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Expression, crystallization and crystallographic analysis of DegS, a stress sensor of the bacterial periplasm

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Regulated proteolysis is a key event in transmembrane signalling between intercellular compartments. In *Escherichia coli*, a protein DegS has been identified as being a periplasmic stress sensor for unfolded or misfolded outer membrane proteins (OMPs). Activation of DegS initiates a proteolytic cascade which results in the transcription of periplasmic genes under σ^{E} control, most importantly chaperones and proteases. DegS has been cloned and expressed as full-length protein and in an N-terminally truncated form. Both proteins were tested for crystallization and two forms of well diffracting crystals of the truncated form were obtained. Crystals of form I diffract to 3.5 Å and belong to space group $P2_13$, while crystals of form II diffract to 2.2 Å and belong to space group $I23$. Crystals of form II were soaked with a consensus peptide representing the C-termini of outer membrane proteins and data to 2.4 Å resolution were collected. Molecular-replacement trials using a homologous protease domain indicate the presence of two molecules in the asymmetric unit of crystal form I. The correctness of the molecular-replacement solution was verified by identifying radiation-damage-induced structural changes.

1. Introduction

Transmembrane signalling between cellular compartments is often accomplished by regulated proteolysis. In *Escherichia coli*, the unfolded protein response towards misfolded or unfolded outer membrane proteins (OMPs) is induced by successive degradation of the inner membrane protein RseA, an anti-σ^{E} factor (Walsh *et al.*, 2003; Kanehara *et al.*, 2003). The proteolytic cascade is initiated by the periplasmic protease DegS, which is attached to the inner membrane and is inactive under normal growth conditions. Protease activation is induced by unfolded OMPs *in vivo* and activation can be mimicked by the addition of small synthetic peptides ending with C-terminal OMP sequences *in vitro* (Walsh *et al.*, 2003). The RseA-destruction process is continued by the integral membrane protease YaeL, which cleaves the substrate protein within or close to the plane of the membrane (Kanehara *et al.*, 2003; Brown *et al.*, 2000). This proteolytic two-step process finally leads to release of the σ^{E} factor, which is otherwise tightly bound to the cytosolic domain of RseA. The release of σ^{E} results in increased transcriptional levels of genes under σ^{E} control (Missiakas *et al.*, 1997).

DegS belongs to the HtrA (high-temperature requirement) class of proteins (Clausen *et al.*, 2002). The structural architecture of these ATP-independent proteases is made up of a serine-protease domain followed by a varying number of PDZ domains. PDZ domains are one of the most commonly observed protein–protein interaction domains in organisms ranging from bacteria to humans. Within this class of proteins, DegS appears unusual in being a membrane-bound enzyme with high substrate specificity. DegS is a 35 kDa protein with an N-terminal hydrophobic and membrane-bound α-helix followed by the protease and a single C-terminal PDZ domain. Activation of full-length and N-terminally truncated DegS and subsequent degradation of RseA *in vitro* can be induced by the addition of ten-residue-long peptides that have been shown to interact with its PDZ domain (Walsh *et al.*, 2003). Moreover, DegS has been implicated in the recognition of proteins marked by *ssra*-degradation signals (Karzai *et al.*, 2000).

The only structures of homologous proteins that have been determined so far are those of DegP from *Escherichia coli* and HtrA from mitochondria (Krojer *et al.*, 2002; Li *et al.*, 2002). However, these proteins have a different mode of activation and function, as their substrate specificity is diverse (Spiess *et al.*, 1999). In our work, we aim to determine the three-dimensional X-ray structure of DegS in a free and in a substrate-complexed form in order to understand its mode of activation by OMP peptides.

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2. Material and methods

2.1. Cloning, expression and purification

The gene encoding full-length DegS ($DegS_{FL}$) and the N-terminally truncated version DegS missing the first 28 residue ($DegS_{\Delta N}$) were amplified by PCR from *E. coli* DNA and subcloned into the pet22B(+) vector (Novagen). The recombinant plasmids were transformed into *E. coli* strain BL21(DE3) (Novagen) and cells were selected on agar plates containing 100 µg ml^{-1} ampicillin. Single colonies were chosen for further inoculation and cultivated in Terrific Broth medium with 100 µg ml^{-1} ampicillin. For $DegS_{FL}$, the bacteria were grown at 310 K to an OD_{600} of 1.5. The cultures were cooled to 293 K, overexpression was induced with 1 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG) and cells were allowed to grow overnight. $DegS_{\Delta N}$ expression strains were treated in essentially the same way as the full-length construct except for a further reduced growth temperature of 289 K after induction with 0.5 m*M* IPTG. Cells were harvested, resuspended in buffer *A* containing 100 m*M* NaCl, 100 m*M* KCl, 3 m*M* $MgCl_2$, 0.5 m*M* *β*-mercaptoethanol, 20 m*M* HEPES pH 7.5 and broken using a French press. For $DegS_{FL}$, the solution was centrifuged at 75 000*g* for 1 h to isolate bacterial membranes, which were homogenized and solubilized using buffer *A* including 3% octyl-polyoxyethylene (o-POE) for 1 h at room temperature (RT). The solution was centrifuged at 75 000*g* and the supernatant incubated with Ni–NTA matrix (Ni–NTA Superflow, Qiagen, Hilden). The matrix was washed with buffer *A* and the protein was eluted with a linear gradient of buffer *A* containing 0.5% o-POE and 300 m*M* imidazole. Protein fractions predominantly containing $DegS_{FL}$ were collected, concentrated to 40 mg ml^{-1} and applied onto a size-exclusion chromatography column (Superdex 200) using buffer *B* (buffer *A* and 0.5% o-POE). The purification procedure was almost identical for $DegS_{\Delta N}$ as for $DegS_{FL}$, except that buffers lacking detergent were used.

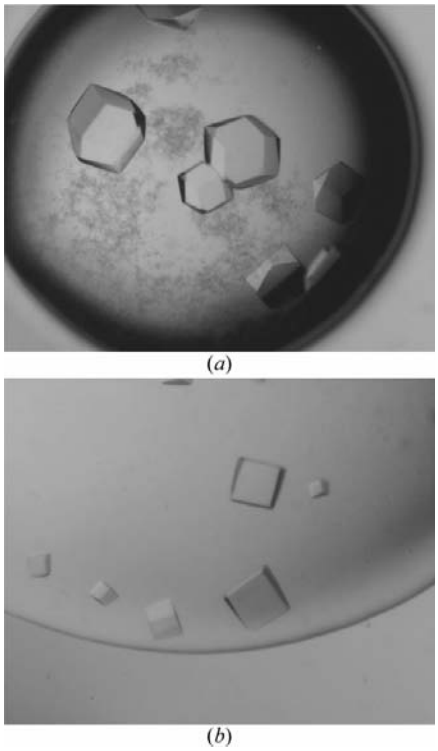

Figure 1
(*a*) Cubic shaped crystals of DegS from *E. coli* with dimensions 0.3 × 0.3 × 0.3 mm that crystallized in space group $P2_13$ (crystal form I). (*b*) Crystals of DegS in space group *I*23 with dimensions of 0.15 × 0.15 × 0.15 mm (crystal form II).

2.2. Crystallization and X-ray crystallographic analysis

Crystallization trials were performed using purified $DegS_{FL}$ at 15 mg ml^{-1} in buffer *C* containing 20 m*M* NaCl, 20 m*M* KCl, 3 m*M* $MgCl_2$, 0.5 m*M* *β*-mercaptoethanol, 10 m*M* HEPES pH 7.5 and 1.2% *β*-D-octylglucoside. Crystallization trials were performed using Crystal Screens I and II from Hampton Research with 1 µl crystallization reagent plus 1 µl protein hanging drops. The truncated protein $DegS_{\Delta N}$ at 10, 20 and 40 mg ml^{-1} in buffer *C* was tested against solutions from Crystal Screens I, II and Index Screen from Hampton Research, and crystals were observed using Crystal Screen II condition No. 4 (35% dioxane, crystal form I) and Index Screen condition No. 55 (0.05 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5, 30% polyethylene glycol monomethyl ether 550, crystal form II). In order to prepare crystals of $DegS_{\Delta N}$ with the activating peptide (OMP_{CT}, peptide sequence DNRDGNVYYF), protein crystals of form II were either co-crystallized with 1 m*M* of the peptide or soaked for 2 h by addition of 0.5 m*M* peptide to the crystallization drops. Prior to data collection, $DegS_{\Delta N}$ crystals of form I were soaked for a few seconds in mother liquor containing 35% glycerol as cryoprotectant and frozen in liquid nitrogen. Crystals of form II (either native, soaked or co-crystallized with the OMP_{CT} peptide) were briefly transferred to drops containing mother liquor plus 5% 2*R*,3*R*-butanediol and frozen in liquid nitrogen. Data were collected at beamline BW6 of the synchrotron-radiation source DESY (Deutsches Elektronen Synchrotron, Hamburg, Germany) on crystals of form I and II as well as on form II crystals soaked with peptide. Data were collected at 100 K and a wavelength of 1.05 Å. Additional data were collected at 100 K and a wavelength of λ = 0.939 Å using the highly intense radiation produced by ID14-4 at the ESRF (European Synchrotron Radiation Facility, Grenoble, France) from crystals of form II either in the free form or co-crystallized with the OMP_{CT} peptide. Diffraction patterns were recorded on a 165 mm MAR CCD (at BW6) and an ADSC Q4R mosaic CCD detector system (at ID14-4). All data were indexed, integrated and scaled using the programs *XDS* and *XSCALE*, respectively (Kabsch, 1988). Molecular-replacement trials were performed using the program *Phaser* (Storoni *et al.*, 2004).

Radiation-damage studies were performed at ID14-4 using type II crystals according to the following protocol. Low-resolution data of crystals diffracting to better than 3 Å were collected using a strongly attenuated beam (∼5% of the full intensity). Data from the same crystal slice were collected to 2.2 Å without further attenuation, followed by a second low-resolution data collection from the crystals under essentially the same conditions as for the initial data collection. The possible MR solutions were verified by examining radiation-damage-induced structural differences (Weik *et al.*, 2000).

3. Results and discussion

Size-exclusion chromatography indicate that both $DegS_{FL}$ and the truncated $DegS_{\Delta N}$ are trimeric and that the N-terminal helix appears to be dispensible for oligomerization (data not shown). The state of oligomerization is different to that of the homologous DegP complex, which is a hexameric, soluble and ATP-independent chaperone (Krojer *et al.*, 2002). We initially set out to structurally investigate DegS from *E. coli* as the full-length protein, including the N-terminal membrane helix. Four different detergents, *β*-D-octylglucoside, LDAO, dodecyl-*β*-D-maltoside and octyltetraoxyethylene (C8E4), were tested for crystallization and small crystals of 20–30 µm in size appeared using *β*-D-octylglucoside. These crystals diffracted to 6 Å using synchrotron radiation (data not shown). Unfortunately, neither the size nor the diffraction quality of the crystals could be improved by conventional crystallization techniques and further crystallization attempts were therefore focused on the truncated protein $DegS_{\Delta N}$.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Crystal form I	Crystal form II	Crystal form II + OMP_{CT} peptide, soaked	Crystal form II + OMP_{CT} peptide, co-crystallized
Beamline	BW6	ID14-4	BW6	ID14-4
Resolution (Å)	3.5 (3.72–3.5)	2.2 (2.3–2.2)	2.4 (2.54–2.4)	3.4 (3.6–3.49)
Wavelength (Å)	1.05	0.939	1.05	0.939
Oscillation angle (°)	0.5	0.5	0.5	0.5
Unique reflections	12978	38296	27834	10570
Completeness (%)	89 (71.4)	98.6 (90.9)	94.3 (88.7)	97.6 (95.4)
Redundancy	4.4	5.4	3.6	4.8
Average $I/\sigma(I)$	7 (2.2)	15.8 (3.0)	12.8 (3.0)	16 (3.6)
R_{merge}†	0.117 (0.61)	0.048 (0.39)	0.052 (0.40)	0.085 (0.35)

† $R_{merge} = \sum_{unique\ reflections}(\sum_{i=1}^{N} I_i - \overline{I}|)/\sum_{unique\ reflections}(\sum_{i=1}^{N} I_i)$, where N represents the number of equivalent reflections and I the measured intensity.

The crystal screens applied to this protein yielded crystals under a variety of conditions. Most of the crystals tested so far did not diffract to better than 6 Å. However, small temperature-sensitive cubic shaped crystals of form I appeared in 35% dioxane after 4–6 weeks incubation at 291 K. These conditions were further refined using additive screens from Hampton Research and the crystal dimensions reached 0.3 × 0.3 × 0.3 mm (see Fig. 1*a*). Cubic shaped crystals of form II either free or in an OMP_{CT} co-crystallized form were obtained using 30% PEG MME 550, 0.05 m*M* $MgCl_2$, 100 m*M* HEPES pH 7.5 and grew over a period of 8–10 weeks. This crystal form was also used for co-crystallization with the OMP_{CT} peptide (see Fig. 1*b*). Data-collection statistics of all data sets collected are summarized in Table 1. Crystals of form I merge in the cubic space group $P2_13$ with unit-cell parameter a = 150 Å and crystals diffracted to 3.5 Å resolution with an R_{merge} of 11.7% and $I/\sigma(I)$ = 7. Crystals of form II diffract to 2.2 Å resolution and belong to the cubic space group $I23$, with unit-cell parameter a = 166.3 Å, an R_{merge} of 4.8% and $I/\sigma(I)$ = 15.8. The form II crystals co-crystallized with the peptide diffracted to 3.4 Å with a = 166.9 Å, an R_{merge} of 8.5% and $I/\sigma(I)$ = 12.8. Crystals of form II soaked with the OMP_{CT} activating peptide diffracted to 2.4 Å with a slightly smaller unit-cell parameter of a = 164.7 Å, with R_{merge} of 5.2% and $I/\sigma(I)$ = 16.

Initial molecular-replacement trials using the protease domain of DegP as a search model (190 out of 320 residues) resulted in a crystallographically useful model arrangement with two molecules per asymmetric unit in crystal form II and a solvent content of 55.2%. However, neither a search with DegP models including the protease and PDZ domain nor a more extensive search with *Phaser* using an averaged model of known PDZ domains (Claude *et al.*, 2004) (PDB codes 1be9, 1ihj, 1pdr, 1qav and 1uhp) led to an unambiguous solution for this domain. The R factor and R_{free} using the protease model alone were 49.6 and 52.3%, respectively. No interpretable difference density could be found at this stage for the PDZ domain. In order to verify the correctness of the MR solution, difference Fourier maps were calculated between low-dose data sets that were collected before and after the high-resolution data set. These maps showed clear negative peaks near to Glu and Asp residues, indicating specific radiation damage. Although these differences were not strong enough to be useful for radiation-damage-induced phasing (Ravelli *et al.*, 2003), they led to an unambiguous assignment of the correct MR solution. Automated model-building programs such as *ARP/wARP* and *RESOLVE* did not succeed in building the structure; therefore, we currently aim to improve the weak model phases manually.

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