The Serine Protease HhoA from *Synechocystis* sp. PCC 6803:

Substrate Specificity and Formation of a Hexameric Complex

are Regulated by the PDZ Domain

Running title: *Synechocystis* serine protease HhoA

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Enzymes of the ATP-independent Deg serine endopeptidase family are very flexible with regard to their substrate specificity. Some family members cleave only one substrate, while others act as general proteases on unfolded substrates. The proteolytic activity of Deg proteases is regulated by PDZ protein interaction domains. Here we have characterized the HhoA protease from *Synechocystis* sp. PCC 6803 *in vitro* using several recombinant protein constructs. The proteolytic activity of HhoA was found to increase with temperature and basic pH and was stimulated by the addition of Mg\(^{2+}\) or Ca\(^{2+}\). We found that the single PDZ domain of HhoA played a critical role in regulating protease activity and in the assembly of a hexameric complex. Deletion of the PDZ domain strongly reduced proteolysis of a sterically challenging resorufin-labeled casein substrate, but unlabeled β-casein was still degraded. Reconstitution of the purified HhoA with total membrane proteins isolated from *Synechocystis* sp. PCC 6803 wild type and a ΔhhoA mutant showed specific degradation of selected proteins at elevated temperatures. We conclude that a single PDZ domain of HhoA plays a critical role in defining the protease activity and oligomerization state, combining the functions that are attributed to two PDZ domains in the homologous DegP from *Escherichia coli*. Based on this first enzymatic study of a Deg protease from cyanobacteria, we further propose a general role of HhoA in the quality control of extracytoplasmic proteins, including membrane proteins, in *Synechocystis* sp. PCC 6803.
INTRODUCTION

Proteolysis is an essential process in every living cell, involved in protein quality control (45) as well as in regulation of diverse cellular events (11). The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which is a widely used model system for studying photosynthesis and acclimation to abiotic stresses, has 77 proteases encoded in its genome (http://merops.sanger.ac.uk/). Of these, three genes encode ATP-independent serine endopeptidases of the Deg (HtrA) family (14, 19, 21, 40). The protease domain of these enzymes harbors the hallmark catalytic triad of His, Asp and Ser responsible for the proteolytic activity and may additionally confer a chaperone function (16, 42). Deg proteases usually contain one or two protein-protein interaction domains of the PDZ type C-terminally to the protease domain (7). Studies on *E. coli* DegP (also named HtrA), *E. coli* DegS (also termed HhoB) and human HtrA2 showed that PDZ domains regulate the proteolytic activity (16, 18, 27, 38, 42, 46), are involved in the formation of homo-oligomeric complexes (16, 18, 38) and play a role in substrate recognition (7, 16, 23, 35).

In prokaryotes, Deg proteases are usually located in the periplasm and are implicated in the response to a variety of stresses such as heat (3, 30, 33), oxidative stress (3, 47) and high light stress (3, 39). Deg proteases are also essential for virulence in several pathogenic bacteria (33, 47). An intriguing feature of this protease family is its functional diversity from general proteases to highly specific regulatory enzymes. *E. coli* DegP prevents aggregation of denatured proteins in the periplasm by acting as a chaperone at lower temperatures and as a general protease for unfolded substrates at higher temperatures (7, 23, 32, 42). DegP has further been implicated in limited proteolysis...
during the maturation of proteins secreted to the periplasm (5, 7). The homologous DegS protease in the same organism is a highly specific enzyme responsible for the primary cleavage inducing a proteolytic signal transduction cascade. Interaction of misfolded outer membrane porins with the PDZ domain activates DegS, which cleaves its only known substrate, the plasma membrane protein RseA in a periplasmic loop (44, 46). This is the first step of a proteolytic cascade which degrades the RseA protein and thus triggers the $\sigma^E$-dependent heat shock response (2, 7, 8).

The crystal structures of *E. coli* DegS (46) and human HtrA2 (27), which both contain only one PDZ domain, showed the association of monomer subunits into homotrimeric complexes by interactions of the protease domains. In human HtrA2, the PDZ domains formed a lid which prevented access to the catalytic centers of the protease domain and deletion of the PDZ domain enhanced proteolysis of $\beta$-casein (28). In *E. coli* DegS the PDZ domains extended sideways, forming a funnel-shaped structure with open access to the proteolytic center, which was present in an inactive conformation (46). Specific interactions with the PDZ domains are necessary to induce a conformational shift to activate the protease, and consequently deletion of the PDZ domain yielded an inactive DegS variant. In contrast to these two homologues, *E. coli* DegP contains two PDZ domains, termed PDZ1 and PDZ2, and showed further assembly of two trimers to a cage-like hexameric structure (23). This hexamer formation was attributed to an extended flexible loop in the N-terminal regions of the protease domains, termed LA loop, which showed complex interactions with the opposing trimer (7, 23). Two different conformations of the hexamer were observed in the crystal: A closed conformation where the PDZ2 domains interacted with the PDZ1 and PDZ2 domains of the opposite trimer...
and an open conformation where the PDZ domains extended sideways and the PDZ2 domains were too flexible to be resolved. Thus, it was suggested that the PDZ domains guarded the lateral entrance to the protease domains and coupled substrate binding and translocation into the DegP hexamer (23). Both forms of crystallized DegP were present in proteolytically inactive conformations where both substrate binding and catalysis were prevented (23). Recently, detailed deletion studies demonstrated that, in addition to the LA loop, both PDZ domains were necessary for hexamer formation (16, 18). Interestingly, only the PDZ1 domain was necessary for proteolysis, but not the formation of the hexameric complex.

In contrast to the detailed information reported for the localization, structure and function of *E. coli* DegP and DegS, less is known about the role of the three Deg proteases in *Synechocystis*. Care should be taken when comparing the Deg/HtrA proteases of these two organisms. Even though *Synechocystis* HtrA (*htrA*, slr1204), HhoA (*hhoA*, slr1679) and HhoB (*hhoB*, slr1427) have been named according to the *E. coli* proteases (19), they are not orthologous enzymes in both organisms. This means that *Synechocystis* proteases are more similar to each other than to any Deg proteases from *E. coli* carrying the same names and do not necessarily share the same protein structure and function (14, 17, 21). For instance, in contrast to the *E. coli* enzymes all three Deg proteases in *Synechocystis* contain only one PDZ domain.

Proteome analyses demonstrated that HhoA from *Synechocystis* is a soluble protein in the periplasm (10) which associates with the plasma membrane (12). However, HhoA might also be located in the thylakoid lumen, because protein sorting in cyanobacteria is poorly understood and no methods exist for reliable separation of the periplasm from the
thylakoid lumen (41). HhoB contains a predicted signal sequence for the translocation to
the periplasm or into the thylakoid lumen (17, 21), but this protease has not yet been
identified in any proteome study. HtrA contains a predicted transmembrane segment at its
N-terminus and has been found in the outer membrane (13). Triple mutants of
*Synechocystis* in which all three Deg proteases were inactivated exhibited a dramatic
growth defect when exposed to high temperatures or high light intensities (3, 39). Based
on these studies it was proposed that *Synechocystis* Deg proteases protect the
extracytoplasmic compartments from the effects of heat and light stress and from
oxidative damage caused by reactive oxygen species which are generated by
photosynthetic electron transport (3). Furthermore, HtrA, HhoA and HhoB were
suggested to overlap at least partially in their function, because no severe phenotype was
found in any of the double mutants (3). In contrast to this report, an earlier study
indicated that a Δ*hhoA* insertion mutant was more sensitive to heat stress than wild type
or a Δ*hhoB* mutant, suggesting different physiological functions (40).

The strongly differing mechanisms and functions of the *E. coli* enzymes, together
with the unresolved function of the Deg proteases in *Synechocystis* encouraged us to
characterize one of these enzymes, HhoA, in biochemical terms *in vitro*. We demonstrate
that mature HhoA protease, expressed as a soluble recombinant His-tag fusion protein,
acted as a general protease against unfolded model substrates. The proteolytic activity of
HhoA increased with temperature and pH and was stimulated by addition of Mg^{2+} and
Ca^{2+}. We show that the PDZ domain of HhoA is essential for the proteolytic activity
against certain substrates. Furthermore, recombinant HhoA formed a hexameric complex
in solution, with two trimeric units assembled into a hexamer depending on the presence
of the PDZ domain. Finally, we show that HhoA degraded only a limited set of proteins when added to isolated *Synechocystis* membrane fractions. Thus, our data demonstrates a dual role of the single PDZ domain in HhoA and supports a role of this protease in the protein quality control in the extracytoplasmic space, including membrane proteins.

**MATERIALS AND METHODS**

**Strains and culture conditions.** A glucose-tolerant strain of *Synechocystis* sp. PCC 6803, referred to as WT, and a deletion mutant in which the HhoA-encoding gene sll1679 was interrupted by a cassette conferring kanamycin resistance, referred to as Δ*hhoA* (I. Adamska, P. F. Huesgen, and C. Funk, unpublished), were cultured in BG-11 medium (36) buffered to pH 8.0 with 20 mM TES and at a photon flux density of 170 μmol photons m⁻² s⁻¹ at 30°C with 1.5% CO₂.

**Plasmids.** Genomic DNA was isolated from *Synechocystis* using a kit (Roche Diagnostics GmbH, Mannheim, Germany). The *hhoA* gene (sll1679) was amplified by PCR from genomic DNA with gene specific primers (Operon Biotechnologies, Cologne, Germany) and ligated into the pET151-D/TOPO expression vector using the Champion directional cloning kit (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer’s instructions. Similar cloning strategy was used for the engineering HhoA deletion constructs using following primers: for full-length HhoA 5´-CAC CAT GAA ATA TCC CAC TTG GTT ACG-3´ and 5´-TTA ACT GGT GGG ATT ACG AAG TTG -3`, for mature HhoAΔN34 5´-CAC CGC GGA CGA CGA TTT GCC CCC G-3´ and 5´-TTA ACT GGT GGG ATT ACG AAG TTG -3` and for HhoAPD lacking the PDZ domain 5´-CAC CGC GGA CGA TTT GCC CCC G-3´ and 5´- GAG TTA TCC CCC CGC AGC ACG...
GAG GGT -3’. To obtain plasmids for the expression of proteolytically inactive mature HhoA (HhoA\textsubscript{S237A}\textsubscript{ΔN34}) and HhoAPD (HhoA\textsubscript{S237APD}), the codon for Ser\textsubscript{237} in the active site was changed to an Ala codon using the primers 5’-CGG AAT GCC GGG GGC CCG TTG C-3’ and 5’-CAA CGG GCC CCC GGC ATT GCC G-3’ and the Quikchange II point mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according to manufacturers instructions. These primers also introduced an additional \textit{ApaI} restriction site through a silent point mutation to facilitate distinction between the two plasmids. The inserted sequences and orientation in the plasmids were confirmed by DNA sequencing (GATC Biotech AG, Konstanz, Germany).

\textbf{Expression and purification of recombinant protein.} \textit{E. coli} BL21(DE3)Star Oneshot chemocompetent cells (Invitrogen GmbH, Karlsruhe, Germany) transformed with the expression plasmids were grown in Lubert-Bertani minimal medium containing 100 \textmu g mg\textsuperscript{-1} Ampicilin at 19°C to OD\textsubscript{600} = 0.4 to 0.6. Expression of the protease constructs was induced by the addition of 0.1 mM isopropyl-1-thio-D-galactoside (IPTG). Cultures were further grown over night at 19°C and then harvested by centrifugation at 5,000 \textit{g} for 10 min at 4°C. For purification of the HhoA constructs, cell pellets corresponding to 1 l culture were resuspended in 9 ml buffer A (50 mM Hepes, 300 mM NaCl, adjusted to pH 8.0) and 1 ml buffer B (50 mM Hepes pH 8.0, 300 mM NaCl, 500 mM imidazol, adjusted to pH 8.0). Cells were lysed on ice by 10 sonication cycles of 10 s with intervening 20 s cooling periods. Unbroken cells, insoluble cell debris and inclusion bodies were pelleted by centrifugation for 1 h at 23,000 \times g and 4°C. The supernatant was sterile filtered and purified by nickel affinity chromatography (HisTrap, GE Healthcare Europe GmbH, Munich, Germany) using an Äkta purifier FPLC system (GE Healthcare Europe GmbH,
Munich, Germany). A typical elution protocol included two washing steps with 10 ml buffer A containing 75 mM imidazol and 100 mM imidazol before the purified protein was eluted with buffer B. The fractions were directly used for activity assays or desalted using a HiTrap desalting column (GE Healthcare Europe GmbH, Munich, Germany). Size exclusion chromatography was performed with a prepacked Superdex 200 column (GE Healthcare Europe GmbH, Munich, Germany). Dynamic light scattering of concentrated size exclusion chromatography elution fractions containing from 0.5 to 2 mg ml\(^{-1}\) protein was measured with the DynaPro instrument (Protein Solutions, Piscataway, NJ, USA) and data was acquired with the Dynamics software version 6 (Protein Solutions, Piscataway, NJ, USA).

**Protease activity assays.** To assay the proteolytic activity of the purified proteins, elution fractions containing 100 pmol purified protein as assayed with the Bio-Rad Protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) were incubated with 8 µg resorufin-labeled casein (Universal Protease Substrate, Roche Diagnostics GmbH, Mannheim, Germany) in 50 mM Hepes, pH 8.0 (adjusted at 20°C) and 20 mM CaCl\(_2\) at 40°C for 2 h when not indicated otherwise. As alternative substrates, 10 µg β-casein or 10 µg bovine serum albumin (BSA, both from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), were used.

**Protease reconstitution assay.** Total membrane proteins containing 200 µg chlorophyll were prepared by rupture with glass beads essentially as described (22). The chlorophyll concentrations of cells or total membrane fractions were determined in methanol as described (29). 100 pmol purified HhoAΔN34 were added to membrane fractions containing 50 µg chlorophyll a in 50 mM Tris pH 7.5 supplemented with 20 mM CaCl\(_2\).
and incubated for 2 h at the temperature indicated in the figure. The bands of interest were excised and identified by mass spectrometry using a commercial service (Proteome Factory AG, Berlin, Germany).

**Protein analysis.** Proteins were solubilized in LDS buffer and separated by SDS-PAGE as described (25). The gels were stained with Coomassie brilliant blue R250 (37).

**Bioinformatics.** Sequence analysis was performed using SMART (26), TargetP (9) and SignalP (34) programs. For the engineering of the truncated constructs, the secondary structure of HhoA was analyzed with PsiPred v2.5 (4) and compared to published structure and sequence alignments (7, 17).

**RESULTS**

**Engineering and heterologous expression of full-length HhoA and its deletion constructs.** Analysis of the primary structure of HhoA from *Synechocystis* (encoded by the gene sll1679) showed that this protein contains a putative N-terminal signal peptide, a protease domain of the trypsin type and a single C-terminal PDZ domain (Fig. 1A). To assess the importance of conserved domains for proteolytic activity and oligomeric complex formation, we expressed full-length HhoA protein and various deletion constructs in *E. coli*. Our attempts to express full-length HhoA in a native form failed due to its insolubility and the accumulation in inclusion bodies (data not shown). Deletion of the predicted periplasmic signal peptide of 34 amino acid residues overcame this problem and yielded the soluble HhoAΔN34 of 41.4 kDa when expressed at 19°C (Fig. 1B, line 1). The purified HhoAΔN34 was prone to slow self-degradation as revealed by the
accumulation of lower molecular mass bands that were observed in the elution fraction separated on a Coomassie-stained SDS-gel (Fig. 1B, line 1). In contrast, HhoA\textsubscript{S237A\textjesus\textjesus N34} construct, in which the Ser\textsubscript{237} of the catalytic triad is replaced by Ala (Fig. 1A) was autoproteolytically inactive since a single band of 41.4 kDa is visible in a Coomassie-stained SDS-gel (Fig. 1B, line 2). To investigate the role of the PDZ domain, HhoAPD (PD for protease domain) and HhoA\textsubscript{S237A}\textjesus\textjesus PD constructs of 29.3 kDa were engineered. In both constructs the C-terminal PDZ domain was deleted by truncation after Gly\textsubscript{282} (Fig. 1A) and in the latter one the catalytic Ser\textsubscript{237} was replaced by an Ala. Also in this case the replacement of the catalytic Ser\textsubscript{237} abolished the autoproteolysis as compared with the HhoAPD construct (Fig. 1B, compare lines 3 and 4).

**The PDZ domain modulates HhoA specificity against unfolded protein substrates.** Purified HhoA deletion constructs were tested for their proteolytic activity against three model substrates, including β-casein, resorufin-labeled casein as a chromogenic substrate and bovine serum albumin (BSA). Elution fractions containing HhoA\textjesus\textjesus N34 readily degraded the naturally unfolded model substrates β-casein and resorufin-labeled casein but not the globular BSA (Fig. 2A and B). Fractions containing purified HhoAPD were active against β-casein but not against resorufin-labeled casein or BSA. As expected, purified HhoA\textsubscript{S237A\textjesus\textjesus N34}, HhoA\textsubscript{S237A}\textjesus\textjesus PD or the elution buffer used as a control showed no proteolytic activity against all three substrates tested (Fig. 2A and B).

**The PDZ domain mediates formation of a homo-hexameric HhoA complex.** In order to investigate whether Synechocystis HhoA forms an oligomeric complex and to estimate the size of such a complex, HhoA\textjesus\textjesus N34 was subjected to analytical size exclusion chromatography. Two main peaks in terms of absorption at 280 nm were observed during
the elution (Fig. 3, solid line). While the first peak contained high molecular mass protein aggregates which could not be solubilized, the majority of HhoAΔN34 was eluted as a broad peak with an apparent molecular mass of approximately 245 kDa as calculated from a calibration curve obtained with marker proteins (Fig. 3). This indicated the formation of a complex composed of six 41.4 kDa monomeric subunits. Consistent with the observation that the purified HhoAΔN34 fraction contained some self-degradation fragments (Fig. 1B, line 1), the peak 2 was asymmetric and showed some tailing as well as smaller peaks containing polypeptides with lower apparent molecular masses (Fig. 3). The distribution of the proteolytic activity within the collected fractions assayed against resorufin-labeled casein followed the elution profile of the main peak (Fig. 3, grey boxes). Size exclusion chromatography of the proteolytically inactive form HhoAS237AΔN34 showed a more symmetric main peak at roughly the same elution volume (data not shown). The more homogeneous HhoAS237AΔN34 complex was further investigated by dynamic light scattering (DLS). The concentrated size exclusion chromatography elution fractions were a mono-disperse solution and contained particles with an apparent Stokes radius of 5.7 nm, corresponding to a molecular mass of 228 kDa (data not shown). Analytical size exclusion chromatography of the PDZ domain deletion construct HhoAS237APD revealed a complex with an apparent molecular mass of 80 kDa, which is somewhat smaller than the theoretical molecular mass of 88.2 kDa expected for a trimer formation (Fig. 3, dotted line). Subsequent analysis of the elution fraction with DLS showed that the protein solution was monodisperse and contained particles with a radius of 4.2 nm, corresponding to a molecular mass of 95 kDa (data not shown). This is consistent with a trimer formation of HhoAS237APD.
**Biochemical characterization of HhoA activity.** We further characterized the proteolytic activity of HhoAΔN34 with resorufin-labeled casein at different temperatures, buffer conditions and salt concentrations. First, we tested the degradation kinetics of resorufin-labeled casein to determine when the amount of substrate becomes limiting step (Fig. 4A). The assay proceeded in a linear manner for the first two hours and therefore we used this incubation time in the consecutive assays. The obtained results are shown as relative activities normalized to the proteolytic activity of a reference point within the series because the observed proteolytic activity of 100 pmol protease varied somewhat for each independent protein preparation. Profiling of the pH preference showed that HhoAΔN34 was active at all pH values tested, although the activity was two-fold higher at pH 8.0 than at pH 6.0 (Fig. 4B). The effect of temperature was more pronounced than the pH value. At a low temperature of 15°C HhoA was barely active, and the activity increased gradually with increasing temperature from 25°C up to 50°C and decreased rapidly at temperatures over 55°C, probably due to the denaturation of the enzyme (Fig. 4C). We tested the requirement of metal ions for the HhoA protease activity. Addition of the divalent cations Mg$^{2+}$ and Ca$^{2+}$ stimulated the HhoA activity two- and three-fold, respectively (Fig. 4D). This effect could be reverted by the addition of an equimolar concentration of EDTA (Fig. 4D). A weak stimulation of the proteolytic activity was also observed when 20 mM EDTA is added to the control reaction, which may be attributed to the capture of Ni$^{2+}$ ions that are eluted with the protein from the Ni$^{2+}$ affinity chromatography column.

**Identification of HhoA substrates.** In order to test the substrate specificity of HhoA, we reconstituted purified recombinant HhoAΔN34 with total membrane proteins isolated...
from *Synechocystis* WT or the deletion mutant Δ*hhoA* prior to incubation of assays at two different temperatures. As a control, mock reactions were performed under the same conditions. After separation of proteins by SDS-PAGE, we observed at least three prominent bands with apparent molecular mass of approximately 70 kDa, 50 kDa and 22 kDa, as judged from a Coomassie-stained gel, that were specifically degraded in samples with added HhoAΔN34, but not in control samples (Fig. 5). The degradation of similar proteins in membrane fractions of WT and Δ*hhoA* confirmed that the added recombinant HhoA mediated this proteolysis. Unfortunately, our attempt to identify these proteins by mass spectrometry failed.

**DISCUSSION**

This work provides the first biochemical characterization of the cyanobacterial protease HhoA. We have shown that purified recombinant mature *Synechocystis* HhoA formed proteolytically active homo-hexameric complexes and degraded unfolded model substrates *in vitro*. Furthermore, we demonstrated that HhoA degraded selected membrane proteins at elevated temperatures *in vitro*. This is consistent with the earlier proposed function in the protein quality control of the extracytoplasmic compartments as suggested by studies of *Synechocystis* mutants (3).

The **PDZ domain of HhoA is essential for the formation of a homohexameric complex**. A similar function in protein quality control has been described for the well studied homolog DegP from *E. coli* (7). However, the comparison of structural and biochemical properties of *Synechocystis* HhoA with *E. coli* DegP showed some remarkable differences. To date, all published structures of Deg proteases with one PDZ
domain showed trimerization by interactions of the protease domains (27, 46). Only *E. coli* DegP, which contains two PDZ domains, showed dimerization of two trimers to a cage-like hexameric structure (23). HhoA from *Synechocystis* was proposed to act as a trimer because it did not contain an extended LA loop as judged by protein sequence alignments and modeled best onto a trimeric template structure (17). However, our experimental data demonstrated that recombinant HhoA formed a proteolytically active hexameric complex (Fig. 3) and that the PDZ domain was essential for the dimerization of two trimers. The deletion construct of HhoA lacking the PDZ domain assembled only into a trimer. A similar behavior was also reported for *E. coli* DegP, which failed to assemble into a hexamer after deletion of either the LA loop or the PDZ2 domain (18). In the same study, the authors demonstrated that also the PDZ1 domain was necessary for hexamer formation, serving as a spacer that allowed the PDZ2 domains to interact with the opposing trimer. Apparently such a spacer is not necessary for hexamerization of HhoA. In this case, the lack of pillars formed by the LA loops may enable the PDZ domains to interact with the opposing trimer. However, such a HhoA hexamer is expected to have a reduced dimension of the inner chamber. Taken together, these findings suggest that the formation of the hexameric HhoA protease complex differs from the complex observed in the crystal structure of *E. coli* DegP, whose formation was attributed primarily to the protease domain (23).

The PDZ domain of HhoA is required for the degradation of sterically challenging substrates. Here we demonstrated that a *Synechocystis* HhoA construct lacking its only PDZ domain retained proteolytic activity against β-casein, but lost the ability to degrade the sterically more complex resorufin-labeled casein substrate (Fig. 2B). Similarly,
deletion constructs of *E. coli* DegP lacking the PDZ2 domain were found to be impaired in the degradation of resorufin-labeled casein (42), but almost as active as the wild type against β-casein (18). DegP deletion constructs lacking the PDZ1 domain or both PDZ domains, however, were proteolytically inactive against both resorufin-labeled casein (38, 42) and β-casein (16). Assuming that the hexameric HhoA complex resembles the structure reported for *E. coli* DegP, this suggests that hexamerization, which is mediated by the single PDZ domain of *Synechocystis* HhoA, is necessary for the cleavage of sterically demanding substrates. Similar to the PDZ1 domain in *E. coli* DegP, the PDZ domain of HhoA may have the additional task to feed the substrate to the protease domain and/or to prevent its premature escape. The constraining cage formed by six protease domains may increase the probability of binding of the sterically hindered substrate to a binding pocket, which would be followed by cleavage of the substrate. In the case of the trimeric HhoAPD the bulky resorufin attached to the side chains of some casein residues would prevent such binding. On the other hand, a sterically less demanding substrate, such as β-casein, may bind to the substrate-binding pocket even in the absence of a constraining cage-like structure and/or without the help of the PDZ domains. The hypothesis that sterically challenging peptides are poor substrates for Deg proteases is additionally supported by the observation that HhoA and HhoAPD could not cleave a range of paranitroanilide (pNA)-labeled tetrapeptide substrates commonly used to determine protease substrate specificities (data not shown).

**The proteolytic activity of HhoA is temperature-dependent.** Our data demonstrated that *Synechocystis* HhoA showed only a residual proteolytic activity at temperatures between 15 and 25°C and this activity increased almost linearly with an increasing
temperature up to 50°C (Fig. 4C). It was shown that at lower temperatures, *E. coli* DegP was proteolytically almost inactive, but promoted refolding of the chemically denatured MalS (42). This chaperone function was also retained by the catalytically inactive mutant DegP$_{S210A}$ (42). The crystal structure of *E. coli* DegP, which showed the protein locked in the proteolytically inactive conformation, supported the suggestion that a conformational change is induced by higher temperatures to activate the protease function (23, 42). It is tempting to speculate that *Synechocystis* HhoA might undergo a similar structural change, but the effect of the temperature-dependent increase of HhoA protease activity may be related to general effects of higher temperatures, such as increased overall reaction rates, increased unfolding of the substrate and/or increased diffusion of the sterically hindered model substrate into the catalytic chamber.

**A conserved general role of HhoA in quality control of protein folding.** Three Deg proteases, named Deg1, Deg5 and Deg8 (1, 14), which are closely related to *Synechocystis* HhoA, have been identified in the thylakoid lumen of *A. thaliana* (14, 21). Of these three proteases, only Deg1 has been characterized in biochemical terms. Comparison of *A. thaliana* Deg1 with *Synechocystis* HhoA is particularly interesting in the light of the cyanobacterial origin of the chloroplast (31). Like HhoA, *A. thaliana* Deg1 also possesses only one C-terminally-located PDZ domain, forms a hexamer and degrades unfolded model substrates (6). Deg1 was further suggested to degrade denatured and mistargeted proteins in the thylakoid lumen (6) and to participate in the degradation of the photodamaged membrane D1 protein from the reaction center of photosystem II (20). Thus, both HhoA and Deg1 appear to fulfill similar physiological tasks in the quality control of membrane proteins. Comparison of the biochemical properties reported...
for Deg1 (6) and for *Synechocystis* HhoA (this work) showed that both enzymes adapted to different environmental conditions. The positive effect of increasing pH on the proteolytic activity of HhoA correlated well with the pH tolerance of *Synechocystis* cells, which are able to survive under slightly acidic conditions but prefer alkaline conditions for growth, and thus with the pH experienced in the periplasm (24). In contrast, *A. thaliana* Deg1 was most active at a low pH of 6.0 and this activity decreased at higher pH, which corresponds to the conditions prevalent in the thylakoid lumen (6). HhoA and Deg1 also differ in their responses to stress conditions both on the transcript and protein level. Deg1 was originally discovered as a transiently heat shock-induced protein which showed two-fold higher accumulation at elevated temperatures (15), suggesting a role in the transient heat shock response in higher plants. Northern Blot analysis showed no significant increase of *hhoA* transcript levels (17) and a combined microarray and proteome analysis revealed that 1.8-fold and 1.28-fold higher levels of HhoA transcripts and proteins, respectively, accumulated in response to heat shock (43).

We demonstrated that HhoA recognized and cleaved a wide range of substrates, supporting the suggestion that HhoA is a general protease involved in the quality control of extracytoplasmic proteins, including membrane proteins (3). The identification of native substrates of HhoA under different stress conditions as well as the identification of the substrates of the HtrA and HhoB proteases constitute interesting future perspectives to understand the role of individual enzymes and their combined action in the protein quality control network and their importance for cell survival under stress conditions. Identification of the molecular determinants for the differences observed in the biochemical properties of *E. coli* DegP, *A. thaliana* Deg1 and *Synechocystis* HhoA will
provide insights into the adaptation of the protein quality control machinery to changing environmental conditions during evolution.

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**Figure Legends**

**FIG. 1.** Engineering and purification of HhoA deletion constructs. A, Domain structure of the full-length HhoA protease and its truncated constructs. Grey oval, putative signal peptides; light grey boxes, protease domains of the S1B subfamily of serine proteases with the amino acid residues of the catalytic triad indicated; dark grey boxes, PDZ domains; His, 6xHis-tag and linker introduced by the expression vector. B, Ni\(^{2+}\)-affinity purified recombinant protein (4 \(\mu\)g) separated by SDS-PAGE and visualized by Coomassie-staining. Lane 1, HhoA\(\Delta N34\), lane 2, HhoA\(S237A\Delta N34\), lane 3, HhoAPD, lane 4, HhoA\(S237A\)PD.

**FIG. 2.** Proteolytic activity of purified HhoA deletion constructs against various model substrates. In all assays 100 pmol purified HhoA protein was incubated with the respective substrate for 2 h at 40°C in 50 mM Hepes pH 8.0 and 20 mM MgCl\(_2\). A, Degradation assays with \(\beta\)-casein and BSA visualized in a Coomassie-stained SDS-PAGE gel. A representative gel of three independent replicates is shown. Lane 0, elution buffer, lane 1, HhoA\(\Delta N34\), lane 2, HhoA\(S237A\Delta N34\), lane 3, HhoAPD, lane 4, HhoA\(S237A\)PD. B, Proteolytic activity against resorufin–labeled casein. The chemical structure of the resorufin-label is shown in as an inlet. Values are means ±S.D. (n=4).

**FIG. 3.** Analysis of HhoA complex formation. The elution diagrams of the size exclusion chromatography of recombinant HhoA\(\Delta N34\) (solid line) and HhoA\(S237A\)PD (dotted line)
are shown. For HhoAΔN34, the proteolytic activity of 10 µl of selected fractions against
resorufin-labeled casein is shown (grey boxes, right axis).

FIG. 4. Characterization of HhoA protease activity. For all assays 100 pmol purified
HhoAΔN34 were incubated with 8 µg resorufin-labeled casein and repeated with at least
three independent protein purifications. A, Degradation kinetics at 40°C in 50 mM Hepes
pH 8.0 and 20 mM CaCl$_2$. Values are means ±S.D. (n=4), normalized to the activity after
4 h. B, Effect of pH. HhoAΔN34 was incubated in 50 mM MES (pH 6.0-6.5) or 50 mM
Hepes (pH 7.0-8.5), supplemented with 20 mM MgCl$_2$ for 2 h at 40°C. Values are means
±S.D. (n=7), normalized to the activity at pH 8.0. C, Effect of temperature. HhoAΔN34
was incubated in 50 mM Hepes pH 8.0 and 20 mM CaCl$_2$ for 2 h. Values are means
±S.D. (n=6). D, Effect of divalent cations. HhoAΔN34 was incubated for 2 h at 40°C in
50 mM Hepes pH 8.0 supplemented with 20 mM MgCl$_2$ or CaCl$_2$ as indicated (black
bars) and with added 20 mM EDTA (grey bars). Values are means ±S.D. (n=6 for black
bars, n=3 for grey bars).

FIG. 5. Activity of HhoAΔN34 against isolated total membrane proteins.
Isolated total membranes of WT or ΔhhoA mutant cells were incubated without (-) or
with (+) recombinant HhoAΔN34 at 15°C and 45°C in 50 mM Tris pH 7.5 supplemented
with 20 mM CaCl$_2$, separated by SDS-PAGE and visualized by Coomassie-staining.
Open arrows indicate bands of the added HhoAΔN34 and its degradation fragments,
filled black arrows indicate protein bands specifically degraded by HhoAΔN34.
A

Elution Buffer

HhoA

∆N3

S237A

PD

BSA

β-casein

BSA

B

[Casein]

activity [ΔA574]

0.00

0.02

0.04

0.06

0.08

0.10

Elution Buffer

HhoA∆N34

HhoA S237A∆N34

HhoAPD

HhoA S237APD