

Allosteric Activation of HtrA Protease DegP by Stress Signals during Bacterial Protein Quality Control**

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The key cellular process of protein quality control (PQC) ensures that all proteins are structurally and functionally intact and properly localized.^[1] Although PQC is in particular demand under stress conditions, such as heat shock, it plays an important role under any environmental situation as protein misfolding occurs even under homeostatic conditions. Deficient PQC often leads to the formation of protein aggregates that are the basis for severe disorders, such as Alzheimer's or Parkinson's disease.^[2] PQC is also critical for bacterial virulence, pathogenic bacteria without these cellular factors are unable to survive in their hosts.^[3] Consequently, selective inhibition of bacterial PQC might represent a promising strategy for developing novel antibiotics.

In bacteria, two periplasmic HtrA (high-temperature requirement) proteases, DegS and DegP, are key players of PQC. The widely conserved HtrA proteases consist of a protease domain adopting a chymotrypsin fold and one or two PDZ domains.^[4] Their proteolytic activity is controlled by cellular stress signals that affect the reversible switch between active and resting states. In DegS, proteolytic activity is allosterically turned on by mislocalized outer-membrane proteins that bind to the PDZ and protease domains.^[5] Proteolytic activation initiates a signal-transduction cascade that ultimately leads to the expression of DegP,^[6] the major

PQC factor that eliminates misfolded proteins with little substrate specificity.

DegP consists of one protease and two PDZ domains and assembles into complex, multimeric particles that switch between chaperone (protein repair factor) and protease functions in a temperature-dependent manner.^[7] While chaperone activity is present at low temperatures, protease function dominates at elevated temperatures, resulting in a distinct proteolytic activity at 37°C. Although the crystal structure of DegP in the chaperone state is available,^[8] the regulation of its catalytic activity is unclear. We hypothesized that besides the known temperature-dependent regulation, an additional chemical mechanism could exist. Since DegP is already proteolytically active at 37°C, this chemical activation would result in an amplification of temperature-induced proteolysis.

We wished to investigate the effect of chemical stress signals on DegP activity, using a chromogenic protease assay. However, no suitable chromogenic substrate is available and the absence of proper peptidic substrates for any member of the pathophysiologically important HtrA family has prevented accurate enzymatic characterization of this protease family. To overcome this limitation, we performed digests of the known DegP substrate citrate synthase and synthesized *para*-nitroaniline (pNA) derivatives of selected cleavage products (compounds **1–3**, Scheme 1). These derivatives were obtained by a combination of 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis on 2-chlorotrityl resin, allowing efficient synthesis of fully protected precursor peptides, followed by solution peptide coupling with commercially available H-Val-pNA. Out of these three pNA-derivatives, **1** containing the SPMFKGV-pNA sequence was the most suitable synthetic substrate, displaying a cooperative binding with a $K_{0.5}$ value (the half-saturation constant in the Hill equation) of 2.7 mM, a $v_{\max}$ of 1.28 $\mu\text{mol min}^{-1}$, a k_{cat} of 0.56 s^{-1} , and a specificity constant ($k_{\text{cat}}/K_{0.5}$) of 0.21 $\text{mM}^{-1} \text{s}^{-1}$, whereas **2** and **3** were not cleaved. We optimized conditions for future protease assays by studying the effect of pH value, buffers, and salts; 50 mM NaH_2PO_4 buffer at pH 8.0 was most appropriate (for thorough biochemical characterization, see Supporting Information).

After having established a suitable assay, we investigated our hypothesis. Initially, we added casein as a model compound for misfolded proteins and monitored (by photometry) degradation of substrate **1** by DegP as we reasoned that some short cleavage products derived from casein might amplify DegP proteolysis. Indeed, 2.5-fold activation was

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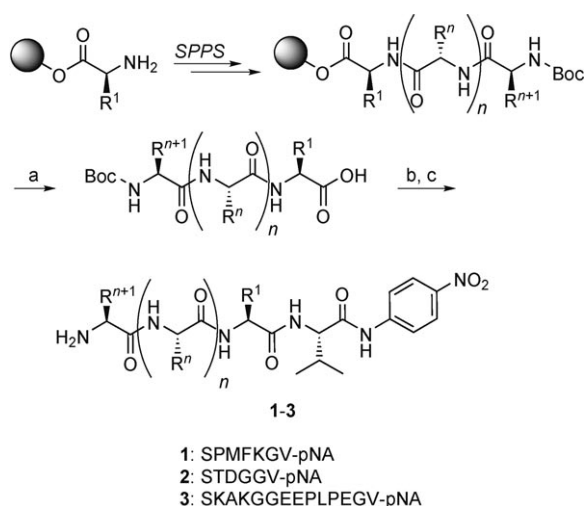
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Scheme 1. Synthesis of chromogenic substrates by solid-phase synthesis on 2-chlorotrityl resin. a) Acetic acid/trifluoroethanol/ CH_2Cl_2 (2:2:6); b) H-Val-pNA-HCl (2 equiv), EDC-HCl (1.5 equiv), HOBT (2 equiv), DIEA (3.5 equiv); c) TFA/ H_2O /ethanedithiol/triisopropylsilane (94:2.5:2.5:1). Boc = *tert*-butoxycarbonyl, EDC = *N*-(3-dimethylamino-propyl)-*N*-ethylcarbodiimide, HOBT = 1-hydroxy-1*H*-benzotriazole, DIEA = *N,N*-diisopropylethylamine, TFA = trifluoroacetic acid.

observed, supporting our assumption of an additional allosteric activation. We then used a native substrate, periplasmic α -amylase (MalS) and found that full-length denatured MalS, as well as MalS fragments and the C-terminus of MalS, amplified DegP proteolysis (Supporting Information).

These data indicated that DegP is activated by oligopeptides. We imagined that the underlying mechanism might be similar to DegS activation. The crystal structure of the DegS-activator complex revealed that activating peptides bind to the PDZ domain and that residue X of the YXF C-terminal motif of activating peptides additionally interacts with a loop of the protease domain, thereby triggering proteolytic activity. The activation of DegS critically depends on the chemical nature of residue X. We therefore prepared decapeptide derivatives of the known stress signal consensus sequence DNRDGNVYXF by solid-phase peptide synthesis, where X was substituted by various residues. We synthesized ten peptides and tested their effect on proteolytic activation of DegP and DegS. In addition, we analyzed decapeptides derived from native C-termini of cell envelope proteins, that is, outer membrane porin C (OmpC), periplasmic alkaline phosphatase A (PhoA), and MalS. Of these, the C-terminus of OmpC is a known DegS activator while PhoA served as a negative control. The proteolytic activity of DegP was measured with substrate **1** (Figure 1) and denatured MalS to ensure that the observed effects were not due to the use of a synthetic substrate (Supporting Information). DegS activity was determined using its native substrate, the anti-sigma factor RseA (Supporting Information). Peptides **4** and **5**, carrying YFF or YWF C-termini, activated DegP by about 2.5- and 2-fold, respectively. Peptide **7** with a C-terminal YQF sequence, corresponding to the C-terminus of the natural stress signal OmpC, induced a 1.5-fold activation. Interestingly, a decamer of the C-terminus of OmpC (**15**) was the best

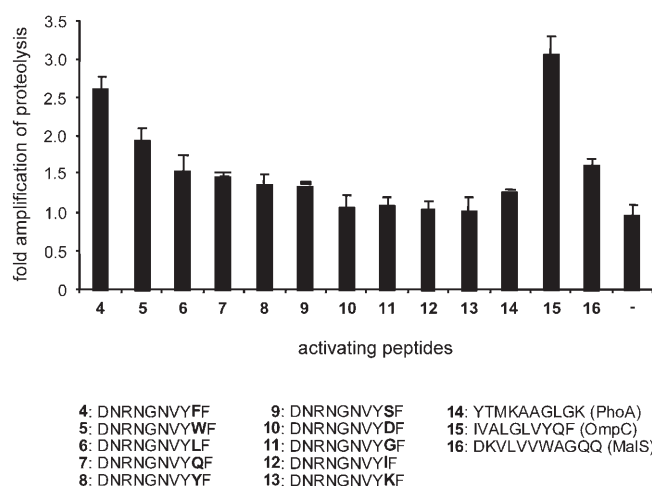


Figure 1. Sequences of activating peptides **4–16** and results derived from proteolysis experiments with **1**. The varied penultimate residue is highlighted in bold. Assay conditions: 0.5 mM **1**, 50 μM activating peptides **4–16**, 0.13 μM DegP, 50 mM NaH_2PO_4 pH 8.0 normalized to activity without addition of activating peptide. Each assay was repeated five times with duplicate samples, and the resulting standard deviations are shown as error bars.

activator, leading to 3-fold activation. Importantly, these experiments supported our hypothesis of an additional allosteric activation of DegP activity. Moreover, a good correlation between sequences activating DegS and DegP was observed with peptides **4–16**, indicating a physiologically relevant mode of regulation.

Thermodynamic binding affinities of peptides **4** and **6**, carrying an activating YFF or a weakly activating YLF C-terminus, were determined by isothermal titration calorimetry. The data could be fitted with a three sequential binding sites model and revealed cooperative binding. For all three binding sites, only slight differences in the dissociation constant K_d between **4** and **6** could be observed (Supporting Information), suggesting an important role of the penultimate residue of the activating peptides.

To elucidate if DegP activation is based on an interaction of the activating peptide with PDZ domains, we constructed two point mutations in PDZ domain 1. Arg262, which is involved in binding of the C-terminus carboxy group of the activating peptide ligands, was replaced by alanine. In the second mutant, Val328 was substituted by serine, thereby changing the spatial arrangement of the ligand binding pocket.^[8] These mutations completely abolished proteolytic activation of even the strongest activating peptide **15**, suggesting that the allosteric binding site is indeed located on PDZ domain 1 (Figure 2, and Supporting Information Figure S5). Furthermore, isothermal titration calorimetry revealed that peptide **4** did not bind to either of these PDZ mutants (data not shown). Therefore, PDZ 1 of DegP is able to transmit information to the protease domain. Given the structural differences between DegP and DegS (different oligomeric states, activation domains, and sensor loops) future studies are required to understand the precise activation mechanism. In addition, peptides **7** and **15**, both carrying

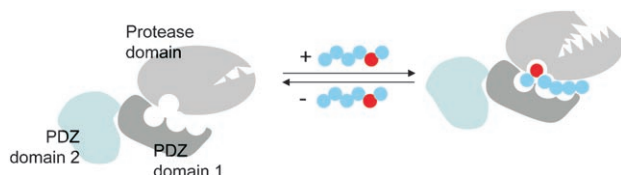


Figure 2. Schematic model of the allosteric activation mode of DegP. Activating peptides (blue circles) bind to PDZ domain 1 and the chemical nature of the penultimate residue (red circle) determines the extent of activation.

the OmpC C-terminus YQF, differed in their activation potential (Figure 1). This indicates that while the last three residues of the ligand are essential, upstream sequences are also contributing to activation; however, the underlying structural basis is not yet understood.

Finally, we asked if activating peptides and upregulated protease activity result in a loss of chaperone function at low temperatures. To address this question, we performed refolding assays with MalS and activating peptide **4** (Supporting Information Figure S6). Interestingly, we detected an increased degradation of MalS and almost no refolding when **4** was added, showing that activating peptides can switch DegP from chaperone to protease at low temperatures. Therefore, chemical activation dominates over regulation by temperature.

We conclude that the bacterial PQC factor DegP is allosterically regulated by model peptides mimicking cellular stress signals. This result implies that besides the known transcriptional activation, an additional allosteric activation of DegP occurs. The simultaneous allosteric regulation of DegP and DegS by peptides significantly contributes to a rapid and robust stress response and a fine-tuned amplification of the stress signal (Figure 3). Consequently, synthesis of non-activating peptidomimetics binding to the allosteric regulation pocket should induce a distinct disturbance of bacterial PQC and could therefore represent a novel strategy for the development of antimicrobials. Moreover, we propose that allosteric activation is a common feature of all members of the HtrA family. This conclusion is in line with other studies reporting binding of short peptides to human HtrA proteases HtrA1 or HtrA2.^[9] Although peptidic stress signals have not yet been identified for human HtrAs, the conserved activation mode might be relevant for a better understanding of protein-folding diseases as HtrA1 has been described as a risk factor for amyloid-diseases including age-related macular degeneration.^[10]

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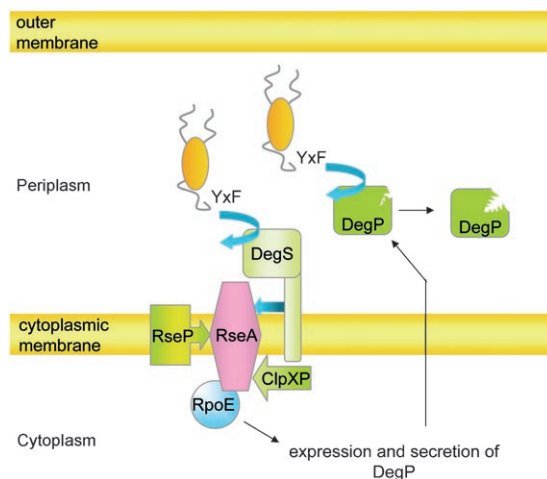


Figure 3. Amplification of bacterial stress response by allosteric activation. DegS senses unfolded proteins and initiates a proteolytic cascade that leads to elevated expression of DegP. In addition, these activating sequences also lead to an allosteric activation of DegP, causing an amplification of the original stress signal by transcriptional and allosteric activation.

Keywords: allosterism · proteases · protein quality control · proteins · stress signal

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