Molecular Basis for the Unique Role of the AAA\textsuperscript{+} Chaperone ClpV in Type VI Protein Secretion\textsuperscript{*}\textsuperscript{5}

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Ring-forming AAA\textsuperscript{+} ATPases act in a plethora of cellular processes by remodeling macromolecules. The specificity of individual AAA\textsuperscript{+} proteins is achieved by direct or adaptor-mediated association with substrates via distinct recognition domains. We investigated the molecular basis of substrate interaction for \textit{Vibrio cholerae} ClpV, which disassembles tubular VipA/VipB complexes, an essential step of type VI protein secretion and bacterial virulence. We identified the ClpV recognition site within VipB, showed that productive ClpV-VipB interaction requires the oligomeric state of both proteins, solved the crystal structure of a ClpV N-domain-VipB peptide complex, and verified the interaction surface by mutational analysis. Our results show that the substrate is bound to a hydrophobic groove, which is formed by the addition of a single \(\alpha\)-helix to the core N-domain. This helix is absent from homologous N-domains, explaining the unique substrate specificity of ClpV. A limited interaction surface between both proteins accounts for the dramatic increase in binding affinity upon ATP-driven ClpV hexamerization and VipA/VipB tubule assembly by coupling multiple weak interactions. This principle ensures ClpV selectivity toward the VipA/VipB macromolecular complex.

AAA\textsuperscript{+} proteins (ATPase associated with various cellular activities) are involved in numerous cellular processes including DNA replication and repair, protein refolding, degradation, and transport, among others (1, 2). AAA\textsuperscript{+} proteins share the conserved AAA domain, which mediates ATP binding and hydrolysis and usually drives AAA\textsuperscript{+} protein oligomerization into hexameric assemblies. ATP hydrolysis fuels conformational changes within AAA\textsuperscript{+} proteins, which generate a mechanical force to unfold or disassemble substrates. The remarkable substrate heterogeneity of AAA\textsuperscript{+} proteins is based on alterations of sequence elements within the AAA\textsuperscript{+} domain and especially the fusion or insertion of additional domains determining specificity. Such extra domains either directly bind to substrates or act indirectly by interacting with cooperating proteins (3, 4).

Hsp100 proteins constitute an AAA\textsuperscript{+} protein subfamily and are key components of quality control systems by either refolding or degrading misfolded and aggregated protein species (5). N-terminal extra domains (N-domains) of Hsp100 proteins play crucial roles in substrate selection and activity control. They usually function as binding platforms for adaptor proteins, which deliver their bound cargo to the cognate Hsp100 proteins (6–9). In addition, N-domains can also contact substrates directly (10, 11). N-domains are connected via flexible linkers to the AAA\textsuperscript{+} ring and are structurally mobile, which may facilitate binding to target proteins (12, 13). Transplantation of N-domains between different Hsp100 proteins allows for transfer of substrate specificity, indicating that N-domains function as independent units in substrate selection (6, 11).

Interestingly, various Hsp100 proteins share homologous N-domains; however, they exert different cellular activities. This is especially true for ClpV, which, in contrast to other Hsp100 proteins, is not involved in protein quality control, but is an essential component of the recently discovered type VI secretion systems (T6SSs)\textsuperscript{4} (11, 14). T6SSs are proposed to act as contractile machineries, targeting effector proteins into bacterial and eukaryotic target cells (15, 16). ClpV binds via its N-domain to the large tubular VipA/VipB complex and disassembles it upon ATP hydrolysis (11). It has been speculated that tubule severing causes the ejection of a T6SS contractile device composed of Hcp and VgrG proteins (17, 18).

The molecular determinants that differentiate the ClpV N-domain (ClpV-N) from other homologous domains are not yet known. Thus, the basis for the unique function of ClpV in protein secretion is not understood. Furthermore, it is unclear by which mechanism ClpV is selectively directed toward its macromolecular substrate but prevented from being jammed by interacting with unassembled or disassembled subunits.

Here, we unravel the molecular details of ClpV substrate specificity, by identifying the ClpV binding site within VipA/VipB tubules, by determining a strong dependence of binding affinity on the oligomeric state of the interacting proteins and

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\textsuperscript{4} The abbreviations used are: T6SS, type VI secretion system; ATP\textsubscript{y}S, adenosine 5\textsuperscript{'}-O-(thiotriphosphate); ClpV-N, ClpV N-domain; ITC, isothermal titration calorimetry; PDB, Protein Data Bank.
by solving the crystal structure of ClpV-N in complex with a VipB peptide representing the ClpV binding site. The comparison of the complex with homologous N-domain structures of other Hsp100 proteins reveals how functional diversity is gained by a limited set of structural alterations.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Supplemental Table IV lists all strains and plasmids used in this study. All bacterial strains were grown in LB medium at 37 °C if not stated otherwise. Antibiotics were applied as described (11). PCRs and cloning procedures followed standard protocols. *Vibrio cholerae* deletion mutants were complemented by pMPM-A4 harboring the gene of interest allowing for arabinose-controlled gene expression. Total protein levels were adjusted to wild type levels by titration of arabinose levels and comparing the signal intensities upon immunoblot analysis.

**Proteins**—His tag-based protein purifications were performed after overproduction from *Escherichia coli* XL-1 Blue and MC4100 cells using Ni-IDA resin following the instructions of the manufacturer (Macherey-Nagel). Proteins were further purified via size exclusion chromatography at 4 °C in reaction buffer (50 mM Tris, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 2 mM DTT) using a Superose 6 HR 10/30 column (Amersham Biosciences). ClpA and ClpP were purified as described (7). SeMet labeling of ClpV-N was performed as described (19). Protein concentrations were determined with the Bio-Rad Bradford assay using BSA as standard.

**Crystallization, Data Collection, Structure Determination, and Analysis**—For crystallization of the ClpV-N apo form, 250 nl of 25 mg/ml SeMet-labeled protein was mixed with 250 nl of 2 mM sodium acetate, pH 7.0, 20% (w/v) PEG 3350 at 18 °C, using the sitting drop method. For the P*-VipB/ClpV-N complex, 17 mg/ml protein was preincubated with 1:1.2 peptide (molar ratio), then 1 μl of protein solution was mixed with 1 μl 0.1 M MES, pH 5.5, 17.5% (v/v) PEG 8000 at 4 °C, using the hanging drop method. Crystals appeared after 2–6 days and were flash frozen in the presence of 20% (v/v) glycerol or ethylene glycol.

Data were collected at 100 K at beamlines ID23h1 and ID29, European Synchrotron Radiation Facility, Grenoble, France. The SeMet crystal of space group I222 diffraction to 1.8 Å, whereas the crystal of the complex was of space group C2 and diffraction to 1.94 Å (supplemental Table I).

The ClpV-N apo structure was solved by multianomalous dispersion method using SeMet data. Data were processed with XDS (20), and the initial model was obtained using Phenix autosol (21). For the complex, data were processed with XDS (20) followed by molecular replacement using the ClpV-N apo as search model and Phaser (CCP4 suite). The initial models of both structures were refined by alternating cycles of model building in COOT (22) and Refmac5 (CCP4 suite) or Phenix (21) for refinement (with TLSMD) (23). Structures were validated using SFCHECK (CCP4 suite) and MolProbity (24), revealing Ramachandran plot values (favored/outliers) of 98.1/0% and 98.2/0%, respectively.

Images were generated using PyMOL (25), the complex interface was analyzed using the PISA server (26) and LIGPLOT (27), the electrostatic surface was analyzed using APBS (28), and structural superpositions were generated by Dalilite (29). Secondary structure predictions were done using Psipred (30).

**Biochemical Assays**—The analysis of the *V. cholerae* secretome was performed as described (11). Size exclusion chromatography of ClpV (10 μM) was performed at room temperature in reaction buffer using a Superose 6 HR 10/30 column. Nucleotide-dependent oligomerization was followed in presence or absence of 2 mM ATP in the running buffer. ATPase hydrolysis rates under steady-state conditions were determined as described previously (31).

VipA/VipB processing by either NVAP/ClpP or ClpA/ClpP was monitored by SDS-PAGE and Coomassie Blue staining. The reactions were carried out at 30 °C in reaction buffer. After mixing of VipA/VipB (2 μM) with NVAP/ClpA (1 μM) and ClpP (1 μM) the reactions were started by addition of an ATP-regenerating system (2 mM ATP, 3 mM phosphoenolpyruvate, 20 ng/ml pyruvate kinase). Degradation of FITC-casein was monitored on a PerkinElmer Life Sciences LS50B spectrofluorometer as described (32).

Peptide libraries were prepared by automated spot synthesis (JPT Peptide Technologies GmbH). ClpV-N (500 nm) was incubated with peptide scans in MP2 buffer (15.5 mM Tris, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.05% (v/v) Tween 20, 5.0% (w/v) sucrose) for 30 min at room temperature. Unbound ClpV-N was removed with TBS (4 °C), and peptide-bound ClpV-N was electrotransferred onto a PVDF membrane as described (33). Transferred ClpV-N was detected with specific antibodies raised against the ClpV N-domain.

Peptide interaction in solution was monitored by incubating ClpV N-domain or full-length ClpV (each 1 μM) with 2-(4′-(iodoacetamido)anilino) naphthalene-6-sulfonic acid (IAANS)-conjugated peptides (1 μM) in reaction buffer for 5 min at 30 °C. Labeling of P*-VipB and P*-VipB-19AAA21 with IAANS was performed according to the instructions of the manufacturer (Invitrogen). Emission spectra were recorded between 340 and 600 nm on a PerkinElmer Life Sciences LS50B spectrofluorometer at a fixed excitation wavelength of 326 nm. The competition of VipA/VipB with P*-VipB for ClpV binding was tested in the same manner, except P*-VipB was preincubated with ClpV in the presence or absence of ATP/γS, and then VipA/VipB was added at increasing concentrations (0–4 μM).

Isothermal titration calorimetry (ITC) experiments were carried out in reaction buffer without DTT using a VP-ITC microcalorimeter (Microcal, Northampton, MA) equilibrated at 20 °C. 120 μM ClpV-N was added to the cell, and 1.2 mM P*-VipB or its mutant derivatives were added to the syringe of the microcalorimeter. Peptide titration was performed by continuous injections of 12-μl aliquots of the titrant into the cell under constant stirring at time intervals of 5 min to ensure that the titration peak returned to the base line. Origin 7.0 software was used for data analysis.

Disassembly of VipA/VipB tubules (0.5 μM) by ClpV wild type or variants (0.125–0.15 μM each) was analyzed at a PerkinElmer Life Sciences LS50B spectrofluorometer by monitoring the decrease of turbidity (excitation and emission wavelength: 550 nm). The reactions were performed at 25 °C in reaction buffer containing an ATP-regenerating system. ClpV wild type and variants were preincubated with ATP at 25 °C for 5
min to allow for hexamerization. Mixtures of ClpV wild type and ClpV-I10R had a final concentration of 0.18 μM and were preincubated with 2 mM ATP at 30 °C for 5 min before addition of VipA/VipB tubules. Kinetic rates (single exponential decay) were determined using GraphPad Prism Version 5.0b.

Binding of 3H-labeled ClpV to biotinylated VipA/VipB-Avi complexes (0.5 μM) was determined by pulldown experiments using streptavidin magnetic beads (NEB). ClpV binding was monitored by determining radioactivity in bound and non-bound fractions by scintillation counting. ClpV labeling was performed using N-succinimidyl [2,3-3H] propionate following the instructions of the manufacturer (Amersham Biosciences).

Electron Microscopy—Visualization of VipA/VipB complexes by electron microscopy was performed as described (11). Micrographs were taken with a Zeiss EM10A electron microscope.

RESULTS

ClpV N-domain Specifically Binds to the N-terminal Region of VipB—To elucidate the substrate binding specificity of ClpV, we first determined whether VipA or VipB is the primary target of ClpV within the VipA/VipB tubule. We made use of the chimera NVAP, which harbors the ClpV N-domain hooked onto the AAA+ ring of ClpA, an AAA+ protein that forms a complex with the peptidase ClpP and acts in regulatory proteolysis (11). NVAP, in contrast to ClpA, can bind to VipA/VipB tubules, and co-expression of NVAP with ClpP in V. cholerae cells leads to reduced levels of VipB and VipA (11). This finding suggests that both VipA and VipB might be recognized as substrates or that one of them becomes unstable upon NVAP/ClpP-mediated degradation of the other component. To differentiate between these two scenarios we monitored VipA/VipB tubule degradation by NVAP/ClpP in vitro (Fig. 1A). NVAP/ClpP specifically degraded VipB, whereas VipA was stable, indicating that ClpV-N recognizes VipB and that the reduced VipA levels observed upon NVAP/ClpP expression in vivo may be an indirect, secondary effect. Because autodegradation of ClpA and NVAP proceeded with similar kinetics, VipB degradation by NVAP/ClpP is not caused by a general higher activity of this proteolytic complex (Fig. 1A). Similarly, ClpA/ClpP degraded FITC-casein, an Hsp100 protein model substrate, faster and more efficiently compared with NVAP/ClpP (supplemental Fig. 1).
To determine the ClpV binding site precisely we screened immobilized peptide libraries, representing the primary sequences of VipA and VipB, for recognition by ClpV (Fig. 1B). The peptide scans were composed of 15-mers that overlap with adjacent peptides by 11 residues. VipA and VipB peptide libraries were incubated with ClpV-N, followed by electrophoresis and immunodetection using ClpV-N-specific antibodies. ClpV-N did not interact with the VipA peptide scan, consistent with the resistance of VipA toward NVAP/ClpP degradation. In case of the VipB peptide scan, ClpV-N did bind to few peptide spots. We focused on two peptides at the N-terminus of VipB (residues Gln13-Pro31) because they were the strongest binding of ClpV-N, suggesting that they harbor a specific recognition site (Fig. 1B). To confirm binding of ClpV-N in solution, we used a fluorescently labeled derivative of the corresponding VipB peptide (P-VipB: KKW15AQGSLDEIMQTGR28(CKK)), termed P*-VipB. Due to poor solubility of the VipB peptide, two lysine residues were attached to its N and C termini. In addition, tryptophan and cysteine residues were incorporated to allow precise determination of peptide concentration and specific labeling with the fluorescent dye IAANS. Addition of ClpV-N to P*-VipB caused an increase in fluorescence intensity, which was partially reduced in presence of a 20-fold excess of nonlabeled peptide, indicating that ClpV-N binds to the VipB peptide (Fig. 1C, upper). Incubation of P*-VipB with full-length ClpV resulted in a comparable signal increase, which was not further affected by addition of ATPγS, indicating that peptide binding is nucleotide-independent (Fig. 1C, lower). The presence of a ClpV variant lacking the N-domain (∆N-ClpV), did not alter P*-VipB fluorescence, demonstrating that P*-VipB is exclusively bound by the N-domain in context of full-length ClpV (Fig. 1C, lower). To validate the specificity of the P*-VipB/ClpV interaction further, we mutated the central 19LDD21 residues of P-VipB to AAA21. Addition of either ClpV-N or ClpV to a fluorescently labeled derivative of this mutant peptide resulted only in a slight increase in fluorescence that was insignificant compared with the wild type peptide, further demonstrating the specificity of the interaction (supplemental Fig. 2A). Furthermore, we incubated the peptide with the N-domain of V. cholerae ClpB or full-length V. cholerae ClpB. In both cases only a marginal increase in fluorescence was observed (supplemental Fig. 2B).

A ClpV-specific N-terminal Helix Creates a Hydrophobic Binding Cleft—To understand how ClpV interacts with VipB, we first determined the crystal structure of the ClpV N-domain (residues 2–159) at 1.8-Å resolution using multiple wavelength anomalous dispersion (Fig. 2A; data collection and refinement statistics are given in supplemental Table I). The ClpV-N structure comprises the complete N-domain and nine residues of the N-terminally fused His6 purification tag and shows an α-helical fold with overall dimensions of 40 × 30 × 25 Å.

The structure of ClpV- N is very similar to the N-domains of other Hsp100 proteins (ClpA, ClpB, ClpC), which consist of eight α-helices (α1–α8) (34–37). The corresponding N-domains can be superimposed with a root mean square deviation of e.g. 2.2 Å for ClpB for corresponding Ca atoms (Fig. 2B and supplemental Table II), indicating the high degree of conservation. The ClpV-N core is formed by the short helices α2, 3, 6, and 8, with the helices α4 and 7 packed against the core on one side and the long helices α1 and 5 on the other side (Fig. 2A).

ClpV-N contains an additional α-helix (α0) at the N terminus, which is missing in other Hsp100 N-domains, such as ClpA, ClpB, and ClpC (Fig. 2, B and D). Secondary structure predictions indicate that α0 is conserved in ClpV proteins (supplemental Fig. 3A). There are only a few contacts between α0 and the core domain, and α0 seems to be anchored to the core domain by interaction with α5. The contact involves Trp99, strictly conserved in ClpV homologs, which interacts with Lys12 (Fig. 2A). α0 is connected to α1 by a short loop arranging the two helices in a V-shaped orientation, creating a slightly concave surface that is lined by conserved hydrophobic residues (including Ile10, Leu21, Ala25, and Phe87) (Fig. 2C and supplemental Fig. 3B). In Hsp100 proteins lacking α0, the surface of the N-domain is rather flat and does not contain a cleft in the corresponding position. We therefore consider the possibility that the N-terminal extension of ClpV-N creates a specific binding site for interacting proteins.

The ClpV-specific Hydrophobic Groove Provides the Binding Site for VipB—To unravel the molecular basis of the VipB-ClpV interaction, we co-crystallized ClpV-N and the VipB peptide that interacts with the ClpV N-domain. The structure was solved at 1.94 Å by molecular replacement with ClpV-N as a search model (Fig. 3A and supplemental Table II). The asymmetric unit contains two heterodimers (A and B) with dimer A (ClpV-N residues 2–158, 14 residues of P-VipB) and dimer B (ClpV-N residues 2–158, 10 residues of P-VipB) differing only in the length of the bound peptide resolved in the structure.

Superposition of the ClpV-N peptide complex with the ClpV-N apo structure shows no major rearrangements upon peptide binding, illustrated by a root mean square deviation of 0.8 Å (155 Ca atoms). Small but noticeable differences are found at the C terminus of helix α1 and in the loops connecting helices α1 and α2 (L1–2) and helices α4 and α5 (L4–5). Whereas α1 and the first residues of L1–2 move toward the peptide in the ClpV-N/P-VipB complex, L4–5 slides by about 1 Å, resulting in a repositioning of residues involved in the binding cleft (including Tyr4, Ala30, Phe87). Although the observed differences upon P-VipB binding are only small, they may contribute to the interaction.

Bound P-VipB forms an amphipathic helix, which interacts with the previously identified hydrophobic groove of ClpV-N. P-VipB contributes 7 residues and ClpV-N 12 residues to the interaction (supplemental Fig. 4A). P-VipB orients nearly antiparallel to α0 and utilizes a predominantly hydrophobic interface of ~500 Å2 with only two hydrogen bonds (Fig. 3B). Ile23 and Met24 from VipB are in the center of the interface and contact two sides of the groove, whereas Leu20 points deep into the pocket (Fig. 3B and supplemental Fig. 4B). The observation that a P*-VipB19AAA21 peptide variant cannot bind to ClpV (supplemental Fig. 2A) supports the importance of Leu20. All interacting residues show a high degree of conservation among VipB homologs, emphasizing the requirement for long hydrophobic residues at these positions (Fig. 3C). Taken together, the crystal structures provide an explanation why ClpV but not other Hsp100 family members interact with VipB because the binding groove is only formed in ClpV by the addition of α0.
The N-terminal VipB Peptide Represents the Dominant ClpV Binding Site in VipA/VipB Tubules—To confirm the interaction mode between the VipB peptide and ClpV-N, we determined the affinity of ClpV-N for P-VipB and peptide variants, in which residues that contribute key interactions in the crystal structure (Leu20, Ile23, Met24) were replaced by alanine, by ITC. ClpV-N and P-VipB formed a 1:1 complex with a $K_{d}$ of 38.8 nM (Fig. 4A). Analysis of P-VipB-M24A displayed strongly reduced, but residual binding to ClpV-N ($K_{d}$: 206 nM), whereas the variants P-VipB-L20A and P-VipB-I23A did not display any detectable affinity for ClpV-N (Fig. 4A and supplemental Fig. 5).

To confirm that the sequence corresponding to P-VipB is the major binding site for ClpV in context of VipA/VipB tubules, we co-produced VipA and VipB (wild type and variants) in E. coli. All VipB variants formed complexes with VipA, and the resulting tubules were structurally indistinguishable from wild type VipA/VipB tubules as judged by electron microscopy (EM) (Fig. 4B, left). Disassembly of VipA/VipB tubules in the presence of ClpV and ATP was monitored by EM and by following the decrease of light scattering intensity. VipB-L20A and VipB-I23A tubules were completely resistant to ClpV-mediated disassembly, and disassembly of VipB-M24A occurred at a significantly reduced rate ($k_{0} = 0.08/min$ compared with 0.9/min for VipB wild type) (Fig. 4B, middle and C). To demonstrate that the observed resistance of VipB-L20A and VipB-I23A toward ClpV-mediated disassembly is directly caused by a loss of interaction we visualized the binding of ClpV to VipA/VipB tubules in presence of nonhydrolyzable ATP$\gamma$S by EM. ClpV association is evident from the appearance of dot-like ClpV hexamers.
at the surface of VipA/VipB tubules (11). Although ClpV hexamers decorated VipA/VipB wild type and VipA/VipB-M24A tubules, no binding to VipA/VipB-L20A and VipA/VipB-I23A tubules was observed (Fig. 4B, right). Together, these findings demonstrate that the N-terminal region of VipB serves as an essential binding site for ClpV. Finally, we analyzed the physiological consequences of a loss of ClpV interaction by testing for the ability of VipB variants to restore T6SS activity in V. cholerae/vipB cells. Functionality of T6SS is commonly tested by monitoring the appearance of Hcp in cell culture supernatants (38). Consistent with our in vitro data, VipB-L20A and VipB-I23A could not complement the Hcp secretion defect of ΔvipB cells, whereas VipB-M24A exhibited partial activity in vivo (Fig. 4D).

The Hydrophobic ClpV-N Binding Groove Is Essential for VipA/VipB Interaction—In a reciprocal approach we set out to confirm the ClpV substrate binding pocket by mutagenesis. The hydrophobic character of the groove was disrupted by
replacing key residues Ile\textsuperscript{10} (α0) and Phe\textsuperscript{87} (loop 4–5) with basic residues, yielding I10R and F87R. We also generated a ClpV-L21K variant, which, however, formed inclusion bodies upon overproduction in \textit{E. coli} and could not be purified in a soluble state. In addition, we constructed a ClpV-A25R variant, in which entry to the binding pocket is predicted to be blocked according to the crystallographic data. We also tested the potential importance of Trp\textsuperscript{99} in positioning α0 by constructing
Molecular Basis of ClpV Substrate Specificity

ClpV-W99A. All ClpV variants formed hexamers in presence of ATP, excluding general structural defects as the basis for potential VipA/VipB disassembly defects (supplemental Fig. 6). All variants exhibited an increased ATPase activity compared with ClpV wild type to varying degrees (supplemental Table III). We measured the ability of ClpV wild type and variants to sever VipA/VipB tubules by light scattering and EM (Fig. 5, A and B). Mutations that disrupted the hydrophobic character of the binding groove (I10R, F87R) or blocked substrate access (A25R) were entirely inactive in VipA/VipB disassembly. ClpV-W99A exhibited partial activity and was 4.8 times less active than ClpV wild type (Fig. 5A). A defect of the inactive ClpV variants in substrate binding could be visualized by EM because no decoration of VipA/VipB tubules by ClpV rings was observed in presence of ATPγS (Fig. 5B). In contrast, ClpV-W99A revealed binding that was, however, less efficient compared with ClpV wild type. Similar results were obtained when binding of the ClpV variants to P*-VipB was monitored (supplemental Fig. 7).

Finally, we tested the ability of the different ClpV variants to restore Hcp secretion in V. cholerae ΔclpV cells. In agreement with our in vitro findings, none of the binding-deficient ClpV variants showed activity in vivo, whereas Hcp secretion was observed upon expression of ClpV-W99A albeit at slightly reduced levels (Fig. 5C).

Multiple Interactions between ClpV-N and VipA/VipB Tubules Increase the Binding Affinity and Are Crucial for ClpV Activity—Because the determined binding affinity of ClpV-N to the VipB peptide is moderate (38 μM), we speculated that the affinity of ClpV hexamers toward VipA/VipB tubules might be significantly higher due to simultaneous interactions of several ClpV N-domains with multiple VipB binding sites. Such a dynamic range in substrate affinity might enable ClpV to selectively interact with VipA/VipB tubules but prevent it from binding to disassembled subunits. In agreement with this possibility, a 10–25-fold excess of either ClpV-N or VipB peptide did not slow the disassembly of VipA/VipB tubules by ClpV as monitored by light scattering and EM (supplemental Fig. 8).

We compared the ability of VipA/VipB tubules to compete for binding of ClpV to P*-VipB in absence and presence of ATPγS, which triggers ClpV hexamerization (Fig. 6A). VipA/VipB tubules were more efficient to outcompete P*-VipB for ClpV interaction in presence of nucleotide, indicating higher affinity of ClpV hexamers for VipA/VipB tubules compared with monomeric ClpV.

To determine the affinity of ClpV-VipA/VipB tubule interaction, we co-produced VipA and a VipB variant harboring a C-terminal Avi tag, allowing for biotinylation in E. coli cells. Biotinylation of VipB-Avi was confirmed by mass spectrometry and corresponding VipA/VipB-Avi tubules were disassembled by ClpV at the same rate as wild type tubules (data not shown). Biotinylated VipA/VipB-Avi tubules were immobilized on streptavadin magnetic beads and incubated with fully active 3H-labeled ClpV in the presence and absence of ATPγS. The binding affinity was calculated by quantifying the amount of 3H-labeled ClpV that was specifically pulled down by VipA/VipB-Avi tubules (Fig. 6B). Analysis in presence of ATPγS revealed a Kd for ClpV-VipA/VipB-Avi interaction of 66 ± 13 nm, whereas in the absence of nucleotide the affinity was 4.94 ± 0.36 μM, revealing a 70-fold difference. The 7-fold difference in affinity in absence of nucleotide compared with the one determined by ITC for ClpV-N/P-VipB interaction might be explained by modification of the synthetic peptide, which harbored additional lysine, tryptophan, and cysteine residues or by the different experimental setups.

The binding of hexameric ClpV (+ATPγS) to VipA/VipB-Avi tubules reached saturation at lower ClpV concentrations compared with monomeric ClpV. The architecture of the cogwheel-like VipA/VipB tubules, which contains 12 cogs, suggests that the structural building unit of the tubule is a dodecameric VipA/VipB complex (11). Based on this assumption and the quantified amount of bound 3H-ClpV, we calculated that monomeric ClpV completely saturated all binding sites in VipA/VipB tubules (1:1 binding ratio), whereas a single ClpV hexamer occupies 1.5 dodecameric VipA/VipB repeat units, representing a binding ratio of 1:3 (ClpV/VipB). We speculate that docking of a ClpV ring onto a VipA/VipB tubule could block binding of further ClpV hexamers in close proximity due to steric hindrance.

Finally, we calculated the number of N-domains that are required for efficient VipA/VipB severing by ClpV by determining the disassembly rates of mixtures of ClpV wild type and ClpV-I10R, which cannot bind to the tubules (Fig. 5B). ClpV hexamerization under the given buffer conditions is strictly ATP-dependent (supplemental Fig. 6), permitting oligomerization to initiate after mixing of the two ClpV variants. Formation of mixed oligomers became evident by increased disassembly activity of low ClpV wild type levels in presence of inactive ClpV-I10R (data not shown). The distribution of ClpV wild type and ClpV-I10R subunits within hexamers was calculated using a binomial distribution function for each mixing ratio (39, 40). Experimentally determined disassembly rates of ClpV wild type/ClpV-I10R mixtures were compared with those estimated from a model that assumes that a hybrid hexamer only displays activity if it contains a certain number of wild type subunits.

**FIGURE 4.** The N-terminal VipB peptide represents the dominant ClpV binding site in VipA/VipB tubules. A, determination of binding affinities for P-VipB interaction with ClpV-N by ITC measurements. Analysis of the titration isotherms resulted in a Kd of 38.8 ± 9.3 μM and a stoichiometry of 1:1 for P-VipB (open circles) and a Kd of 206 μM ± 81 μM (1:1 stoichiometry) for P-VipB-M24A (squares) binding. No heat change was associated with injections of P-VipB-L20A (blue circles) and P-VipB-123A (red circles). B, morphology of VipA/VipB wild type and mutant complexes monitored by EM. Wild type and mutant VipA/VipB tubules (left) were incubated with ClpV in presence of an ATP-regenerating system (middle) or ATPγS (right) for 15 min at 30 °C. Arrows indicate dot-like structures at the surface of VipA/VipB tubules, representing bound ClpV hexamers. Scale bars, 100 nm top, 25 nm bottom. C, disassembly of VipA/VipB complexes by ClpV. Tubule disassembly was monitored as a decrease of sample turbidity at 550 nm over time. VipA/VipB complexes analyzed were: VipB wild type (red), VipB-L20A (light blue), VipB-123A (green), VipB-M24A (dark blue). The black curve represents turbidity measurement of VipA/VipB wild type without ClpV addition. D, effects of VipB mutations on type VI protein secretion in V. cholerae. The deficiency of V. cholerae ΔvipB mutant cells in Hcp secretion and its complementation by pMPM-A4 encoded vip8 wild type and vip8 mutant derivatives was analyzed by immunoblot analysis using Hcp-specific antibodies. Production of equal VipB levels was ensured by Western blotting using VipB-specific antibodies. S, culture supernatant; T, total cell extract; wt, V. cholerae wild type; mock, empty pMPM-A4 plasmid.
The experimental values lay between the curves, which indicates that a given ClpV hexamer is only active when harboring 3–5 active subunits. This analysis therefore suggests that the presence of 3–5 N-domains in ClpV is required for efficient VipA/VipB tubule disassembly, supporting the necessity of multiple contacts between ClpV and its substrate.
DISCUSSION

We have dissected the substrate specificity of ClpV at the molecular level by identifying the ClpV binding site within the N-terminal region of VipB and determining the crystal structure of the ClpV N-domain complexed to a VipB peptide encompassing the binding site. The ClpV N-domain is similar in structure to N-domains of other Hsp100 proteins including ClpA, ClpB, and ClpC, confirming structural predictions (41). N-domains of ClpA and ClpC function as binding platforms for specific adaptor proteins (ClpS and MecA, respectively), which deliver bound substrates to the cooperating Hsp100 protein. Remarkably, N-domains of both ClpA and ClpC employ the same binding platform, formed by helices 2 and 5, for adaptor interaction (Fig. 3). In both cases the interaction is limited to a single /H9251-helix in either ClpS (Ala78-Asn95) or MecA (Thr171-Leu183), respectively. Adaptor specificity of N-domains is achieved via distinct residues, leading to a primarily electrostatic interface in case of ClpA-ClpS interaction, whereas intermolecular hydrogen bonds confer ClpC-MecA specificity (34, 35, 42). Remarkably, the binding surface between ClpV and its substrate VipB also seems to be restricted to a single helix of VipB, because mutational alterations of key residues within this helix completely abrogated ClpV binding to full-length VipB. In this case, however, the interaction involves a predominantly hydrophobic interface. Moreover, the addition of /H9251o in ClpV-N creates a specific binding site, which enables ClpV to exert its unique cellular activity. Specific residues of the ClpV-N core domain contributing to the hydrophobic binding groove, for example Phe477, are highly conserved among ClpV homologs but not in other Hsp100 N-domains (Fig. 2).

The creation of a binding site at a novel location in ClpV-N causes the bound substrate helix to be turned by 90° compared with other Hsp100 complexes (Fig. 3). This might reflect the different roles of the interacting proteins: although VipB represents the direct substrate of ClpV, ClpS and MecA act as adaptor proteins that deliver substrates to their cognate AAA^+ partners. We speculate that the different roles of the Hsp100 N-domain contacting proteins demand a different positioning relative to the AAA^+ protein ring.

Notably, the limited binding interfaces in all three complexes (ClpA-N/ClpS, ClpC-N/MecA, ClpV-N/P-VipB) have different consequences on the regulation of AAA^+ protein activity.
Molecular Basis of ClpV Substrate Specificity

Whereas in case of ClpA, binding to a single ClpS molecule is entirely sufficient to direct the activity of ClpA hexamers toward its substrates (43), ClpV activity demands multiple interactions between its N-domains and assembled VipA/VipB complexes. Thus, isolated N-domains do not reduce ClpV activity toward VipA/VipB tubules even if present in excess. A dramatic increase in ClpV substrate affinity depends on both ATP-dependent hexamerization of ClpV and VipA/VipB tubule assembly. A comparable increase in substrate affinity in presence of ATP has been reported for the AAA + protein katanin, which severs microtubules, indicating a conserved principle to control substrate binding of AAA + proteins engaged in the disassembly of macromolecular complexes (44). Based on the analysis of mixed oligomers we calculate that the simultaneous binding of 3–5 ClpV N-domains is required for efficient VipA/VipB tubule disassembly. In the physiological context of T6S this mechanism enables ClpV to distinguish between its substrate, the VipA/VipB tubule and VipB protomers, which are the product of the disassembly reaction. Such a mechanism likely ensures both undisturbed VipA/VipB tubule formation and subsequent ClpV-mediated disassembly by (i) preventing ClpV from abrogating the VipA/VipB assembly process by sequestering unassembled VipB subunits and by (ii) preventing jamming of the AAA + protein by VipB monomers, thereby directing ClpV activity exclusively toward the VipA/VipB tubule. Our in vivo analysis of VipA/VipB tubule variants that are resistant to ClpV-mediated severing and of ClpV mutants that cannot bind to the VipA/VipB complex, illustrates the necessity of tubule disassembly for T6S. We propose that ClpV-mediated pulling at the N-terminal VipB binding site leads to partial or complete unfolding of VipB, which breaks intermolecular interactions within the VipA/VipB tubule and ultimately drives tubule breakdown.

In conclusion, our findings illustrate how small structural alterations in an extra domain can direct a molecular machine to function in a novel cellular context. The limited binding site between the interacting protomers ensures that association only occurs in the context of the respective oligomeric assemblies, allowing for the controlled assembly and disassembly of a macromolecular complex.

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