Structural and Molecular Mechanism for Autoprocessing of MARTX Toxin of Vibrio cholerae at Multiple Sites

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The multifunctional autoprocessing repeats-in-toxin (MARTX) toxin of Vibrio cholerae causes destruction of the actin cytoskeleton by covalent cross-linking of actin and inactivation of Rho GTPases. The effector domains responsible for these activities are here shown to be independent proteins released from the large toxin by autoproteolysis catalyzed by an embedded cysteine protease domain (CPD). The CPD is activated upon binding inositol hexakisphosphate (InsP₆). In this study, we demonstrated that InsP₆ is not simply an allosteric cofactor, but rather binding of InsP₆ stabilized the CPD structure, facilitating formation of the enzyme-substrate complex. The 1.95-Å crystal structure of this InsP₆-bound unprocessed form of CPD was determined and revealed the scissile bond Leu⁴⁴²⁸–Ala³⁴²⁹ captured in the catalytic site. Upon processing at this site, CPD was converted to a form with 500-fold reduced affinity for InsP₆, but was reactivated for high affinity binding of InsP₆ by cooperative binding of both a new substrate and InsP₆. Reactivation of CPD allowed cleavage of the MARTX toxin at other sites, specifically at leucine residues between the effector domains. Processed CPD also cleaved other proteins in trans, including the leucine-rich protein YopM, demonstrating that it is a promiscuous leucine-specific protease. Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins are a family of large bacterial protein toxins with conserved repeat regions at the N and C termini that are predicted to transfer effector domains located between the repeats across the eukaryotic cell plasma membrane (1). The best characterized MARTX is the >450-kDa secreted virulence-associated MARTX of Vibrio cholerae. This toxin causes disassembly of the actin cytoskeleton and enhances V. cholerae colonization of the small intestine, possibly by facilitating evasion of phagocytic cells (2, 3). The central region of the V. cholerae MARTX toxin contains four discrete domains: the actin cross-linking domain (ACD) that introduces lysine-glutamate cross-links between actin protomers (4, 5), the Rho-inactivating domain (RID) that reduces small Rho GTPases (6), an αβ hydrolase of unknown function (1), and an autoprocessing cysteine protease domain (CPD) (7, 8).

The CPD is a 25-kDa domain found in all MARTX toxins located just before the start of the C-terminal repeats (7, 8). This domain is activated for autoproteolysis upon binding inositol hexakisphosphate (InsP₆) (7), a molecule ubiquitously present in eukaryotic cell cytosol (9–11), but absent in extracellular spaces and bacteria. Thus, autocatalytic processing would not occur until after translocation of the CPD and effector domains is completed. In the context of the holotoxin, catalytic residue Cys³⁵⁶⁸ was found to be essential for the toxin to induce efficient actin cross-linking by the ACD and Rho inactivation by the RID, demonstrating that autoprocessing is essential for MARTX to induce cell rounding (8).

While it is clear that InsP₆ activates the CPD and that autoprocessing is essential for MARTX function (7), the mechanism by which InsP₆ activates CPD is not well understood. Furthermore, only one processing site at Leu⁴⁴²⁸–Ala³⁴²⁹ has been identified, although multiple processing events would be required to release each effector independently. In fact, after autoprocessing at Leu⁴⁴²⁸–Ala³⁴²⁹, CPD is reported to adopt a conformation with reduced affinity for InsP₆ (7), raising questions as to how the protease might process MARTX at other sites.

We present here the structure of the pre-processed form of the V. cholerae MARTX CPD bound to InsP₆. Our results dem-

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‡1 The atomic coordinates and structure factors (code 3F2Y) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–6.

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onstrate that autoprocessing is activated by rearrangement of a β-hairpin loop upon InSp₆₆ binding that locks the N terminus of the CPD in the active site, facilitating hydrolysis of the Leu₃⁴₂⁸–Ala₃⁴₃⁰ peptide bond. After autoprocessing, CPD adopts a post-processing form that has poor affinity for InSp₆₆ and thus must be cooperatively reactivated for high affinity binding of InSp₆₆ by association of a new substrate. As a consequence, we are able to demonstrate how CPD cleaves MARTX toxin between effector domains and releases them from the large toxin resulting in increased catalytic activity of the effectors.

EXPERIMENTAL PROCEDURES

Reagents and Supplies—Chromatography columns for the ÄKTA Purifier FPLC system were purchased from Amersham Biosciences; common reagents from Fisher Biotech; chloromethyl ketone (CK) inhibitors, InSp₆₆ (phytic acid), and G-actin from Sigma; N-ethylmaleimide (NEM) from Pierce; beef pancreas trypsin (3× crystallized) and chicken egg white trypsin inhibitor from MP Biomedical; and SYPRO Orange dye from Invitrogen. Enzymes for recombinant DNA experiments were from New England Biolabs and Invitrogen. Crystallization trays were purchased from Fisher and crystallization screens from Qiagen.

Cloning and Protein Purification—pHisCPD, pHisCPD/C-S, and pMCSG7-cpdΔ51 for overexpression of rCPD, rCPD/C-S, and pro-CPD, respectively, have been described (7, 8) and are diagrammed in supplemental Fig. 1. Using the Stratagene QuikChange II XL mutagenesis kit, a C3568S substitution was introduced into pMCSG7-cpdΔ51 for overexpression of pro-CPD/C-S as previously described (8). Other amino acid substitutions were introduced into pHisCPD (or pMCSG7-cpdΔ51) using primers listed in supplemental Table 1.

rCPD and mutant variants were purified as previously described (7), pro-CPD and pro-CPD/C-S were expressed in Escherichia coli BL21(ADE3), and soluble protein extracts were prepared by sonication in Buffer A (20 mM Tris, 500 mM NaCl (pH 8.0) containing 5 mM imidazole as previously described (8). Proteins were loaded onto a 5-ml HisTrap HP column and collected in the flow through or separated using a Superdex 200 10/30 column.

For cloning of YopM, primers as listed in supplemental Table 1 were used to amplify the DNA of the rtxA gene corresponding to amino acids 2635–3909 from N16961 genomic DNA. The resulting product was cloned into EcoRI-HindIII sites of pET28a (Novagen) creating pRTX2635. The DNA corresponding to amino acids 1580–2635 with flanking Ncol sites was then amplified. The resulting product was cloned into Ncol-EcoRI sites of pRTX2635 creating pRTX1580.

For RtxA₁₅₈₀₋₃₉₀₉ purification, primers as listed in supplemental Table 1 were used to amplify the DNA of the rtxA gene corresponding to amino acids 2635–3909 from N16961 genomic DNA. The resulting product was cloned into EcoRI-HindIII sites of pET28a (Novagen) creating pRTX2635. The DNA corresponding to amino acids 1580–2635 with flanking Ncol sites was then amplified. The resulting product was cloned into Ncol-EcoRI sites of pRTX2635 creating pRTX1580.

RESULTS

Analysis of Protease Activity and Cleavage Products—Autoprocessing of 2 µg of rCPD, pro-CPD, or mutant variants or 10
µg of RtxA1580–3909 in 20 mM Tris, 150 mM NaCl (pH 7.4) was initiated by addition of InsP₆ in 20-µl reaction volumes. When indicated, proteins were incubated with 100 µM NEM or CK inhibitors except for inhibition of RtxA1580–3909, which was performed with 1 mM inhibitors. For some studies, proteins were dialyzed against 20 mM Tris, 150 mM NaCl (pH 7.4) to remove excess inhibitor from final reactions. After addition of InsP₆ at concentrations indicated in the figure legends, reactions were incubated at 37 °C, stopped by boiling in sample buffer, separated by SDS-PAGE, and stained with Coomassie Blue R250. Fourier transform mass spectrometry (FT-MS) analysis of cleavage reaction mixes was performed as previously described (8).

Cleavage in trans of rCPD by post-CPD was performed as described above except that post-CPD was first mixed with 2 µg of CPD mutant protein and processing was initiated by addition of 100 µM InsP₆. Cleavage of other proteins was performed similarly with 10 µg of RtxA1580–3909 or YopM.

**Table 1**

**Data collection and refinement statistics**

| Crystal 1 | Space group | P2₁ 2₁ 2₁ | Cell dimensions, a, b, c (Å) | 46.06, 66.37, 137.96 |
|-----------|-------------|------------|-----------------------------|----------------------|
| Resolution (Å) | 25.00-1.95 (1.98-1.95)* | R<sub>work</sub>/R<sub>free</sub> | 0.076 (0.448) |
| Completeness (%) | 99.4 (95.3) |
| Redundancy | 6.7 (3.9) |

**Refinement**

- Resolution (Å): 23.67-1.95
- No. reflections: 29,932
- No. atoms: Protein 3,484, Ligand/InsP₆ 41.5/52.0, Water 45.8
- B-factors: Protein, Chain A/Chain B 39.5/41.2, Ligand-InsP₆, Chain A/Chain B 41.5/52.0, Water 45.8
- Root mean square deviations: Bond length (Å) 0.013, Bond angle (°) 1.7
- Ramachandran statistics
  - Most favored: 89.7%
  - Additional allowed: 9.8%
  - Generously allowed: 0.5%

* Values in parentheses are for highest resolution shell.

As defined in Ref. 19.

µg of RtxA1580–3909 in 20 mM Tris, 150 mM NaCl (pH 7.4) was initiated by addition of InsP₆ in 20-µl reaction volumes. When indicated, proteins were incubated with 100 µM NEM or CK inhibitors except for inhibition of RtxA1580–3909, which was performed with 1 mM inhibitors. For some studies, proteins were dialyzed against 20 mM Tris, 150 mM NaCl (pH 7.4) to remove excess inhibitor from final reactions. After addition of InsP₆ at concentrations indicated in the figure legends, reactions were incubated at 37 °C, stopped by boiling in sample buffer, separated by SDS-PAGE, and stained with Coomassie Blue R250. Fourier transform mass spectrometry (FT-MS) analysis of cleavage reaction mixes was performed as previously described (8).

Cleavage in trans of rCPD by post-CPD was performed as described above except that post-CPD was first mixed with 2 µg of CPD mutant protein and processing was initiated by addition of 100 µM InsP₆. Cleavage of other proteins was performed similarly with 10 µg of RtxA1580–3909 or YopM.

**Thermal Shift Assays**—25-µl solutions containing 0.5 mg/ml protein in 20 mM Tris, 150 mM NaCl (pH 7.4), SYPRO® Orange dye diluted 1:625, and InsP₆ from 0 to 5 mM were set up in 96-well trays. Samples were heated from 25 to 75 °C in 0.5 °C/12-s steps. Fluorescence intensity was measured at excitation/ emission wavelengths of 495/519 nm using a Bio-Rad iCycler iQ5. T<sub>m</sub> was calculated by fitting a Boltzmann sigmoidal nonlinear regression curve using Prism4 for Mac from GraphPad Software (La Jolla, CA).

**Limited Trypsin Digestions**—300 µl of 0.5 mg/ml protein in 20 mM Tris, 150 mM NaCl (pH 7.4) were incubated with or without InsP₆ for 1 h at 25 °C. Trypsin was then added to 20-µl aliquots for 30 min at 37 °C followed by addition of 100 µg/ml trypsin inhibitor. For some studies, post-CPD was preincubated with 100 µM CK inhibitors for 30 min at 25 °C. Proteins were analyzed either by SDS-PAGE or FT-MS as previously described (8). Cleavage fragments were identified by comparing mass peaks to fragments predicted by FindPept (21, 22).

**N-terminal Sequencing**—Autoprocessed RtxA1580–3909 fragments were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. N-terminal sequences were determined by Edman degradation according to standard protocols using the automated protein sequencing system Procise (Applied Biosystems).

**Actin Cross-linking**—In vitro actin cross-linking assay was performed at 25 °C for 25 min as described previously (23) with 3 µM actin and 0.09 µM RtxA1580–3909.

**Accession Code**—The coordinates and the structure factors for pre-processed CPD have been deposited in PDB with the accession code 3FZY.

**RESULTS**

**Crystal Structure of the Pre-processed Form of MARTX CPD**—For this study, the uncleaved form of V. cholerae MARTX CPD (residues 3428–3637 according to the original rtxA gene annotation by Lin et al. (24)) with a catalytically inactive C3568S mutation (pro-CPD/C-S) was purified. This protein has a His₆ tag and TEV protease recognition site upstream of the processing site with no additional sequences on the C terminus (see diagram in supplemental Fig. 1). The binding affinity of pre-CPD/C-S for InsP₆ was determined as 0.18 ± 0.02 µM (supplemental Fig. 2). This enhanced binding affinity compared with previously determined dissociation constants likely reflects the absence of long N- or C-terminal extensions present on previously studied CPD proteins (7, 25).

To define the conformation that is adopted upon InsP₆ binding, we determined a 1.95-Å resolution crystal structure of pro-CPD/C-S bound with InsP₆, capturing the scissile bond in the active site prior to autoproteolysis of the N terminus. The core (Fig. 1A, green) formed by residues Phe<sup>3458</sup>-Ser<sup>3600</sup> is nearly identical to the previously determined structure of the post-processing form of CPD, here referred to as post-CPD (25), and consists of 7 β-strands and 3 α-helices. A C-terminal subdomain (Fig. 1A, magenta) from Ser<sup>3601</sup> to the end of the protein forms a 5-stranded β-structure, previously designated the β-flap (25). The N terminus of CPD was predicted by the algorithm DisoPred2 (26) to be disordered and, consistent with this prediction, the N terminus (Fig. 1A, blue) is a loose strand wrapped around the outside of the protease. This strand is anchored to the core by the P1 residue Leu<sup>3428</sup> inserted in the S1 site and by Trp<sup>3442</sup> and TEV protease recognition site upstream of the processing site.

**Structure of the Catalytic and Substrate Binding Sites**—At P3′ residue Gly<sup>3431</sup> (Fig. 1A, orange), the N terminus turns toward the catalytic site. The enzyme is clearly autocatalytic with its own N terminus in the active site. The catalytic dyad residues, His<sup>3519</sup> and Ser-substituted Cys<sup>3568</sup>, are found at the ends of parallel strands, β5 and β6 (Fig. 1B). The catalytic residues are separated by ~6 Å with the scissile bond inserted between them. This arrangement is similar to that in the
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enzyme-substrate complexes of Clan CD peptidases, including caspases and gingipains (27, 28). Consistent with classification in this clan (29, 30), it is proposed that Cys3568 is not activated by His3519, but rather is substrate-activated by close alignment of the scissile bond, as previously proposed for the caspase interleukin-1-converting enzyme (28). This initial step of the processing reaction would then be followed by protonation of the leaving group by His3519.

Concerning other residues involved in catalysis, we previously determined that mutation of either Glu3467 or Asp3469 does not affect autoprocessing, but a double Glu3467/Asp3469 mutant is partially defective (7). Assessment of the structure (Fig. 1B) shows that both residues function to properly align the catalytic residue His3519. On the other side of the catalytic site, Glu3602 is reoriented in our structure compared with the previous determined structure (25), and in this location appears to correctly position the Ser-substituted catalytic cysteine (Fig. 1B). However, the presence of Glu3602 is not critical, because mutation of Glu3602 to Ala did not affect proteolysis (data not shown).

The most significant difference in this pre-processing structure compared with the previous structure of post-CPD is that the P3, P2, P1, and P1’ residues of the N terminus are rigidly aligned in a binding cleft (Fig. 1C). The P1 Leu3428 is inserted in the hydrophobic S1 site where it interacts directly with residues Val3517 and Val3566 on the parallel β5 and β6 strands of the Clan CD peptidase fold. Other residues that form the hydrophobic pocket are Val3472, Ala3475, and Leu3603 and Val3605 of β8 of the β-flap. Interestingly, the P3 Asn residue that is part of the TEV protease recognition site is not accessible, accounting for the presence of the N-terminal tag in the structure despite attempts to remove it during crystallization (see “Experimental Procedures”).

Autoprocessing Is Specific for Leu-Xaa Bond—All Clan CD proteases have specificity for cleavage at a single recognition residue (29, 30). Specificity for Leu as the P1 residue was demonstrated by altering Leu3428 to Ala in rCPD, a recombinant variant of CPD with a longer 75 residue extension facilitating easy detection of autoproteolysis (7) (diagrammed in supplemental Fig. 1). This protein was autoprocessed, but the site of hydrolysis shifted primarily to the P10 Leu residue (Fig. 2, A and C). At lower concentrations of InsP6, other sites were also cleaved resulting in a banding pattern that likely corresponds to...

FIGURE 1. Structural model of pro-CPD/C-S reveals enzyme-substrate complex. A, pro-CPD/C-S with N terminus (blue), protease core (green), β-flap (magenta), and InsP6 (red). Key residues (orange) and catalytic residues (yellow) are labeled according to annotation of Lin et al. (24). B, schematic representation of the Clan CD fold catalytic site with P1 Leu3428 (magenta) inserted into S1 site. Distances (in angstroms) of key bonds are shown as dashed lines. C, stereo view of the active site of pro-CPD/C-S as a stick model with surrounding Fo-Fc map contoured at 1 sigma (green) and the N terminus residues, surrounded with omit Fo-Fc map contoured at 4 sigma level (blue; omitted residues are Ala-Leu-Ala). For B and C, carbon of the active site, carbon of the substrate, oxygen and nitrogen atoms are colored in green, yellow, red, and blue, respectively.
processing at any of five other leucine residues in the extension on rCPD.

Changing the Ala residues in either the P1’ or the P2 position of rCPD to Ile had no effect on processing (Fig. 2, B and C). Consistent with this finding, these side chains are directed to the solvent (Fig. 1C). Furthermore, in the rCPD L3428A mutant, the new P10 processing site is flanked by histidine and glutamine (Fig. 2C), also confirming that the P2 and P1’ residues do not contribute to substrate recognition. Within the structure of activated CPD, the Asp residue in position P2/H11032 is pointed toward solvent, and mutation of this residue did not affect autoprocessing (data not shown). At the P3 position, the normally present Glu residue is replaced by Asn from the TEV protease recognition site in pro-CPD, and thus, there is no specificity at this position. Overall, recognition of the CPD cleavage site depends only on Leu3428.

Binding of InsP₆ Increases CPD Tₘ and Trypsin Resistance—

Twelve residues previously identified to contact InsP₆ (7, 25) are dispersed between the N-terminal leader, the core, and the β-flap, but come together in the tertiary structure (Fig. 3A). This global requirement for binding suggests a primary role of InsP₆ may be to stabilize CPD after translocation as an unfolded or partially folded protein. Consistent with this proposal, ITC experiments revealed a high negative enthalpy and entropy upon ligand binding that would be consistent with the protein becoming more ordered (ΔH = −59 kcal/mol; TΔS = −50 kcal/mol at 300 K, supplemental Fig. 2). Yet, no change in quaternary structure occurred upon InsP₆ binding as determined by dynamic light scattering, chemical cross-linking studies, and native gel electrophoresis (data not shown). Thus, the entropy change upon InsP₆ binding is most consistent with the monomeric protein becoming more ordered, which should result in a more stable structure.

To determine to what extent protein stability changes, Tₘ was determined by SYPRO Orange thermal shift assays (31–33). In the absence of InsP₆, half-maximal denaturation of pro-CPD/C-S occurred at 38 °C, but increased by 14 °C with addition of InsP₆ (Fig. 3B). As further evidence of increased protein stability, trypsin-sensitive pro-CPD/C-S became resistant to trypsin digestion after InsP₆ was added (Fig. 3C).

It is possible that the low Tₘ of pro-CPD/C-S is due to electrostatic repulsion when the positively charged binding site is not occupied by InsP₆. Consistent with this, high concentrations of phosphate and citrate also increased Tₘ. However, pro-CPD in phosphate had only
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FIGURE 4. Susceptibility of pro-CPD to inhibition by NEM depends on position of the N terminus. A and B, space-filling models of pro-CPD/C-S with N terminus (blue), protease core (green), β-flap (magenta), and InsP₆ (red). Models are based on the B molecule. In A, P1 Leu₃₄₂₈ (L) is buried in the hydrophobic S1 site (formed by the protease core (aqua) and the β-flap (medium blue)). The catalytic cysteine (CS) is covered by the N terminus at the scissile bond (dashed line) and by Glu³⁶⁰² (E). In B, removal of the N terminus to the P3 residue exposes the catalytic cysteine. Other labeled residues are the catalytic His³⁵¹⁹ (H), S1 residues from the α₁ helix (Val¹⁴⁷² (αⅠ) and Ala¹⁴⁷⁵ (αa)) or the β8 strand (Leu³⁶⁰³ (βL) and Val³⁶⁰⁵ (βV)) and the N terminus residues in the binding cleft identified by single letter code. C, pro-CPD with intact cysteine was incubated without (−) or with 100 μM NEM for time indicated at temperature indicated after which the protein was dialyzed at 4 °C to remove excess inhibitor. Processing was then initiated by addition of 100 μM InsP₆ and processing of full-length protein (FL) to the processed form (P) was assessed by SDS-PAGE. D, pro-CPD was incubated without NEM (−) or with 100 μM NEM for 30 min (30′) at 25 °C after which InsP₆ as indicated was added. In lanes marked 0′, NEM and InsP₆ were first mixed and then added simultaneously (0 min preincubation). Reactions were monitored after 1 h at 37 °C by SDS-PAGE.

50% processing capability within 3 h, and citrate stimulated no processing above controls, even after 24 h (supplemental Fig. 3). Thus, InsP₆ is required both to stabilize the protein and to generate an active conformation for efficient cleavage.

Arg³⁵⁹⁹, Arg³⁶¹⁰, and Lys³⁶¹¹ Are Exposed to Trypsin in pro-CPD/C-S in Absence of InsP₆—To define the region that is structurally altered upon InsP₆ binding, we used FT-MS to identify peptides after limited proteolysis in 0.1 μg/ml trypsin (supplemental Fig. 4). Arg³⁵⁹⁹, Arg³⁶¹⁰, and Lys³⁶¹¹ were identified as exposed residues by detection of peptides originating from the site of cleavage to both the N and C terminus, indicating they were generated by a single cleavage event. Each of these residues contact InsP₆; moreover, Lys³⁶¹¹ is known to be absolutely essential for InsP₆ binding (7). By contrast, other Arg and Lys residues that form the InsP₆ binding pocket were protected, indicating that there is not complete exposure of the InsP₆ pocket.

The location of these residues within the structure revealed that the antiparallel β8/β9 hairpin structure of the β-flap that directly contacts both InsP₆ and P1 Leu³⁴₂₈ (Fig. 3D) could be improperly folded or have an altered arrangement in the absence of InsP₆. In particular, access of trypsin to Arg³⁵⁹⁹ would require the absence of an interaction of the loop between strands β7 and β8 with the loop that contains the catalytic cysteine. Furthermore, changes in the structure of β8 just after Arg³⁵⁹⁹ would affect the S1 site. This is consistent with InsP₆ contributing to proper structure of β8/β9 and consequently the active site.

N Terminus Is Tightly Bound in the Active Site of pro-CPD upon Binding of InsP₆—Because a structural rearrangement upon InsP₆ binding may alter the S1 pocket, it was considered whether the N terminus would require InsP₆ to enter the binding cleft. As seen if Fig. 4A, when Leu³⁴₂₈ occupies the active site, cysteine proteases inhibitor NEM would only have access to Cys³⁵⁶⁸ when the N terminus transiently departs from the active site. By contrast, if the N terminus is not in the S1 site, Cys³⁵⁶⁸ is exposed to solution and should be rapidly inactivated. When pro-CPD was preincubated with NEM at 25 °C followed by dialysis at 4 °C to remove the inhibitor prior to addition of InsP₆, long preincubation times (up to 30 min) were required for 100% inactivation (Fig. 4C). If NEM incubation was performed at 37 °C, inactivation was more rapid. These data indicated that the N terminus normally occupies the S1 site independent of InsP₆ binding, but may transiently exit the site such that NEM has access to the catalytic cysteine over time.

Further evidence that the N terminus is not tightly bound within InsP₆-free pro-CPD was obtained from the analysis of fragments after limited trypsin proteolysis of pro-CPD/C-S (supplemental Fig. 4). A 3240.50 mass unit fragment corresponding to trypsin cleavage after Lys³⁴³₂ in pro-CPD/C-S indicated that the N-terminal loop between the Trp³⁴⁴₂ anchor and the P1 Leu³⁴₂₈ is accessible to trypsin. However, because the InsP₆-bound form was trypsin-resistant (Fig. 3C), the N terminus becomes more tightly bound, or locked, in the active site by InsP₆. If this is correct, NEM should not inhibit autoprocessing if added simultaneously with 100 μM InsP₆, because rapidly locking the N terminus in a high concentration of InsP₆ should
block access of inhibitors to the cysteine. Indeed, when added simultaneously, NEM poorly inhibited InSp₆-induced autoprocessing (Fig. 4D). By contrast, at an InSp₆ concentration below the Kₘ, when <50% of pro-CPD would be bound by InSp₆ immediately upon addition, inhibition was >90% (Fig. 4D).

Therefore, NEM and trypsin accessibility data support the view that binding of InSp₆ to pro-CPD causes ββββ to adopt an appropriate structure for proper orientation of the scissile bond relative to the catalytic cysteine. Hydrophobic residues on β8 would then fully contribute to the S1 site. The resulting rigid structure would then be amenable to substrate-activated autoprocessing initiated by Cys₃⁵⁶⁸.

After Processing, CPD Loses Its High Affinity for InSp₆—A previous study suggested that post-CPD has reduced affinity for InSp₆ (7). Determination of the binding affinity of InSp₆ for purified post-CPD revealed that it does indeed have reduced affinity (Kₐ = 100 ± 20 μM, n = 6). Notably, this dissociation constant is above the physiological concentration of InSp₆ (40–60 μM) indicating that only a fraction of processed CPD would bind InSp₆ in vivo. This result suggests that, upon processing, CPD adopts a conformation distinct from the pre-processing form. However, a structure similar to pro-CPD can be adopted upon incubation in very high concentrations of InSp₆ as was done to determine the structure of post-CPD (25).

Post-CPD Is Biochemically Distinct from pro-CPD—In the absence of an InSp₆-free post-CPD structure, the structural alteration resulting in reduced InSp₆ binding affinity was investigated using biochemical assays. Limited proteolysis of post-CPD revealed trypsin cleavage at lys₃⁶¹¹, further indicating poor binding of InSp₆ to post-CPD (Fig. 6A and supplemental Fig. 5). However, in contrast to the pre-processed form, access of trypsin to Arg₃⁶¹⁰ likely occurred only after prior cleavage at Arg₃⁶¹⁰ or lys₃⁶¹¹ (supplemental Fig. 5). In addition, thermal shift assays showed that post-CPD has a Tₘ of 47 °C independent of InSp₆ concentration (supplemental Fig. 6). Altogether, these data indicate that ββββ remained folded after processing and post-CPD did not simply revert to the less stable, pre-processed form.

Reoccupation of the S1 Site Reactivates CPD for High Affinity Binding of InSp₆—To model the effect of N-terminal processing, the pro-CPD/C-S structural model was re-examined with the N terminus removed (Fig. 4B). Departure of Leu₃⁴²⁸ from the S1 site would expose the S1 site creating an 8–9 Å hydrophobic crevice in the protein. We postulated that the exposed hydrophobic S1 site could be partially covered by a shift of β8 toward α1. A simultaneous shift of the antiparallel β9 would weaken contact of β9 residues Arg₃⁶¹⁰, and particularly lys₃⁶¹¹, with InSp₆, releasing InSp₆ from the binding pocket. Lys₃⁶¹¹ would then become susceptible to trypsin as observed (Fig. 5A).

If this model is correct, then re-occupation of the S1 site could re-activate CPD by shifting ββββ back toward the InSp₆ pocket, thereby facilitating high affinity binding of InSp₆ and formation of new active enzyme-substrate complex.

To test this model, CK inhibitors were used as a surrogate for a new substrate. CK inhibitors are known to inactivate Clan CD proteases by alkylating the catalytic cysteine when the attached amino acid or peptide binds within the S1 site (34). When post-CPD was pre-treated with inhibitor L-LeuCK or z-Gly-Leu-Leu-Phe-CK, InSp₆ did bind with Kₐ = 0.9 ± 0.3 μM and Kₐ = 1.1 ± 0.3 μM, respectively (Fig. 5B). These data indicate that the CKs successfully mimic a new substrate and re-activate post-CPD for InSp₆ binding. Furthermore, these proteins regain resistance to trypsin, indicating reformation of a structure resembling an active enzyme-substrate complex (Fig. 4A), which was not reached even by addition of 2 mM InSp₆ without CKs.

T-LysCK did not restore binding of InSp₆ to a detectable range under standard conditions indicating that reoccupation of the S1 site by Leu is specifically required for reactivation of post-CPD. These data thus support a cooperative model for reactivation of CPD requiring both a new substrate and InSp₆.

Reactivated post-CPD Can Process rCPD Mutant Proteins in Trans—Because post-CPD can be reactivated for InSp₆ binding, it should be able to process other substrates. Previously, cleavage of other proteins was discounted, because the Leu₃⁴²⁸-Ala₃⁴²⁹ bond in the rCPD/C-S protein used as a substrate was not processed during co-incubation with pro-CPD (7). However, our data presented here indicate that, in the presence of InSp₆, Leu₃⁴²⁸ in the rCPD/C-S mutant would be locked within the S1 site and thereby protected. To determine if cleavage of rCPD/C-S by post-CPD occurs when the N terminus is freely available, rCPD/C-S with a mutation of hydrophobic S1 pocket residue Leu₃⁴⁷⁹ preventing insertion of the N terminus was used as a substrate. Indeed, rCPD L3⁴⁷⁹D/C3⁵⁶⁸S was cleaved by post-CPD in an InSp₆-dependent manner, demonstrating in trans processing. rCPD/C-S, which can bind InSp₆, was fully protected, while mutant proteins that cannot bind...
0.4M urea was included in processing reaction mix. pro-CPD Leu residues (36), YopM was purified from inclusion bodies and composed of 21% leucine (35). To expose the normally buried leucine-rich repeats family of type III secretion effectors and is substrate for in

Post-CPD can process rCPD/C-S and unrelated proteins in trans. A, Coomassie-stained gels of full-length mutant rCPD/C-S proteins (rCPD FL) that cannot autoprocess. If the S1 site (L3479D) or InsP6 binding pocket (K3611A and K3482A) are modified to prevent Leu from occupying its own active site, mutants can be processed (P) by post-CPD dependent upon InsP6. B, full-length proteins (FL) were incubated without inhibitor (−) or with 100 μM NEM (N) or L-LeuCK (L) for time specified at 25 °C after which proteins were dialyzed to remove excess inhibitor, and 100 μM InsP6 was added when indicated to initiate processing of rCPD L3479D/C3568S by post-CPD. Processing for both assays was determined by SDS-PAGE after 2-h incubation at 37 °C. For C purified YopM was incubated with pro-CPD in 0.4 M urea and with or without 100 μM InsP6 for the times indicated.

InsP6 were cleaved by post-CPD, confirming that binding of InsP6 locks the N terminus in the S1 site (Fig. 6A). Post-CPD processing of rCPD L3479D/C3568S was rapidly inhibited by NEM and L-LeuCK, thereby demonstrating that the cysteine in post-CPD is freely exposed (Fig. 6B). Together, these data demonstrate that cooperativity through binding of a new substrate along with a new molecule of InsP6 can reactivate CPD to process other proteins. These data also show that the cysteine is freely accessible in the absence of the N terminus, and thus activation of CPD does not involve an allosteric structural conversion to expose a previously buried cysteine to substrate, as suggested by others (25).

Post-CPD Degradates the Leucine-rich Protein YopM—To test if CPD is a specific protease able to cleave only the Leu3428–Ala3429 site of MARTX or if it is able to promiscuously cleave other sites, YopM of Yersinia pseudotuberculosis was used as a substrate for in trans processing. YopM is a member of the leucine-rich repeats family of type III secretion effectors and is composed of 21% leucine (35). To expose the normally buried Leu residues (36), YopM was purified from inclusion bodies and 0.4 M urea was included in processing reaction mix. pro-CPD without InsP6 or InsP6 alone did not affect YopM. When pro-CPD and InsP6 were added to YopM together, pro-CPD first autoprocessed then extensively cleaved YopM within 5 min, and YopM was completely degraded by 1 h (Fig. 6C). Therefore, CPD is a promiscuous enzyme able to cleave non-MARTX substrates.

A distinct difference in the band patterns between in trans processing (Fig. 7A, lane 6) and autoprocessing (lane 9) of RtxA1580–3909 was noted. In particular, an in trans processing product (marked by an asterisk) is absent from the autoprocessing reaction. This fragment corresponds to the αβ hydrolase (see below), indicating that Leu3428–Ala3429 is poorly cleaved during in trans processing but readily cleaved by autoprocessing. This presumably occurred because Leu3428–Ala3429 is locked in the active site of the inactivated RtxA1580–3909 when InsP6 is added and is thus not accessible for in trans cleavage, in agreement with our observations with rCPD/C-S as a substrate.

RtxA1580–3909 preincubated with NEM was not capable of autoprocessing, indicating that autoprocessing depends on the catalytic cysteine of CPD (Fig. 7B). By contrast, in the presence of L-LeuCK, autoprocessing of RtxA1580–3909 was only partially inhibited. Notably, protein that was processed was predominantly cleaved into two fragments of an estimated 59 and 185 kDa, a processing event that corresponds to cleavage at Leu3428–Ala3429 followed by processing at other sites. In the presence of L-LeuCK, the cysteine is readily inhibited once Leu3428–Ala3429 departs the active site, confirming results with post-CPD.

Autoprocessing of RtxA1580–3909 Results in Releasing of Individual Domains—To define the processing sites within RtxA1580–3909, Edman degradation was performed to identify the five predominant fragments that arose after 1-min autopro-

A Longer Form of MARTX Can Be Both Autoprocessed and Processed in Trans—Because the CPD can be reactivated and can recognize apparently any exposed leucine residue, it was considered likely that CPD can process MARTX at additional sites. To test this, recombinant protein RtxA1580–3909 was purified. This protein contains all of the effector domains and part of the protein upstream and downstream without the repeat regions.

As the first experiment (Fig. 7A, lanes 1–6), RtxA1580–3909 was treated with NEM for 30 min to inactivate its CPD, and then this protein was dialyzed and used as a substrate for in trans processing by pro-CPD. After InsP6 was added, pro-CPD cleaved itself generating post-CPD, and then processed RtxA1580–3909 (lane 4). The processing pattern differed over time, but within 1 h, full-length RtxA1580–3909 was absent, and stable processing products were generated (lanes 4–6). To test for autoprocessing, InsP6 was added to RtxA1580–3909 alone. This protein was also processed to stable fragments (lanes 7–9).
The N-terminal sequences indicated processing at four sites: Leu\(^{1958}\)–Ser\(^{1959}\), Leu\(^{2434}\)–Gly\(^{2435}\), Leu\(^{3085}\)–Ser\(^{3086}\), and Leu\(^{3428}\)–Ala\(^{3429}\) (Fig. 7, C and D).

The largest fragment F1 (92 kDa) corresponded to the N terminus of RtxA\(_{1580-3909}\) through the ACD ending at Leu\(^{2434}\) (Fig. 7D). The smaller 52 kDa fragment (F4) corresponding to Ser\(^{1959}\)–Leu\(^{2434}\) would also contain the ACD. These results indicate the naturally occurring ACD is a 480-amino acid protein, larger than the functionally active 412-amino acid protein (5). It is also notable that the pattern of RtxA\(_{1580-3909}\) processing sometimes varied between experiments. In some cases, ACD was found predominantly as the 92-kDa F1 fragment with Leu\(^{1958}\)–Ser\(^{1959}\) uncleaved, whereas in other experiments, it was mostly present as the F4 52-kDa fragment. This variability indicated that the region upstream of ACD may not be consistently properly folded and interferes with the processing reaction. However, in the context of the holotoxin, this structure would presumably be

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**FIGURE 7.** CPD domain autoprocesses RtxA\(_{1580-3909}\) in multiple sites. A and B, NEM-treated RtxA\(_{1580-3909}\) can serve as a substrate for CPD (lanes 1–6), but also untreated RtxA\(_{1580-3909}\) can undergo autoprocessing (lanes 5–7). For lanes 1–6, RtxA\(_{1580-3909}\) was first incubated with 1 mM NEM for 30 min at 25 °C, after which protein was dialyzed to remove excess of inhibitor. Then, pro-CPD was mixed with NEM-pretreated RtxA\(_{1580-3909}\) and incubated for 5 min, after which processing was initiated by addition of 100 \(\mu\)M InsP\(_6\) for the time indicated at 37 °C. The asterisk in lane 6 marks a fragment absent after autoprocessing (lane 9). For lanes 7–9 and B, autoprocessing reactions were initiated by addition of 100 \(\mu\)M InsP\(_6\) and terminated at the times indicated. In B, protein was incubated with 1 mM of the indicated inhibitor for 30 min at 25 °C prior to addition of InsP\(_6\). C and D, N termini of marked fragments (F1–F5) arising from autoprocessing for 1 min were identified by Edman degradation and are marked with the first five amino acids (C) and correspond to fragments diagrammed in D. E, actin cross-linking reactions were performed with commercial G-actin in the presence of InsP\(_6\) and/or 5 mM EDTA as indicated. The arrowhead marks RtxA\(_{1580-3909}\). F, alignment of all processing sites identified in this study.
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properly folded and thus consistently recognized for cleavage by CPD.

The N-terminal sequence of the 75-kDa F2 fragment revealed a processing site at Leu1454–Gly1455, and its size would suggest it corresponds to the RID domain extending to Leu1508. Thus, the naturally occurring size of the RID is 651 amino acids, larger than the 547-amino acid functional peptide (6). The N-terminal sequence of the 36-kDa F5 fragment indicated the processing at Leu3085–Ser3086 encompassing the αβ hydrolase to the known CPD processing site at Leu3428–Ala3429.

Concerning the CPD itself, at 1 min after initiation of the autoprocessing reaction (Fig. 7A, lane 8), the CPD from RtxA1580–3909 was identified as the 59-kDa F3 fragment, which corresponds to the theoretical size of 53.1 kDa, from the Leu3428–Ala3429 cleavage site to the C terminus of RtxA1580–3909, including the His6 tag. As an independent verification, the presence of a His6 tag on the F3 fragment was demonstrated by Western blotting using anti-His antibody (data not shown). This fragment disappears within 60 min (lane 9), and Western blotting with anti-CPD antibody indicated the CPD was C-terminally processed to shorter fragments suggesting processing at any of the 19 leucine residues downstream of the CPD in RtxA1580–3909 (data not shown). The variability of cleavage at these sites indicates this represents nonspecific processing events in the partially unstructured ends of a recombinant protein.

Altogether, from these analyses, it was found that autoprocessing of MARTX results in processing of at least four sites resulting in release of the three effector domains. If autoprocessing is left unchecked, the promiscuous CPD may also apparently cleave other exposed leucine residues.

Releasing of ACD Increases Its Activity—If the effectors are normally bound in a larger structure, then releasing them may be essential for these proteins to become catalytically functional. To investigate this, in vitro actin cross-linking catalyzed by the ACD within RtxA1580–3909 was performed with and without addition of InsP6 (Fig. 7E). RtxA1580–3909, in the absence of InsP6, induced some actin cross-linking, but the activity was increased by the addition of InsP6. The cross-linking of actin was inhibited by NEM, demonstrating the CPD was essential, and by EDTA, demonstrating the cross-links are due to the Mg2+-dependent ACD.

DISCUSSION

In this study, we determined the crystal structure of the enzyme-substrate complex of V. cholerae MARTX CPD and used the structural information to suggest biochemical experiments that probe the site and mechanism for InsP6-induced autoproteolysis both at Leu3428–Ala3429 and at other sites of the MARTX toxin. The results support a model of controlled activation in which InsP6 is important for stabilization of the enzyme prior to autoprocessing specifically at leucine residues, and secondly, functioning as a cooperative factor along with a new substrate for reactivation to process other sites. The subsequent autoprocessing would release the effector domains ACD, RID, and αβ hydrolase to independently access different targets. The final result will be actin destruction due to cross-linking of cytosol localized monomeric G-actin by the ACD and membrane localized Rho GTPases by the RID. Consistent with the important role of autoprocessing, both in vitro (Fig. 7) and in vivo (8), the ability of MARTX to efficiently cross-link actin was dependent upon it first being autoprocessed to release the ACD as an independent domain.

In this study, we sought to more clearly understand the mechanism of InsP6-induced autoprocessing. The key structure for coordination of the interdependence between the peptide to be processed and InsP6 was identified as a β-hairpin structure (βββ9) that contacts the target peptide through interaction of hydrophobic side chains on β8 with P1 Leu3428 and with InsP6 through arginine and lysine side chains on β9. Prior to InsP6 binding, the protein exists in a conformation that is trypsin-sensitive, most notably in a portion that may coordinate proper alignment of the catalytic cysteine. Upon InsP6 binding, the β-flap undergoes a structural alteration that locks the substrate in the S1 pocket in a rigid structure amenable to substrate-activated processing. Subsequent to autoprocessing, the β-flap may shift resulting in release of InsP6, although cooperative binding of a new substrate and InsP6 results in reactivation.

Previously, a role for the β-flap in coupling binding with activation was postulated and Asp3606 and Trp3620 were identified as residues within the β-flap that would affect folding and thereby establish contact with the protein core (25). In this work, the key structure that is altered is identified as βββ9 by partial trypsin digestion.

This model for activation of CPD by formation of a stable enzyme-substrate complex is quite distinct from a recently proposed model for activation by an allosteric structural conversion to expose a previously buried catalytic cysteine to substrate (25). That allosteric mechanism was suggested when it was observed that a 1-min exposure of pro-CPD to NEM followed by addition of InsP6 poorly inhibited autoprocessing, initially suggesting the catalytic cysteine is buried, a result we confirm in Fig. 4C. However, we found that a longer incubation of pro-CPD or RtxA1580–3909 with NEM fully inhibited processing indicating transient exposure of the cysteine (Figs. 5 and 7). Inspection of the pre-processed structure suggests the N terminus frequently, if not predominantly, occupies the S1 site, thus, inhibition by NEM occurs only over time, targeting the cysteine only when the N terminus transiently departs the active site. This time dependence was reduced by increasing temperature to promote more dynamic motion of the N terminus and, most definitively, by complete removal of the N terminus by autoprocessing. The free accessibility of the cysteine in the processed form of CPD is also strongly supported by the observation that a fluorescent maleimide reacts with the cysteine of post-CPD, but not the pre-processed form, when it is added to a CPD autoprocessing assay (25).

The previously proposed allosteric mechanism was further suggested by the observation that autoprocessing of pro-CPD was inhibited when exposed to NEM and InsP6 simultaneously (25). These data directly conflict with our results that simultaneous addition of NEM with 100 μM InsP6 resulted in a protein that was ~80% processed (Fig. 4D). It is notable that the experiment cited as supporting a structural transition was carried out with pro-CPD that had previously been exposed to NEM for
1 min, a treatment expected to inhibit ~50% of CPD (Fig. 4C). Thus, the pro-CPD in that experiment was probably inactivated prior to NEM and InsP₆ co-injection, preventing autoprocessing. Thus, all data support a cooperative mechanism for activation, as opposed to the allosteric structural transition model, for pro-enzyme activation and reactivation both in the context of small recombinant CPD proteins (Fig. 6) and the larger recombinant protein RtxA₁₅₈₀₋₃₉₉₀ (Fig. 7).

In the context of the MARTX holotoxin, we propose that translocation of the ~185-kDa central region of MARTX to the cytosol likely involves at least partial unfolding of the toxin, because translocation of a fully folded protein would require a substantial pore and it has been demonstrated that there is no leakage of cytosol contents due to MARTX (37). Upon CPD translocation, Leu³⁴²⁸ moves into the CPD S1 site facilitating high affinity binding of InsP₆. Upon binding, βββ adopts the proper conformation locking the scissile bond into the active site where it is cleaved. Reactivation of CPD then occurs by reoccupation of the S1 site by a new substrate and binding of InsP₆. This reactivation results in cleavage at other leucine residues located between the effector domains releasing them from the larger toxin. Secondary structure predictions have shown that these leucines are present in unstructured regions that flank the effectors, explaining the specificity of preferred cleavage sites despite the fact that CPD is apparently a promiscuous protease able to process at almost any exposed leucine. Interestingly, autoprocessing of RtxA₁₅₈₀₋₃₉₉₀ by its own CPD was more efficient than in trans processing by pro-CPD (Fig. 7A). This result suggests that close proximity to other processing sites may create the necessary high local concentration of substrate facilitating immediate reoccupation of the active site after departure of Leu³⁴²⁸—Ala³⁴²⁹. As an extension, this observation might indicate that the effector domains are associated with CPD until processing is completed.

The specificity of this enzyme for leucine is of keen interest. All Clan CD proteases are recognized for having high specificity for processing: for example, eukaryotic caspases at Asp residues, the gingipain K and R proteases of Porphyromonas gingivalis at Arg and Lys, respectively, and mammalian legumain at Asn (29, 30). The identification of specificity for Leu of the MARTX CPD indicates that this and similar proteases represent a unique family of proteases, although the structure of the catalytic site clearly places it in Clan CD. All the processing sites defined in this study are shown in Fig. 7F in the context of 10 amino acids on each side of the scissile bond. By analyzing these peptides, no conservation of residues other than Leu in P1 site is evident, and mutation analysis indicated any of the two neighboring residues can be altered. Yet, among the preferred processing sites, there is a preference for a small amino acid residue on each side of the Leu (Fig. 7F). Extension of this analysis to the Ala-Leu-Gly or Ser-Leu-Gly recognition sites for Clostridium difficile Toxin B further indicates preference for small-Leu small suggesting a conserved preference among this family of proteases (38).

As a conclusion, we have described a new model for delivery of multiple toxin effectors where, upon translocation of the central region into eukaryotic cells, CPD binds InsP₆, cleaves itself and then other sites in MARTX, releasing the functional domains that then reach their targets and contribute to pathogenesis of cholera.

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