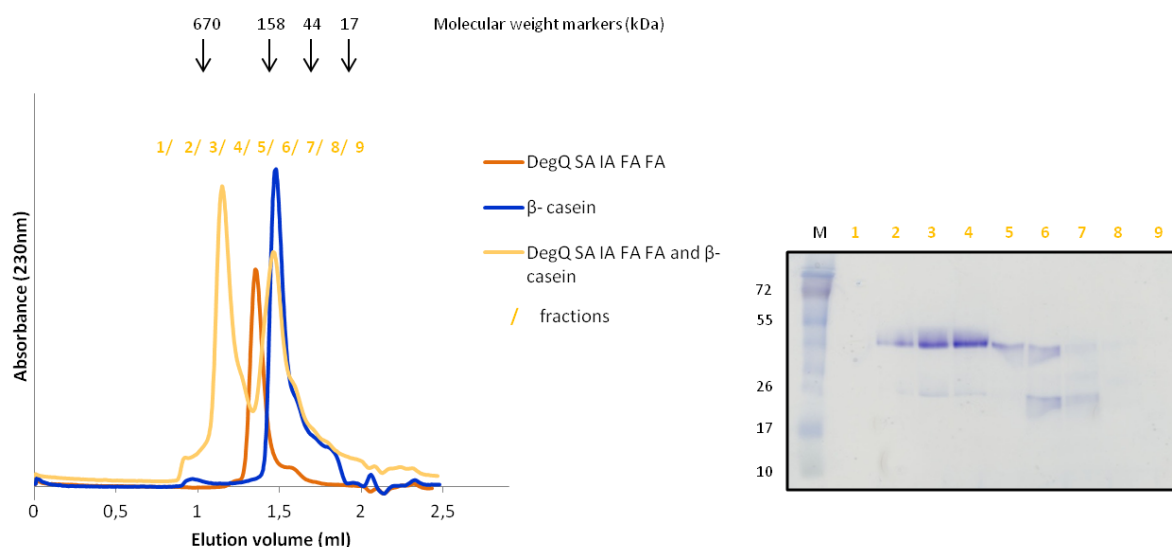


Fig. 4.17- Interaction studies: SEC with DegQ SA IA FA FA and β -casein.

The data obtained demonstrated that the DegQ SA IA FA FA mutant was able to interact with β -casein, which triggered a shift of the curve to higher order particles. The complex was

eluted at 1.15 ml. Moreover, the SDS-PAGE analysis showed that the mutant and β -casein interacted and were collected in the same fractions 3 and 4, confirming the complex formation.

6.6.3.3 Interaction studies with β -casein and DegQ SA FE FE mutant

The ability of the mutant DegQ SA FE FE to interact with β -casein was tested under the same conditions as formerly mentioned. The mutant and the substrate were pre-incubated and then potential complex formation was analyzed by analytical SEC and SDS-PAGE, as shown in figure 4.18.

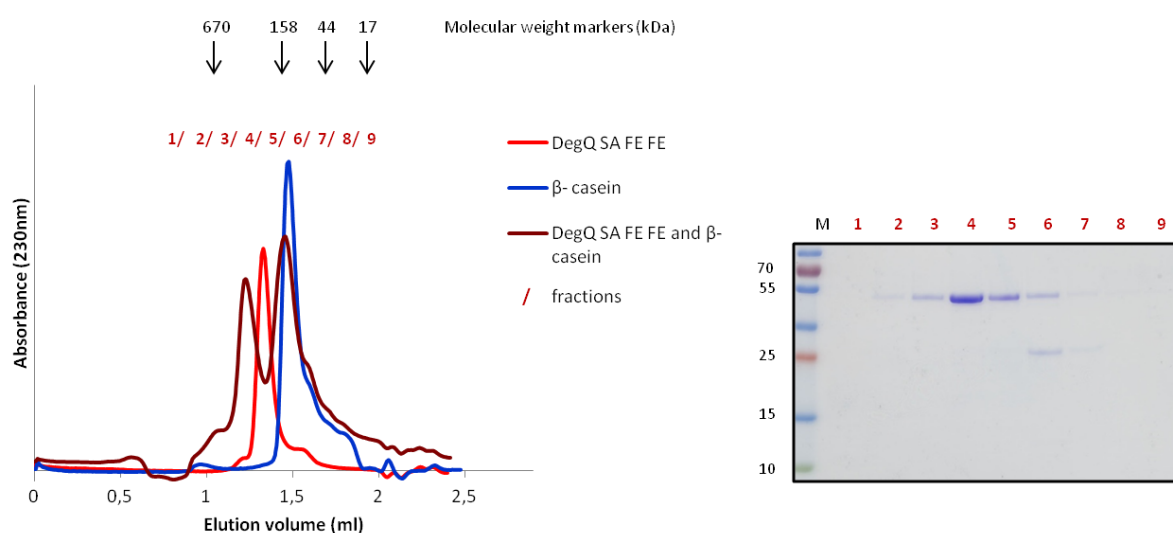


Fig. 4.18- Interaction studies: SEC with DegQ SA FE FE and β -casein.

The results obtained indicated that β -casein interacted with the DegQ SA FE FE mutant. In the analytical SEC, the DegQ SA FE FE mutant shifts to higher molecular weight. The potential complex between the DegQ SA FE FE mutant and β -casein was eluted at 1.22 ml. However, the SDS-PAGE analysis demonstrated that the DegQ SA FE FE mutant and the substrate were eluted separately in fractions 3 to 5 and 6, 7 respectively - not confirming the complex formation.

6.6.3.4 Interaction studies with β -casein and DegQ SA FE FR mutant

Interaction studies between DegQ SA FE FR mutant and β -casein were also carried out. The assays obeyed the same previously mentioned conditions. The substrate β -casein was pre-incubated with the mutant and the sample was submitted to analytical SEC and SDS-PAGE analysis (figure 4.19).

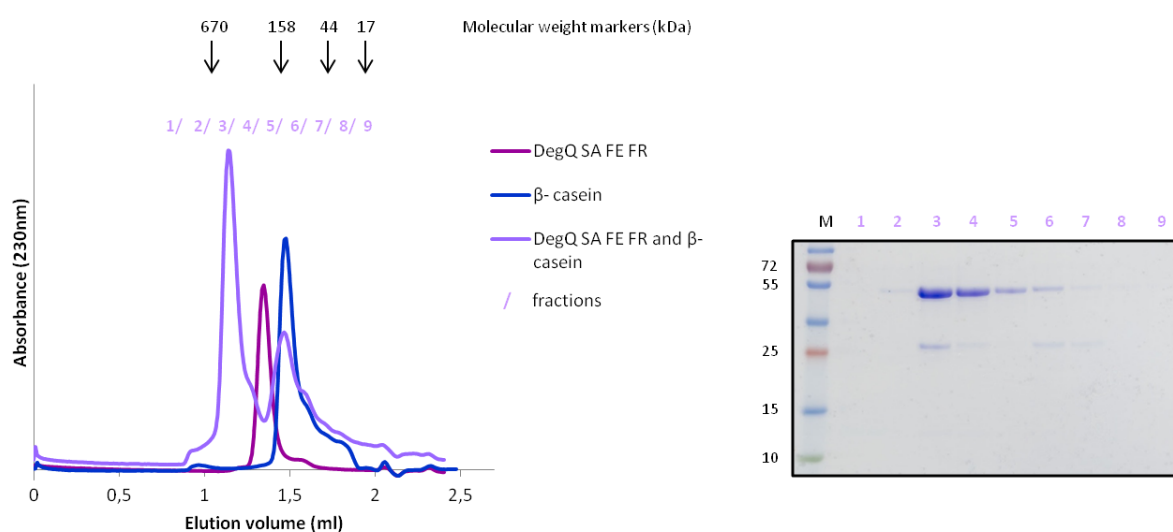


Fig. 4.19- Interaction studies: SEC with DegQ SA FE FR and β -casein.

The results showed that the DegQ SA FE FR mutant interacted with β -casein. The interaction triggered a shift of the curve to higher molecular weight. The complex between DegQ SA FE FR mutant and the substrate was eluted at 1.14 ml. Additionally, SDS-PAGE analysis showed that both proteins were collected concurrently in fractions 3 and 4, confirming that complex formation between the mutant and β -casein had occurred.

mutant	unfolded lysozyme	unfolded α -lactalbumin	β -casein
DegQ SA	clear interaction 12-mer formation (elution volume: 1.15 ml)	clear interaction 24-mer formation (elution volume: 1.0 ml)	clear interaction 24-mer formation (elution volume: 0.93 ml)
DegQ SA IA FA FA	no interaction no oligomerization	no interaction no oligomerization shoulder observed at the elution volume corresponding to the DegQ 12-mer, however no co- elution of the proteins observed in the SDS-PAGE	interaction 12-mer formation (elution volume: 1.16 ml)
DegQ SA FE FE	no interaction no oligomerization	no interaction no oligomerization shoulder observed at the elution volume corresponding to the DegQ 12-mer, however no co- elution of the proteins observed in the SDS-PAGE	potential interaction shift in elution volume to 1.22 ml observed, corresponding to the elution volume of the DegQ hexamer however, co-elution of β -casein and DegQ FE FE not confirmed in the following SDS-PAGE
DegQ SA FE FR	no interaction no oligomerization	no interaction no oligomerization shoulder observed at the elution volume corresponding to the DegQ 12-mer, however no co- elution of the proteins observed in the SDS-PAGE	interaction 12-mer formation (elution volume: 1.15)

Table 4.1- The table shows a resume of the performed interaction studies with DegQ SA variants and different unfolded substrates

6.7 Analysis of the DegQ chaperone activity - MaIS refolding assay

In order to evaluate the effect of the performed mutations on DegQ chaperone activity, a chaperone assay was performed.

A well-described chaperone assay used to further characterize the ATP-independent chaperone activity of DegQ SA and its mutants is the MaIS refolding assay. MaIS is a periplasmic α -amylase protein present in *E. coli* which cleaves long maltodextrins prior to transport to the cytoplasm (Spiess *et al.*, 1997). In addition, MaIS is a natural substrate of DegP (Spiess, Beil and Ehrmann, 1999). MaIS refolding assay was performed as described in the attached publications (Spiess *et al.*, 1997; Malet *et al.*, 2012). The assay is based on indirectly measuring the chaperone activity of a protein through measurements of MaIS activity. Unfolded MaIS is not able to degrade its substrate p-nitrophenylhexaoside (PNP6). For the assay, chemically unfolded MaIS was pre-incubated with DegQ SA and the MaIS substrate was subsequently added to the reaction. The activity of folded MaIS was monitored by the levels of the cleaved substrate chromogenic *p*-nitrophenol, which absorbs at 405nm (figure 4.20 A). Finally, the rate of MaIS refolding in the presence of a chaperone could be detected and compared to rates of MaIS spontaneously refolding, as shown in figure 4.20 B.

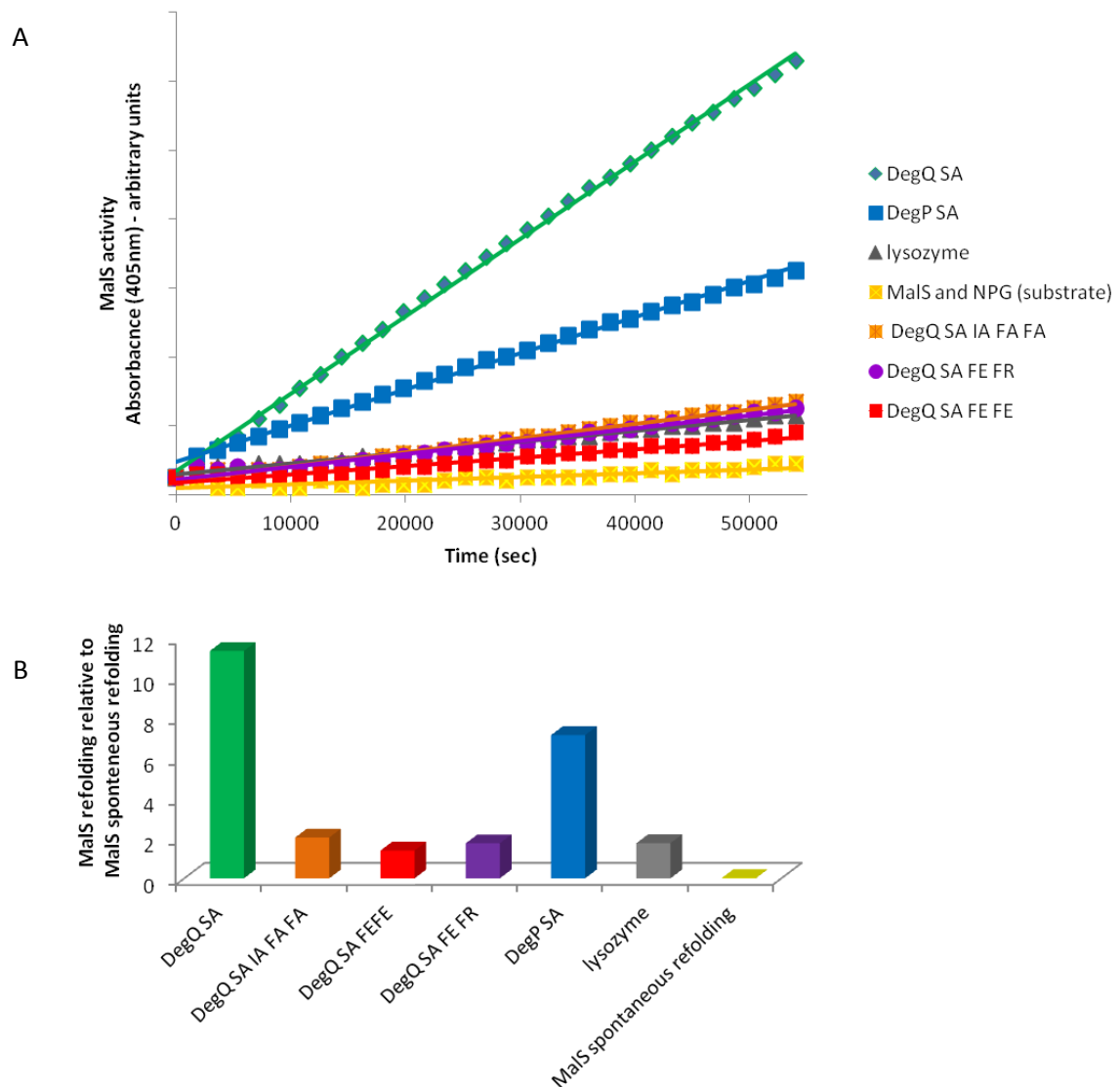


Fig. 4.20- MaIS refolding assay. (A) The graph shows the absorbance levels of the colorimetric reactions. (B) Bar chart corresponding to MaIS refolding levels in relation to MaIS spontaneously refolding.

As seen in figure 4.20 A, the results showed that DegQ SA was the most efficient chaperone in refolding MaIS, followed by DegP SA. In addition, it was also possible to observe that compared to the negative control lysozyme, all DegQ SA mutants showed very low and similar levels of chaperone activity. Furthermore, the same results were observed when the levels of MaIS refolding, in the presence of DegQ variants, were compared to levels of MaIS spontaneous refolding (figure 4.20 B). DegQ SA showed the highest levels of chaperone

activity in comparison to MalS spontaneously refolding. DegP SA also demonstrated significant levels of chaperone activity. However, the mutants DegQ SA IA FA FA, DegQ SA FE FE and DegQ SA FE FR showed comparable but unimportant levels of chaperone activity as the negative control lysozyme.

6.8 Analysis of the protease activity of DegQ

Since the experiments performed had shown that the mutations in the PDZ1 domain affected substrate binding and chaperone activity of DegQ, the protease activity was also evaluated. With the aim of investigating whether the mutations in the PDZ1 domain would affect the protease activity of DegQ, degradation assays with different unfolded substrates were performed. For the protease assays the chemically unfolded lysozyme and α -lactalbumin and the intrinsically unfolded β -casein were used as substrates. The degradation assays were carried out as described in the attached publication (Sawa *et al.*, 2011). For this assay, only the proteolytically active mutants were used. DegQ wt or the DegQ FE FR mutant was incubated with the unfolded substrates and their degradation was monitored by SDS-PAGE over time.

6.8.1 Degradation assay using unfolded lysozyme as substrate

The first model substrate to be tested was lysozyme. Chemically unfolded lysozyme was incubated with DegQ wt at 37°C and samples were collected. The same was done for the DegQ FE FR mutant. Finally, each of the collected fractions was submitted to SDS-PAGE analysis, as shown in figure 4.21.

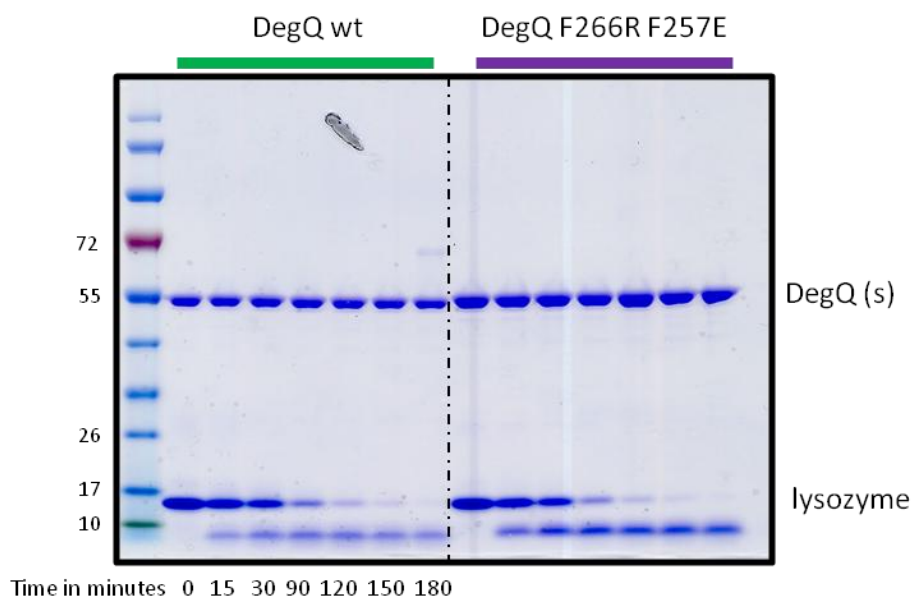


Fig. 4.21- Degradation assay. DegQ and DegQ FE FR were used at 2.5 μ M and lysozyme was used as substrate at 20 μ M. The reaction was supplied with 20mM DTT.

The results of SDS-PAGE analysis demonstrated that DegQ wt and the DegQ FE FR mutant were able to degrade unfolded lysozyme at comparable rates. In addition, it was possible to observe that in the lanes where the DegQ FE FR mutant was present, a protein band corresponding to 10kDa accumulated over time. The same band accumulated in the lanes corresponding to DegQ wt. However, the accumulated levels of the 10kDa protein band were lower in the presence of DegQ wt than in the presence of the DegQ FE FR mutant.

6.8.2 Degradation assay using unfolded α -lactalbumin as substrate

The degradation of chemically unfolded α -lactalbumin in the presence of DegQ wt or DegQ FE FR mutant was analyzed. For this assay the same experimental conditions as already mentioned were applied. The unfolded substrate was incubated with DegQ wt or the mutant at 37°C and fractions containing the reaction were collected at specific time points. Subsequently, these samples were analyzed by SDS-PAGE as shown in figure 4.22.

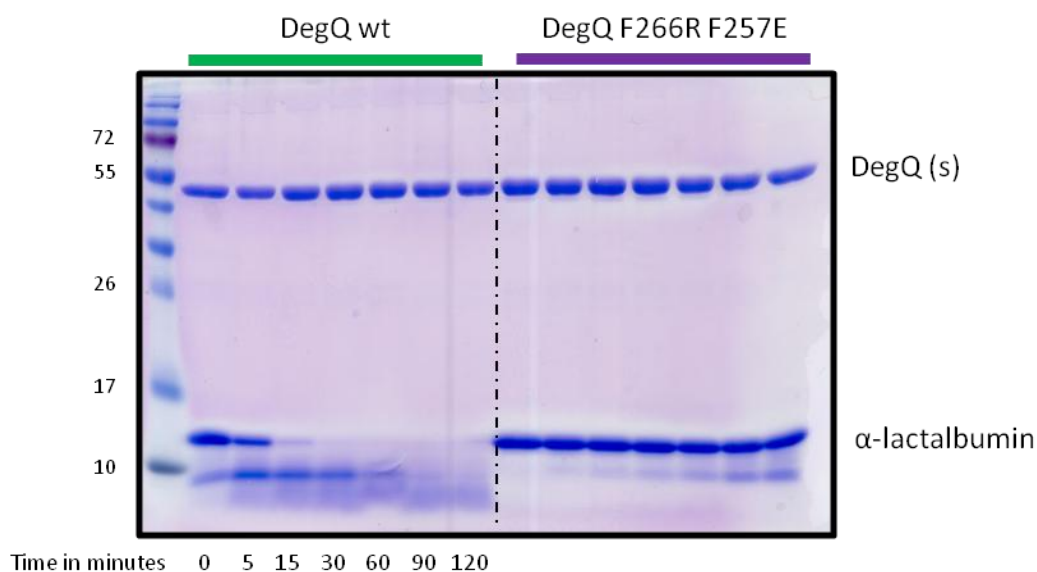


Fig. 4.22- Degradation assay. DegQ and DegQ FE FR were used at 2.5 μ M and α -lactalbumin was used as substrate at 20 μ M. The reaction was supplied with 40mM DTT.

It was observed that the unfolded α -lactalbumin was degraded in the presence of DegQ wt. In contrast, the Deg FE FR mutant degraded the unfolded substrate at negligible levels. In addition, the SDS-PAGE analysis revealed that a 10kDa protein band in the presence of DegQ wt was completely digested over time. However, in the presence of the DegQ SA FE FR mutant, the equivalent protein band accumulated during the incubation period.

6.8.3 Degradation assay using β -casein as substrate

The model substrate β -casein was used in this degradation assay. The degradation of the substrate in the presence of DegQ wt or DegQ FE FR was monitored by SDS-PAGE analysis. As mentioned before, β -casein was incubated with DegQ wt or the mutant and fractions of the reaction were collected at defined time points. Finally, the fractions were analyzed by SDS-PAGE (figure 4.23).

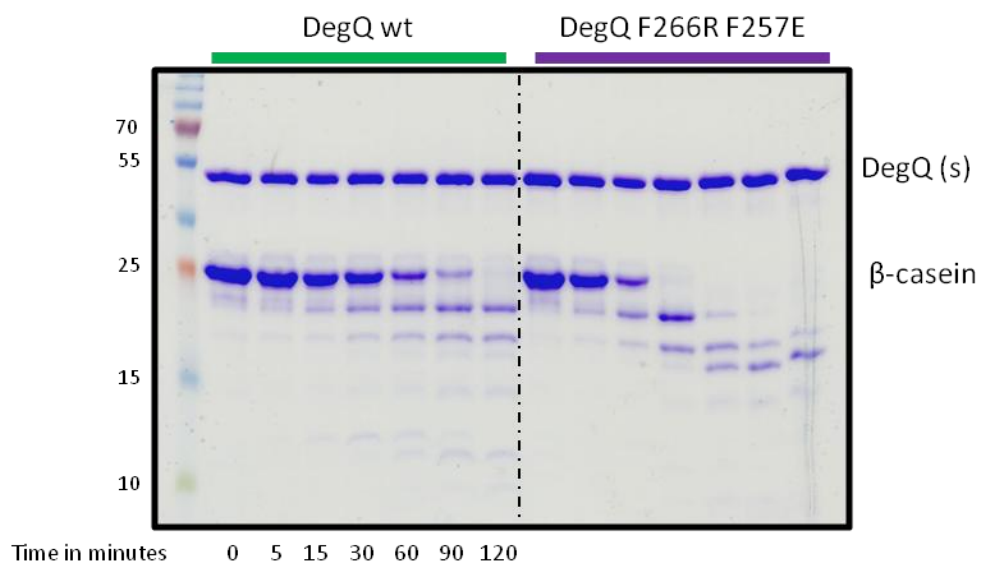


Fig. 4.23- Degradation assay. DegQ and DegQ FE FR were used at 2.5 μ M and β -casein was used as substrate at 20 μ M.

The SDS-PAGE analysis revealed that β -casein was degraded by wild-type DegQ and by the DegQ FE FR mutant. Furthermore, the formation of degradation intermediate products which degraded over time in the presence of both proteins was detected. However the wild-type DegQ and the Deg FE FR mutant exhibited different degradation patterns when degrading β -casein. Additionally, it was possible to observe that the mutant was more efficient than DegQ wt in degrading β -casein.

7 Discussion

DegQ, a protein which resides in the periplasm of Gram-negative bacteria, is a member of the HtrA protein family (Bass *et al.*, 1996; Waller and Sauer, 1996). Proteins belonging to this family have the exceptional ability to switch between protease and chaperone activity thereby performing protein quality control in many organisms. The proteins DegP and DegS also belong to the HtrA family and play together with DegQ an important role in maintaining protein quality in the bacterial periplasm (Korjer *et al.*, 2008; Sawa *et al.*, 2011; Spiess, Beil and Ehrmann 1999; Walsh *et al.*, 2003; Wilken *et al.*, 2004). Whereas DegP and DegS are proteins which were extensively studied, the structure and function of DegQ remained elusive (Krojer *et al.*, 2002; Wilken *et al.*, 2004; Kim and Kim, 2005). Therefore, the first work performed in this study aimed to structurally and functionally characterize DegQ with regards to its protease activity, see attached publication (Sawa *et al.*, 2011). In the subsequent study, the focus was on the characterization of the ATP-independent chaperone activity of DegQ. This work provided the structural framework for the chaperone activity of DegQ, see attached publication (Malet *et al.*, 2012). In this thesis, the overall aim was to further characterize the molecular details of the chaperone activity of DegQ. The data summarized in the publications by Sawa *et al.* and Malet *et al.* as well as additional data provided in this thesis are discussed in the following sections.

7.1 The mutations in the PDZ 1 domain lead to impairment of interactions with unfolded substrates

The electron microscopy (EM) study performed by Malet *et al.* showed that in the DegQ SA dodecamer in complex with folded lysozyme three parts of DegQ were interacting with the

substrate (Malet *et al.*, 2012). The corresponding parts are the residues 33 to 63 of the protease domain, the residues 408 to 413 of the PDZ 2 domain and the residues 251 to 257 of the PDZ 1 domain. Furthermore, the same regions were also suggested to be located in close proximity to the density of β -casein in the DegQ 24-mer structure (figure 5.1). It is therefore supposed that these different hydrophobic patches in DegQ might be important for substrate binding. Due to the close proximity to the folded substrate within the dodecameric DegQ complex, the identified regions might be involved in substrate binding to the DegQ SA hexamer.

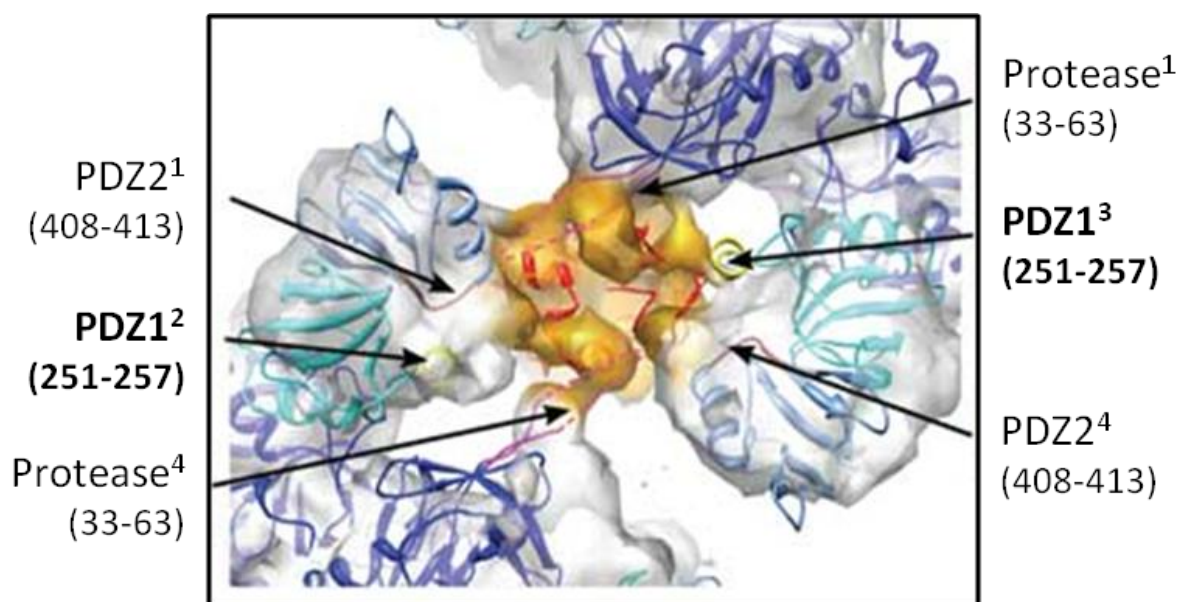


Fig. 5.1- Interaction between DegQ dodecamer and lysozyme. The figure shows a zoomed-in view corresponding to the interactions between DegQ domains from different protomers and folded lysozyme (figure adapted from Malet *et al.*, 2012).

The identified regions could be substrate binding sites in DegQ mediating the interaction of this periplasmic protein with different substrates. In the amino acid sequence corresponding to the loop LA, for example, are three conserved phenylalanine residues (F45, F48 and F49 in DegQ) which are also present in DegP and are possibly involved in substrate binding in DegQ. As already mentioned, the size of the loop LA is one the most pronounced features which distinguishes DegP from DegQ. The loop LA of DegP is 20 amino acids longer than the one in

DegQ. In fact, the loop LA is longest in DegP among other members of the HtrA family which have much shorter LA loops as DegQ (Kim and Kim, 2005). Since the loop LA is projected into the active site of the opposite trimer, the size of the loop determines the distance between the two trimers of one hexamer. In this way, the loop LA in DegP provides hydrophobic docking platforms in the inner cavity of the DegP hexamer. Its height (15Å) restricts the access to single secondary structure elements (Clausen *et al.*, 2002). As the loop LA of DegQ is much shorter, the access to the hydrophobic patch within the DegQ hexamer might offer different restrictions concerning the size of the substrates. Therefore, it is tempting to suggest that the loop LA might confer different substrate specificity to DegQ and DegP.

In addition to the loop LA, another interactive region mapped by the EM studies is the PDZ 2 domain. The PDZ domains are conserved protein units which mediate specific protein-protein interactions (Doyle *et al.*, 1996). Among the HtrA proteins present in *E. coli*, only DegP and DegQ have two PDZ domains. In DegP, the PDZ 2 domain was suggested to be mostly involved in the maintenance of the hexameric state and higher oligomer assembly (Sassoon *et al.*, 1999; Iwanczyk *et al.*, 2007; Meltzer *et al.*, 2008). Similarly, it was shown that the PDZ 2 domain of DegQ is dispensable for protease activity and substrate binding in other organisms, as shown for DegQ from *L. fallonii* (Sawa *et al.*, 2011; Wrase *et al.*, 2012). In addition, it was also demonstrated that a DegQ mutant lacking the PDZ 2 domain was able to form higher order oligomers (Sawa *et al.*, 2011). Due to all these reasons, the PDZ 2 domain was not considered as attractive as the PDZ 1 domain to be further analyzed as potential substrate binding site.

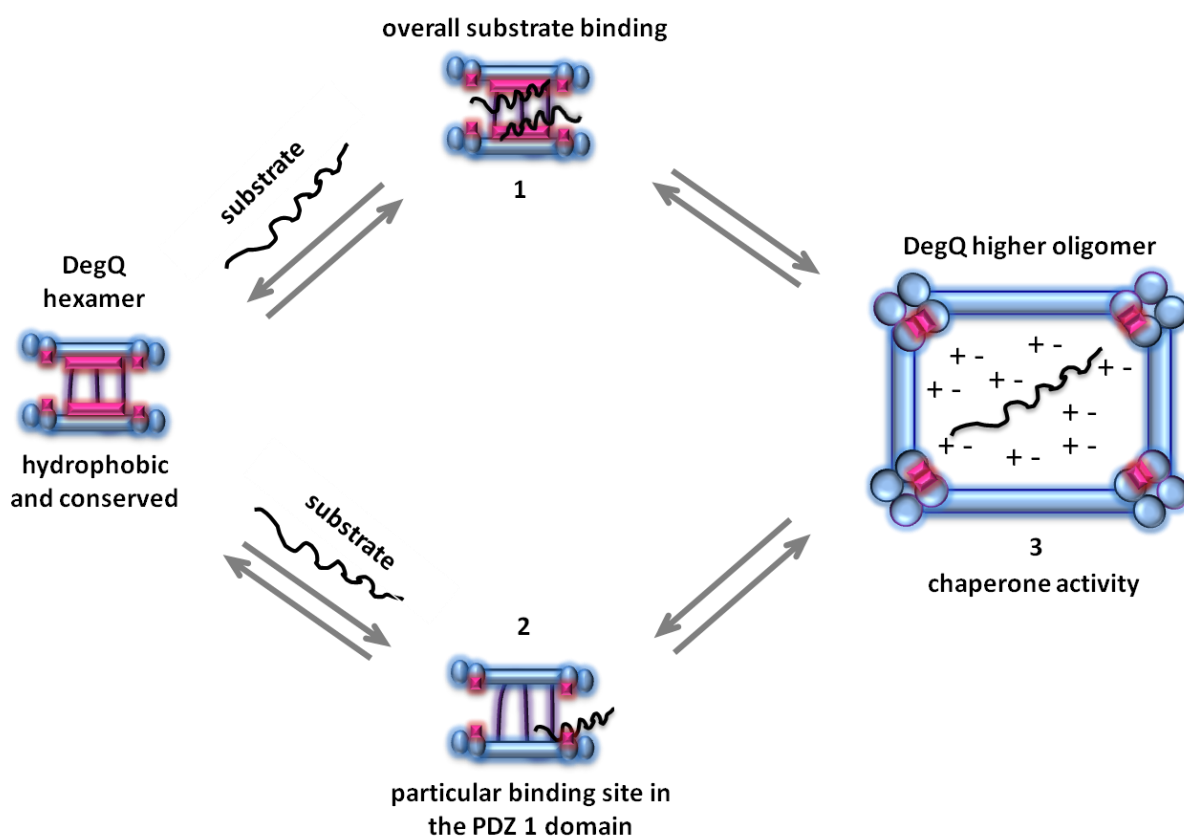


Fig. 5.2- Overall DegQ binding sites. (1) Substrate binding regions of DegQ (blue) in the protease and PDZ 1 domains are shown in pink. (2) Only the hydrophobic patch in the PDZ 1 domain interacting with unfolded substrate is highlighted in pink. (3) The oligomerization of DegQ after substrate binding is shown. The hydrophobic patches in the PDZ 1 domain do not interact with the substrate upon oligomerization but are rather buried within the wall of the DegQ higher oligomer.

In DegP, the PDZ 1 domain was described as a mediator for substrate and activator peptide binding. Similar to DegP, peptide binding to the DegQ PDZ 1 domain was sufficient to trigger oligomer conversion. It is therefore suggested that the PDZ 1 domain of DegQ might also be involved in substrate binding. Thus, in combination with the obtained EM data by Malet *et al.*, the PDZ 1 domain was chosen for mutational analysis in order to further evaluate its role in substrate binding.

As already mentioned, a mutational analysis of the PDZ 1 domain of DegQ was performed. In an attempt to explore potential substrate binding sites in DegQ, the ability of DegQ variants to interact with unfolded substrate was evaluated. For this, proteolytically inactive DegQ

mutants and different model substrates were used. All the interaction studies were performed by analytical SEC and SDS-PAGE analysis. As expected, DegQ SA was able to interact with all tested unfolded substrates (lysozyme, α -lactalbumin and β -casein). In addition, DegQ SA assembled to higher order oligomers forming a complex with the tested unfolded substrates (figures 4.8, 4.12, 4.16). However, the DegQ SA IA FA FA, DegQ SA FE FE and DegQ SA FE FR mutants did not interact with the chemically unfolded substrates lysozyme and α -lactalbumin and therefore, no complex formation was observed (figures 4.9 to 4.11 and 4.13 to 4.15, respectively). Nevertheless, it is important to state that in the presence of α -lactalbumin a “shoulder” was observed in the SEC analysis indicating the formation of higher oligomers of the mutants. Although this small shift was observed in the SEC, the SDS-PAGE analysis did not show that complex formation occurred. Perhaps, a more sensitive gel staining method as silver staining would be able to reveal the interaction between the DegQ SA IA FA FA, DegQ SA FE FE and DegQ SA FE FR and α -lactalbumin. However, in order to show that higher order oligomer assembly occurred, EM analysis of the complex with unfolded α -lactalbumin is required. Furthermore, all the DegQ SA variants in the trimeric state did not oligomerize to higher order oligomers in the presence of unfolded lysozyme.

On the other hand, the selected DegQ SA mutants were able to interact with the intrinsically unfolded model substrate β -casein. Although a shift corresponding to higher order oligomers was observed in the SEC analysis when the DegQ SA IA FA FA and DegQ SA FE FR mutants were in the presence of β -casein, electron microscopy would be required for validation (figures 4.17 and 4.19). In the case of the DegQ SA FE FE mutant, interaction with β -casein also occurred but since the observed shift was less pronounced in comparison to the other mutants, interaction might have occurred in lower levels (figure 4.18).

To summarize, these data demonstrated that the DegQ SA IA FA FA and DegQ SA FE FR DegQ FE FE might have retained the ability to interact with β -casein to different degrees. Additionally, the interaction studies also showed that a weak interaction with unfolded α -

lactalbumin might have occurred. Furthermore, the tested DegQ SA mutants did not interact with unfolded lysozyme.

A possible explanation for the sustained interaction with β -casein could be that β -casein either interacts with DegQ by the mutated residues, other hydrophobic patches or another not yet determined binding site. In contrast, the substrates lysozyme and α -lactalbumin might need specific interactions with the residues F257 and F266 of the PDZ 1 domain to bind and trigger the oligomerization of DegQ. Therefore, the interaction with these unfolded substrates might have been strongly or completely impaired.

The obtained results lead to the conclusion that additional unfolded substrates should be tested in the future in order to further evaluate the role of the mutated hydrophobic patch in the PDZ 1 domain concerning substrate binding.

Whereas DegQ SA was purified as a hexamer and no presence of OMPs was detected, the DegQ SA FE FR mutant was purified as trimers and dodecamers in complex with OMP-A. This data indicate a pre-disposition of the DegQ SA FE FR mutant to interact with native substrates. Native substrates of DegQ should be investigated and used for *in vitro* assays as interaction studies, for example. Thereby, the role of the residues F257 and F266 in the PDZ 1 domain would be more suitably evaluated concerning substrate binding to the suggested DegQ SA mutants.

7.2 The selected mutants show impaired chaperone activity

The previous experiments had shown that the mutations introduced in the PDZ 1 domain impaired the interaction with the unfolded substrates lysozyme and α -lactalbumin. The following experiments were performed to evaluate the ATP-independent chaperone activity

of DegQ and the selected mutants. For this purpose, a well-established chaperone assay was applied.

The MaS refolding assay aimed to indirectly measure the chaperone activity of DegQ, as already mentioned in the attached publication (Malet *et al.*, 2012). MaS is a periplasmic protein and was characterized as a natural substrate of DegP (Spiess *et al.*, 1997; Spies, 1999). Since DegP and DegQ are homologous proteins, it was expected that DegQ is also able to refold MaS. Thus, in an attempt to further investigate the ATP-independent chaperone activity of DegQ SA and variants, the MaS refolding assay was carried out (figure 4.20). As expected, the results showed that DegQ SA efficiently refolded MaS. Similarly, but in lower levels DegP also showed chaperone activity, suggesting that DegQ is a more efficient chaperone in refolding MaS than DegP. The DegQ SA FE FE, DegQ SA IA FA FA and DegQ SA FE FR mutants were not able to refold MaS. The exhibited levels of chaperone activity were comparable to the negative control, lysozyme. Consequently, it is suggested that the chaperone activity was impaired in these mutants. In order for MaS to regain its full activity it has to first be bound by DegQ and then fold in the hydrophilic chamber provided by the chaperone upon oligomerization. It should be also noted that unlike the DegQ SA FE FE and DegQ SA IA FA FA mutants the DegQ SA FE FR mutant oligomerized in the presence of OMP-A, a natively co-purified protein. Therefore, it is suggested that the mutations resulting in opposite charged residues (glutamate and arginine) allowed the oligomerization of the DegQ SA FE FR mutant to higher order oligomers. Thus, the impaired chaperone activity of this mutant might be due to a disruption in the specific substrate binding sites (F257 and F266) that MaS might require to interact with DegQ. Furthermore, in the case of the substrates lysozyme and α -lactalbumin, the interaction was simply abrogated and therefore no evident oligomerization occurred.

It has been shown for many chaperones that the folding of the substrate must occur inside the provided hydrophilic cavity. In the case of GroEL/GroES, the oligomeric structure is required for protein folding (Weber *et al.*, 1998; Brinker *et al.*, 2001). However, for other

chaperones, like HdeA, oligomerization is not required (Tapley *et al.*, 2009). HdeA is a holdase chaperone, as other sHSPs (small Heat Shock Proteins) (Lee *et al.*, 1997; Tapley *et al.*, 2009). Besides, HdeA is one of the smallest known chaperones (Tapley *et al.*, 2009). Holdase chaperones bind partially unfolded polypeptides so that their capability to fold is preserved (Lee *et al.*, 1997; Sun and MacRae, 2005). Hence, HdeA releases its clients in a nonnative but folding-competent state (Tapley *et al.*, 2010). Thus, holdase chaperones can protect cells from protein losses or protein aggregation. However, the holdases must pass their aggregation-prone substrates on to the downstream chaperones that facilitate folding (Sun and MacRae, 2005).

HdeA is a periplasmic protein that senses low pH conditions leading to the activation of its chaperone function. Under normal conditions, HdeA is an inactive dimer. Upon activation by acidic conditions, HdeA becomes a monomer exposing its hydrophobic dimer interface suitable for substrate binding and capable of preventing the irreversible aggregation of the substrate. Finally, pH neutralization triggers the release of the substrate from HdeA (Tapley *et al.*, 2010). Since DegQ was already shown to be an active protease under acidic conditions, it would be interesting to analyze the effect of pH variation on its chaperone activity.

It is important to note that DegQ could act as a holdase avoiding aggregation of misfolded proteins. It has been shown that DegQ from *Legionella fallonii* was able to exert its chaperone activity independent of oligomerization to dodecamers – the trimers (substrate-capturing state) were active as chaperones. Therefore, considering DegQ as a holdase chaperone, it is suggested that the DegQ SA IA FA FA, DegQ SA FE FE and DegQ SA FE FR mutants show impaired chaperone activity due to their inability to properly interact with unfolded substrates. The results furthermore indicate that the residues F257 and F266 might be required for specific recognition and interaction with unfolded MalS.

As mentioned before, there are also chaperones that can only refold their substrates inside the hydrophilic compartment that those chaperones can provide. The chaperonin GroEL/GroES is one of the well-studied chaperones that facilitate the folding of a substrate within its hydrophilic cavity. To interact with unfolded substrates, the chaperonin GroEL exposes its hydrophobic binding sites in the substrate-capturing state. However, upon ATP and GroES binding, GroEL undergoes a huge conformational change leading to release of the substrate into the GroEL/GroES chamber (see figure 1.3). Additionally, the hydrophobic sites which were previously interacting with the substrate are buried during the conformational change, generating a hydrophilic environment for the substrate to fold (Horwich *et al.*, 2007). Similarly in DegQ, it was also observed that in the substrate-capturing state - the hexameric form – DegQ also exposes its hydrophobic patches, as seen in the bis-ANS binding assay (figure 4.7). It was furthermore shown that in the dodecameric form of DegQ, the hydrophobic binding sites are internalized and consequently not available for interaction. Thus, it is suggested that DegQ, if acting as a folding chaperone would expose its hydrophobic binding sites in the substrate-capturing state. In addition, upon substrate binding, DegQ would undergo oligomerization into higher order oligomers. The oligomerization would promote substrate release into the DegQ cage. Furthermore, burial of the hydrophobic patches would occur providing a hydrophilic environment which would favor substrate folding.

Taking together these observations, it is tempting to suggest that DegQ could act as a holdase and as a folding chaperone. Perhaps, substrate specificity would determine in which chaperone mode DegQ would exert its ATP-independent chaperone function. The mutations in the PDZ 1 domain were shown to affect the efficiency of DegQ in refolding the substrate MalS. While DegQ SA was able to competently refold MalS, the mutants DegQ SA IA FA FA, DegQ SA FE FE and DegQ SA FE FR showed impaired chaperone activity due to the fact that a proper interaction with the unfolded substrate did not occur, preventing DegQ to act either as a holdase or as a folding chaperone, concerning MalS.

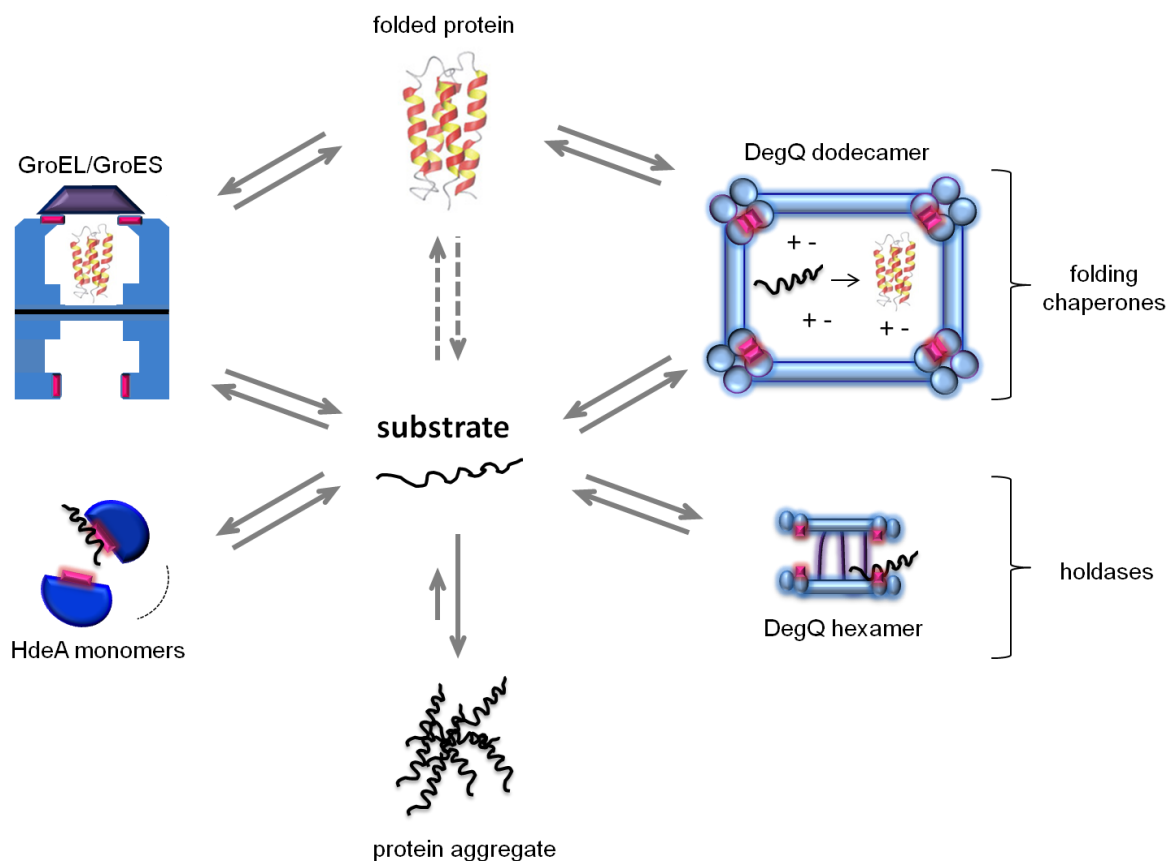


Fig. 5.3- Schematic representation of DegQ chaperone activity. The cartoon shows how DegQ could act as a holdase chaperone (similar to HdeA) preventing unfolded protein aggregation - or as a folding chaperone (similar to GroEL/GroES) providing an encapsulated hydrophilic environment to promote substrate folding.

To clarify whether DegQ act as a holdase, a folding chaperone or both, further experiments are required. Nevertheless, the obtained data suggest that the hydrophobic patch in the PDZ 1 domain is required for substrate binding of specific substrates. Consequently, a proper interaction with the unfolded substrate would allow DegQ to act as holdase preventing protein aggregation - or promote DegQ oligomerization to finally be converted into an active folding chaperone.

7.3 The protease activity of DegQ is affected by mutations in the PDZ 1 domain in a substrate-dependent manner

To investigate the effect of the PDZ 1 domain mutations on the protease activity of DegQ, degradation assays were performed. For the assays, the proteolytic activity of wild-type DegQ and its variant DegQ FE FR were tested using different model substrates. It is important to note that while wild-type DegQ is a hexamer, DegQ FE FR was purified as a trimer (figure 4.4). The working mechanism of the protease activity of DegQ is described in figure 5.4.

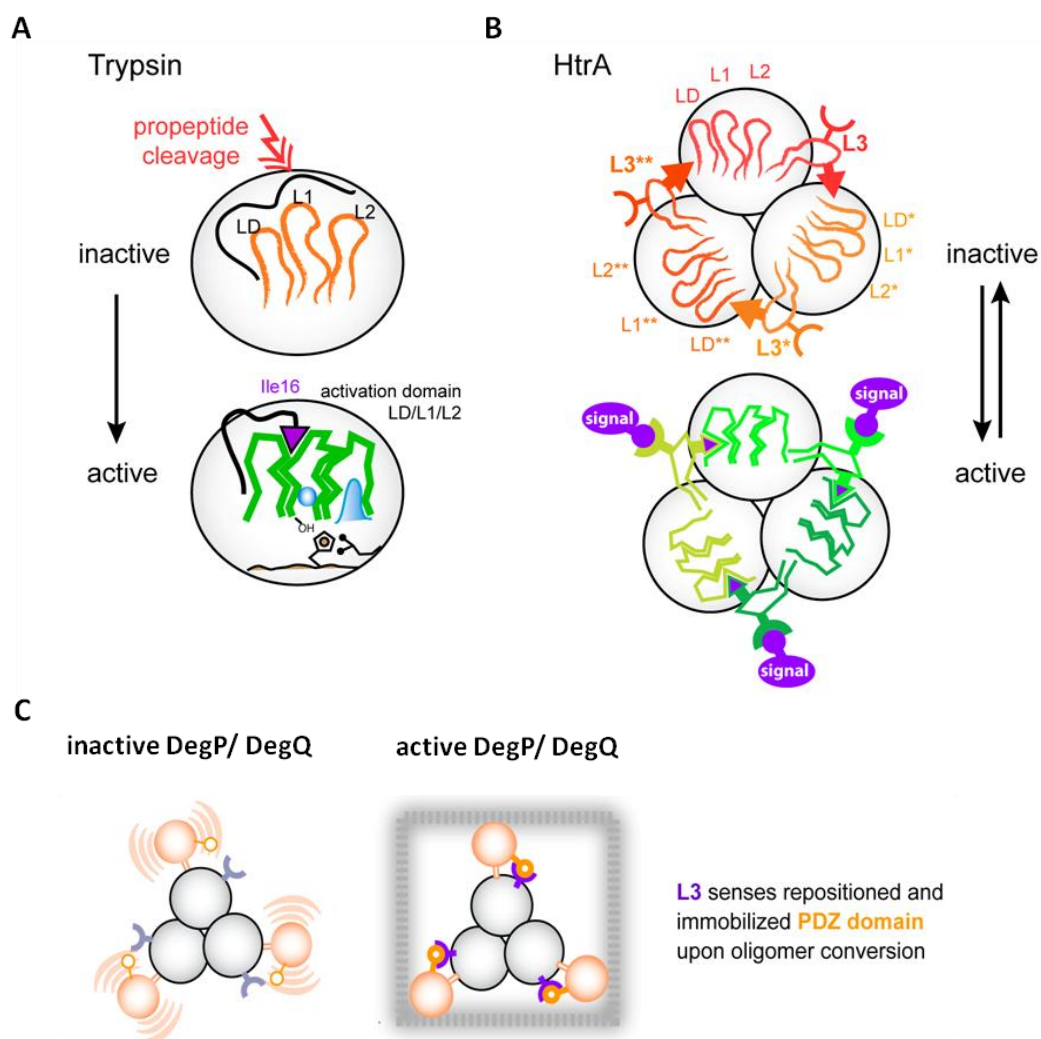


Fig.5.4- Regulation of HtrA protease activity. (A) The activation of trypsin, a canonical serine protease is shown. A cleavage in a propeptide (lilac) of the inactive trypsinogen generates a new amino terminus at isoleucine 16 (Ile16, shown by arrowhead) which binds to a hydrophobic pocket of trypsin leading to its protease activation. During trypsin activation, the loops L1, L2 and LD undergo a conformational change from a disordered to an ordered state. This conformation is characterized by a functional catalytic triad (the side chains of serine, histidine and aspartate are indicated), an oxyanion hole (blue sphere) and a substrate-specificity pocket, shown in blue. **(B)** The protease activation mechanism of HtrA proteins is illustrated. Similarly to trypsin activation, the loops L3, LD, L1 and L2 transit from a disordered to an ordered state but with a reversible activation. A specific arginine residue in loop L3 interacts with the activation loop LD of an adjacent subunit (indicated by * and **), leading to the adjustment of the activation domain. **(C)** The substrate-induced oligomer conversion triggering the transition from an inactive to an active DegP/DegQ protein leads to the re-localization and immobilization of the PDZ domains. The loop L3 interacts with the re-oriented PDZ 1 domain, stabilizing the active state of the protein (figure adapted from Clausen *et al.*, 2011).

Unfolded lysozyme was used as substrate and degraded by both proteins. However, the DegQ FE FR mutant was less efficient in degrading lysozyme (figure 4.21). It was observed that a 10kDa protein fragment, which might be an intermediate degradation product, accumulated in the presence of both proteins. Nevertheless, comparing both reactions, it was noticed that the amount of the corresponding protein band was higher in the presence of the mutant than in the presence of wild-type DegQ. This leads to the conclusion that the proteolytic activity of DegQ towards unfolded lysozyme was affected by the mutations in the PDZ 1 domain, resulting in a decrease of the degradation rates.

When β -casein was used as substrate, the DegQ FE FR mutant was faster in degrading it than wild-type DegQ (figure 4.23). Additionally, DegQ FE FR mutant and wild-type DegQ exhibited different degradation patterns. The SDS-PAGE analysis showed that β -casein was degraded by the proteases in a different manner regarding the size of the cleavage products. In the presence of the DegQ FE FR mutant an accumulation of a degradation product corresponding to approximately 17kDa was observed during the degradation process of β -casein. However, in the presence of wild-type DegQ the accumulation of this 17kDa degradation product was not observed. Furthermore, the appearance of smaller degradation products (lower than 15kDa) only in the presence of wild-type DegQ could indicate that the initial cleavage products were further degraded. Hence, the mutations in the PDZ 1 domain

affected the protease activity of the DegQ FE FR mutant in degrading β -casein. Precisely, the mutations in the residues F257 and F266 affected the digestion patterns generated by the DegQ FE FR mutant possibly due to the altered substrate binding and oligomerization behavior. As already mentioned, while wild-type DegQ is a hexamer with internal hydrophobic binding sites, the DegQ FE FR is a trimer in the resting state. Additionally, the mutant was faster than the wild-type in degrading β -casein. Whereas the wild-type DegQ had to disassemble its hexamers into trimers in order to oligomerize to higher order oligomers, the DegQ FE FR mutant was already in the trimeric form. Therefore, it is suggested that the trimeric form of the DegQ FE FR mutant facilitated a faster degradation rate. Regarding the different degradation pattern exhibited by the DegQ FE FR mutant, it is also suggested that the mutations in the PDZ 1 domain affected the interaction with β -casein. The interaction indeed occurred but other binding sites might have been accessed. Therefore, the substrate-induced oligomer conversion which leads to re-localization and immobilization of the PDZ domains might have been affected by the mutations in the PDZ 1 domain. This effect could account for the different degradation patterns generated by the DegQ FE FR mutant.

Unfolded α -lactalbumin was also tested as a model substrate in protease assays (figure 4.22). The results showed that wild-type DegQ completely degraded unfolded α -lactalbumin. However, the DegQ FE FR mutant was not able to degrade α -lactalbumin as efficient as wild-type DegQ. The obtained results showed that in the presence of wild-type DegQ, a 10kDa protein fragment accumulated over time but was further degraded. However, in the presence of the mutant DegQ FE FR, the same protein band also accumulated during the assay but without being further degraded. Additionally, it was observed that wild-type DegQ was much faster in degrading α -lactalbumin than the DegQ FE FR mutant. At the time when the 14 kDa α -lactalbumin band is completely degraded in the presence of DegQ, a faint band (10kDa protein fragment) for the intermediate degradation product appears in the presence of the DegQ FE FR mutant. Thus, it is suggested that the mutations in the PDZ 1 domain altered the protease activity of DegQ in degrading α -lactalbumin by altering pattern and the rate of the degradation.

Finally, these data led to the conclusion that the protease activity of DegQ is affected by the introduced mutations in the PDZ 1 domain. In addition, it is suggested that the mutations affected the degradation pattern and rate of the DegQ protease. The observed effects are in agreement with the work from Sawa *et al* where it has been shown that the protease and PDZ 1 domains are important for DegQ protease activity (Sawa *et al.*, 2011). However, the data furthermore suggest that the observed effects in the protease activity are substrate-dependent. Three different model substrates were tested and different outcomes were obtained. Whereas β -casein was faster degraded by the DegQ FE FR mutant, unfolded lysozyme was degraded at lower rates and unfolded α -lactalbumin was degraded in insignificant levels by the mutant protein. Furthermore, based on degradation of β -casein the data suggest that DegQ unlike DegP is not able to processively degrade the tested substrates. Processive proteases as DegP retain their substrates until they are cleaved into small peptides (Krojer *et al.*, 2008). Thus, degradation patterns of processive proteases remain constant. In contrast, in the presence of wild-type DegQ, the degradation pattern of β -casein changed with time.

8 Conclusions

The work presented in this thesis provides important insights into DegQ structure and function. The biochemical and structural data reveal that DegQ, like DegP, is able to oligomerize into higher order particles upon substrate binding. The work, performed with DegQ lacking the PDZ 2 domain, showed that trimers can reassemble into larger complexes. Since the PDZ 1 domain is a feature present in most of the HtrA proteins, this finding represents a common activation mechanism among HtrA protein family members. In addition, the provided crystal structure of DegQ in combination with mutational analysis led to the characterization of the protease activation mechanism of DegQ. It was shown that a conserved activation cascade is required for protease activation. The mechanism consists of re-location and immobilization of the PDZ domains upon substrate-induced oligomer conversion. The re-located PDZ 1 domain is thereby able to interact with loop L3 and to stabilize the active protease state (see figure 3, Sawa *et al.*, 2011; Clausen *et al.*, 2011).

Furthermore, consistent *in vivo* and *in vitro* functional data revealed that DegQ is a pH-sensitive protease providing protein quality control in the bacterial periplasm. The pH-sensitive proteases assume their active conformation upon pH change. In the case of DegQ, acidic conditions (pH 5.5) are sufficient to trigger oligomer conversion and proteolytic activity (see figure 6, Sawa *et al.*, 2011). DegQ is not the only HtrA protein which is pH-regulated. The Deg1 and Deg2 proteins reside in chloroplasts of *Arabidopsis thaliana* and were also shown to be pH regulated (Chassin *et al.*, 2002; Haussuehl *et al.*, 2001). Protease activation upon pH variation is an extremely useful adaptation to promote protein quality control in the periplasm. Since the periplasmic space is highly subjected to pH variation due to the membrane porosity, DegQ can work as a protease to deal with pH-mediated misfolded proteins (Sawa *et al.*, 2011). In addition, the fact that the protease activity of DegQ is activated by acidic pH provides extra protection against protein denaturation under

acidic conditions. Thus, this adaptation might also be important for *E. coli* when entering a host gut, which would place the bacteria at acidic conditions. On a gene-regulation level, the gene *degQ* is located directly upstream of the *degS* gene (Ehrmann, 2006). The *degQ* and *degS* genes are transcribed in the same direction, but seem to be separately regulated. Unlike *degP*, none of these genes are heat inducible (Waller and Sauer, 1996). The expression of DegP is regulated on the gene level by the stress-response pathway, whereas DegQ is rather expressed as a house-keeping protein. These data provide indications that although DegP and DegQ share 60% sequence similarity, they exhibit functional differences.

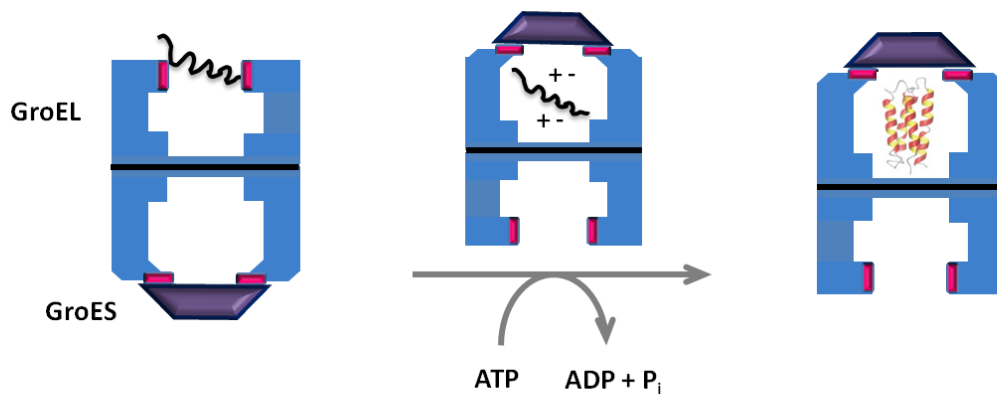
As already mentioned, DegQ and DegP have similarities and disparities. Since DegP had been shown to function as a protease and chaperone in the bacterial periplasm, the potential chaperone activity of DegQ was also investigated. Initially, structural studies using electron microscopy were performed with DegQ in complex with different substrates. The obtained results provided the structure of the DegQ dodecamer and 24-mer in complex with lysozyme and β -casein, respectively. Despite the low resolution, it was possible to detect folded lysozyme inside the DegQ chamber, giving first structural evidence that DegQ indeed also functions as a chaperone. Additionally native mass spectrometry analysis revealed the stoichiometry of the DegQ dodecamer-lysozyme complex in which up to six lysozymes can fit inside the DegQ dodecameric chamber (see figure 3, Malet *et al.*, 2012).

Finally, in an attempt to characterize the molecular aspects of the DegQ ATP-independent chaperone activity, biochemical studies and mutational analysis were performed. The results showed that mutations in the PDZ 1 domain impair the interaction with particular unfolded substrates. When the residues F257 and F266 are mutated to alanine or to oppositely charged amino acids (glutamate and arginine, respectively), DegQ is not able to interact with the unfolded substrates lysozyme and α -lactalbumin. These data, in combination with the electron microscopy data provided by Malet *et al.* directly implicate the mutated DegQ residues in substrate binding. It was also revealed that the chaperone activity of the DegQ SA FE FR mutant was impaired. The data suggest that the mutations in the PDZ 1 domain

impaired substrate binding and consequently oligomerization of DegQ, affecting its holdase and/or folding chaperone function(s). In addition, the protease activity of DegQ in the presence of the described mutations was also analyzed. The obtained data showed that the protease activity of the DegQ FE FR mutant was affected. The DegQ FE FR mutant was able to degrade the substrates, lysozyme at lower rates and α -lactalbumin in insignificant rates. In addition, in a substrate specific-manner, the mutant also exhibited a different degradation pattern in comparison to wild-type DegQ.

Ultimately, to finally show that the periplasmic protein DegQ is able to refold misfolded proteins as chaperonins do, further experiments are required. Interaction studies with MalS, for example, could be used to corroborate that the chaperone activity of the DegQ SA FE FR mutant is impaired due to inappropriate interaction with the substrate protein. Further evidence that the DegQ SA FE FR mutant can oligomerize would be also supportive. For example, electron microscopy of the dodecameric DegQ SA FE FR mutant would be extremely helpful to finally prove that the mutant can indeed oligomerize. Once these experiments are performed, it will be possible to correlate the DegQ ATP-independent chaperone function in the bacterial periplasm with the ATP-dependent chaperonins in the cytoplasm. A suggested model for the mechanism of the ATP-independent chaperone activity of DegQ is shown (figure 6.1).

ATP-dependent



ATP-independent

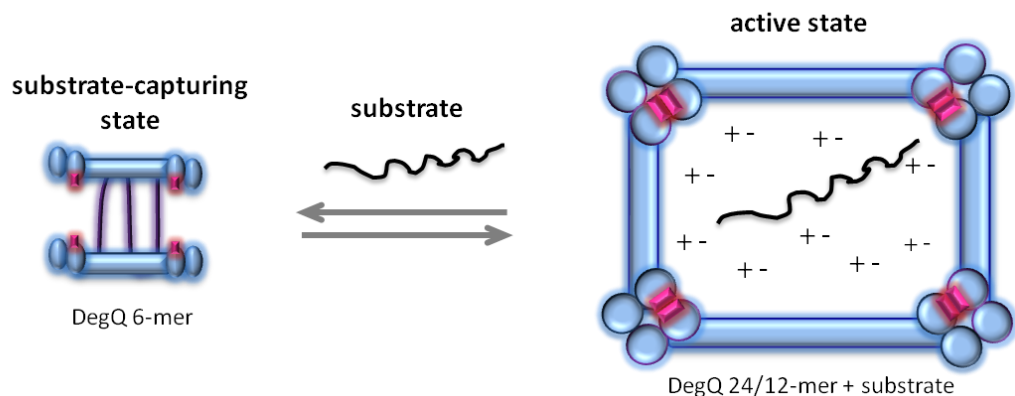


Fig. 6.1- Suggested working model for the ATP-independent chaperone activity of DegQ. Similarly to the GroEL chaperonin, DegQ exposes its hydrophobic binding sites for substrate interaction in the substrate-capturing state. Finally, when oligomerization is completed the substrate is released into the DegQ chamber which is now a hydrophilic environment favoring substrate folding. While ATP is the source of energy for GroEL/GroES conformational change and subsequent folding activity, the oligomerization of DegQ seems to “drive” its ATP-independent chaperone activity.

9 References

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10 Curriculum Vitae

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11 Appendix

11.1 Publication 1

Molecular adaptation of the DegQ protease to exert protein quality control in the bacterial cell envelope

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Author contributions: Protein purification was performed by J.S. and biochemical analyses were done by J.S., F.C., and H.M. under supervision of T.C. The X-ray studies were performed by J.S., T.K. and T.C. The project was supervised by T.C. and M.E. The manuscript was written by J.S. and T.C.

Molecular Adaptation of the DegQ Protease to Exert Protein Quality Control in the Bacterial Cell Envelope^{*[S]}

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To react to distinct stress situations and to prevent the accumulation of misfolded proteins, all cells employ a number of proteases and chaperones, which together set up an efficient protein quality control system. The functionality of proteins in the cell envelope of *Escherichia coli* is monitored by the HtrA proteases DegS, DegP, and DegQ. In contrast with DegP and DegS, the structure and function of DegQ has not been addressed in detail. Here, we show that substrate binding triggers the conversion of the resting DegQ hexamer into catalytically active 12- and 24-mers. Interestingly, substrate-induced oligomer reassembly and protease activation depends on the first PDZ domain but not on the second. Therefore, the regulatory mechanism originally identified in DegP should be a common feature of HtrA proteases, most of which encompass only a single PDZ domain. Using a DegQ mutant lacking the second PDZ domain, we determined the high resolution crystal structure of a dodecameric HtrA complex. The nearly identical domain orientation of protease and PDZ domains within 12- and 24-meric HtrA complexes reveals a conserved PDZ1 → L3 → LD/L1/L2 signaling cascade, in which loop L3 senses the repositioned PDZ1 domain of higher order, substrate-engaged particles and activates protease function. Furthermore, our *in vitro* and *in vivo* data imply a pH-related function of DegQ in the bacterial cell envelope.

The accumulation of misfolded and aggregated proteins hampers important biological processes and can lead to cellular malfunctions and even cell death (1, 2). To cope with conditions that interfere with protein structure and function, all cells employ molecular chaperones that support the refolding of non-native polypeptides or cooperate with proteases to eliminate irreversibly damaged proteins (3, 4). The protein quality control system constituted by these factors is not only important for survival under stress but also to perform important housekeeping functions in various cellular compartments. The key factors promoting protein quality control in extracytoplas-

mic compartments belong to the family of high temperature requirement (HtrA) proteases (5, 6). Prokaryotic HtrAs have been implicated in tolerance to various folding stresses as well as to pathogenicity (7–14), whereas defects in human HtrAs are correlated with protein folding diseases including Alzheimer and Parkinson diseases, arthritis, and neuromuscular disorders (15–22). HtrA proteases have a chymotrypsin-like protease as their catalytic domain and one or two accessory PDZ domains (23) implicated in substrate binding and controlling protease function. HtrA proteases form large molecular assemblies that range from trimers of 100 kDa to 24mers of 1.2 MDa (24–30). They either function as regulatory proteases cleaving specific substrates with pronounced specificity or act as general proteases reducing the levels of misfolded proteins (5, 31).

Even though all HtrA proteases exhibit a similar domain architecture, share a common trimeric building block, and are controlled by a conserved activation mechanism (32), they are involved in diverse biological pathways including protein quality control, outer membrane protein biogenesis, unfolded protein response, apoptosis, cell growth, tumor progression, and the metabolism of amyloid precursor protein (5, 16, 31, 33–34).

The functionality of proteins in the cell envelope, the periplasm, of *Escherichia coli* is monitored by three HtrA proteases, namely DegS, DegP, and DegQ. DegS is a regulatory protease that is tethered to the cytoplasmic membrane via one transmembrane segment. It senses and binds mislocalized outer membrane proteins. The bound outer membrane proteins function as allosteric activators triggering the DegS-mediated cleavage of RseA, thereby initiating the bacterial unfolded protein response (30, 35–38). The DegP protease chaperone is a heat shock protein that represents the key protein quality control factor in the bacterial cell envelope, eliminating severely damaged proteins (39–41) while in parallel promoting outer membrane protein biogenesis (26). In contrast with the membrane anchored DegS that occurs as a stable trimer (30), DegP can reversibly switch between different oligomeric forms that represent inactive (6-mer) and active (12- and 24-mer) protease states (24, 26). The third HtrA protease of the *E. coli*, DegQ, is homologous to DegP comprising one protease and two PDZ domains. Both proteases have similar substrate specificities and cleave misfolded protein substrates (42). Consistently, it has been shown that overproduction of DegQ rescues the temperature-sensitive growth defect of a *degP* null strain (43). Because sequence comparison of active site loops suggests that general HtrA proteases are most closely related to DegQ (31), DegQ appears to be the ideal model system to study

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

The atomic coordinates and structure factors (codes 3sti and 3stj) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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the general principles of HtrA protease regulation. To address this point and to delineate the precise function of DegQ in the bacterial cell envelope, we performed a detailed structural and biochemical analysis of DegQ. Our data suggest that HtrA proteases involved in protein quality control are under control of substrate-induced oligomer reassembly, irrespective whether they have one or two PDZ domains. Moreover, we present structural data illustrating the molecular architecture of a catalytically active dodecamer. This DegQ12 structure suggests that the signaling cascade leading to the protease activation of 12- and 24-mer HtrA complexes is conserved and depends on the precise positioning of the PDZ1 domain upon formation of substrate-engaged HtrA particles.

EXPERIMENTAL PROCEDURES

Construct Design—The *degQ* constructs (DegQ full-length residues 1–438, QProtPDZ1 residues 1–337, and QProt residues 1–237) lacking the native signal sequence were PCR-amplified from the genomic DNA of strain DH5 α and cloned into pET26b(+) (Novagen) vector encoding a N-terminal *pelB* signal sequence for periplasmic localization of the recombinant protein and an additional C-terminal His₆ tag for affinity purification. All of the point mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene). All of the constructs were verified by DNA sequence analysis.

Protein Expression and Purification—All of the recombinant DegQ variants were overexpressed in the *E. coli* strain BL21(DE3). Cells were grown at 37 °C in LB medium and induced with 1 mM isopropyl β -D-thiogalactopyranoside for 4 h at an A₆₀₀ of 0.6. Harvested cells were resuspended in 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0, and disrupted by sonication. The cleared lysate was loaded on a nickel-nitrilotriacetic acid column (Qiagen), and DegQ was eluted by applying a stepwise imidazol gradient. The eluate fraction containing 150 mM imidazole was concentrated using VIVASPIN (Vivascience) concentrators (molecular mass cut-off, 50 kDa) and applied to a Superdex 200 column (prep grade; GE Healthcare) equilibrated with 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl. Protein purity and monodispersity was judged by SDS-PAGE and dynamic light scattering (DynaPro-801; Protein Solutions Inc.), respectively. Recombinant DegP used in the pH-dependent casein degradation assay was purified as described previously (40).

Crystallization and Structure Solution—A deletion construct of DegQ lacking the second PDZ domain (QProtPDZ1) was crystallized at 19 °C using the sitting drop vapor diffusion method by mixing 2.5 μ l of a 30 mg/ml protein solution with 2.5 μ l of a reservoir solution containing 24% PEG600, 5% PEG1000, 10% glycerol, 0.1 M MES/NaOH,² pH 5.4. Crystals appeared within a few days and could be directly flash frozen in liquid nitrogen because of the high content of cryoprotectants in the crystallization solution. The diffraction data were collected at the European Synchrotron Radiation Facility (Beamline 14-4, Grenoble, France). The data were integrated using DENZO and scaled with SCALEPACK (44). The crystals belonged to space

group P3₁ with cell parameters $a = b = 115.3$ Å, $c = 287.4$ Å and contained 12 protomers in the asymmetric unit. The structure was determined by molecular replacement using the program Phaser (45) and the DegP protease and PDZ1 domain as separate search models (Protein Data Bank code 1ky9). Parts of the loop LA (residues 35–57), the first 10 N-terminal residues, and the last three C-terminal residues were not resolved and omitted in the final structure. In addition, two oligopeptides were observed in the peptide-binding sites of the protease and PDZ1 domain and built-in as six-residue polyalanine models.

The QProt crystals were grown using the same method by mixing 3 μ l of a 35-mg/ml protein solution with 1.5 μ l of a reservoir solution containing 1.6 M ammonium sulfate, 3% PEG400, 0.1 M HEPES/NaOH, pH 7.0. The data were collected in-house on a MarResearch image plate at room temperature with crystals mounted in a glass capillary. Data were integrated using DENZO and scaled with SCALEPACK (44). Crystals belonged to space group P3₁ with cell parameters of $a = b = 70.9$ Å and $c = 152.0$ Å and three QProt protomers per asymmetric unit. The crystal structure was solved by molecular replacement using the program Phaser (45) and the DegP protease domain (Protein Data Bank code 1ky9) as a search model. Because of the lack of interpretable electron density, parts of the loop LA (residues 30–63), loop L2 (residues 207–212), and the first ten N-terminal residues are absent in the final model.

In both cases, the models were built with O (46) and refined with CNS (47). Data collection, phasing, and refinement statistics are summarized in Table 1. Accordingly, the two protein structures exhibit good stereochemistry and have no outliers in the Ramachandran plot (48). Coordinates of the QProt and QProtPDZ1 crystal structures have been deposited at the PDB Data Bank with accession codes 3sti and 3stj, respectively. All of the graphical presentations were prepared using the program Pymol (49).

Characterization of DegQ and DegQ-Substrate Complexes by Gel Filtration—Complex formation was analyzed using the proteolytically inactive mutants (S187A) of all DegQ variants tested. We incubated 50 μ M of full-length DegQ variants, 120 μ M QProtPDZ1 or 120 μ M QProt with either 160 μ M casein or 200 μ M lysozyme in 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl supplied with 10 mM DTT in the case of the lysozyme assay. Prior to the experiment lysozyme was denatured by preparing a 50 mg/ml protein solution in 4 M urea and 10 mM DTT. Assays were incubated for 10 min at 37 °C before samples were injected on a Superdex 200 gel filtration column (PC3.2/30; GE Healthcare). Comparison with marker proteins and SDS-PAGE analysis revealed the size and composites of the individual complexes.

To follow the dose-dependent DegQ12 and DegQ24 formation, DegQ (50 μ M) was incubated with various amounts of lysozyme (120, 300, 450, or 950 μ M) at 37 °C for 10 min. The resulting complexes were analyzed by size exclusion chromatography (SEC).

To survey the complex formation in the presence of the activating peptide, 30 μ M DegQ was incubated with 200 μ M SPMFKGVLDMMYGGMRGYQV peptide in 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl buffer for 15 min at 37 °C. Subsequently, the sample was injected on the gel filtration column

² The abbreviations used are: MES, 4-morpholineethanesulfonic acid; SEC, size exclusion chromatography; ITC, isothermal titration calorimetry.

pre-equilibrated with corresponding buffer supplied with 200 μM of the peptide activator.

To analyze the pH-dependent change in the oligomeric state of DegQ, we dialyzed 50 μM DegQ aliquots against 50 mM MES/NaOH, pH 5.5, 150 mM NaCl, or 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl at 4 °C with slow stirring. After 3 h, the samples were directly applied on the gel filtration column pre-equilibrated with the respective buffer.

Isothermal Titration Calorimetry—The thermodynamic values of the interaction between DegQ and the activating peptide were determined using an isothermal titration microcalorimeter (MCS-ITC; Microcal). All of the experiments were conducted in overflow mode at 30 °C. 1.8 ml of solution of 20 μM DegQ was placed in the temperature-controlled sample cell and titrated with 200 μM peptide loaded in the 300- μl mixing syringe. For the experiment 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl was used as isothermal titration calorimetry (ITC) buffer. Injections of 10 μl peptide were dispensed into the sample cell using a 120-s equilibration time between injections and stirring at 300 rpm. Control experiments were carried out to measure and correct the heat of dilution upon buffer addition. Finally, the data were analyzed using the program ORIGIN following the instructions of the manufacturer.

Characterization of the Proteolytic Activity—The effect of the peptide activator on the activity of DegQ was measured using the pNA-chromogenic peptide substrate (SPMFKGV-pNA). The 0.8-ml reaction mixtures containing 0.5 mM pNA substrate in 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, and the 0.3 mM of activating peptide were preincubated for 15 min at 37 °C. The cleavage reaction was initiated by the addition of DegQ to a final concentration of 5 μM . The continuous increase in the absorbance at 405 nm at 37 °C was monitored.

To follow the degradation of the model substrate casein, we mixed 2.5 μM of DegQ or derivatives thereof with 20 μM casein in 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl buffer or 4 μM of DegQ with 20 μM lysozyme in the same buffer supplied with 10 mM DTT. Prior to the assay, lysozyme was denatured in the 4 M urea and 10 mM DTT. The samples were incubated at 37 °C. The reaction was stopped at the indicated time points by adding SDS loading buffer and boiling the samples for 10 min at 95 °C. Subsequently, the aliquots were analyzed by SDS-PAGE followed by Coomassie Blue staining.

The pH-dependent proteolytic activity of DegQ and DegP was determined with resorufin-labeled casein (Roche Applied Science). 15 μl of 0.4% (w/v) resorufin-labeled casein was added to 100 μl of incubation buffer of a respective pH containing 3 μM of DegQ or DegP and incubated at 37 °C for 3 h (DegP) and 12 h (DegQ). The reaction was stopped by precipitating casein with 480 μl of 10% (w/v) TCA. The samples were again incubated for 10 min at 37 °C and subsequently centrifuged (10 min, 10000 $\times$ g, room temperature). 400 μl of the supernatant was mixed with 600 μl of 1 M Tris/HCl, pH 8.8, and the absorbance at 574 nm was determined. In the pH screen, we used the following 50 mM buffers supplemented with 150 mM NaCl: acetic acid (pH levels 4.5 and 5.0), MES/NaOH (pH levels 5.5, 6.0, and 6.5), HEPES/NaOH (pH levels 7.0 and 7.5), Tris/HCl (pH levels 8.0, 8.5, and 9.0). The pH was adjusted at 37 °C.

Recording Bacterial Growth Curves—The unbuffered overnight cultures of *degP* null (CLC198, *degP::Tn10*) (40), *degQ* null (MG1655 *degQ::Tn5 Kan^R* strain ordered from *E. coli* Genome Project) and their parental strains were standardized to equal A_{600} and used to inoculate 100 ml of fresh LB medium. In the case of *degQ* null strain, kanamycin was added to a final concentration of 25 $\mu\text{g}/\text{ml}$. 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/NaOH, pH 7.5, or 10 ml of 0.5 M MES/NaOH, pH 5.5. Cells growth and pH were monitored at 30-min time points until the stationary phase was reached.

RESULTS

Oligomer Conversion and Activation of DegQ Does Not Depend on the Second PDZ Domain—Recent studies with DegP from *E. coli* suggested that substrate-induced oligomer conversion and activation is of central importance for HtrA protease regulation (24, 26). To explore whether DegQ employs a similar mechanism, we first analyzed complex formation with the unfolded model substrates lysozyme and β -casein. For this purpose, we used a catalytically inactive DegQ mutant, in which the active site serine Ser-187 was replaced by alanine. SEC and SDS-PAGE analysis revealed that incubation of DegQ with lysozyme and casein leads to the formation of higher order, substrate-engaged DegQ12 and DegQ24 multimers, respectively (Fig. 1*a*), with the size of the generated particle depending on the amount of substrate. At lower substrate concentration, the 12-mer is predominantly formed, whereas substrate at increasing concentration is preferentially captured in DegQ24 (Fig. 1*b*).

To extend these studies to HtrAs containing a single PDZ domain, we performed complex formation analyses with a DegQ variant lacking the second PDZ domain (QProtPDZ1). In the presence of substrate, we could observe the formation of higher order assemblies, however exclusively of 12-meric particles (Fig. 1*c*). The inability to form 24-mers is not surprising because the PDZ2 domain is essential to mediate intertrimer contacts in this higher order oligomer as suggested by the architecture of DegP24 (24, 26). Moreover, in the absence of substrate, QProtPDZ1 occurred as a trimer, which should represent the resting state of the mutant. With regard to protease activity, QProtPDZ1 retained the ability to degrade casein, demonstrating that PDZ2 is dispensable for protease activity (Fig. 1*d*). Conversely, removal of both PDZ domains (the QProt variant) resulted in proteolytically inactive trimers that are incapable of forming higher order oligomers (Fig. 1, *c* and *d*). Together, these data indicate that only PDZ1 is essential to couple substrate binding with the formation of proteolytically active higher order DegQ oligomers.

Crystal Structure of the QProtPDZ1 Dodecamer and the QProt Trimer—Because of the failure to crystallize full-length DegQ (*i.e.* DegQ6 and DegQ12/24 complexes with substrate), we crystallized suitable deletion variants including the QProtPDZ1 mutant that retained the capability to form 12-mer complexes in the presence of substrate. This dodecamer is also seen in the crystal structure, which was solved by molecular replacement at 2.6 Å resolution (Table 1). The crystal structure of the QProtPDZ1 dodecamer shows a 400-kDa hollow particle with

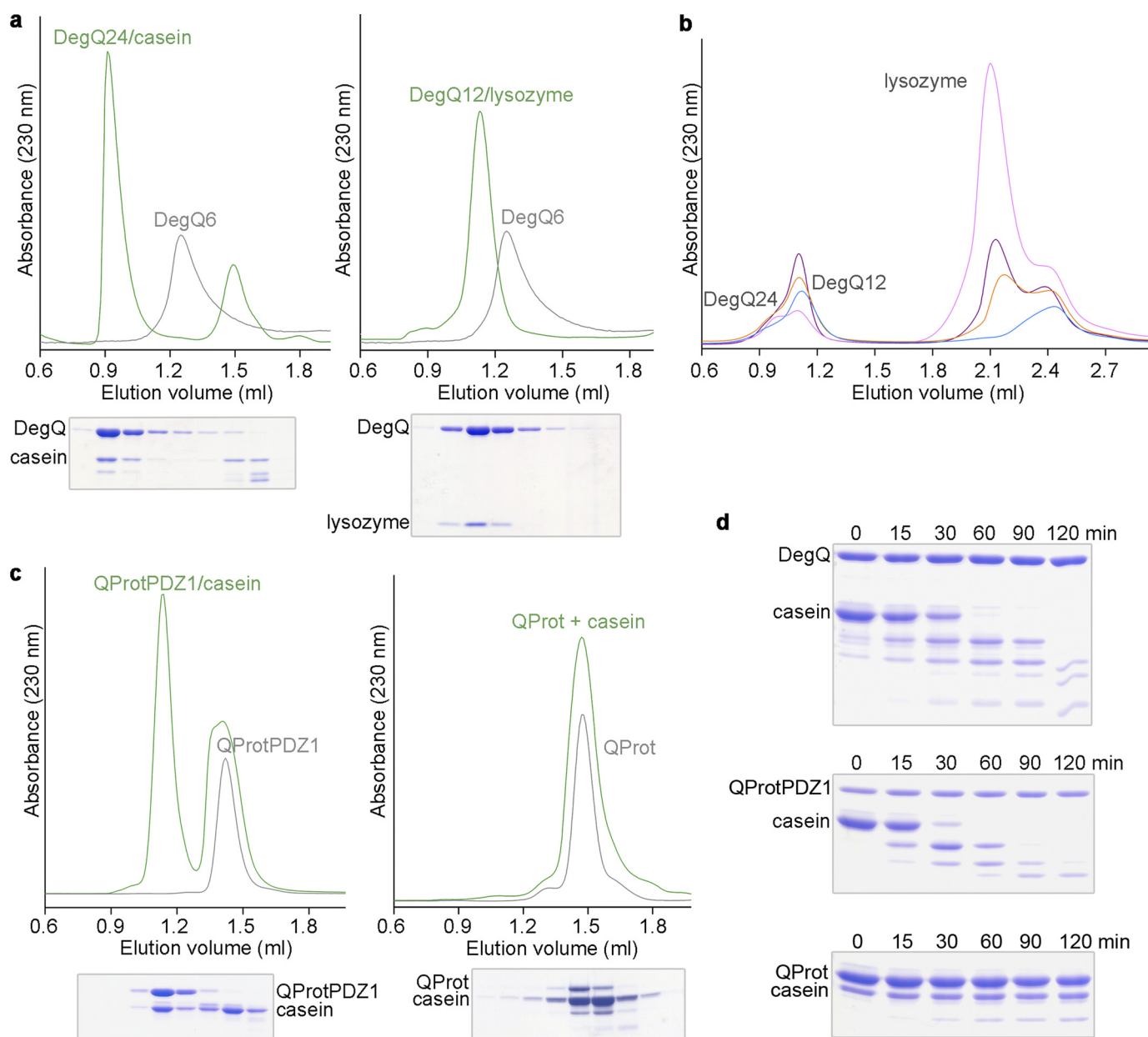


FIGURE 1. Substrate-induced oligomer reassembly of DegQ. *a*, full-length DegQ (gray line) was incubated with casein (left panel) or unfolded lysozyme (right panel), and complex formation was monitored by SEC and SDS-PAGE. Substrate binding transforms the DegQ hexamer (DegQ6) into DegQ24-casein and DegQ12-lysozyme complexes, respectively. *b*, incubation of DegQ6 (50 μ M) with increasing lysozyme concentrations (120 μ M, blue; 300 μ M, red; 450 μ M, purple; 950 μ M, magenta) reveals that the distinct higher order oligomers are formed depending on the amount of substrate. For lysozyme concentrations higher than 1 mM, the 24-mer starts to be preferentially formed. The absorbance peak at 2.4 ml can be attributed to the reducing agent used to facilitate lysozyme unfolding. *c*, the QProtPDZ1 mutant occurs in its latent state as a trimer (left, gray line) as well as the QProt mutant (right, gray line). Incubation with an unfolded substrate (casein) triggers formation of a dodecameric QProtPDZ1-substrate complex (blue line), whereas no higher order oligomer is formed with QProt. *d*, SDS-PAGE analysis of the proteolytic activity of the different DegQ variants (DegQ wild type, top panel; QProtPDZ1, middle panel; QProt, bottom panel) against casein at distinct time points.

dimensions of 115 Å × 115 Å × 110 Å (Fig. 2*a*). The four trimers are located at the vertices of a tetrahedron and assemble a protein shell that encloses an internal cavity of ~50 Å diameter. The contacts between the trimers are mainly mediated by the PDZ1 domains yielding a rigid molecular cage. The proteolytic sites are sequestered within this cage and open up into the interior. Therefore cleavage products have to leave the particle through one of the four ~20 Å-wide pores, which are bordered by the protease domains of adjacent trimers. The spatial organization of the trimers resembles a planar triangle with cen-

tered protease and PDZ domains at the vertices (Fig. 2*b*). The peripheral PDZ1 domains contact each other via the interaction clamp, an HtrA signature motif that is important to form higher order oligomers by mediating contacts between juxtaposed trimers (23, 25–26). The interaction clamp of QProtPDZ1 comprises a hydrophobic region (residues 249–266) that interacts with the corresponding region of PDZ1* (the asterisk denotes a neighboring molecule) of the adjacent trimer, thereby constituting the hydrophobic core of the 12-mer interface (Fig. 2*c*).

TABLE 1

Data collection and refinement statistics

	QProtPDZ1	QProt
Data collection		
Space group	P3 ₁	P3 ₁
Unit cell parameters (Å)	<i>a</i> = 115.3 <i>b</i> = 115.3 <i>c</i> = 287.4	<i>a</i> = 70.9 <i>b</i> = 70.9 <i>c</i> = 152.0
Resolution (Å) ^a	20–2.6	20–2.5
Completeness (%)	95.4 (87.8)	96.4 (93.8)
<i>R</i> _{sym} (%) ^b	8.0 (33.9)	9.3 (67.8)
<i>I</i> / <i>σ</i> (<i>I</i>)	15.1 (1.5)	11.2 (1.2)
Redundancy	2.3 (1.9)	3.7 (3.2)
Refinement		
Resolution	20–2.6	20–2.6
Number of reflections <i>R</i> _{work} / <i>R</i> _{free}	118,403/6,289	22,399/1,204
Number of protein atoms	26,507	4023
Number of ligand atoms		
<i>R</i> _{cryst} / <i>R</i> _{free} ^c	18.5/21.2	22.2/25.7
Average B-factor (Å ²)	60.3	51.9
Root mean square deviations of bond length (Å)/angles (°)	0.010/1.3	0.010/1.3
Ramachandran statistics (%) most favored, allowed, and disallowed region ^d	91.7, 8.3, 0.0, 0.0	91.3, 7.8, 0.4, 0.4

^a Numbers in parentheses refer to the highest resolution shell.^b *R*_{sym} is the unweighted *R* value on *I* between symmetry mates.^c *R*_{cryst} = $\sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl}) - k|F_{\text{calc}}(\text{hkl})| / \sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl})|$ for the working set of reflections; *R*_{free} is the *R* value for 5% of the reflections excluded from refinement.^d The stereochemistry of the model was validated with PROCHECK (48).

In addition, we determined the crystal structure of the protease domain alone (QProt) representing the inactive state. This structure enabled us to delineate the function of the PDZ domains in mediating the switch in activity. In contrast with QProtPDZ1, the QProt mutant could not form higher order complexes and remained as trimer in solution under all of the conditions tested. Consistently, QProt was observed as trimer in the crystal lattice (Fig. 2*d*). The assembly of the protease trimer is similar to QProtPDZ1, in which intersubunit contacts are exclusively established between residues of the protease domain, most of which originate from the N-terminal α -helix.

QProtPDZ1 and QProt Reflect the Proteolytically Active and Inactive States of DegQ—Comparison of the two DegQ crystal structures (root mean square deviation value of 1.9 Å for 175 aligned C α atoms) revealed that QProtPDZ1 and QProt show characteristic differences in their active site architecture. In QProt, the activation domain (L1, L2, and LD) as well as loop L3 (loop that typically mediates interaction with the PDZ domain) are highly flexible as indicated by the elevated crystallographic temperature factors (Fig. 3*a*) and the absence of interpretable electron density for residues 207–212 of loop L2. The conformational flexibility within the active site impedes proper adjustment of the catalytic triad, oxyanion hole, and S1 specificity pocket and thus explains the drastically reduced catalytic activity of the PDZ-less mutant. Conversely, in QProtPDZ1, the activation domain is well defined by electron density and adopts a strikingly different conformation (Fig. 3*b*, with detailed views shown in [supplemental Fig. S1](#)): First, a functional catalytic triad is set up between His-82, Asp-112, and Ser-187 with the hydroxyl, imidazol, and carboxylate group being properly aligned to hydrogen bond each other. Second, a peptide flip of the amide linkage between residues Gly-185 and Arg-184 enables the Arg-184 carbonyl oxygen to interact with the amide nitrogen of Phe-148 of loop LD, thereby allowing formation of the oxyanion hole constituted by residues 184–

187 of loop L1. Third, the residue Ile-182 together with Ile-205 and Ala-204 of loop L2 are properly oriented to establish the S1 specificity pocket, whereas residues Thr-203, Ala-204, Ile-205, Leu-206, and Ala-207 adopt a β -strand conformation required to bind the main chain of the incoming protein substrate by β -augmentation. The shallow S1 hydrophobic pocket selects for small hydrophobic residues, which is consistent with the previously described specificity of DegQ cleaving model substrates at discrete Val/Xaa or Ile/Xaa sites (42). Based on our structural data, we conclude that QProt and QProtPDZ1 represent the inactive and active states of DegQ. Therefore, protease activity depends on the regulated folding of the activation domain, a process that is under control of loop L3 and its interaction with the PDZ1 domain. Similarly to other serine proteases of the chymotrypsin family, this activation process is connected with a disorder-to-order transition of the activation domain (Fig. 3*b*).

Peptide Binding to PDZ1 Triggers Formation of Proteolytically Active Higher Order Oligomers—The activation of HtrA proteases is known to be a reversible process that can be triggered by distinct molecular cues. For example in DegS, peptides that signal folding stress are recognized and bound by the PDZ domain. These bound peptides are capable of inducing rearrangement of the sensor loop L3, which in turn triggers the remodeling of the activation domain into its functional state capable of cleaving the substrate protein RseA (30, 36, 38). In contrast with this transactivation mechanism, allosteric regulation of DegP depends on the substrate itself. Substrate binding to the first PDZ domain of DegP, PDZ1, induces oligomer conversion from DegP6 to DegP24 that leads to a repositioning and immobilization of the PDZ domains such that they can induce rearrangement of loop L3, thereby activating protease function (26, 32, 50). To test which activation mechanism is employed by DegQ, we assessed its interactions with various synthetic peptides. Similarly to DegP, DegQ showed the highest affinity to peptides having a C-terminal valine residue, whereas an interaction with the preferred peptide ligand of DegS having a C-terminal phenylalanine could not be observed by means of ITC. For the SPMFKGVLDMMYGGMRGYQV peptide, a described allosteric activator of DegP (32, 51), ITC measurements revealed a *K_D* value of 16 μ M (Fig. 4*a*). Further SEC analysis demonstrated that this peptide is capable of inducing the transformation of DegQ6 into DegQ12 (Fig. 4*b*), which is correlated with an enhanced proteolytic activity against a chromogenic model substrate (Fig. 4*c*). In contrast, effector peptides with a C-terminal glutamate residue did not bind to DegQ and did not stimulate protease activity (data not shown).

Consistent with these biochemical data, two peptide ligands were observed in the electron density map of the QProtPDZ1 dodecamer. One peptide is accommodated in the binding groove of the PDZ1 domain. The built-in polyalanine model illustrates that the peptide is attached via β -augmentation to the core of the PDZ1 domain allowing the C-terminal residue to penetrate a shallow hydrophobic pocket lined by residues Phe-298, Leu-242, Ile-244, Leu-301, Arg-302, and Ile-305 (Fig. 4*d*). The second peptide is accommodated in the proteolytic site, where it is tethered to the β -strand formed by residues Thr-203,

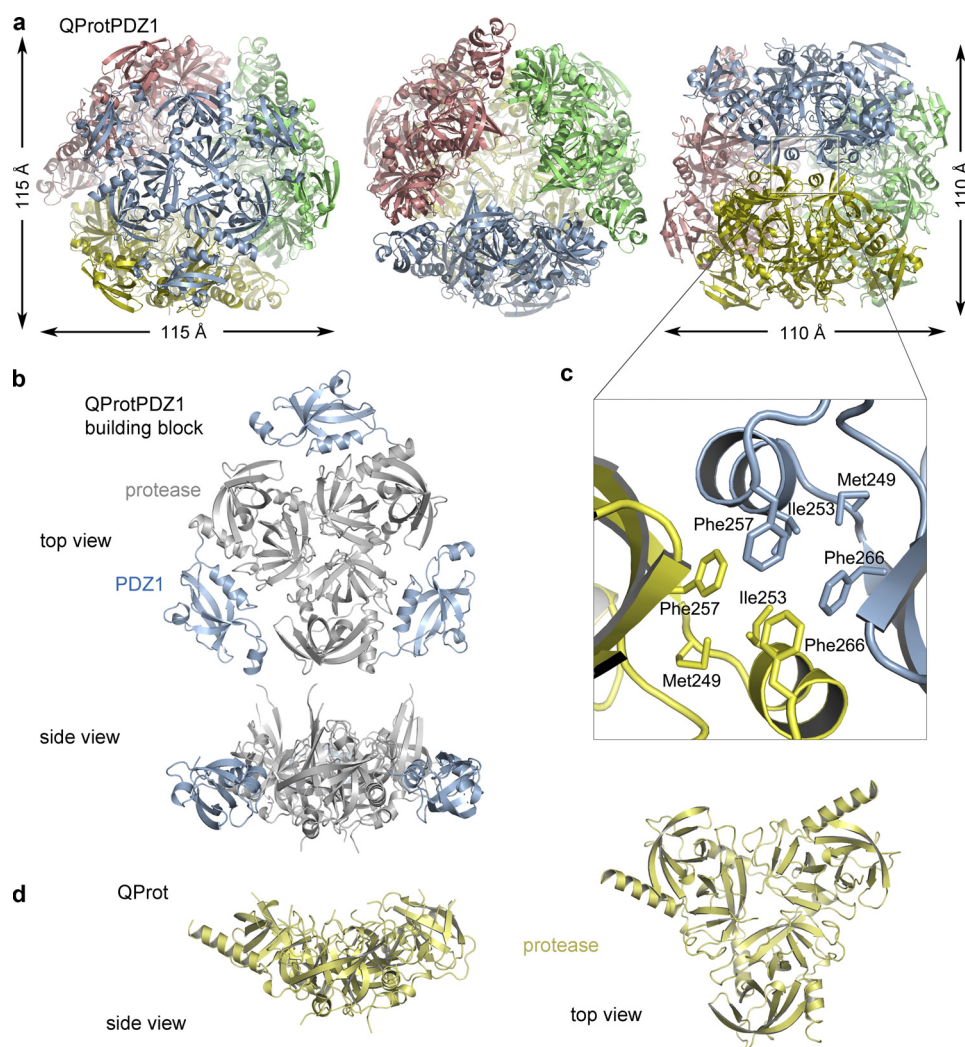


FIGURE 2. Crystal structures of the QProtPDZ1 dodecamer and the QProt trimer. *a*, ribbon plot of the QProtPDZ1 particle illustrating its overall dodecameric architecture. The constituting trimers (colored differently) occupy the vertices of a tetrahedron yielding a proteolytic cavity of ~ 50 Å in diameter and defined exit pores. The particle is shown along the molecular 3-fold (left and middle panels) and the 2-fold axis (right panel). *b*, side and top view of one QProtPDZ1 trimer of the dodecamer with the protease domain colored gray and the PDZ1 domain colored blue. *c*, the intertrimeric contacts in the dodecamer are mediated mainly by the hydrophobic “interaction clamp” of the PDZ1 domain. The clamp residues involved in the interactions between two adjacent PDZ1 domains (yellow and blue) are depicted in stick mode. *d*, side and top view of the QProt trimer.

Ala-204, Ile-205, Leu-206, and Ala-207 (in orange in supplemental Fig. S2), thus allowing the side chain of the P1 residue to protrude in the S1 specificity pocket (supplemental Fig. S2). Because QProtPDZ1 is not subject to autodegradation, the bound peptides cannot result from autocleavage, as seen for example in *Mycobacterium tuberculosis* HtrA (28), and should thus represent co-purified and co-crystallized oligopeptides that mimic potential cleavage intermediates. A similar scenario has been reported for DegP, in which a variety of oligopeptides were captured in the PDZ1 binding groove of the functionally active DegP24, but not in the resting DegP6. These bound peptides appear to rearrange the carboxylate binding loop of the PDZ1 domain and the adjacent interdomain linker segment, thereby inducing a domain rearrangement such that DegP6 is transformed into DegP12/24 (32). Given the similarity of DegP and DegQ in their peptide binding modes and activation mechanism, we thus presume that the peptides bound to QProtPDZ1 orient and immobilize the PDZ1 domain, thereby allowing crystallization of the functionally active dodecamer. Taken

together, our biochemical and structural data demonstrate that peptide binding to PDZ1 stimulates the protease activity of DegQ by triggering formation of catalytically active higher order oligomers.

The PDZ1 Domain and Loop L3 Constitute a Molecular Switch Regulating Protease Function—As shown for the DegP protease, the PDZ1 domain plays an essential role in the activation process. Upon substrate binding and DegP12/24 oligomer formation, the repositioned PDZ1 domain induces rearrangement of the protease loop L3, which in turn stabilizes the functional state of the proteolytic site. The high resolution structure of QProtPDZ1 enabled us to explore whether a similar PDZ1 $\rightarrow$ L3 $\rightarrow$ LD/L1/L2 protease activation cascade occurs in DegQ (Fig. 5a). To test whether the interplay between PDZ1 domain and the L3 loop is critical to activate protease function in the dodecameric scaffold, we disrupted this interaction by introducing the R302A mutation (Arg-302 of PDZ1 forms a hydrogen bond with the carbonyl oxygen of Gly-171 in loop L3; Fig. 5a). Indeed, when we assayed the catalytic activity of the

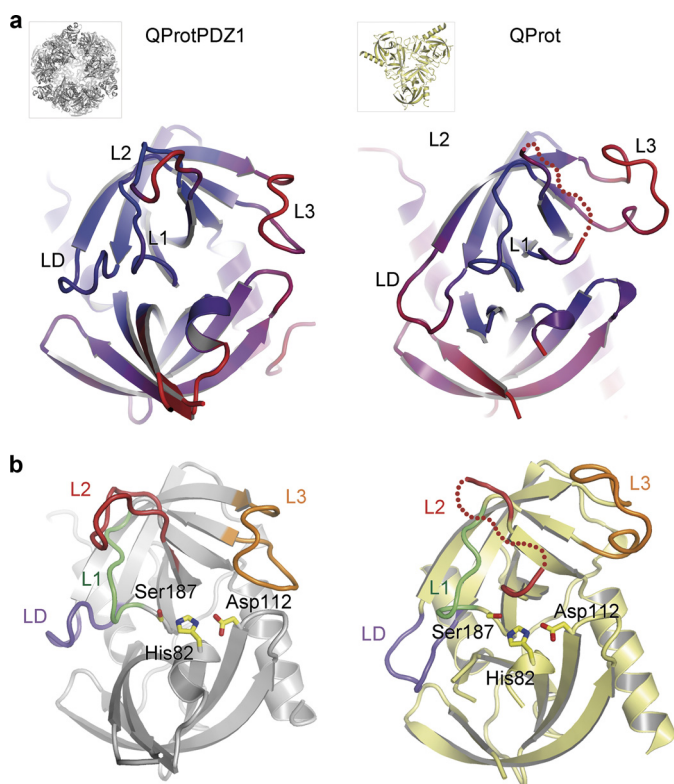


FIGURE 3. Comparison of the active QProtPDZ1 and the inactive QProt protease folds. Ribbon plots of the protease domains of QProtPDZ1 (left panels) and QProt (right panels) are shown. *a*, structures are color-coded by the crystallographic temperature factors (rigid portions, blue; disordered regions, red) with the active site loops being labeled. *b*, highlighted active site loops that adopt strikingly different conformations in the two DegQ variants representing the active (QProtPDZ1) and the inactive state (QProt). The active site residues (His-82, Asp-112, and Ser-187) are shown in stick mode.

mutant, we could observe that the degradation of lysozyme was strongly reduced (Fig. 5*b*). To show that the abolished activity results from the disrupted PDZ1-L3 communication and not from impaired substrate binding or hindered 12/24-mer formation, the R302A mutant was subjected to SEC analysis. The R302A mutant retained the ability form higher order complexes in the presence of substrate (Fig. 5*c*), indicating that the reduced protease activity is the direct consequence of the abrogated PDZ1-L3 interaction. Once initiated by loop L3, the signaling cascade results in the remodeling of the activation domain of the protease. This step is mediated by a conserved arginine residue (Arg-164 in DegQ) that is located on the N-terminal stem segment of loop L3 (Fig. 5*a*). To test the importance of Arg-164 for transferring the activation signal from the PDZ1 domain to the proteolytic site, we exchanged it to an alanine, thereby preventing its interaction with the main-chain carbonyl of Gln-152 and the hydroxyl group of Thr-153 of loop LD. In the SDS-PAGE assay, the proteolytic activity of the R164A mutant against unfolded lysozyme was significantly reduced, highlighting the importance of the L3-LD interaction for protease activation (Fig. 5*b*). Moreover, comparison with the inactive QProt structure revealed that a flexible loop L3 that is not tethered by PDZ1 is not capable of interacting with loop LD. As a consequence, the remodeling of the activation domain into the active state is prevented, explaining the abolished protease activity of the QProt mutant. In sum, these data indicate the preservation of the intramolecular PDZ1 → L3 → LD/L1/L2 signaling module, suggesting that loop L3 functions as a conserved molecular switch in regulating HtrA proteases in both 12- and 24-meric HtrA oligomers.

DegQ Is a pH-Sensitive HtrA Protease—Because it is known that HtrA proteases are regulated by different molecular cues

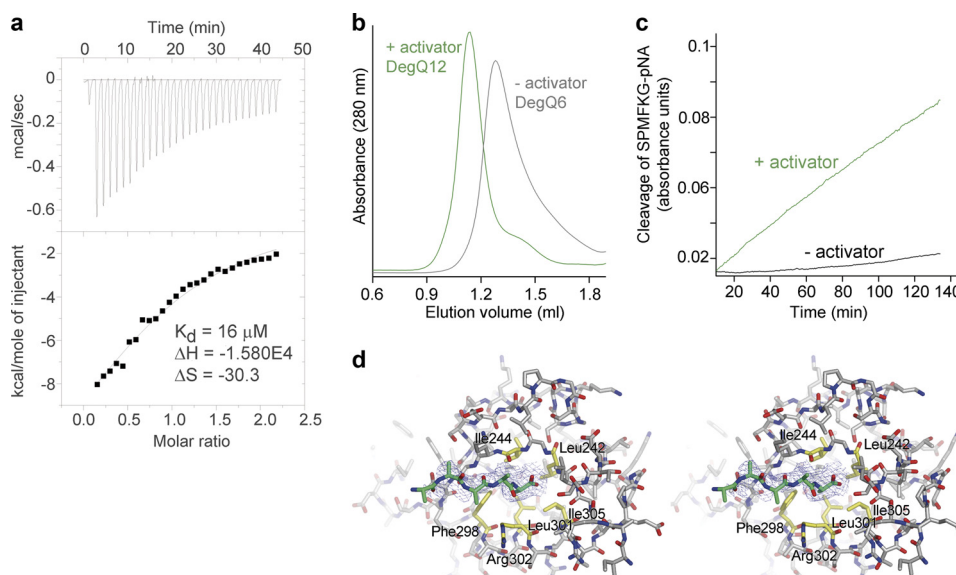


FIGURE 4. Oligomer conversion and proteolytic activity of DegQ is regulated by peptide binding to the PDZ1 domain. *a*, ITC measurement of the binding of the activating peptide to DegQ6. The area under each peak was integrated and plotted against the molar ratio of peptide to DegQ inside the sample cell (lower panel). The black line represents the fit to a binding isotherm, assuming one binding site per protomer. The indicated thermodynamic values were calculated using the protomer concentration of DegQ. *b*, SEC analysis illustrating conversion of DegQ6 (Q6, gray) into DegQ12 (Q12, green) in the presence of the activating peptide (200 μM). *c*, cleavage of the chromogenic SPMFKGV-pNA substrate by DegQ in the absence (black) and presence of the activating peptide (green). *d*, cartoon representation of the peptide binding to PDZ1 in stereo view. The polyaniline chain (green) binds to the PDZ1 domain (gray) via β -augmentation. The $2F_o - F_c$ electron density map, which was calculated at 2.6 Å resolution without contribution of the peptide ligand, is contoured at 1.2 σ . Residues of the PDZ1 domain accommodating the C-terminal side chain of the peptide are highlighted in yellow and labeled.

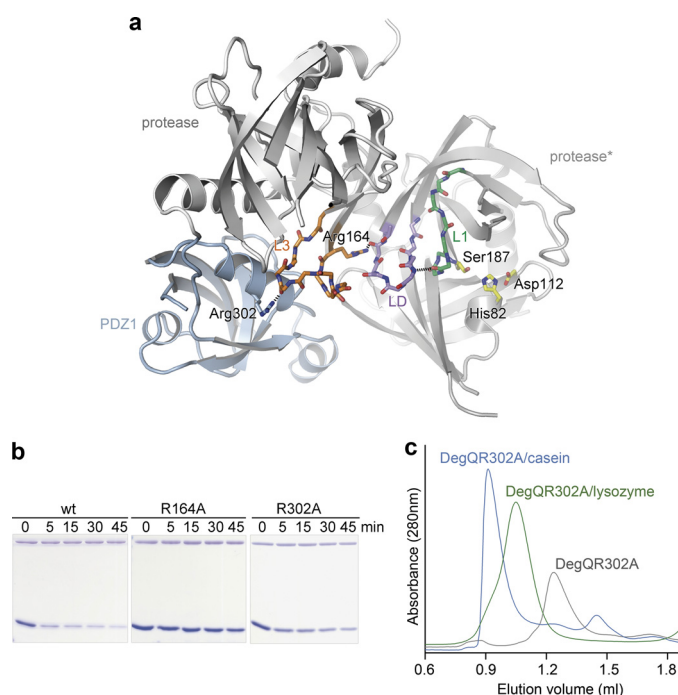


FIGURE 5. Activation cascade in DegQ. *a*, illustration of the key interactions involved in signaling from loop L3 to the proteolytic site as seen in the QProtPDZ1 crystal structure. The fixed position of PDZ1 (blue) orients loop L3 (orange) by interactions mediated via Arg-302 (depicted in stick mode). As a result, Arg-164 of the loop L3 interacts with the main chain carbonyl of residue Gln-152 and the hydroxyl group of Thr-153 of loop LD (lilac) in the adjacent protomer (protease*), which in turn induces remodeling of the proteolytic sites (loop L1 shown in green and functional catalytic triad in yellow). *b*, SDS-PAGE analysis of the cleavage of unfolded lysozyme by DegQ wild type and R164A or R302A mutants indicating that both mutations render the DegQ protease inactive. *c*, SEC analysis reveals that, although proteolytically inactive, both mutants (R302A mutant shown here as representative) can form higher order oligomers with casein (24-mer, blue) and lysozyme (12-mer, green).

(38, 40, 52, 53), we systematically analyzed the effect of different physical and chemical stimuli on the activity of DegQ and found that DegQ digests substrates in a pH-dependent manner. We monitored the proteolytic activity of DegQ at different pHs in a colorimetric assay using resorufin-labeled casein and observed that the degradation is most efficient at pH values between 4.5 and 6 with an optimum at pH 5.5 (Fig. 6*a*). Remarkably, SEC analysis revealed that pH had an additional effect on the oligomeric state of DegQ. Whereas the hexamer is the dominant form at pH 7.5, the equilibrium shifts to the dodecamer at pH 5.5 (Fig. 6*b*). Despite these different oligomeric states, casein was encapsulated at both pH 5.5 and pH 7.5 in the 24-mer particle, suggesting that the pH does not interfere with substrate engagement (data not shown). Although the exact molecular mechanism of the pH effect is still elusive, it is apparent that the distinct oligomeric states of DegQ occur in a dynamic, pH-dependent equilibrium. We presume that at slightly acidic pH, the activation barrier to transform the 6-mer into the 12/24-mer is decreased. Accordingly, substrate encapsulation and degradation in DegQ12/24 should be facilitated.

To examine the physiological importance of the pH-dependent protease activity, we compared the growth rate of a *degP* null mutant exposed to slightly acidic (pH 5.5) and neutral (pH

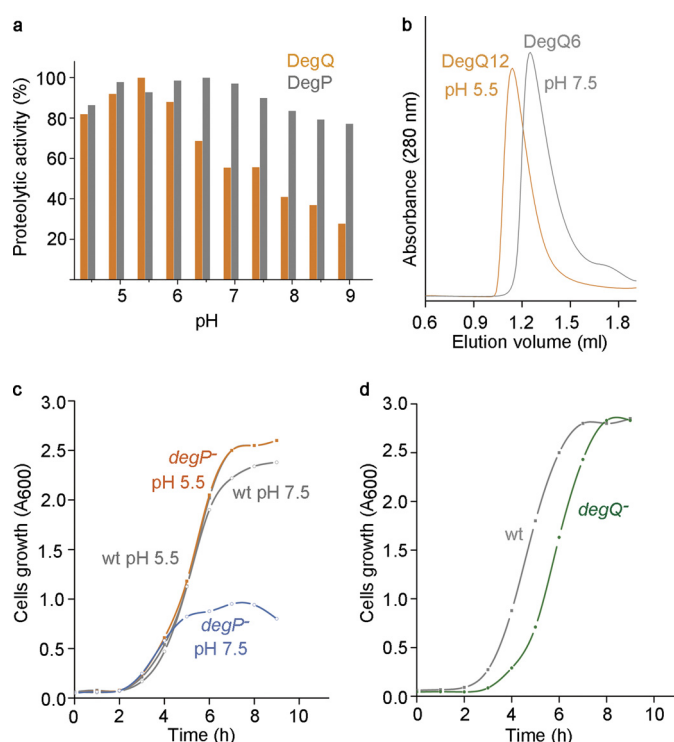


FIGURE 6. DegQ function is affected by pH. *a*, effect of pH on the proteolytic activity of DegQ (orange) and DegP (gray) against casein in the pH range from 4.5 to 9. The relative proteolytic activities were calculated by standardization to the highest obtained value, which was regarded as 100%. *b*, SEC analysis of the oligomeric state of DegQ in pH 5.5 (orange line) and 7.5 (gray line) revealed pH-dependent DegQ6 to DegQ12 conversion. *c*, growth of *degP*-null and wild type strains in LB medium buffered to either pH 5.5 or 7.5. The growth rate of the wild type strain was not affected by pH (gray lines), whereas *degP*-null cells cultured in pH 7.5 (blue line) entered the stationary phase 3.5 h earlier compared with pH 5.5 (orange line) and grew to lower cell density. *d*, growth of *degQ*-null and wild type strains in LB media buffered to pH 7.5. The mutant strain (green line) shows extended lag time after inoculation compared with the wild type (gray line).

7.5) medium and observed a striking correlation with the pH-dependent activity of DegQ. At pH 5.5, the growth of the *degP* null mutant was identical to wild type, whereas at pH 7.5, cells stopped dividing and entered stationary phase 3.5 h earlier (Fig. 6*c*). These data indicate that DegQ is capable of taking over the function of DegP in the *degP* null mutant strain. However, it can only reconstitute the wild type situation at slightly acidic pH values, at which the protease activity of DegQ is the highest. Analyzing the growth rates of a *degQ* null strain did not reveal a similar pH dependence (data not shown), thus confirming that the activity of DegP is largely pH-independent in the range analyzed. However, when we compared the initial growth phase of the *degQ* null mutant with the wild type strain, we noticed that the adaptation of the mutant cells was prolonged (Fig. 6*d*). In the absence of DegQ, the *degP* expression has to be up-regulated in response to the accumulation of unfolded proteins in the cell envelope. Because of the time required to sense the stress situation and to trigger the corresponding unfolded protein response (43), the initial growth phase appears to be delayed until DegP is produced in sufficient amounts. Together, these findings reveal that DegQ functions as pH-sensitive protease in the cellular envelope, establishing the initial proteolytic response against misfolded proteins.

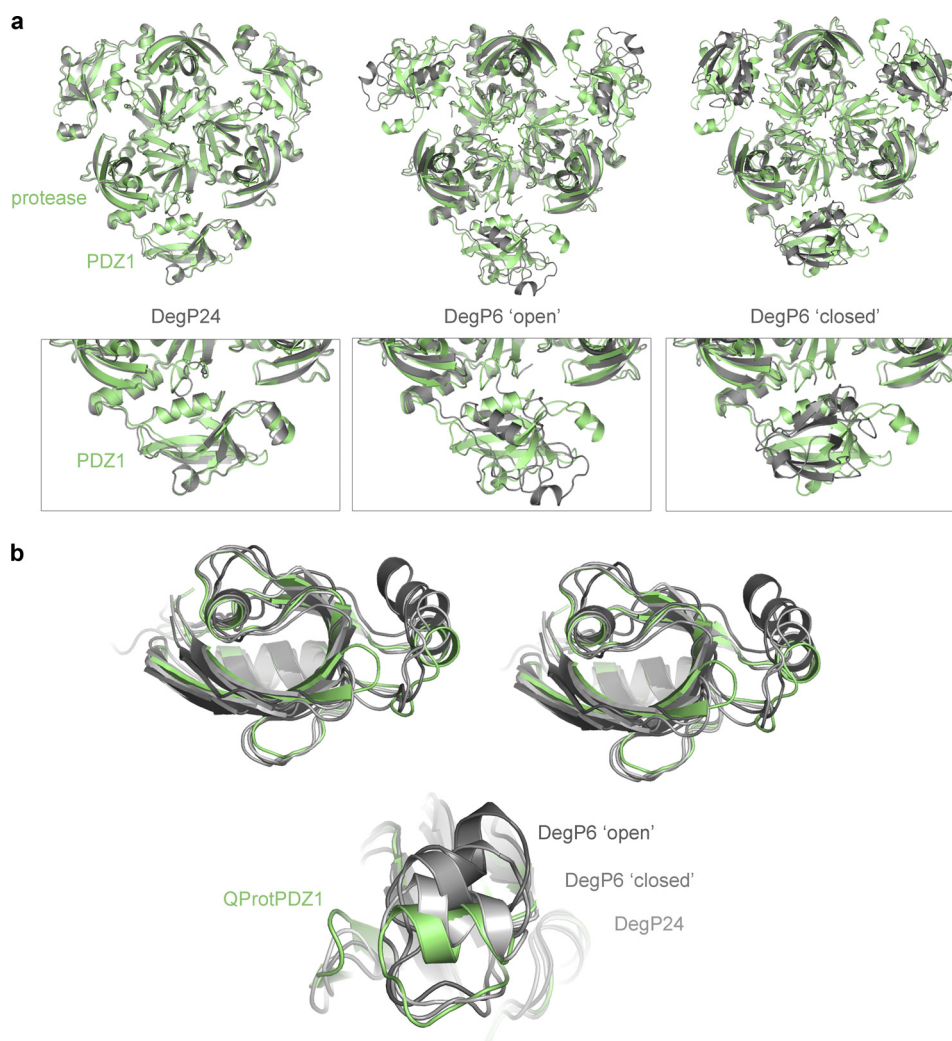


FIGURE 7. Immobilization of the PDZ1 domain in higher order HtrA oligomers is crucial for protease activation. *a*, structural alignment of one QProtPDZ1 (green) trimer to trimers of DegP24 (left panel, gray), DegP6 open conformation (middle panel, gray), and DegP6 closed conformation (right panel, gray). The enlarged picture of the protease/PDZ1 interface illustrates that QProtPDZ1 and DegP24 have strikingly similar domain orientations, whereas the PDZ1 domain of QProtPDZ1 and the two DegP6 forms is oriented differently relative to the protease domain. *b*, structural alignment of the PDZ1 domain of QProtPDZ1 (green), DegP24 (light gray), DegP6 closed (medium gray), and DegP6 open (dark gray) illustrating that the interaction clamp (helix on right side) is the structural element showing the highest en-bloc flexibility within the PDZ scaffold of HtrA proteases.

DISCUSSION

HtrA Proteases with a Single PDZ Domain Are Capable of Forming Proteolytically Active Higher Order Particles—Our biochemical and structural analysis revealed that a DegQ mutant lacking the second PDZ domain can form higher order oligomers to encapsulate and degrade substrate proteins. In analogy to DegP (24, 26), binding of an unstructured polypeptide to the PDZ1 domain induces the conversion of the resting (DegQ-hexamer and QProtPDZ1-trimer) into the catalytically active state (DegQ-12/24mer and QProtPDZ1-12mer). Consistently, it was recently shown that human HtrA1, an HtrA protease containing a single PDZ domain and carrying out protein quality control in the extracellular matrix (34), encapsulates misfolded proteins in higher order complexes (54). Given that the majority of HtrA proteases encompass only a single PDZ domain, the reassembly of trimers into higher molecular weight complexes can be considered as a conserved mechanism regulating the activity of most HtrA proteases. However, it should be noted that activation by oligomer conversion is only

relevant for soluble HtrAs, because membrane anchored HtrA proteases like DegS from *E. coli* (30) do not form oligomers larger than a trimer. Moreover, the membrane-anchored HtrA family members are often regulatory proteases, in which activation and proteolytic cleavage occur separately (30, 36). Therefore, the regulatory mechanism linking substrate binding with protease activation and oligomer conversion should be relevant for all HtrA proteases having one or two PDZ domains and acting on a broad range of misfolded proteins during protein quality control. More specialized members like the DegS stress sensor appear to be under control of more specific regulatory mechanisms that act in *trans* and that are not directly coupled with substrate binding.

The Activation Cascade PDZ → L3 → LD/L1/L2 Is Conserved in 12- and 24-meric HtrA Oligomers—Recent structural work on DegP24 uncovered key aspects of how HtrA proteases involved in protein quality control recognize, bind, and processively cleave substrate and how they are regulated by the mechanism of substrate-induced oligomer conversion (24, 26). How-

ever, to fully understand the regulation of HtrA proteases, which form distinct substrate engaging oligomers (24, 26, 29), high resolution data of a dodecameric form of a HtrA protease is required. Our structural studies of the truncated version of DegQ (QProtPDZ1) provide this information and present a detailed view of the molecular architecture of a functional HtrA dodecamer. Based on the structural information, we could verify that the molecular mechanism underlying regulation of HtrA 24-mers (with DegP24 as best characterized representative) (32, 50) is conserved in substrate engaged 12-mer particles (QProtPDZ1; this work). A structural alignment demonstrates that the relative position of PDZ1 and protease domains observed in QProtPDZ1 fits remarkably well to DegP24 (root mean square deviation 1.1 Å for 298 Ca atoms of protease and PDZ1 domain), whereas it is strikingly different from the domain arrangement in the resting DegP6 (Fig. 7a). The similar domain orientation in the 12- and 24-mer particles is even more surprising, because the two states were derived from two different proteins, DegQ and DegP, respectively, and from protein variants that differ in their domain composition. Therefore, the present data implicate that a precisely aligned PDZ1 domain is key to trigger protease activation in both HtrA 12- and 24-multimers. These multimers are held together by the PDZ interaction clamp that mediates contacts between adjacent trimers. Because of the en-bloc mobility of this motif within the PDZ fold (Fig. 7c), HtrA proteases can form different oligomeric assemblies while maintaining the critical loop L3-PDZ1 interaction required for protease activation.

DegQ Is a pH-sensitive Protease in the Bacterial Cell Envelope—Our functional *in vitro* and *in vivo* studies reveal that DegQ is a pH-related protease that maintains protein homeostasis in the bacterial cell envelope. Given the porous and thus highly permeable character of the outer membrane, all periplasmic proteins are exposed to rapid environmental changes such as changes in the pH. It is evident that resultant protein damage has to be counteracted immediately. Because of its pH-dependent activity, DegQ appears to be the “first-in-place” protease to react on pH-mediated protein misfolding. Only when the protease-chaperone system of the periplasm is overloaded and damaged proteins accumulate, is the production of DegP up-regulated. Under such stress conditions, DegP would function as the primary protease reducing the levels of misfolded proteins (55, 56). Therefore, DegQ and DegP appear to closely collaborate with each other in the bacterial cell envelope, ensuring high fidelity protein quality control under mild and severe stress conditions, respectively.

DegQ is not the only HtrA protease whose proteolytic activity is affected by pH. Deg1 and Deg2 from *Arabidopsis thaliana* chloroplasts also cleave substrates in a pH-dependent manner. The distinct pH optima of Deg1 and Deg2 appear to reflect the adaptation of the two proteases to their individual compartments (52, 53). Deg1 resides in the thylakoid lumen (57), whereas Deg2 is located at the stromal side of the thylakoid membrane (53). The light-induced pH gradient between the two adjacent compartments sustains a low pH (pH 4.5–6.0) in the lumen and an alkaline pH (above 8.0) in the stroma (58, 59). Consistently, the luminal Deg1 protease has an optimum at pH 6, whereas the stromal Deg2 protease most efficiently degrades

proteins at pH 8 (52, 53). Accordingly, the regulatory mechanism employed by DegQ could be associated with the changes of external pH in the enteric habitat of *E. coli*, which can vary between pH 5 and 8 (60). Evolving a pH-regulated protease such as DegQ would help bacteria to deal with mild pH alterations and to avoid energy- and time-consuming processes required to up-regulate the stress response machinery.

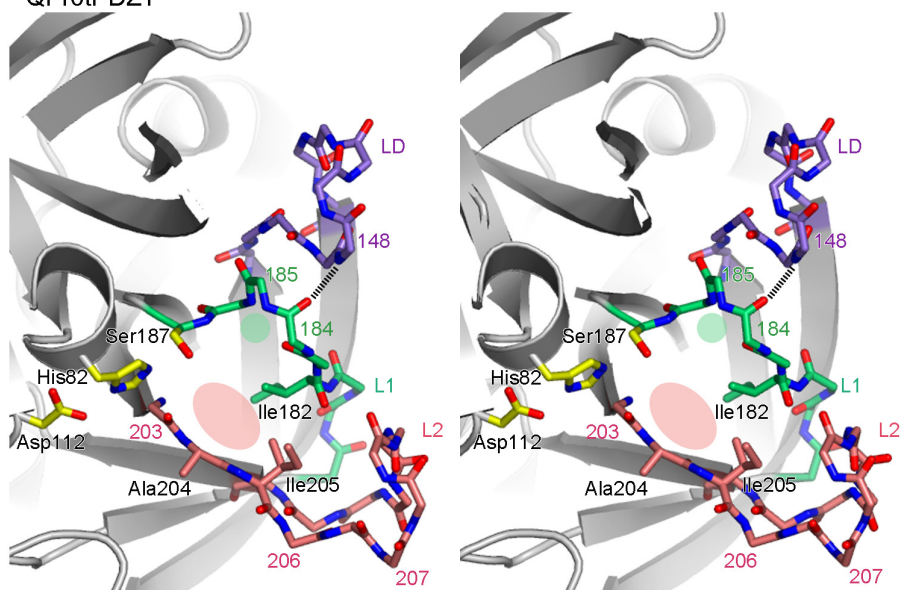
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Figure S1 Sawa et al.

QProtPDZ1



QProt

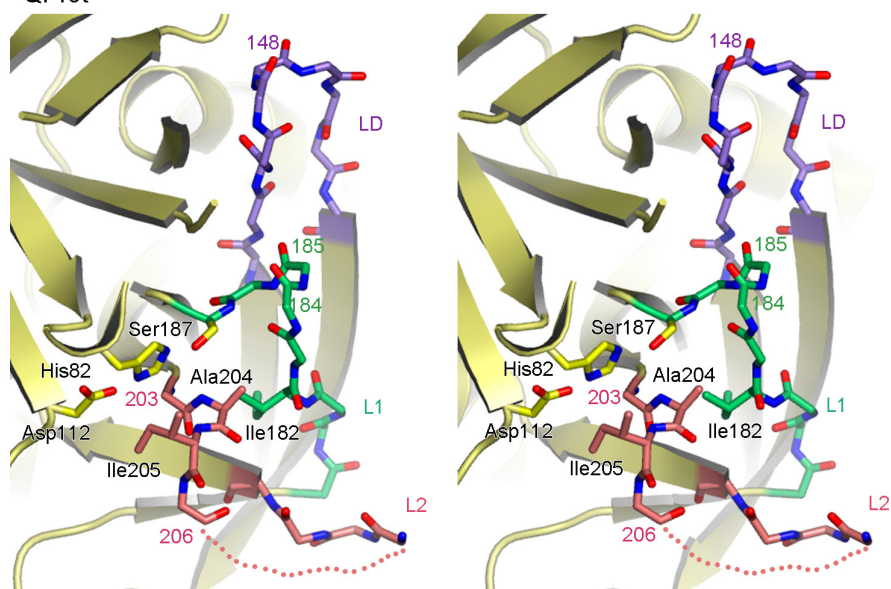


Fig. S1. **Structural details of the active site loops in proteolytically active QProtPDZ1 and inactive QProt.** Stereo view of QProtPDZ1 (top panel, grey) and QProt (bottom panel, light yellow). The backbone of the activation domain (loops L1, L2 and LD in green, light red and lilac, respectively) as well as the side chains of residues forming the S1 pocket formation (Ile182, Ala204 and Ile205, yellow) and the catalytic triad (His82, Asp112 and Ser187, yellow) are shown in stick mode. The position of the oxyanion hole is depicted as a green circle and residues engaged in its stabilization are labeled (Arg184, Gly185 and Phe148). For clarity, side chains of residues that are not discussed are omitted from the illustration.

Figure S2 Sawa et al.

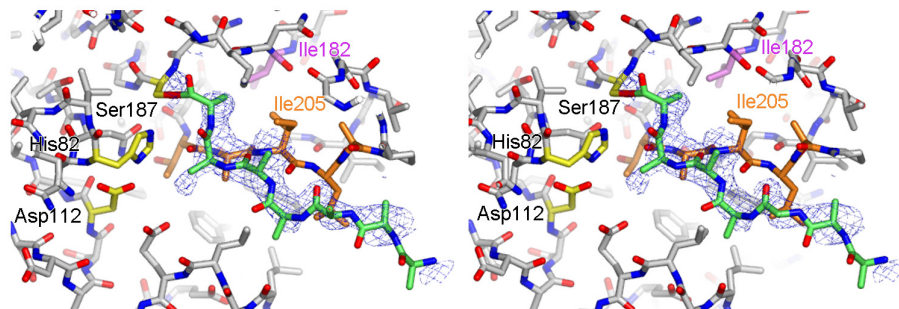


Fig. S2. **Peptide binding to the proteolytic site of QProtPDZ1.** Detailed stereo view of the proteolytic site with the bound peptide (green) that is attached via β -augmentation to the L2 loop (orange). The peptide P1 residue forms a covalent bond with the active site Ser187 (yellow, stick mode) and its side chain protrudes into the S1 specificity pocket defined by the side chains of Ile205 (orange) and Ile182 (magenta). The 2Fo–Fc electron density map, which is contoured at 1.3 σ , was calculated at 2.6 Å resolution without contribution of the bound peptide ligand.

11.2 Publication 2

Newly folded substrates inside the molecular cage of the HtrA chaperone DegQ

Malet H, Canellas F, Sawa J, Yan J, Thalassinou K, Ehrmann M, Clausen T, Saibil HR

Author contributions: EM data collection and processing, fitting of atomic coordinates into EM maps and tryptophan fluorescence were carried out by H.M. under the supervision of H.R.S. Protein purification and complex formation were done by F.C., J.S. and H.M. Mass spectrometry experiments were performed by J.Y. under the supervision of K.T. The refolding assays were carried out by J.S. and F.C. under the supervision of T.C. and M.E. H.R.S. and T.C. supervised the project. H.M., H.R.S., and T.C. wrote the manuscript.

Newly folded substrates inside the molecular cage of the HtrA chaperone DegQ

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The HtrA protein family combines chaperone and protease activities and is essential for protein quality control in many organisms. Whereas the mechanisms underlying the proteolytic function of HtrA proteins are well characterized, their chaperone activity remains poorly understood. Here we describe cryo-EM structures of *Escherichia coli* DegQ in its 12- and 24-mer states in complex with model substrates, providing a structural model of HtrA chaperone action. Up to six lysozyme substrates bind inside the DegQ 12-mer cage and are visualized in a close-to-native state. An asymmetric reconstruction reveals the binding of a well-ordered lysozyme to four DegQ protomers. DegQ PDZ domains are located adjacent to substrate density and their presence is required for chaperone activity. The substrate-interacting regions appear conserved in 12- and 24-mer cages, suggesting a common mechanism of chaperone function.

Cell viability depends on the proper structure and function of the proteome. For protein quality control, all cells have developed elaborate systems of molecular chaperones and proteases^{1,2}. Failure of protein homeostasis leads to the accumulation of misfolded or aggregated proteins, a malfunction associated with fatal protein-folding diseases³. Members of the high temperature requirement A (HtrA) family have a central role in protein quality control in a wide range of organisms, as they combine proteolytic and remodeling activities of aberrant proteins in a highly regulated and ATP-independent mechanism⁴. Disturbances in the function of human HtrA proteins (HTRA1 and HTRA2) are associated with severe disorders, including Alzheimer's and Parkinson's diseases and cancers^{5–7}. Prokaryotic HtrAs are essential for bacterial virulence and survival after exposure to various environmental and cellular stresses⁸. In *E. coli*, three HtrA proteins contribute to maintenance of protein quality in the periplasm (DegP, DegQ and DegS)^{9–13}, with DegQ considered as a model of the HtrA family, due to its high sequence identity with many HtrA members¹⁴.

HtrA proteins are composed of a chymotrypsin-like protease domain and one (DegS, HTRA1 or HTRA2) or two PDZ domains (DegP or DegQ)¹⁴. Three protease domains interact tightly to form the trimeric building blocks of all HtrA complexes. Whereas membrane-anchored HtrA proteases such as *E. coli* DegS and human HTRA2 are active as trimers^{13,15}, several soluble HtrA proteins have been shown to form larger oligomers. Human HTRA1 trimers assemble into 12-mers in the presence of non-native polypeptides¹⁶, whereas *E. coli* DegQ switches between a hexameric resting state without substrate to active 12-, 15-, 18-, 24- and 30-mer states in the presence of a substrate that can be refolded or degraded^{9,17–20}. Similarly to *E. coli* DegP, *E. coli* DegQ

changes its oligomeric state from hexamers to either 12- or 24-mers, depending on the concentration of unfolded substrate. In addition, *E. coli* DegQ forms 12-mers in the absence of substrate at acidic pH (ref. 10).

Two types of higher oligomeric structures have been described for *E. coli* DegP: soluble cages (12- and 24-mer) and bowl-shaped structures bound to liposomes (12-, 15- and 18-mers)^{9,17,18,20}. For *E. coli* DegQ, full-length 12- and 24-mers remain uncharacterized, but the DegQ Δ PDZ2 12-mer has been described as a cage-like structure of 135 Å in diameter¹⁰. This structure provides a model for the dodecameric forms of soluble HtrA proteins containing only one PDZ domain, such as human HTRA1. In addition, the recent crystal structure of a *Legionella fallonii* DegQ dodecamer shows a divergent organization and a smaller size compared to *E. coli* HtrA proteins (140 Å in diameter for DegQ_{Lf} versus 165 Å for DegP_{Ec})²¹.

Although some HtrA cage-like structures have been obtained in the presence of substrates to be folded or degraded, the protein ligands are not visible in crystallographic structures and symmetrized cryo-EM maps, probably because of conformational and positional flexibility^{9,17,18,22}. Only short peptides bound to PDZ1 and protease domains have been resolved in X-ray crystal structures. An asymmetric cryo-EM reconstruction of the DegP 12-mer showed a folded outer membrane protein (OMP) encapsulated within the cage, but its low resolution (28 Å) precluded analysis of the DegP-OMP interaction⁹.

How the HtrA proteins bind and fold their substrates, which is central to understanding their chaperone activity, thus remains to be characterized. In order to probe HtrA chaperone function, we carried out a structural and biochemical analysis of the protease-deficient S187A mutant of *E. coli* DegQ in complex with several model

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substrates. Here we present cryo-EM structures of DegQ 12- and 24-mer cages encapsulating these substrates. Unexpectedly, the DegQ 12-mer can accommodate and fold up to six lysozymes. The interaction of a bound lysozyme in close-to-native conformation with the DegQ cage is revealed by an asymmetric reconstruction of the DegQ 12-mer–lysozyme complex. PDZ domains are located close to substrate density, and they are required for DegQ chaperone activity.

RESULTS

Cryo-EM structure of DegQ24– β -casein complex

To gain insights into the chaperone function of DegQ, we determined the cryo-EM structures of DegQ–substrate complexes. DegQ 24-mers were formed by incubating proteolytically inactive hexameric DegQ_{S187A} with β -casein (a 24-kDa natively unstructured protein used as a model substrate)¹⁰ (Supplementary Fig. 1). Imaging the purified samples by EM revealed mainly large particles approximately 215 Å in diameter and minor complexes of 165 Å diameter (Supplementary Fig. 2). The large complexes correspond to 24-mers and the smaller ones represent 12-mer cages. Cryo-EM images of the 24-mers show four-, three- and two-fold symmetry (Supplementary Fig. 2), and a three-dimensional (3D) reconstruction with octahedral symmetry was obtained by angular reconstitution at 7.5 Å resolution (Supplementary Fig. 3). Overall, the cryo-EM map shows a hollow spherical shape formed by eight trimeric building blocks, similar to the DegP 24-mer^{9,17}. The quality of the map allows localization of secondary structure elements (Fig. 1a).

To obtain a pseudoatomic model of the complex, a homology model of the DegQ PDZ2 domain was generated, based on the DegP crystal structure, and combined with the DegQ protease–PDZ1 trimer crystal structure^{10,19}. Eight copies of the resulting trimer model were docked into the cryo-EM map and their positions refined by flexible fitting, allowing hinge movements between domains²³. The pseudoatomic model reveals a strong interaction between PDZ1 and PDZ2 from neighboring trimers, mediated mainly by hydrophobic residues. PDZ1 and PDZ2 of the same protomer are around 24 Å apart, separated by an extended linker eight amino acids in length (residues 333–341) (Fig. 1b).

Whereas DegP and DegQ 24-mers share a similar global organization, the DegQ complexes are slightly expanded, with a diameter of 210 Å compared to 195 Å for DegP and larger pores on the two- and four-fold axes (Supplementary Fig. 4). These differences arise from the divergence of domain orientations in DegP and DegQ. When the protease domains of the two structures are superimposed, DegP PDZ1 must be rotated by 11° to fit the DegQ24 cryo-EM map (Fig. 1c).

Figure 1 The DegQ 24-mer cryo-EM map reveals a potential β -casein binding site. **(a)** Two-fold view of the DegQ24 atomic model fitted into the 7.5-Å DegQ24–casein cryo-EM reconstruction with octahedral symmetry. Each trimer is shown in a different color. **(b)** Enlargement of contact regions. Cage formation is mediated by a tight interaction between PDZ1 and PDZ2' domains from adjacent trimers. PDZ1 and PDZ2 domains from the same protomer are connected by an elongated linker spanning 24 Å. Density that is not accounted for by the fitted DegQ atomic model is shown in orange. **(c)** Overlay of DegP and Q protomers from the 24-mer cages, aligned through their protease domains. DegQ is colored by domain with PDZ1 in cyan, PDZ2 in blue and protease in dark blue, and DegP is shown in light green. The orientation differences between DegP and DegQ PDZ1 and PDZ2 domains are indicated. **(d)** Enlargement of the boxed area in **b**. DegQ is colored by domain as in **c**. Residues in the vicinity of the additional orange density belong to domains of three different protomers (labeled domain, domain' and domain''), namely helix 251–257 of PDZ1 (in yellow), loop 408–413 of PDZ2' (in brown) and, potentially, the LA loop of the protease'' domain (residues 31–33 and 58–62 in magenta and the LA loop shown as a magenta dashed line).

The PDZ2 domain position is substantially different, with a 70° rotation and a 5-Å translation observed between DegP and DegQ. The PDZ domain orientations found in DegQ, unambiguously identified in the cryo-EM map, create a cavity defined by protease, PDZ1 and PDZ2 domains from three different protomers. The cleft thus created is surrounded by helix 251–257 of PDZ1, loop residues 408–413 from PDZ2 and residues 31–33 and 58–62 from the protease domain (Fig. 1d). Additional density (colored in orange in Fig. 1b,d) is present in this cavity and cannot be accounted for by the DegQ model. Its volume is 7,300 Å³, corresponding to 6 kDa. We propose that this density corresponds to either (i) the LA loop comprising residues 34–57 of the protease domain, previously shown to be an important regulator of the protease activity but not modeled in DegQ 24-mer owing to its flexibility or (ii) part of the β -casein substrate. Visualization of only 6 kDa out of 24 kDa for β -casein would be consistent with the disordered, natively unstructured state of β -casein. Given that the additional density is buried in a cavity, we suggest that it is more likely to correspond to the ligand density.

Cryo-EM structure of a DegQ12–peptide complex

To discriminate between ligand and DegQ densities, we determined 3D reconstructions of the DegQ cage with and without a high-molecular-mass substrate. To obtain a homogeneous preparation of DegQ 12-mers devoid of substantial ligand density, we incubated DegQ hexamers with a peptide that was 20 amino acids long and that was previously shown to bind to the DegQ PDZ1 domain (SPMFKGVLDMMYGGMRGYQV)^{10,24}. DegQ12–peptide complexes were purified by size-exclusion chromatography (SEC) and imaged by EM, revealing a preparation of hollow, round particles with a diameter of ~165 Å (Supplementary Figs. 1 and 2). The tetrahedral symmetry of the 12-mer complexes, consistent with the observation of two- and three-fold views in cryo-EM particle averages (Supplementary Fig. 2), was unambiguously identified by angular reconstitution. A 3D cryo-EM map was reconstructed at 7.5 Å resolution (Supplementary Fig. 3), allowing precise fitting of the domains and identification of secondary structure elements (Fig. 2a,b). Previous crystallographic analyses

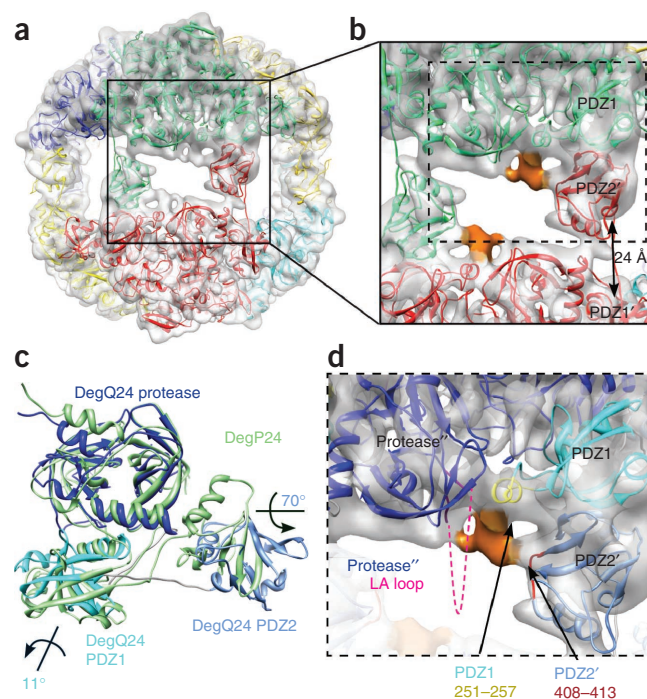


Figure 2 DegQ 12-mer complexes with a peptide and lysozyme. (a,b) Three-fold (a) and two-fold (b) views of the DegQ12 atomic model, colored by trimer, fitted into the 7.5-Å resolution DegQ12–peptide cryo-EM map with tetrahedral symmetry. Regions in the vicinity of the suggested β -casein density of DegQ 24-mer are shown in red. No substrate is visible at this position in the DegQ12–peptide complex, indicated by dashed orange ellipses. (c,d) Three-fold (c) and two-fold (d) views of the tetrahedral map of DegQ12–lysozyme at 13-Å resolution. The extra density relative to DegQ12–peptide is colored in orange. Six folded lysozymes are fitted into this additional density, colored in different shades of purple, magenta and orange. (e,f) Cutaway of three-fold (e) and two-fold (f) view, highlighting the lysozyme fitting.

suggest that polypeptides are cooperatively bound by PDZ1 and protease domains, based on the observation of short segments of peptide binding^{10,18,24}. Our cryo-EM map is compatible with this binding mode, but the peptide was omitted from the model, as the map resolution prevents accurate peptide positioning.

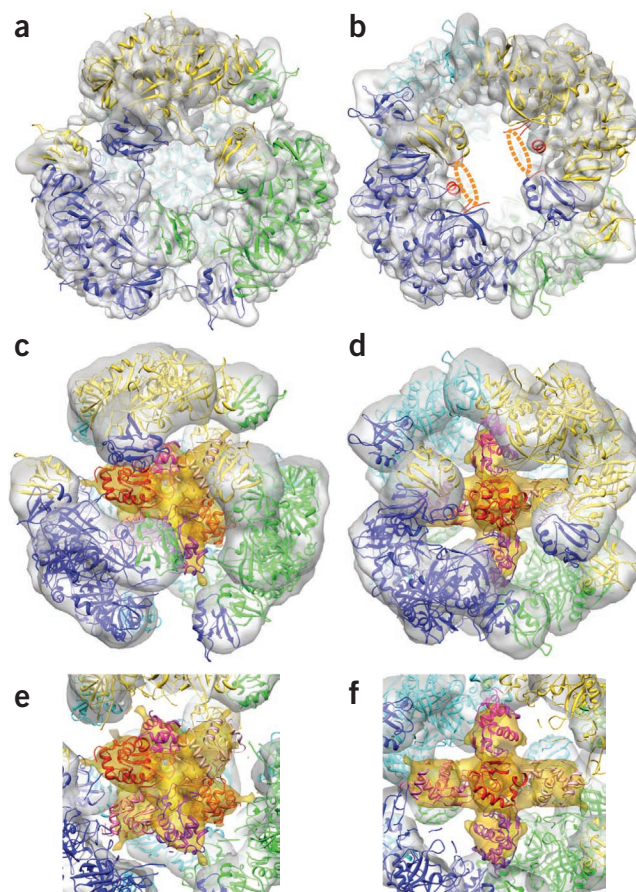
DegQ 12-mer formation is mediated by the interaction of four DegQ trimers through PDZ1 and PDZ2' domains from neighboring trimers. The cage is thus formed of four structural units, each of them containing protease-PDZ1 domains of three subunits tightly bound through hydrophobic interactions to three PDZ2' domains from neighboring protomers. The overall organization of the DegQ 12-mer is reminiscent of the arrangement previously observed in a DegP12–lysozyme cryo-EM map¹⁷. However, there are marked differences in PDZ positions between DegP and DegQ. As in the DegQ 24-mer, PDZ2 is rotated by 70° relative to DegP (Supplementary Fig. 5). In addition, the DegQ 12-mer PDZ1 domain is rotated and shifted 7 Å toward the interior of the cage, compared to its position in DegP, with appreciable flexibility of PDZ1 helix 251–257. En-bloc movement of PDZ1 relative to the protease domain is observed in both DegQ 12- and 24-mers. This conformational change was unexpected, as PDZ1 was observed in a conserved position in all previously determined *E. coli* HtrA cage structures^{9,10,18} (Supplementary Fig. 6).

Comparison of DegQ 12- and 24-mer cages reveals that the DegQ regions adjacent to the suggested β -casein density in the DegQ 24-mer (34–57 from the protease domain, 251–257 from PDZ1 and 408–413 from PDZ2) are close to each other in the DegQ12–peptide map (Fig. 2b). They protrude toward the interior of the cage, in a position compatible with substrate binding, near the predicted peptide binding site. The DegQ12–peptide map is devoid of additional density, further suggesting that the additional density in the DegQ 24-mer corresponds to β -casein rather than to the LA loop.

Five or six folded lysozymes bind inside the DegQ 12-mer

We then investigated the positioning of substrates within the DegQ_{S187A} 12-mer, using reduced and chemically denatured lysozyme (14.3 kDa) as a model substrate. Upon incubation with purified hexameric DegQ_{S187A} at 37 °C, DegQ12–lysozyme complexes formed, which were subsequently purified by SEC (Supplementary Fig. 1). Negative stain and cryo-EM images revealed the presence of cages similar in size to the ones observed for the DegQ12–peptide (Supplementary Fig. 2). The volume of the 12-mer cage appears independent of substrate composition and molecular mass.

The DegQ12–lysozyme images show additional density filling the cages, probably representing the substrate (compare Supplementary Fig. 2b,c). A cryo-EM 3D reconstruction with tetrahedral symmetry was obtained at 13 Å resolution and shows the same DegQ structure as in the peptide complex (Fig. 2c,d and Supplementary Fig. 3). The protease domains remain in the same positions, and only a slight opening of the PDZ1–PDZ2' domain contact is observed. A difference map between DegQ12–lysozyme and DegQ12–peptide reveals the



substantial lysozyme density inside the cage, interacting with the inner surface of DegQ (Fig. 2c–f). The additional density has a volume of 80,000 Å³, corresponding to 66 kDa, indicating the binding of about five folded lysozymes (total molecular mass ~70 kDa).

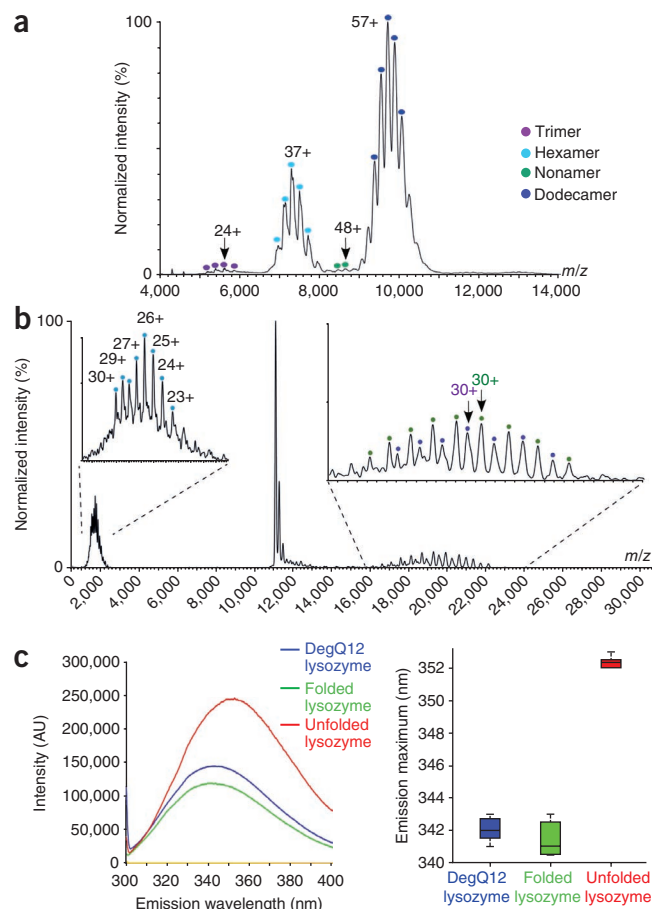
To establish the exact number of lysozymes bound inside the DegQ 12-mer, we conducted MS experiments. We initially recorded a denaturing MS spectrum of DegQ12–lysozyme to determine the mass of the DegQ monomer. The most abundant species has a molecular mass of $44,835.4 \pm 11.0$ Da, which is smaller than the theoretical mass, probably because of proteolysis at the unstructured termini of DegQ. To determine the precise stoichiometry of DegQ–lysozyme assemblies, we analyzed the apo- and substrate-bound DegQ 12-mer complexes by native MS. To obtain DegQ 12-mers in the absence of substrate, we incubated DegQ at pH 5.5, yielding an equilibrium mixture of hexamers and 12-mers, as shown by SEC¹⁰ and negative stain EM (Supplementary Fig. 2). Native MS revealed components with the expected masses for DegQ hexamers and 12-mers, $(270,135.0 \pm 29.2$ and $553,105.8 \pm 156.5$ Da, respectively) (Fig. 3a). To determine the number of bound lysozymes, MS spectra of the DegQ12–lysozyme complexes were initially recorded but showed a very broad peak preventing unambiguous mass determination (data not shown). To overcome this, a tandem MS approach was applied. Tandem MS has previously been used to resolve overlapping charge states arising from polydisperse samples and from the presence of different substrate-bound complexes^{25–27}. The peak at 11,050 *m/z* from the DegQ12–lysozyme precursor was selected using the quadrupole mass filter and subjected to collision-induced dissociation (Fig. 3b). The peak series in the low *m/z* region corresponds to a highly charged, ejected DegQ monomer subunit, and the peak series at higher *m/z* corresponds to the charge-stripped DegQ 11-mer with lysozyme bound. At this region there is a greater separation between

Figure 3 Five or six folded lysozymes are bound to the DegQ 12-mer. (a) Mass spectrum of the DegQ protein without substrate bound. Peaks corresponding to different multimeric states of apo DegQ are labeled in different colors. The most abundant charge state for each of the peak series is indicated. (b) Tandem MS spectrum of the DegQ protein bound to lysozyme. The peak at $m/z = 11,050$ corresponds to the precursor ion that was selected for dissociation. The peak series in the lower m/z region corresponds to the ejected DegQ monomer. The two peak series at higher m/z represent charge-stripped DegQ 11-mer bound to six (major) and five (minor) lysozyme molecules, and are labeled in green and purple, respectively. (c) Tryptophan fluorescence spectra (left) and box plot of the maximum emission wavelength (right) for folded lysozyme (green), unfolded lysozyme (red) and DegQ12-lysozyme (blue). As expected, there is no detectable fluorescence from DegQ alone (yellow, on x axis), indicating that the DegQ12-lysozyme signal corresponds to lysozyme fluorescence. AU, arbitrary units.

charge states facilitating mass assignment²⁸. Two predominant charge state series are present in the charge-stripped complex region, corresponding to a mass of $596,555 \pm 249$ Da and $585,788 \pm 365$ Da, with the former being the dominant species. These masses correspond to DegQ 11-mer bound to six or five lysozymes, respectively.

Consistently, six folded lysozyme molecules can be fitted into this density without clashes (Fig. 2c–f). In order to confirm that the bound lysozyme substrates are folded as implied by the cryo-EM density, we took advantage of the absence of tryptophan in DegQ to monitor the average tryptophan fluorescence of bound lysozymes^{29,30}. The maximum emission of DegQ12-lysozyme is observed at 342.5 nm, whereas the maximum emission is 341.5 nm for folded lysozyme and 352.5 nm for unfolded lysozyme (Fig. 3c). The fluorescence intensity is also increased in the unfolded state (Fig. 3c, with the same concentration of lysozyme used for all spectra). Therefore, the spectra suggest that the bound lysozymes are in a state that is close to native. The small difference in emission maximum and intensity between folded lysozyme and DegQ12-lysozyme could arise from modification of the tryptophan environment by an interaction with DegQ or from a not completely native conformation of the lysozyme. Because cage formation is triggered by denatured but not by folded lysozyme, these results indicate that lysozymes fold within the DegQ cage.

In the tetrahedrally symmetric map, the substrate density level is only about one-third that of DegQ, suggesting that the lysozyme arrangement is most likely asymmetric or disordered. The calculation of an asymmetric reconstruction of DegQ12-lysozyme is thus important for a more accurate description of lysozyme density in the DegQ 12-mer cage.



Substrate-binding regions in DegQ cages

An asymmetric reconstruction of DegQ12-lysozyme was obtained at 14.2-Å resolution (Fig. 4 and Supplementary Fig. 3), revealing the presence of two separate substrate densities. One density is present in the middle of the complex and does not show any direct interaction with DegQ (Fig. 4a,b, in purple). Its volume corresponds to 2.4 folded lysozymes and it probably represents mobile substrates sequestered by the DegQ 12-mer cage. The second density is in contact with DegQ and has a volume corresponding to 10 kDa (Fig. 4a,b, colored orange). It presents a two-lobed shape, compatible with the two domains of a folded lysozyme (Fig. 4c).

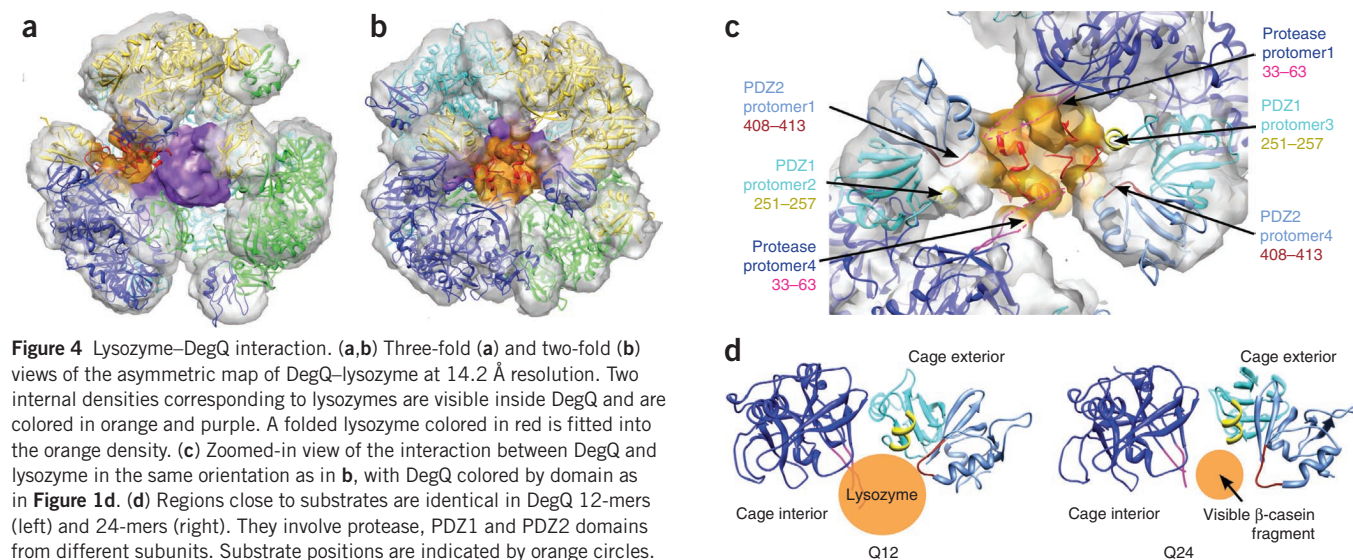
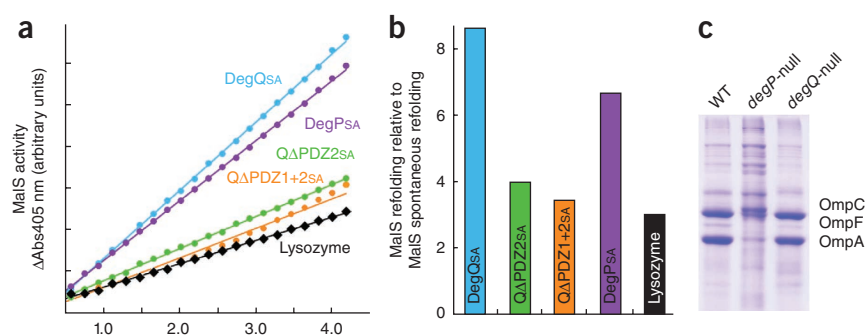


Figure 4 Lysozyme–DegQ interaction. (a,b) Three-fold (a) and two-fold (b) views of the asymmetric map of DegQ-lysozyme at 14.2 Å resolution. Two internal densities corresponding to lysozymes are visible inside DegQ and are colored in orange and purple. A folded lysozyme colored in red is fitted into the orange density. (c) Zoomed-in view of the interaction between DegQ and lysozyme in the same orientation as in b, with DegQ colored by domain as in Figure 1d. (d) Regions close to substrates are identical in DegQ 12-mers (left) and 24-mers (right). They involve protease, PDZ1 and PDZ2 domains from different subunits. Substrate positions are indicated by orange circles.

Figure 5 Chaperone activity of DegQ. (a) MaS refolding assay. The effects of protease-deficient DegP_{S210A} and DegQ_{S187A} constructs on MaS refolding were examined. Lysozyme was used as a nonspecific solute for the negative control. The linear time course indicates that the rate of MaS refolding is constant over time. (b) MaS refolding activity of different DegQ and DegP constructs, and lysozyme control, relative to spontaneous MaS refolding in buffer. (c) Steady-state levels of OmpC, OmpF and OmpA in wild-type (WT), *degP*-null and *degQ*-null mutant strains. Outer membranes were prepared from equivalent numbers of cells.



This can be fitted into this density without creating clashes, although it cannot be precisely positioned at the resolution of this EM map. On the other hand, the DegQ domain positions are well defined by the comparison with DegQ12-peptide. Therefore, the reliable fitting of DegQ reveals the sites of its interaction with the lysozyme density. The ordered parts of the protease domain LA loop are oriented toward the lysozyme, suggesting involvement of the LA loop in lysozyme binding (Fig. 4c). In addition, lysozyme is adjacent to the protease and PDZ2 domains of two DegQ subunits as well as the PDZ1 domains of another two DegQ subunits. The PDZ regions that appear to be involved in lysozyme binding in DegQ 12-mer, namely helix residues 251–257 of PDZ1 and loop 408–413 of PDZ2, are the ones located close to the additional density attributed to β -casein in the DegQ 24-mer (Fig. 4d).

PDZ domains are needed for DegQ chaperone activity

The proximity between PDZ regions and substrate prompted us to investigate the role of PDZ domains in DegQ chaperone function. Refolding of α -amylase (MaS), a natural periplasmic substrate of DegP, was used to monitor the chaperone activity of several DegQ constructs. In this assay, DegQ was incubated with chemically denatured MaS and MaS substrate *p*-nitrophenylhexaamide (PNP6). When folded, MaS cleaves PNP6, yielding the chromogenic *p*-nitrophenol that absorbs at 405 nm. The rate of MaS folding in the presence of HtrA chaperones can thus be readily monitored and compared to its spontaneous folding. Under the *in vitro* conditions used, the protease-deficient DegQ_{S187A} mutant has a slightly higher chaperone activity than DegP_{S210A} (Fig. 5a,b). Deletion mutants of DegQ_{S187A} (Δ PDZ2 and Δ PDZ1+2) have a much lower chaperone activity than DegQ_{S187A}. A role for PDZ domains in chaperone activity is in accordance with the observation of substrate densities adjacent to these domains in the cryo-EM structures of *E. coli* DegQ cages. The low chaperone activity of DegQ_{S187A} Δ PDZ1+2 may also arise from its inability to form cages¹⁰.

We then attempted to determine which PDZ domain residues are implicated in substrate binding and folding. PDZ2 loop 408–413 contains poorly conserved hydrophobic residues. Therefore, PDZ2 might be dispensable for substrate binding in other organisms, as reported for *L. fallonii* DegQ²¹. We thus focused our analysis on the PDZ1 helix 251–257, which contains two hydrophobic residues, namely Ile253 and Phe257. In addition, we noticed that residue Phe266 was correctly oriented to potentially interact with substrates. Unfortunately, the triple mutant I253A F257A F266A, the double mutant I253A F257A and the three corresponding single mutants interfere with 12-mer formation in the presence of lysozyme substrate, preventing the analysis of DegQ12 mutant chaperone activity (Supplementary Fig. 1).

DISCUSSION

The DegQ-substrate complexes presented here reveal new information about the chaperone function of HtrA proteins. The combination

of single-particle cryo-EM, native MS and fluorescence analyses provides strong evidence for the folding of five or six lysozymes inside the DegQ 12-mer. The results suggest that in the context of the cell, DegQ can capture and enclose multiple small, unfolded substrates that are subsequently refolded within its cavity. It is instructive to compare DegQ12-lysozyme with the structure of a closed chaperonin cage containing a newly folded substrate, GroEL-gp31 bound to the T4 bacteriophage capsid protein gp23 (gp31 is the T4 bacteriophage homolog of GroES)³¹. The mechanisms of cage assembly differ: chaperonin cage formation is regulated by ATP binding, whereas DegQ cage assembly is ATP-independent and triggered by substrate binding. The cage architecture is also different, with two compartments alternately used for folding in chaperonins, whereas DegQ forms a single, larger cage. Substrate packing inside the molecular chaperone cages is very dense for both chaperonins and DegQ. The fraction of substrate that is visible, presumably because of ordered packing in a restricted volume, is also comparable, around 70% for GroEL-gp23 and 78% for DegQ12-lysozyme. A similar packing density has been observed for tubulin inside the CCT chaperonin³². However, chaperonins encapsulate only one substrate at a time per compartment, in contrast to the present finding of up to six lysozymes inside one DegQ cage. Folding of multiple substrates within the same compartment, along with the combination of proteolytic and chaperone activities, might have evolved in response to the direct exposure of the periplasm to environmental stresses.

The asymmetric map of DegQ12-lysozyme identifies regions adjacent to the lysozyme (Fig. 4c). They originate from protease, PDZ1 and PDZ2 domains of four different protomers. In addition, we show that PDZ-deletion mutants of DegQ have low chaperone activity in MaS refolding assays (Fig. 5a,b). These data suggest that cage formation and/or interaction of PDZ domains with the substrates are required for chaperone activity. It is notable that the regions of DegQ close to the lysozyme (helix 251–257 of PDZ1, loop 408–413 of PDZ2 and loop LA of the protease domain) are also adjacent to the proposed β -casein density in DegQ 24-mer (Fig. 4d). Thus, our maps suggest that not only the global structural organization but also the binding mode of chaperone substrates are conserved in the two cages. The cryo-EM maps of DegQ assemblies reveal a conserved organization of cage-like complexes in *E. coli* DegP and DegQ. PDZ1 and PDZ2' from different protomers are involved in 12- and 24-mer cage assembly. In a previous study, we proposed a different domain arrangement, based on the fitting of OMP-bound DegP into a low-resolution, asymmetric cryo-EM map. In light of the results presented here and the DegP 12-mer structures^{9,17}, we have revised our fitting of the OMP-bound DegP 12-mer, leading to a consensus for *E. coli* DegP and DegQ cage architecture (Supplementary Fig. 7) (PDB accession code of the new fit is 4A8D).

Although the overall organization of *E. coli* DegQ and DegP 12-mer is conserved, the recently published DegQ 12-mer structure of *L. fallonii* shows a different assembly²¹. Whereas a PDZ2 domain

of DegQ_{EC} only interacts with a PDZ1' domain of another protomer, a PDZ2 domain in the 12-mer of DegQ_{LF} additionally interacts with two PDZ2 and one protease domain of neighboring protomers. Consequently, the DegQ_{LF} 12-mer forms a smaller cage (140 Å in diameter versus 165 Å for DegQ_{EC}). We think it will be interesting to determine the physiological implications of these architectural differences between DegQ_{LF} and DegQ_{EC} in future studies.

Although the global structure of *E. coli* DegP and DegQ cages is preserved, the positions of their PDZ domains differ. PDZ1 domains of DegQ 12- and 24-mer deviate from their positions in DegP cages, with marked rotation relative to the protease domain. The PDZ1 densities are less well defined in the DegQ 12-mer asymmetric map, implying that they are mobile. PDZ2 orientation differs by 70° between DegP and DegQ. As a consequence, DegP and DegQ cages differ slightly in shape, size and electrostatic potential. These structural divergences might be related to the differences in DegP and DegQ function, for example, regarding OMP biogenesis in *E. coli*. Indeed, we observe that isolated outer membranes from the *degQ*-null strain show no detectable alterations of OMP composition compared to wild-type *E. coli* cells, unlike the *degP*-null mutant, in which the levels of some OMPs, including OmpA, OmpC and OmpF, are markedly decreased (Fig. 5c). Consistent with this observation, DegQ is also reported to be dispensable for OMP folding in *Neisseria meningitidis*³³. Our data thus support the model of divergence of substrate specificity between DegP and DegQ and suggest directions for further investigation.

In conclusion, this study pinpoints substrate-binding regions within the cavity of *E. coli* DegQ cages. As many HtrA members are DegQ homologs, the results presented here provide insights into how members of the HtrA protein family encapsulate and fold substrates.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Accession codes. Cryo-EM maps and C α traces of the corresponding fitted atomic structures have been deposited in the Electron Microscopy Data Bank and Protein Data Bank, respectively, with accession codes EMD-1981 and PDB 4A8A for asymmetric DegQ12-lysozyme, EMD-1982 and PDB 4A8B for tetrahedral DegQ12-lysozyme, EMD-1983 and PDB 4A8C for DegQ12-peptide, EMD-1984 and PDB 4A9G for DegQ24-casein. The C α trace of the modified DegP12-OMP fit has been deposited in the PDB with accession code 4A8D.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

EM data collection and processing, fitting of atomic coordinates into EM maps and tryptophan fluorescence were carried out by H.M. under the supervision of H.R.S. Protein purification and complex formation were done by F.C., J.S. and H.M. MS experiments were conducted by J.Y. under the supervision of K.T. The refolding assays were carried out by J.S. and F.C. under the supervision of T.C. and M.E. H.R.S. and T.C. supervised the project. H.M., H.R.S. and T.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Specimen preparation and electron microscopy data collection. DegQ24-casein, DegQ12-lysozyme and DegQ12-peptide were prepared as described previously³⁴. For cryo-EM data collection, DegQ24-casein, DegQ12-lysozyme and DegQ12-peptide were diluted to 0.2 mg ml⁻¹ in a buffer containing 20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl. The buffer was supplemented with 100 µM of the PDZ1-binding peptide for the DegQ12-peptide sample. Four-microliter samples were applied to glow-discharged C-flat grids (CF-2/2-4C-100, Protochips). After 30 s, excess solution was blotted and the grid frozen in liquid ethane. Cryo-EM was collected on a Tecnai F20 microscope (FEI), operated at 200 kV under low-dose conditions. Micrographs were recorded on Kodak SO-163 film at 50,000 magnification, with defocus ranging from 1 to 3 µm.

Image processing. Micrographs were digitized on a SCAI microdensitometer (Zeiss) at 1.4 Å per pixel. A total of 12,312 (DegQ24-casein) and 16,790 (DegQ12-lysozyme) particles were manually picked in Ximdisp³⁵. Using BOXER³⁶, 36,790 particles of DegQ12-peptide were semi-automatically picked. The defocus and astigmatism of the images were determined using CTFIND3 and corrected for the effect of the contrast transfer function (CTF) by phase flipping³⁷. Full CTF correction was applied at the final stage for DegQ12-peptide and DegQ24-casein reconstructions. Images were filtered between 230 and 4 Å (DegQ24-casein) and between 165 and 4 Å (DegQ12-lysozyme, DegQ12-peptide) and normalized using SPIDER³⁸. Image processing and 3D reconstructions were done in SPIDER and in IMAGIC-5 (ref. 39). Multivariate statistical analysis and eigenimage analysis revealed the presence of four-, three- and two-fold symmetry in DegQ24-casein particles and three- and two-fold symmetry in DegQ 12-mer complexes. Angular reconstitution 3D models clearly indicated that Deg 12-mer is tetrahedral and DegQ 24-mer is octahedral. The 3D maps were refined using angular reconstitution and projection matching. The asymmetric 3D map of DegQ12-lysozyme was calculated by projection matching using the final DegQ12-lysozyme symmetrized map as a starting model. Resolution of the reconstructions was assessed by Fourier shell correlation at 0.5 correlation. A more comprehensive description of data processing procedures can be found in the **Supplementary Methods**.

Fitting. *E. coli* DegQ protease-PDZ1 crystal structure and a homology model of DegQ PDZ2 were used during the fitting procedure (homology model generated with MODELLER⁴⁰-based sequence-structure alignment with *E. coli* DegP PDB 3CS0 (ref. 41)). Rigid body and flexible fitting were carried out using UCSF Chimera⁴² 'fit-in-map module' and Flex-EM⁴³, as described in the **Supplementary Methods**. Improvement of cross-correlation between atomic models and cryo-EM maps is shown in **Supplementary Table 1**. Z-scores revealing uniqueness of fits are indicated in **Supplementary Table 2**.

Mass spectrometry. For native MS experiments, DegQ complexes with and without bound lysozyme were buffer exchanged into 100 mM ammonium acetate and concentrated to 15 µM using Amicon Ultra 0.5-ml centrifugal filters (Millipore). For denaturing MS experiments, DegQ complexes were buffer exchanged into 49:49:2 (v/v/v) water/methanol/acetic acid.

MS experiments were carried out on a Synapt HDMS (Waters) Quadrupole-TOF mass spectrometer⁴⁴. Samples (2- to 3-µl aliquots) were introduced to the mass spectrometer by means of nanoelectrospray ionization using gold-coated capillaries that were prepared in house. Typical instrumental parameters were as follows: source pressure, 6 mbar; capillary voltage, 1.0–1.3 kV; cone voltage, 150–200 V; trap energy, 20 V; transfer energy, 10 V; bias, 2.0 V; and trap pressure, 3.6×10^{-2} mbar. For tandem MS experiments, the bias voltage was increased to 80 V.

Mass spectra were smoothed and peak-centered in MassLynx v4.1 (Waters). Mass assignment was achieved by a previously described method⁴⁵, whereby the

charge is iterated over the measured mass value and the s.d. for a given charge state series is calculated each time. The solution is the series that gives rise to the lowest s.d.

Fluorescence. Intrinsic tryptophan fluorescence was excited at 295 nm (to exclude tyrosine fluorescence) and monitored between 300 and 400 nm, with a slit width of 0.7 mm, using a FluoroMax-3 spectrofluorometer (Horiba). The same concentration of lysozyme was used for all the experiments (0.01 mg ml⁻¹). Unfolded lysozyme was prepared by incubation with 10 mM DTT and 8 M urea at 37° for 30 min. Folded lysozyme and SEC-purified DegQ12-lysozyme were buffered with 10 mM HEPES-NaOH, pH 7.5, in 150 mM NaCl. The low background signals from the buffers were subtracted for analysis of the spectra, and each measurement was repeated six times.

MalS refolding assays. MalS, DegP and DegQ purifications and MalS refolding assays were carried out as described previously^{34,41,46}. The point mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene) and the constructs verified by DNA sequence analysis. To determine the effect of DegQ and DegP on MalS refolding, we preincubated 2 µM of DegQ wild type, DegQ_{S187A}, DegP_{S210A}, DegQ_{S187A}ΔPDZ1+2 or DegQ_{S187A}ΔPDZ2 with 2 mM PNP6 in 250 mM NaH₂PO₄, pH 7.5. After 5 min, unfolded MalS was added to a final concentration of 0.13 µM. The activity of the refolded amylase was determined using PNP6 (2 mM final concentration) as a substrate. The release of *p*-nitrophenol from PNP6 by MalS was monitored at 405 nm with a microplate reader. Assays were carried out in a total volume of 100 µl at 22 °C. Lysozyme was used as a negative control, as it supports a lower rate of MalS refolding in a concentration-independent manner, thus reflecting nonspecific interactions.

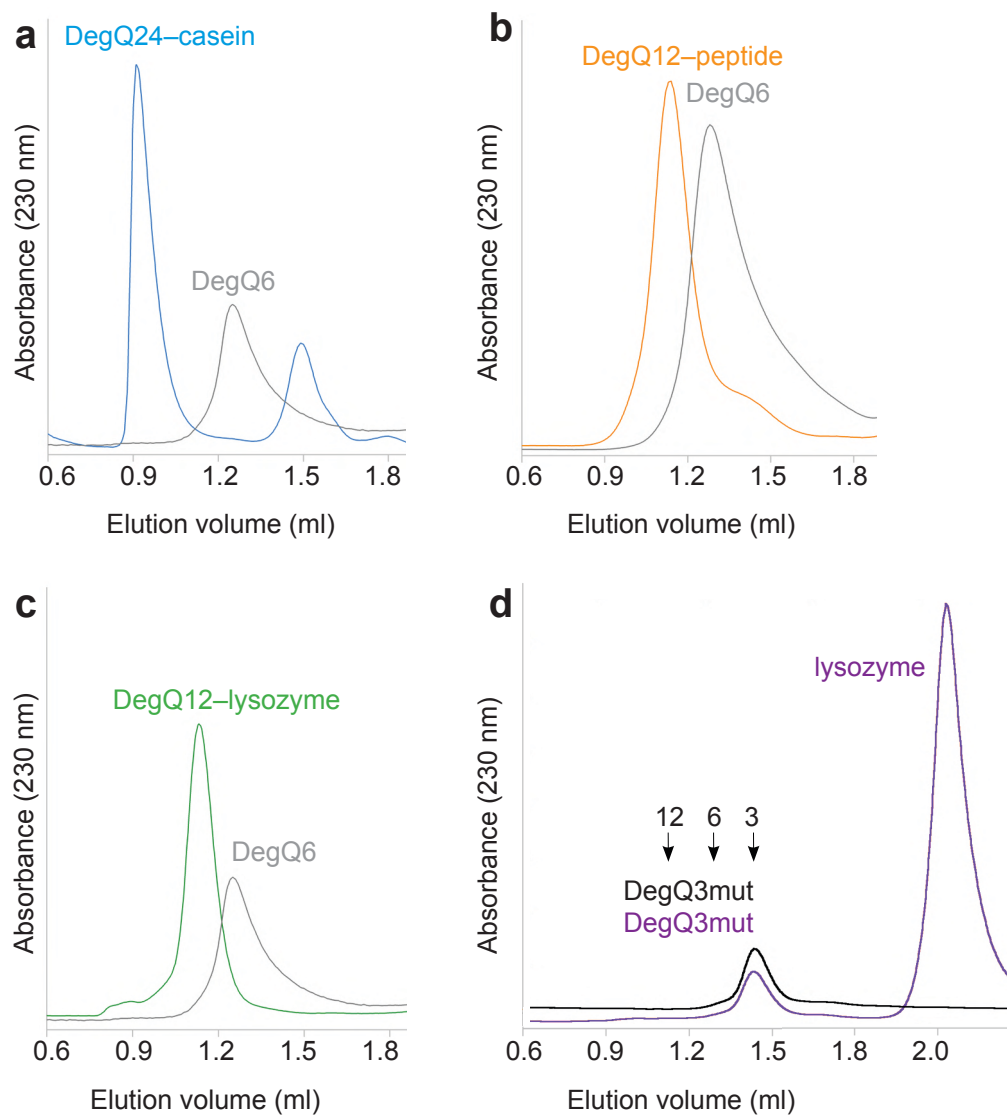
Outer membrane isolation. Outer membranes of *E. coli* wild type, *degQ*-null (MG1655 *degQ::Tn5 KanR*) and *degP*-null (CLC198, *degP::Tn10*) mutant strains were prepared as described previously⁴⁷, with minor modifications as detailed in the **Supplementary Methods**.

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Supplementary Information

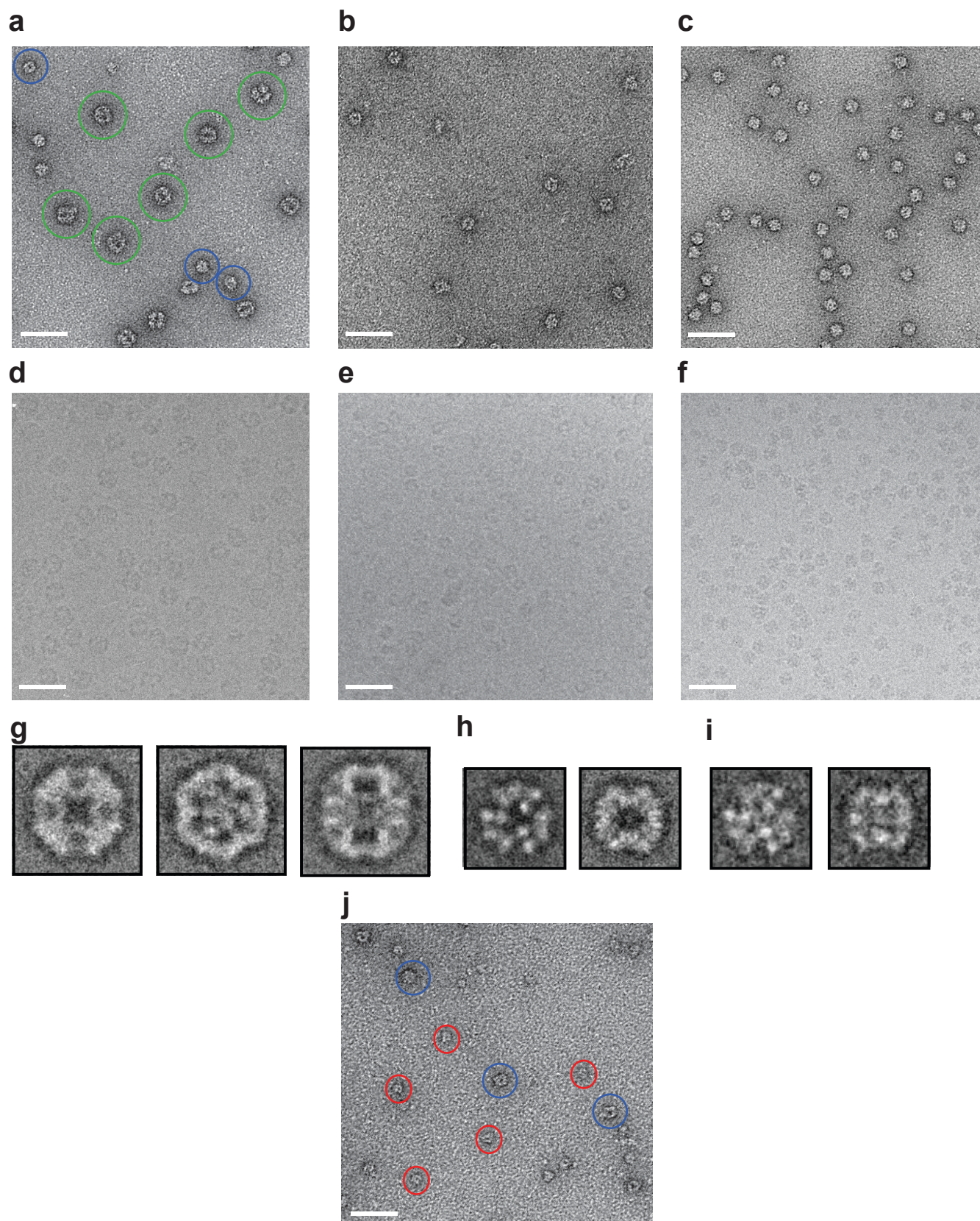
Newly folded substrates inside the molecular cage of the HtrA chaperone DegQ

Hélène Malet, Flavia Canellas, Justyna Sawa, Jun Yan, Konstantinos Thalassinou, Michael Ehrmann, Tim Clausen, Helen R Saibil



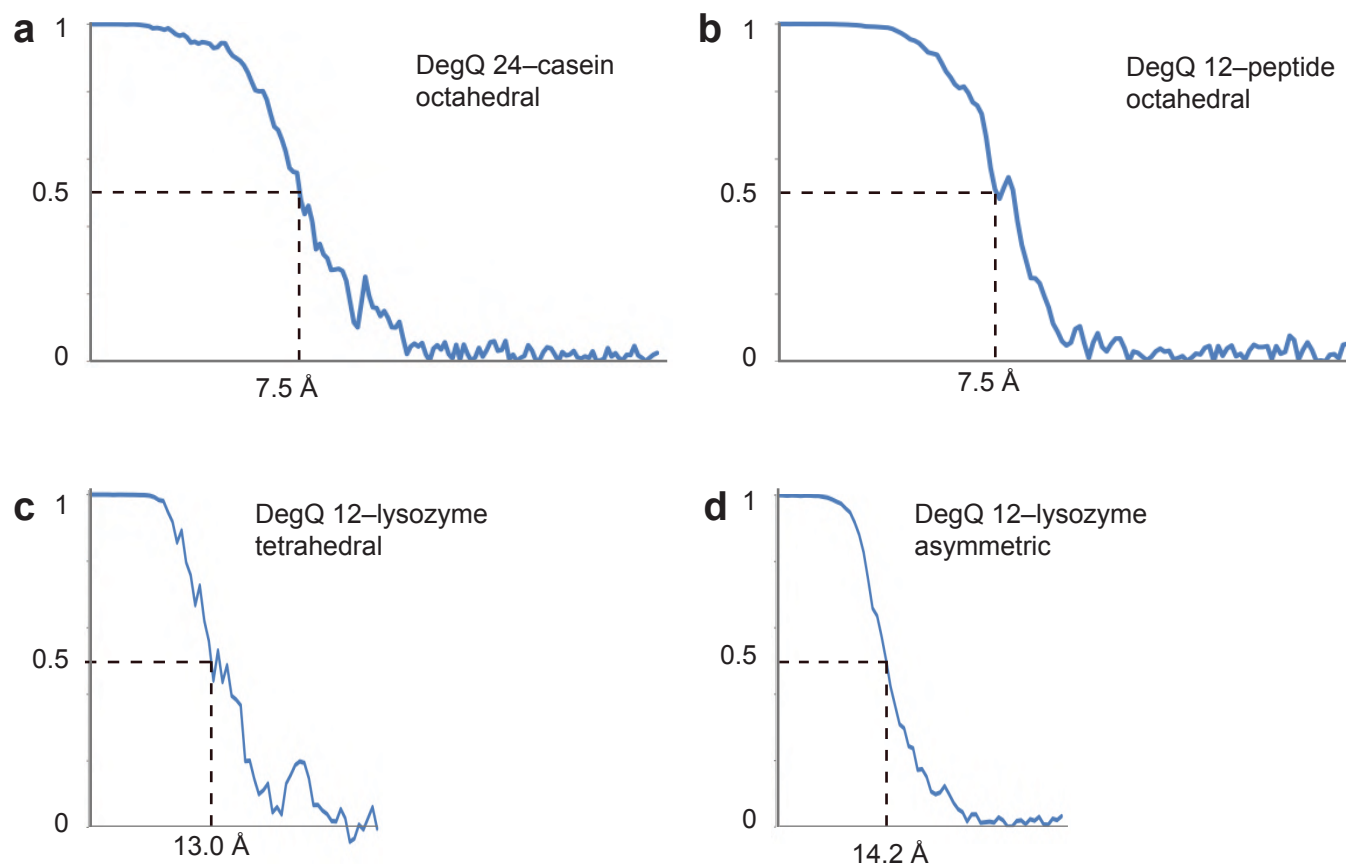
Supplementary figure 1. SEC analysis of DegQ in the presence of different substrates

(a-c) SEC analysis of DegQ in the presence of β -casein **(a)**, a binding peptide **(b)** and lysozyme **(c)**. Substrate binding triggers oligomer conversion from hexamer to 12- (lysozyme, peptide) or 24-mer (β -casein). **(d)** SEC of the DegQ triple mutant I253A F257A F266A. The mutations prevent hexamer formation in the absence of substrate and 12-mer formation in the presence of lysozyme. Expected elution volumes of trimers (3), hexamers (6) and 12-mers (12) are indicated.



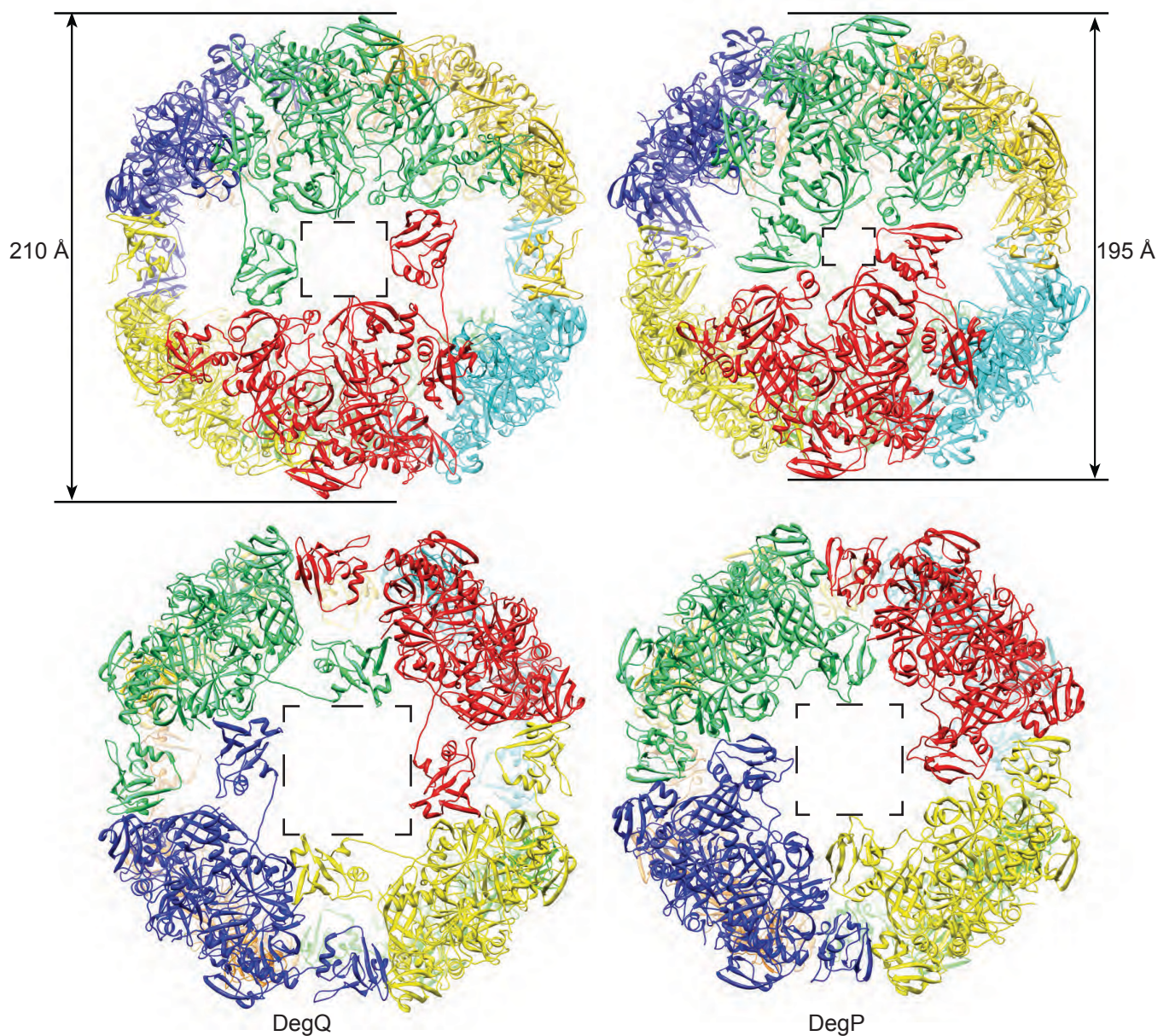
Supplementary figure 2. DegQ micrographs and class averages

(a) Negative stain EM of DegQ24–casein complexes (circled in green). Some DegQ 12-mers (circled in blue) are also visible. Scale bar: 50 nm. **(b)** Negative stain EM of DegQ12–peptide. **(c)** Negative stain EM of DegQ 12-mer bound to lysozyme. Whereas peptide-bound DegQ particles appear empty, lysozyme-bound DegQ complexes are filled with substrate density. **(d–f)** Cryo micrographs of DegQ24–casein **(d)**, DegQ12–peptide **(e)** and DegQ12–lysozyme **(f)**. **(g)** Averages corresponding to 4-, 3- and 2-fold views of DegQ24–casein particles. **(h)** 3- and 2-fold view averages of DegQ12–peptide. **(i)** 3- and 2-fold view averages of DegQ12–lysozyme. **(j)** Negative stain image of apo DegQ at pH 5.5. A mixture of hexamers (circled in red) and 12-mer (circled in blue) is observed.



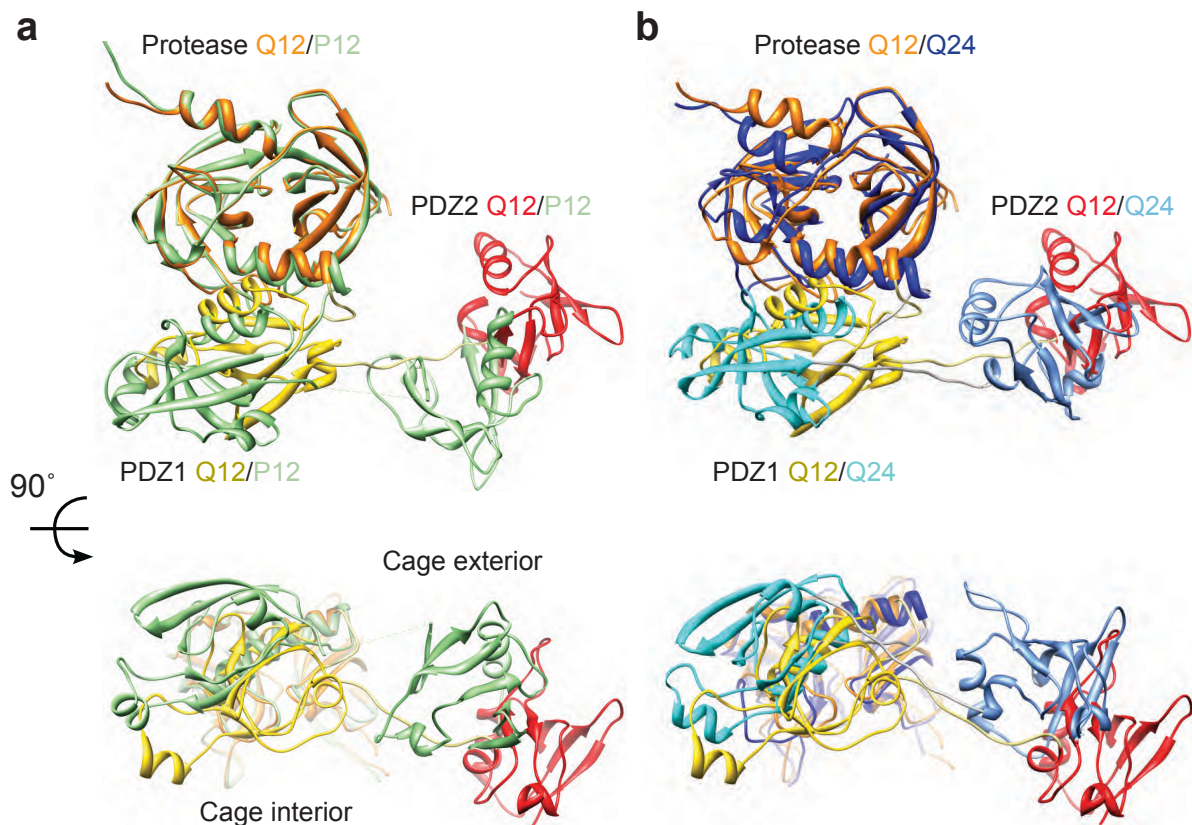
Supplementary figure 3. Resolution estimation by Fourier shell correlation

(a–d) Fourier shell correlations (FSC) of cryo-EM reconstructions of DegQ24–casein **(a)**, DegQ12–peptide **(b)** and DegQ12–lysozymes (symmetric **(c)** and asymmetric **(d)**). The resolution values at 0.5 correlation are indicated.



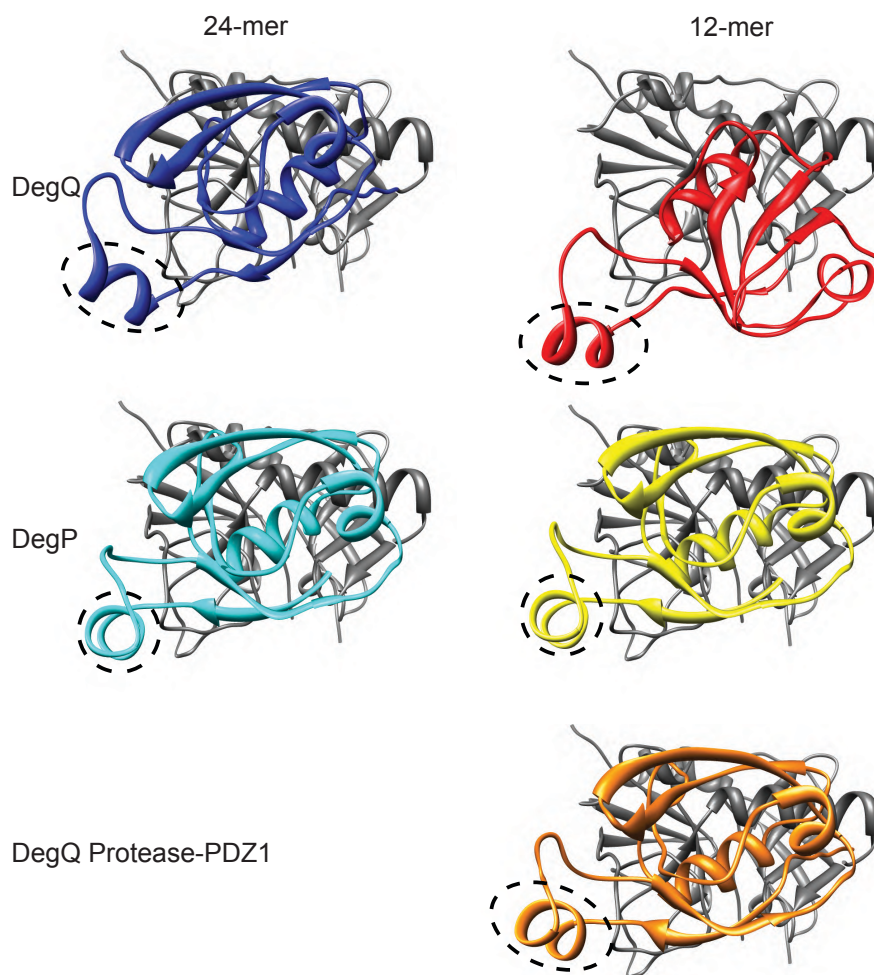
Supplementary figure 4. Comparison of DegQ and DegP 24-mers

DegQ (left) is slightly bigger than DegP (right) (210 vs 195 Å). DegQ pores located on the 4-fold and 2-fold axes are bigger than in DegP.



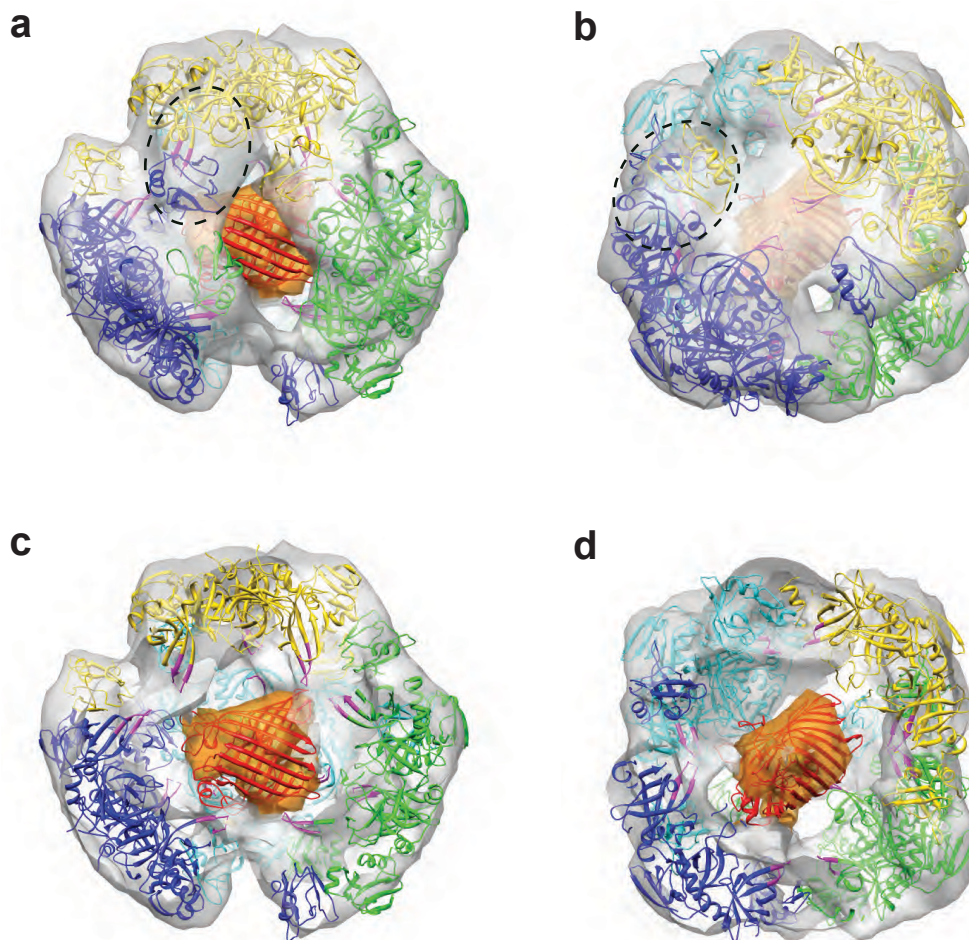
Supplementary figure 5. Domain orientations in the DegQ 12-mer

(a) Comparison of DegP and DegQ protomers in the context of the 12-mer cage. DegP protease, PDZ1 and PDZ2 domains are displayed in orange, yellow and red. DegP is displayed in light green. **(b)** Comparison of DegQ protomers in the context of the 12-mer and 24-mer cage.



Supplementary figure 6. Comparison of PDZ1 domain positions in DegP/DegQ 12- and 24-mers

Positions of PDZ1 domains from DegP/DegQ 12- and 24-mer relative to the protease domain (shown in grey). PDZ1 domains of DegP 24- and 12-mer are colored in blue and cyan respectively. PDZ1 domains of DegQ 24-, 12- and 12 Δ PDZ2 are colored in red, yellow and orange respectively. The PDZ1 position varies in DegQ12 compared to the other structures solved. In particular, the loop containing helix 251-257 (outlined with a dotted circle) is variable in position.



Supplementary figure 7. Revised fitting of DegP–OMP

The DegP12–OMP fit into the 28 Å resolution asymmetric DegP12–OMP cryo-EM map¹ has been substantially revised on the basis of the DegQ cryo-EM maps presented here and the currently available DegP structures^{2,3}. **(a,b)** Three-fold **(a)** and two-fold **(b)** views of DegP–OMP. DegP trimers are colored in blue, cyan, green and yellow. The density corresponding to OMPs is colored in orange with fitted OmpC shown in red. PDZ1 and PDZ2' from neighboring trimers form an assembly contact (dashed line), as seen in DegP12–lysozyme^{2,3} and DegQ12–lysozyme structures (presented here). **(c, d)** Cut-away of the three-fold **(c)** and two-fold **(d)** views of DegP–OMP fitted map. The LA loops are colored in magenta.

Supplementary Tables

		Range of rotation/translation	
		Up to 10 Å translation, up to 30° rotation	No translation, all possible rotations
Map	Rigid body	Z-score (p-value)	
DegQ24-casein	Trimer	2.957 (0.002)	2.171 (0.015)
DegQ12-peptide	Trimer	2.778 (0.003)	2.564 (0.005)
DegQ12-lysozyme	Lysozyme	4.971 (<0.001)	3.137 (0.001)
DegP12-OmpC	Structural unit	4.049 (<0.001)	2.933 (0.001)

Supplementary Table 1. Z-score and p-values of the rigid fits

Z-scores were calculated by comparing each rigid fit with fits of 1000 randomly rotated and translated models. Two ranges of rotation-translation were tested: (left) up to 10 Å and 30° to test the overall placement of the model in the density, (right) no translation, all rotations permitted (up to 360° for lysozyme, up to 120° for degQ trimer/structural unit as the model is on a 3-fold symmetry axis) to test if the final rigid fit is significantly better than fits with alternative rotations around the symmetry axis. Z-scores statistically assess the uniqueness of all the overall fits proposed (p-value < 5%).

	Rigid body fitting		Flexible fitting	
	Rigid bodies used	Cross-correlation	Rigid bodies used	Cross-correlation
DegQ24–casein (7.5 Å resolution)	DegP 24-mer crystal structure	0.424	DegQ24 individual domains	0.501
DegQ12–peptide (7.5 Å resolution)	DegP12-peptide crystal structure	0.405	DegQ12 individual domains	0.513
DegQ12–lysozyme symmetric map (13 Å resolution)	DegP12-peptide crystal structure	0.474	DegQ12 individual domains, six lysozymes	0.673
DegQ12–lysozyme asymmetric map (14.2 Å resolution)	DegP12-peptide crystal structure	0.445	DegQ12 individual domains, one lysozyme	0.654
DegP12–OMP (28 Å resolution)	Previous fit of DegP-OmpC (PDB entry 2ZLE ¹)	0.525	DegP12 trimers, OmpC	0.696

Supplementary Table 2. Molecular modeling and flexible fitting improve the cross-correlations between cryo-EM maps and atomic models

Supplementary methods

Negative stain specimen preparation

DegQ24–casein, DegQ12–lysozyme, DegQ12–peptide and apo DegQ (pH 5.5) at 0.02 mg.ml⁻¹ were imaged by negative stain EM. Samples were applied for 30s to a glow discharged continuous carbon grid, blotted and then stained for 30s with 2% uranyl acetate. They were imaged on a T10 microscope (FEI) operated at 100 kV.

Cryo-EM image processing of DegQ24–casein, DegQ12–lysozyme and DegQ12–peptide

Particles were initially centered against a rotationally averaged total sum and classified using multivariate statistical analysis (MSA) as implemented in IMAGIC-5⁴. Subsets of around 10 classes were selected (based on the visual match between the class average and the individual particles) as references for multi-reference alignment (MRA). After three rounds of MRA/MSA, 3D models were calculated by angular reconstitution. Octahedral symmetry was used for the DegQ 24-mer in accordance with the 4, 3, and 2 fold symmetry observed in the class averages. 23 and 32 symmetry were tested for the DegQ 12-mers, and the results clearly supported tetrahedral symmetry. Particle orientations were refined by multiple cycles of MRA, MSA and angular reconstitution, gradually incorporating more particles. All particles were included in the final class average calculation, with an average of 5 particles per class. Final models were obtained by projection matching (using the AP SH command in SPIDER⁵) with 1° angular spacing between projections. When the alignments had stabilized, more than 95% of the images aligned to the same references in consecutive alignments. Particles were separated into defocus groups and 80% of the best particles of each group (according to their cross-correlation) were selected for 3D

reconstruction (IMAGIC-5). The final reconstructions comprise 9848, 13432 and 29432 particles for DegQ24–casein, DegQ12–lysozyme and DegQ12–peptide, respectively, resulting in resolutions of 7.5, 13.0 and 7.5 Å according to Fourier shell correlation (FSC) at 0.5 correlation. The DegQ12–lysozyme map was used as a starting model for asymmetric reconstruction by projection matching. After five cycles, 95% of the images had stable assigned angles. To confirm the extra densities assigned as ligands, internal densities were deleted from the DegQ12–lysozyme reconstructions. Projection matching was carried out using these maps as starting points, and after only a few iterations, the extra densities reappeared.

In the final reconstructions of DegQ12–peptide and DegQ24–casein, full CTF correction was done in SPIDER. An envelope parameter of 0.23 was used to model the amplitude fall-off of the CTF. The individual images were multiplied by their corresponding CTF functions, band-pass filtered and normalized as before. The alignment determined for the phase flipped images was then applied, images aligning to the same 3D projection were averaged and this average was then divided by the sum of the squared CTFs plus a Wiener filter value of 0.1 to complete the full CTF correction.

Fitting crystal structures into DegQ EM maps

A model of the DegQ trimer was generated using the *E. coli* DegQΔPDZ2 trimer crystal structure⁶ and the DegQ PDZ2 homology model. The resulting trimer was rigidly fitted into DegQ12–peptide and DegQ24–casein cryo-EM maps using UCSF Chimera⁷ ‘fit-in-map module’. The symmetry operation (also implemented in Chimera) was used to obtain 12- and 24-mer rigid fits. The statistical significance of

all the rigid fits is assessed by calculating a Z-score for each unique fit (Supplementary Table 1).

Analysis of the rigid fits and cryo-EM maps clearly indicate that DegQ 12-/24-mer are composed of 4/8 structural units, each of them comprising protease-PDZ1 domains from three subunits and PDZ2' domains from three neighbouring subunits. Next, a rigidly fitted structural unit and its corresponding EM map segment were extracted and used for flexible fitting with Flex-EM⁸. The model was divided into rigid bodies corresponding to protease (residues 11-234), PDZ1 (residues 243-311) and PDZ2 domains (residues 341-427), leaving inter-domain linkers flexible. Careful inspection of the map indicated the additional flexibility of regions containing the N-terminal residues 1-25 and the PDZ1 helix 251-257. To improve the fitting of these regions an additional Flex-EM refinement was performed using 5 rigid bodies per monomer (15-25, 30-234, 243-247 and 266-311, 251-257, 341-427), leaving inter-rigid bodies linker flexible. The fitting procedure improves the cross-correlation between the maps and the models as shown in Supplementary Table 2.

The final pseudo-atomic model of DegQ12-peptide was used as an initial model for the fitting of DegQ12-lysozyme symmetric and asymmetric maps. Lysozymes were rigidly fitted into the density using the Chimera fit-in-map module (Z-scores shown in Supplementary Table 1) and the fitting was refined with Flex-EM using lysozyme and the DegQ domains defined as rigid bodies.

Revised fitting of the DegP-OMP asymmetric cryo-EM map

The DegP-OMP map was originally fitted using a trimer of DegP subunits as the structural unit. However, comparison with DegP12-lysozyme² and DegQ12-lysozyme cryo-EM maps from this work indicates that the structural unit consists of

protease-PDZ1 from three subunits and PDZ2' domains from three neighboring subunits. For the fitting procedure, an atomic model of this revised structural unit was derived from the DegP-peptide crystal structure³ and used for automated rigid body fitting using the CHIMERA fit-in-map module. The OmpC crystal structure (PDB entry 2J1N⁹) was manually fitted into the central density.

Outer membrane isolation

E. coli wild-type, *degQ*-null and *degP*-null mutant strain strains were grown in Luria-Bertani medium until they reached the stationary phase. To obtain pellets of equal cell numbers, standardized aliquots were taken according to measured optical density. The cells were pelleted and resuspended in 500 μ l of 100 mM Tris-HCl pH 8.0 and 10 mM EDTA. Cell walls were digested with lysozyme (100 μ g.ml⁻¹) on ice for 10 min and the spheroplasts were lysed by three freeze-thaw cycles in the presence of 50 mg.ml⁻¹ DNase. The cell debris were pelleted at 2,500g for 15 min and the cleared supernatant was centrifuged for 15 min at 20,000g. The pellet (crude membrane fraction) was washed with 500 μ l of 20 mM NaPO₄ at pH 7.0. The cytoplasmic membrane was solubilized with 50 μ l of 0.5% sarcosyl in 20 mM NaPO₄ at pH 7.0 at room temperature for 30 min. The insoluble outer membranes were pelleted by centrifugation at 20,000g for 15 min, washed once with sarcosyl solution, centrifuged again, and resuspended in 50 μ l solution containing 1.25% SDS and 1.25% β -mercaptoethanol. OMPs were analyzed on a 12% polyacrylamide SDS gel in the presence of 4 M urea to resolve OmpC and OmpF.

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