Thermodynamic properties of the effector domains of MARTX toxins suggest their unfolding for translocation across the host membrane

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SUMMARY

MARTX (multifunctional autoprocessing repeats-in-toxin) family toxins are produced by *V. cholerae*, *V. vulnificus*, *A. hydrophila* and other Gram-negative bacteria. Effector domains of MARTX toxins cross the cytoplasmic membrane of a host cell through a putative pore formed by the toxin's glycine-rich repeats. The structure of the pore is unknown and the translocation mechanism of the effector domains is poorly understood. We examined the thermodynamic stability of the effector domains of *V. cholerae* and *A. hydrophila* MARTX toxins to elucidate the mechanism of their translocation. We found that all but one domain in each toxin are thermodynamically unstable and several acquire a molten globule state near human physiological temperatures. Fusion of the most stable cysteine protease domain to the adjacent effector domain reduces its thermodynamic stability ~1.4 fold (from $\Delta G^{\text{H}_2\text{O}}$ 21.8 to 16.1 kJ/mol). Precipitation of several individual domains due to thermal denaturation is reduced upon their fusion into multi-domain constructs. We speculate that low thermostability of the MARTX effector domains correlates with that of many other membrane-penetrating toxins and implies their unfolding for cell entry. This study extends the list of thermolabile bacterial toxins, suggesting that this quality is essential and could be susceptible for selective targeting of pathogenic toxins.
INTRODUCTION

Multifunctional autoprocessing repeats-in-toxin (MARTX) toxins are produced by several Gram-negative bacteria among which are *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio anguillarium*, *Vibrio splendidus*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, and other pathogens of human, animal, and marine life infectious diseases. MARTX toxins belong to a superfamily of RTX (repeats-in-toxin) toxins, yet they differ substantially from other members of the family in several important aspects. MARTX toxins, with their 350-550 kDa size, are not only substantially larger than other RTX toxins, but are also the largest single polypeptide chain toxins discovered to date (Fig. 1A). Next, the Ca\(^{2+}\)-binding GD-rich 9-aa repeats (consensus motif G-xx-G-x-N/D), which the entire RTX family was named after, are substituted in MARTX toxins with closely related 18-aa repeats (called C-repeats) with a predicted ability to bind one Ca\(^{2+}\) ion per repeat (Satchell, 2011). Additionally, MARTX toxins have two other unique glycine-rich types of repeats named type A and type B (Satchell, 2007). All of these repeats share a common G-7x-G-xx-N motif and are therefore likely to have structural similarity. In a typical MARTX toxin, 14 copies of 20-aa A repeats are located at the N-terminus, followed by 38 copies of 19-aa B repeats, whereas the GD-rich RTX-like C-repeats are located at the C-terminus (Fig. 1A) (Satchell, 2011). The number and the sequences of all three repeats are well conserved in the majority of MARTX toxins with only few minor exceptions (Satchell, 2007). These repeats have been proposed to cooperate for delivery of several (1 to 5) effector domains followed by a conserved cysteine protease domain (CPD) (Satchell, 2011).

By analogy with pore-forming RTX toxins (Lally et al., 1999), it has been proposed that the repeat domains of MARTX form a pore-like structure utilized for translocation of the MARTX effector domains into the cytoplasm of a host cell (Satchell, 2007). The pathogenic effects of MARTX toxins stem mainly from the specific activities of the respective effector domains. Composition of the effector domains varies between MARTX toxins of different species as well as different biotypes of a same species due to genetic recombination with other MARTX toxins encoded either genomically or in mobile elements (Kwak et al., 2011, Roig et al., 2011). CPD is the only invariable internal domain shared by all MARTX toxins. CPD is a cysteine protease that is activated only upon crossing the cytoplasmic membrane via interaction with cytoplasmic inositol hexakisphosphate (InsP\(_6\)); once activated, CPD cleaves off other effector domains and releases them from the membrane-bound repeats.
Apart from CPD, other effector domains of \textit{V. cholerae} MARTX toxin (MARTX\textsubscript{Vc}) include: actin crosslinking domain (ACD), Rho GTPase inactivation domain (RID), and a domain with a characteristic \(\alpha/\beta\)-hydrolase fold (ABH). The \textit{A. hydrophila} MARTX toxin (MARTX\textsubscript{Ah}) also has ACD and ABH domains, but no RID. Other MARTX\textsubscript{Ah} domains absent in MARTX\textsubscript{Vc} include PMT (similar to the C-terminal region of \textit{Pasteurella multocida} toxin) and MCF (similar to “makes caterpillars floppy” toxin) (Fig 1). Both RID and ACD target the actin cytoskeleton. RID indirectly inhibits Rho family GTP-ases by an unknown mechanism (Sheahan \& Satchell, 2007, Pei \& Grishin, 2009, Ahrens \textit{et al.}, 2013), whereas ACD covalently crosslinks monomeric actin via an isopeptide bond (Kudryashov \textit{et al.}, 2008a, Kudryashova \textit{et al.}, 2012) resulting in the accumulation of actin oligomers incapable of polymerization (Kudryashov \textit{et al.}, 2008b). Therefore, both domains induce disassembly of the actin cytoskeleton in affected cells (Cordero \textit{et al.}, 2006, Sheahan \& Satchell, 2007) and a subsequent neutralization of the immune potential of macrophages and neutrophils (Queen \& Satchell, 2012). The functional roles of other effector domains are currently unknown.

Effector domain translocation is not affected by inhibition of endocytosis (Sheahan \textit{et al.}, 2004) and therefore is likely to occur directly across the host cytoplasmic membrane and not through the endosomal pathway. However, neither the host cell receptor elements targeted by MARTX toxins are currently recognized, nor are the mechanisms of effector domain delivery to the cytoplasm known. In the absence of direct experimental evidence, analogy with pore-forming RTX toxins has been made to postulate that the repeat domains of MARTX form a pore-like structure in the host cytoplasmic membrane used as a tunnel for translocation of the effector domains directly to the host cell cytoplasm (Satchell, 2007). The size and permeability of the tentative pore for substances other than effector domains is a subject of controversy. Thus, no open pore was reported for \textit{V. cholerae} MARTX toxin (Fullner \& Mekalanos, 2000); whereas formation of an open pore with a diameter of \(~1.6\) nm was suggested for \textit{V. vulnificus} MARTX toxin based on 50\% inhibition of hemolytic activity of the toxin by polyethylene glycol molecules of this size (Kim \textit{et al.}, 2008). It has been proposed that along with the effector domain composition, the size of the formed pore may account for difference in toxicity between MARTX toxins (Kim \textit{et al.}, 2008).

As the mechanism of translocation is unknown, it is not clear whether the effector domains are transported unfolded or in their folded state, and what is the thermodynamic
gradient fueling this translocation. Membrane structures of MARTX toxins have never been observed, and direct investigation of the molecular mechanisms of the effector domain delivery across the host membrane is hindered by limited availability of full-length MARTX toxins. Indeed, the toxins are difficult to express ectopically due to their enormous size (~0.5 MDa) and are problematic to purify in substantial amounts from toxin-producing microorganisms due to small quantities produced, and sophisticated regulation of expression initiated only upon direct contact with the host cells and ceased soon after (Kim et al., 2008, Lee et al., 2008). We postulate that a mechanism of effector domains’ delivery is encoded in and, therefore, can be deduced from their structural/thermodynamic properties, even though the structure of the transportation machinery per se (e.g. MARTX transmembrane repeats) is not known. A high degree of thermodynamic instability often indicates that the domains are transported in partially or completely unfolded states (e.g. LF and EF of anthrax toxin (Krantz et al., 2004, Krantz et al., 2006, Thoren et al., 2009, Feld et al., 2010, Wynia-Smith et al., 2012), Clostridium difficile TcdA and TcdB toxins (Salnikova et al., 2008)). On the other hand, thermodynamically stable proteins or protein-ligand complexes would likely get transported in their fully folded states (e.g. by the twin arginine translocation (TAT) pathway across bacterial membranes (Lee et al., 2006)). Therefore, considering the aforementioned limitations, we focused on probing thermodynamic properties of MARTX effector domains from V. cholerae, a mesophilic pathogen (Miller et al., 1984), and A. hydrophila, a psychrotrophic bacterium that can thrive under moderately cold (5-15°C) conditions (Grau, 1981), in order to recognize their common characteristics. We reasoned that using two toxins from organisms optimized for growth at different temperature conditions should provide a broader context for testing the proposed hypothesis.

We found that all effector domains of MARTX are notably unstable compared to typical mesophilic proteins and several effector domains, particularly the peripheral ones, melt via molten globule intermediates. Furthermore, fusion of two or more individual domains into a single polypeptide chain decreases precipitation upon thermal unfolding/denaturation of the effector domains, increases the cooperativity of melting, strictly reduces variability in the transition points, and decreases the melting temperature and thermal stability to a value demonstrated by the least stable domains. Intriguingly, although CPD domain is relatively stable on its own, it is destabilized upon fusion to adjacent domains. Thus, free energy change of $\text{CPD}_{Vc}$ unfolding decreases ~1.4 fold upon its fusion to the adjacent $\text{ABH}_{Vc}$ domain. Therefore, our data support the hypothesis that low thermodynamic stability of the MARTX effector domains bestows their unhindered unfolding near host physiological
temperatures needed for the efficient translocation across the host membrane. Notably, thermodynamic instability demonstrated by the effector domains of MARTX toxins is shared with at least several other membrane-penetrating toxins (LF and EF of *Bacillus anthracis* (Krantz *et al.*, 2004, Krantz *et al.*, 2006), TcdA and TcdB of *Clostridium difficile* (Salnikova *et al.*, 2008), Listerialysin O of *Listeria monocytogenes* (Schuerch *et al.*, 2005), and CTA1 subunit of cholerae toxin (Pande *et al.*, 2007)), but not by extracellular host proteins. We speculate that this property is essential for a wide range of membrane-penetrating/pore-forming toxins and can be targeted for toxin inactivation.

**RESULTS**

*Activity of the ACD effector domains of MARTX reaches maximum at sub-physiological temperatures.* To test the hypothesis that a common mechanism of translocation of MARTX effector domains across the host membrane calls for similarities in their thermodynamic properties, we chose to examine two MARTX toxins produced by Gram-negative bacteria adapted to different temperature environments - mesophilic *V. cholerae* (MARTX<sub>Vc</sub>) and psychrotrophic *A. hydrophila* (MARTX<sub>Ah</sub>). To streamline identification of the properties in common among these effector domains, we first selected to focus on one of the most functionally and structurally characterized ACD domains present in both MARTX toxins as a model protein for detailed analysis. The ACD<sub>Vc</sub> functional enzymatic activity is well portrayed (Kudryashova *et al.*, 2012, Kudryashov *et al.*, 2008b, Kudryashov *et al.*, 2008a, Cordero *et al.*, 2006); and the crystal structure of the closely related ACD domain of VgrG1 toxin of *V. cholerae* has been recently solved (Durand *et al.*, 2012).

Upon delivery to the cytoplasm of the host immune cell, ACD works as an actin-specific protein ligase that phosphorylates the E270 residue on one actin molecule (Kudryashova *et al.*, 2012) and then crosslinks it to K50 on another actin molecule via an amide (isopeptide) bond (Kudryashov *et al.*, 2008b, Kudryashova *et al.*, 2012). This crosslinking leads to formation of polymerization-incompetent actin oligomers and disrupts the cytoskeleton (Cordero *et al.*, 2006, Kudryashov *et al.*, 2008a, Kudryashov *et al.*, 2008b). We determined the rates of enzymatic activity of ACD<sub>Vc</sub> and ACD<sub>Ah</sub> in vitro at different temperatures by following the accumulation of crosslinked actin species during the linear stage of the reaction (Fig. 2A). We found that the rate of activity has a characteristic bell-shaped temperature dependence profile with the optimal activity between 31 - 34°C for ACD<sub>Vc</sub> and 22 - 25°C for ACD<sub>Ah</sub> (Fig. 2B). Remarkably, ACD<sub>Ah</sub> retains at least 50% of its activity in the broad range
from 5 to 34°C, which likely reflects the adaptation of *A. hydrophila* to life in both cold and warm waters. Typically, an enzyme activity peaks at or slightly above the environmental temperature (Somero, 1995) and, therefore, the ACD<sub>Vc</sub> activity seems to be optimized not to the human body, but to other hosts. Alternatively, higher than expected thermal sensitivity of ACD<sub>Vc</sub> may reflect that its structural flexibility was acquired for a reason other than thermal adaptation. To test this hypothesis, we analyzed chemical and thermal denaturation of ACD and other effector domains of MARTX toxins from *V. cholerae* and *A. hydrophila*.

**Chemically induced denaturation of ACD<sub>Vc</sub>**. Eight tryptophan residues of ACD<sub>Vc</sub> are evenly spaced throughout the sequence and therefore can serve as reliable reporters of protein denaturation. To determine free energy change (ΔG<sub>o</sub>) of the ACD<sub>Vc</sub> unfolding, we monitored intrinsic Trp fluorescence of ACD<sub>Vc</sub> in the presence of increasing concentrations of guanidinium hydrochloride (GdnHCl) and urea (Fig. 3). Exposure of Trp residues to solvents due to denaturant-induced unfolding of a protein results in a red shift of the wavelength maximum (λ<sub>max</sub>) of Trp emission spectra (Fig. S1A,B). In both denaturants, a prompt shift of λ<sub>max</sub> was detected that reached the maximum at 1.5 M GdnHCl and 3 M urea, reflecting exposure of Trps to solvent due to the protein unfolding (Fig. 3). The Trp λ<sub>max</sub> transition curves fit well to a single-step sigmoidal equation (equation 5 with sloping baselines in Experimental Procedures) with transition midpoints at 0.87 M and 1.67 M for GdnHCl and urea, respectively. Assuming a two-state transition model, free energy changes of ACD<sub>Vc</sub> unfolding for a given denaturant concentrations (ΔG) were calculated (Fig. 3A,B – inserts) and free energy differences of unfolding in the absence of denaturant (ΔG<sup>H2O</sup>) converged well to 11.5 kJ/mol and 11.4 kJ/mol for GdnHCl- and urea-induced denaturation, respectively. Given that ΔG<sup>°</sup> for most proteins is in the 20-65 kJ/mol range (Park & Marqusee, 2004), it can be concluded that ACD<sub>Vc</sub> is substantially (~ 2 - 6 fold) less stable than an average mesophilic protein and similar in this sense to proteins produced by psychrophilic (adapted to cold) organisms (Feller, 2010, Feller & Gerday, 2003).

**Thermal unfolding of tertiary and secondary structures of ACD<sub>Vc</sub>**. To discriminate between unfolding of secondary and tertiary structures of ACD<sub>Vc</sub>, a combination of far- and near-UV circular dichroism (CD) spectroscopy was employed. Whereas far-UV CD signals report changes in secondary structure elements (α-helices, β-sheets, loops), the near-UV CD signals detect changes in aromatic residues and disulfide bonds and can reveal perturbations of tertiary structure in proximity to these elements (Kelly & Price, 2000).

At sub-transition temperatures, the far-UV spectrum of ACD<sub>Vc</sub> showed two characteristic
minima at 208 nm and 222 nm typical for helical proteins (Fig. 4A; S2A). The α-helical content calculated from our experimental CD data using K2D3 software program (Louis-Jeune et al., 2011) correlates well with the X-ray data obtained from a homologous X-ray structure of V. cholerae VgrG1 ACD (pdb: 4DTH) (Durand et al., 2012) (31.13% versus 31%, respectively); whereas β-sheet content differs from the X-ray data by 5.6% (15.44% versus 21% determined from the crystal structure). The small discrepancy in the latter case may reflect a difference between the homologous ACD domains of MARTX Vc and VgrG1 Vc or stem from differences in experimental techniques and/or conditions.

Monitoring the ACD Vc thermal unfolding via far-UV CD at 208 nm revealed relatively minor non-cooperative changes of low amplitude between 30 and 50°C, at which point a steep transition occurred with an apparent half-transition point at 53.6°C (Fig. 4B). Recording the entire far-UV spectra at various temperatures confirmed that the major changes in the ACD Vc secondary structure happened at >52°C with only minor changes detected at lower temperatures (Fig. 4A). Simultaneous rise in dynode voltage showed that the steep secondary structure transition coincided with protein precipitation (Fig. 4B – black line) (Benjwal et al., 2006).

The near-UV CD spectrum of ACD Vc is characterized by two prominent dips at 283 and 290 nm related to signals from Tyr and Trp residues (Fig. 4C, S2B). Both dips were greatly diminished upon transition from 34 to 42°C, and completely leveled at 46°C, before the entire spectra collapsed at 48°C (Fig. 4C) due to the protein aggregation (as evidenced from the simultaneous increase of dynode voltage; Fig. 4D - black line). Even though the 290 nm dip was mostly or completely leveled before the aggregation occurred, only the onset temperature of tertiary structure unfolding (~34°C) and not the half-transition point was extracted from this experiment to avoid over interpretation. Therefore, in the range of 30 - 45°C (before the occurrence of precipitation) the perturbations in the tertiary structure of ACD Vc were substantial, whereas the secondary structure remained mainly preserved. This suggests that under physiological temperature of human body (~37°C) ACD Vc partially exists in a molten globule state, i.e. with a tertiary structure less defined than that in the fully folded protein, but more compact than in a random coil state (Ohgushi & Wada, 1983). Interestingly, the difference between thermal transitions in far- and near-UV is preserved in the homologous A. hydrophila ACD Ah protein even though both far- and near-UV melting profiles of this protein were shifted to 10-15°C lower temperatures (Fig. S3A,B; S4A,B), likely reflecting the adaptation of this pathogen to cold conditions.
Noteworthy, our Trp fluorescence chemical denaturation data imply a two-state (native and denatured) transition for ACD$_{Vc}$ unfolding, while unfolding through a molten globule intermediate imposed by the CD results suggests three states (native, molten globule, and denatured). Applying the three-state model to fit the Trp data generated nearly identical curves, albeit with much greater errors for the fit parameters (Fig. S1C,D). A possible explanation for this discrepancy is that Trp fluorescence characteristics of two of the ACD$_{Vc}$ transition states (native and molten globule, or molten globule and unfolded states) are not sufficiently different and therefore evade detection.

**Effector domains of MARTX toxins from two different microorganisms are thermolabile.** If low thermodynamic stability indeed plays an important role in transport of ACD across the host membrane, then other MARTX effector domains should also have similar thermodynamic properties. To test this hypothesis, we examined individual effector domains of MARTX toxins from *V. cholerae* and *A. hydrophila* using far- and near-UV CD spectroscopy (Fig. 5A,B; S2-4; Table 1).

Interestingly, all six analyzed effector domains of MARTX toxins showed onsets of thermal denaturation at or below 35°C either in the near-, far-, or both UV regions; i.e. demonstrated thermal instability. CD data did not allow for confident detection of a molten globule state for MARTX effector domains other than ACD. We reasoned that this might reflect a context dependence and insufficient sensitivity of near-UV CD for revealing tertiary structure perturbations due to their dependence on particular positioning of Trp, Tyr, and disulfide bonds in a protein. To overcome this drawback, we employed differential scanning fluorimetry (DSF) as an alternative method for monitoring protein unfolding.

DSF is based on a rise of fluorescence of an environmentally sensitive dye (e.g. 8-anilinonaphthalene-1-sulfonic acid (ANS) or, in our case, SYPRO Orange) upon interaction with a denaturation-exposed hydrophobic interior of a protein (Niesen et al., 2007). DSF showed that most (five out of six) of the individual effector domains of both MARTX toxins melt at temperatures close to that of the human body ($T_m \approx 34-44$°C) (Fig. 6A,B; Table 1); whereas the sixth domain (ACD$_{Ah}$) undergoes transition at a substantially lower temperature of ~27°C. Notably, for four domains (all the effector domains of MARTX$_{Vc}$ and ACD$_{Ah}$) the phase transition temperatures detected by DSF were 8-14°C lower compared to those measured by far-UV CD with the largest difference observed for both ACD$_{Ah}$ and ACD$_{Vc}$ domains (13 and 14°C, respectively). The observed differences suggest that DSF reports
tertiary structure changes that precede the unfolding of secondary structure elements detected by far-UV CD.

Importantly, far-UV CD spectra of ACD\textsubscript{Vc} and both CPD domains acquired in the presence of SYPRO Orange showed that there is only marginal (0 - 2\degree C) destabilizing effect of the dye on the unfolding of their secondary structure (Fig. S5), which cannot be held responsible for the observed differences between the melting temperatures detected by DSF and CD approaches. Although we cannot rule out that SYPRO Orange can destabilize native conformation of a protein by binding and stabilizing a partially unfolded state, our data implies that the concentration of the dye used in the study is not sufficient to cause strong destabilization effect. This assumption is supported by our finding that DSF melting points for MPT\textsubscript{Ah}, ABH\textsubscript{Ah}, and PMT-CPD\textsubscript{Ah} were nearly identical to those detected by far-UV CD in the absence of the dye. It is also possible that different effector domains vary in their sensitivity to the destabilizing effects of the dye, which can therefore magnify the difference between the native to molten globule and molten globule to unfolded state transitions.

Therefore, at the human body physiological temperature all effector domains of MARTX\textsubscript{Vc} and at least one domain of MARTX\textsubscript{Ah} (ACD\textsubscript{Ah}) exist in equilibrium between fully folded and partially unfolded molten globule states. The observed low thermodynamic stability of individual domains agrees with a hypothesis that at least partial unfolding of the effector domains is required for their translocation across the host membrane (Satchell, 2007, Egerer & Satchell, 2010).

**Thermostability of CPD domains.** Formally speaking, MARTX CPD is not an effector domain as it does not directly exert toxicity to host cells. Yet, its delivery to the cytoplasm of host cells should obey the same constraints. Interestingly, CPD of both \textit{V. cholerae} and \textit{A. hydrophila} demonstrate substantially higher thermal stability (T\textsubscript{m} = 54/48\degree C and 60/51\degree C, as detected by far-UV CD/DSF, respectively) (Table 1). We verified that this stability is not due to the buffer compositions used in our CD or DSF experiments, since neither sodium phosphate (50 mM) nor sodium chloride (150 mM) had any effect on DSF T\textsubscript{m} of CPD and only marginally (1\degree C difference) affected its CD T\textsubscript{m} (Fig. S6). Therefore, such high stability of CPD implies that either unfolding is not required for its translocation, or that some unaccounted factors adversely affect the stability of the CPD domains upon their cytoplasmic delivery. Indeed, it has been reported earlier that CPD of \textit{V. cholerae} is thermodynamically unstable and undergoes thermal denaturation at about 38\degree C (detected by DSF) unless stabilized by inositol hexakisphosphate (InsP\textsubscript{6}) – a cytoplasmic activator of the CPD protease.
activity (Prochazkova et al., 2009). However, a “post-cleavage” conformation of the enzyme has been found to be substantially more stable than a “pre-cleavage” (and subsequently a “pre-transition”) state (Prochazkova et al., 2009). CPD constructs used in our study do not contain the key Leu residue in the self-cleavage substrate recognition site at the N-terminus of the protease domains. Identical DSF melting points (47°C) of our CPD construct and the processed post-CPD (Prochazkova et al., 2009) under identical buffer conditions, strongly suggest that they both represent the “post-cleavage” CPD state even though prepared in entirely different ways.

If the above supposition is correct, CPD should adopt a more pliable “pre-cleavage” conformation in conjunction with MARTX effector domains (substrates of CPD) and in the absence of InsP₆, and CPD’s unfolding should proceed with a similar minimal investment of energy as the unfolding of other domains. Indeed, in agreement with this assumption the tertiary and secondary structures of most larger CPD-containing constructs undergo thermal denaturation at 36 - 38°C and 42 - 44°C, respectively; whereas the five-domain *A. hydrophila* construct melts at even lower temperatures of 28°C and 37°C (Fig. 5C,D; 6C,D; Table 1).

**Mutual effects of adjacent domains on their stability.** Since all MARTX effector domains are transported across the host membrane together as a single polypeptide chain, we tested whether the domains exhibit mutual influence (stabilization or destabilization) when expressed as fusion proteins (Fig. 1B-D; S7; S8). First, we found that fusion of two or more individual domains into a single polypeptide chain decreased or abolished precipitation upon thermal unfolding/denaturation, so that RID-ABHₜₐ and ACD-RID-ABH-CPDₜₐ constructs showed no signs of precipitation (Table 1, Fig. S9). Next, CD and DSF both showed that none of the tested constructs were more stable than their individual components (Fig. 5; 6; S7-9; Table 1). On the contrary, most of the tested fusion domains of both MARTX toxins underwent thermal transitions near the melting temperatures of the least stable domains, whereas in some cases (ACD-ABHₜₐ) the domains appeared to be mutually destabilized (i.e. melted at the temperature slightly lower than that of the least stable element). Tertiary structure of all fusion domains of MARTXₜₐ was melted at a nearly identical temperature of 37°C; whereas unfolding of secondary structure elements detected by far-UV CD occurred at 43-44°C with the single exception of ACD-RIDₜₐ, for which secondary elements melted at ~48°C (Fig. 5; Table 1). Next, melting profiles of the fused ACD-containing effector domains of MARTXₜₐ were less stable (Tₘ≈25-30°C) than other constructs.
We recognize that the accurate detection of individual transitions in multidomain protein toxins can be hindered by a higher affinity of SYPRO Orange to the least stable domain (in DSF) or by protein precipitation (both in DSF and CD) and therefore can lead to misinterpretation of the melting profiles. However, we contend that aggregation cannot be held accountable, at least for the observed low stability of the CPD$_{Vc}$-containing constructs (ABH-CPD$_{Vc}$ and ACD-RID-ABH-CPD$_{Vc}$; Fig. S9E,I; Table 1), since these constructs undergo thermal unfolding without detectable precipitation.

To further verify that the observed destabilization is not due to the limitations of the experimental techniques discussed above, we compared $\Delta G_{H_2O}$ of CPD$_{Vc}$ alone with that of the same domain in a fusion construct ABH-CPD$_{Vc}$ using CPD-specific spectroscopic probes. First, we took advantage of the intrinsic fluorescence of three tryptophans present in CPD$_{Vc}$, whereas ABH$_{Vc}$ is naturally void of Trp residues. In agreement with our DSF/CD results, we found that CPD$_{Vc}$ alone ($\Delta G_{H_2O}=21.8$ kJ/mol) is 1.4 times more stable than in the fusion form with ABH$_{Vc}$ (16.1 kJ/mol) (Fig. 7A). These results confirmed that indeed, CPD$_{Vc}$ is destabilized by the adjacent ABH$_{Vc}$ domain. Next, we selectively modified CPD$_{Vc}$ by two different thiol-specific reagents in attempt to benefit from a single Cys residue present in CPD$_{Vc}$, but not in ABH$_{Vc}$. However, introduction of either pyrene or acrylodan to label Cys residue strongly destabilized CPD as revealed by $\Delta G_{H_2O}$ differences calculated from changes in Trp or acrylodan/pyrene fluorescence upon urea denaturation (Fig. 7B; S10). Based on this similarity, we speculate that a substrate-mediated destabilization of CPD (as well as its elevated affinity to InsP$_6$ (Prochazkova & Satchell, 2008)) may also be triggered by direct interaction of a substrate with the catalytic cysteine.

Furthermore, in agreement with our far-UV CD and DSF data, we found that fusion of ABH$_{Vc}$ (which has no Trp residues) to RID$_{Vc}$ (has 10 Trp residues) results in mild but statistically significant destabilization of RID$_{Vc}$ ($\Delta G_{H_2O}=11.5$ kJ/mol, compared to $\Delta G_{H_2O}$ of RID$_{Vc}$ alone 13.2 kJ/mol; Fig. S11). It can be noted that while fusion of ABH$_{Vc}$ to RID$_{Vc}$ reduces its stability to the identical value calculated for ACD$_{Vc}$ (11.5 kJ/mol), the $\Delta G_{H_2O}$ values for ABH-CPD$_{Vc}$ and ACD$_{Vc}$ still vary by 4.6 kJ/mol (16.1 versus 11.5 kJ/mol, respectively) and therefore they might still melt as separate domains in the 4-domain fusion construct, but with melting characteristics very similar to each other. Alternatively, the CPD domain can be further destabilized by other N-terminal domains (e.g. ACD) by unexplored mechanisms.

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In the current work we analyzed thermodynamic properties of the effector domains of *V. cholerae* and *A. hydrophila* MARTX toxins and found that they have low thermostability. It can be recognized that the low thermodynamic stability of MARTX effector domains correlate with that of many membrane-penetrating toxins studied earlier and therefore likely became evolved to facilitate their transition through the host membrane in a partially or fully unfolded state.

The majority of tested individual MARTX effector domains melt via a molten globule intermediate as demonstrated by a significant (up to 14°C) difference between unfolding of tertiary and secondary structures detected by DSF and far-UV CD, respectively (Table 1). It has been theoretically predicted that due to its conformational plasticity and ability to accommodate to both polar and nonpolar environments, molten globules can be crucial for protein transport across a membrane (Bychkova *et al.*, 1998). Indeed, molten globule states have been demonstrated for several bacterial toxins, including LF and EF subunits of anthrax toxin (Krantz *et al.*, 2004, Krantz *et al.*, 2006), *Escherichia coli* colicin A (van der Goot *et al.*, 1991, Lakey *et al.*, 1992), diphtheria toxin (Zhao & London, 1986, Ren *et al.*, 1999), botulinum neurotoxin A (Cai *et al.*, 2006, Hasani *et al.*, 2009), and *E. coli* α-hemolysin (Herlax & Bakas, 2007). Remarkably, similar to the effector domains of MARTX toxins, many of these, as well as other bacterial toxins, are known to unfold at or near the human physiological range of temperatures. The list of other thermolabile toxins includes Listeriolysin O of *L. monocytogenes* (Tm≈37°C; (Schuerch *et al.*, 2005)), TcdB (Tm≈40°C) and TcdA (Tm≈45°C) toxins of *C. difficile* (Salnikova *et al.*, 2008), CTA1 subunit of cholera toxin (Tm≈37°C; (Pande *et al.*, 2007)), and lethal factor (LF) of *B. anthracis* (Tm≈42°C with an onset of unfolding at 30°C; (Gupta *et al.*, 2001)).

Thermodynamically, a fully unfolded protein appears to be an even better substrate for translocation through a narrow pore than molten globules. However, we rationalize that compared to the fully unfolded proteins, molten globules have better chances to withstand aggregation and harsh environmental conditions (such as those created by proteases and defense peptide attacks orchestrated by the host immune system). We speculate that there are evolutionary advantages for bacterial toxins to adopt molten globule rather than the unfolded state and, therefore, molten globules play an important role in the pathophysiology of bacterial toxins. Hence, it is reasonable to propose that the tendency of MARTX domains to form a molten globule at near physiological temperatures is an essential part of the
translocation mechanism. Conversely, it appears that the formation of a molten globule is not a common property of all MARTX effector domains and, therefore, the domains, which unfold through a molten globule state, may play a priming role in other domains’ unfolding required for translocation. Thus, ACD domains of both MARTX toxins form very prominent molten globules ($\Delta T_m=13 - 14^\circ C$; Table 1). It is noteworthy that whenever present in MARTX toxins, ACD is always the most N-terminally located effector domain (Satchell, 2011) and tentatively might play an important role in unfolding of the entire structure and/or formation of a molten globule, thus aiding in the translocation process.

ACD domains of both toxins share characteristic shapes of far- and near-UV CD spectra (Fig. S2A,B and S3A,B), well defined molten globule states, nearly identical differences between tertiary and secondary structure melting temperatures (13 and 14°C; Table 1), and overall shapes of phase transition profiles (Fig. S4A and B). Remarkably, despite a high sequence similarity between the two ACD domains (80%), ACD of A. hydrophila has lower optimal activity temperature (22 - 25°C versus 31 - 34°C) and is even less stable than its V. cholerae counterpart as its tertiary and secondary structures melt at 12 - 13°C lower temperatures (27.4 versus 39.7°C and 40.2 versus 53.6°C, respectively). Although not psychrophilic (as it has an optimum of growth temperature >15°C (Morita, 1975)), A. hydrophila is a typical psychrotrophic microorganism capable of growth at 5°C (Grau, 1981). We speculate that the adaptation to cold renders A. hydrophila strains expressing MARTX toxin (Grim et al., 2013, Tekedar et al., 2013) less efficient towards humans than to fish and amphibians, although immune-compromised people can still be infected by this pathogen (Davis et al., 1978). With the activity optimum steeply dropping around 37 - 40°C, ACD$_{Ah}$ is unlikely to be effective under the physiological conditions of human body. This correlates with our unpublished observations that while being toxic to cultured mammalian cells under 37°C, A. hydrophila (strain ATCC 7566) does not cause any noticeable crosslinking of cytoplasmic actin. It is tempting to speculate that the ability to tune the thermodynamic properties of the adjacent effector domains by replacing/modifying its single element enables fast evolutionary adaptation to new temperature conditions. Thus, low stability of ACD$_{Ah}$ seems to extend to other effector domains when in a fused state and is thereby likely to confer the efficient translocation of these otherwise too rigid domains across the host membrane under cold conditions. Yet, being cleaved off by CPD upon cytoplasmic delivery, ACD would not undermine the stability of other effector domains under mesothermal conditions. This also implies that the cellular effects of MARTX$_{Ah}$ toxin might vary depending on temperature conditions. In a broader context, it is tempting to speculate that the cassette type of a
synchronized delivery of several domains exploited in MARTX toxins not only allows simultaneous delivery of several, often synergistic domains (e.g. ACD and RID), but may also confer a remarkable flexibility in transportation of toxins that otherwise would be too rigid to cross the membrane. To what degree this applies to different MARTX-based and other cassette delivery mechanisms remains to be explored.

In agreement with the hypothesis that unfolding of MARTX effector domains occurs under the human physiological conditions compatible with translocation across the host membrane, fusion of the domains reduces precipitation and narrows the heterogeneity of thermal transitions when compared to individual domain melting. The latter effect is particularly prominent for the CPD-containing fusion proteins, all of which melt at temperatures 10 - 23°C lower than the CPD domains alone (Table 1). Remarkably, $\Delta G_{H_2O}$ of CPDVc is reduced 1.4 fold when the domain is fused to the adjacent ABHVc domain (Fig. 7A). Melting properties of the CPD domains are noteworthy for several reasons. Thus, the CPDVc domain is stable in the absence of a substrate (e.g. in the “post-cleavage” stage, when the key recognition Leu residue is cleaved off (Prochazkova et al., 2009) or simply not included in the construct (this work)). However, it is substantially (by 11°C) destabilized upon interaction with Leu of a substrate (e.g. when N-terminal Leu is included in the CPD sequence (Prochazkova et al., 2009) or when CPD is fused to other domains (this work)). Intriguingly, Cys fluorescence probes (pyrene maleimide and acrylodan) destabilized CPD as revealed by comparison of labeled and unlabeled CPDs’ $\Delta G_{H_2O}$ calculated based on Trp or acrylodan/pyrene fluorescence upon urea denaturation (Fig. 7B). We believe that the reduced CPD stability caused by modification of the catalytic cysteine may reflect the mechanism of CPD destabilization by its substrate (adjacent effector domains). Indeed, the Leu-containing region of the substrate also interacts with CPD in the catalytic region (Prochazkova et al., 2009) and might perturb the protein stability/conformation by affecting the catalytic cysteine. We speculate that before cell entry, interaction with the substrate destabilizes CPD and allows it to easily cross the membrane in an unfolded state. However, if the same interaction happens inside the host cell, binding of InsP$_6$ stabilizes the structure and activates the catalytic activity of CPD (Prochazkova et al., 2009). Given the even higher stability of CPDAh in the post-cleavage stage and its even greater destabilization when in complex with other domains (Fig. 5 and 6; Table 1), the same general rules apply to both CPD domains.
Overall, our data support the hypothesis that the effector domains of MARTX toxins are transported across the cytoplasmic membrane in their partially or fully unfolded states. This supposition is further indirectly supported by an obvious depletion of all known MARTX toxins by cysteine residues. Thus, both ~0.5 MDa MARTX toxins from *V. cholerae* and *A. hydrophila* have only two cysteine residues located in each of the CPD domains, as well as in RID<sub>Vc</sub> and MCF<sub>Ah</sub> domains. Analysis of all twelve known MARTX toxins showed that effector domains may contain from one (in CPD only) to a maximum of four cysteines. Under oxidizing conditions of the extracellular environment, cysteines are prone to disulfide crosslinking, which can efficiently prevent protein translocation through a narrow pore (Maher & Singer, 1986). This strong evolutionary pressure applies only to unfolded proteins because disulfide crosslinking can be efficiently evaded if the cysteines are hidden in the protein interior, but it can hardly be avoided upon protein unfolding. Therefore, the only cysteines that could not be negatively selected by evolution are those in the active sites of the enzymes. Indeed, cysteine is essential for catalytic activity of CPD domains (Shen *et al.*, 2009, Prochazkova *et al.*, 2009), and it has been recently demonstrated that the catalytic triad of RID domains also includes a cysteine (Ahrens *et al.*, 2013). The detailed molecular mechanisms of the MCF apoptotic toxicity (Dowling *et al.*, 2007) are not known, but it can be predicted that cysteine plays an important if not primary role in the activity of this protein as well as in the catalytic function of most or all other Cys-containing MARTX effector domains.

The effector domains of the two MARTX toxins tested in the current study extend the list of thermolabile, conformationally pliable bacterial toxins, suggesting that this quality is essential for many pore-forming and membrane-penetrating toxins. As such, we propose that thermodynamic instability can be utilized to create broad-spectrum antidotes for moderately selective targeting and elimination of various classes of bacterial toxins. This type of antidote can be particularly valuable for targeting infectious conditions when the causative agent/toxin is unknown (e.g. in the case of emerging diseases or bioterrorist attacks). It appears also that natural mechanisms targeting thermodynamic stability of bacterial toxins are already in place in mammals and include hyperthermia and potentially humoral defense factors.

**EXPERIMENTAL PROCEDURES**

**Recombinant protein expression and purification.** DNA fragments encoding effector domains of MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub> were PCR-amplified from genomic DNA (gDNA) of the *V. cholerae* N19691 strain (gDNA<sub>Vc</sub> - generously donated by Dr. Satchell; Northwestern
University) and *A. hydrophila* (purified from ATCC 7966 cells using Axyprep Bacterial Genomic DNA MiniPrep kit; Axygen). The amplified fragments encoding effector domains of the toxins were cloned into the pCold I vector (Clontech) using In-fusion HD kit (Clontech). All constructs were cloned in-frame with the N-terminal 6His-tag. Transformed BL21(DE3)pLysS cells were grown in rich bacterial cell growth medium containing 1.25% tryptone, 2.5% yeast extract, 125 mM NaCl, 0.4% glycerol, 50 mM Tris-HCl, pH 8.2 at 37°C. When the cells reached OD$_{600}$=1-1.5, the induction of protein expression was achieved by cooling the culture down to 15°C and adding 1 mM IPTG. Expression was carried out overnight at 15°C with 250 rpm agitation. Proteins were purified using TALON metal affinity resin (Clontech) according to the manufacturer instructions. Briefly, cells were lysed by sonication in 50 mM phosphate buffer, pH 7.5, containing 300 mM NaCl, 5 mM imidazole, 10 mM PMSF, and protease inhibitor cocktail (Sigma). The cell lysates were clarified by centrifugation (30 min at 90,000 g at 4°C) and applied to TALON column. Unbound material was removed by washing the column with the lysis buffer containing 10-20 mM imidazole. Elution of 6His-tagged proteins was achieved with the lysis buffer containing 50-250 mM imidazole. Proteins were concentrated using Amicon centrifugal filters and dialysed either in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM PMSF or 50 mM phosphate buffer, pH 7.5, 0.1 mM PMSF; aliquots were flash frozen in liquid nitrogen and stored at -80°C.

Bacterial cell strains, plasmid DNA, and primer sequences used in this study are presented in Table S1. Information about the recombinant proteins cloned, expressed, and purified in the present study is summarized in Table 1. SDS-PAGE of purified proteins is shown in Fig. 1B. We were unable to purify MCF$_{Ah}$ domain in its native state as it was insoluble. Five-domain MARTX$_{Ah}$ fusion protein was purified in the presence of N-ethylmaleimide, which helped to protect it from degradation. Activities of ACD and CPD were monitored to ensure proper protein folding of the constructs (Fig. 1C,D). Conditions for actin crosslinking activity of ACD are described below. Protease activity of CPD and autoprocessing activity of CPD-containing constructs were assessed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl after the addition of 0.1 mM InsP$_6$ for 90 min and analyzed by SDS-PAGE.

**Actin purification.** Skeletal muscle actin was prepared from acetone powder from rabbit skeletal muscles (Pel-Freez Biologicals) as described (Spudich & Watt, 1971) and stored in G-buffer (5.0 mM TRIS, pH 8.0, 0.2 mM Ca$^{2+}$-ATP, 5.0 mM β-mercaptoethanol) on ice for 2 weeks or flash frozen in liquid nitrogen for prolonged storage.

**Temperature dependence of ACD activity.** ACD-catalyzed crosslinking was performed...
in a reaction buffer containing 2.0 mM MgCl₂, 0.2 mM EGTA, 0.5 mM ATP, 10 mM HEPES, pH 7.5 as described previously (Kudryashova et al., 2012). Briefly, actin was pre-incubated with 1.5 molar excess of latrunculin B (Enzo Life Sciences) for 15 minutes to prevent actin polymerization and crosslinking was initiated by addition of MgCl₂ (2.0 mM) to a mixture of actin and ACD (500-250:1 molar ratio of ACD to actin) in the reaction buffer lacking MgCl₂. Reactions were incubated at corresponding temperatures and the crosslinking was stopped within 1 - 3 minutes of initiation by adding SDS-PAGE sample buffer and boiling. Crosslinked actin species were resolved on 7.5% SDS-gels and stained with Coomassie Brilliant Blue R-250. Images were obtained using Perfection V600 EPSON scanner. Densitometry was performed using ImageJ image-processing software (http://rsb.info.nih.gov/ij/). The rates of ACD activity were expressed in percent of the maximum activity of the enzyme.

**Intrinsic fluorescence measurements.** Fluorescence emission spectra of protein denaturation induced by GdnHCl and urea were recorded using multifunctional plate-reader Infinite-M1000 Pro (Tecan). Excitation wavelength was set to 295 nm; excitation and emission slits were 2.5 and 5 nm, respectively. 5 μM of the sample protein in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) was mixed with increasing concentrations of GdnHCl (0 - 4 M) or urea (0 - 6 M) and incubated for 1 h at room temperature (23°C) to attain equilibrium before measurements. The maximum wavelength of emission (λₘₐₓ) was determined and the data were analyzed according to the two-state transition model (Pace, 1986, Greene & Pace, 1974).

Assuming a two-state transition model, free energy change of ACDᵥc unfolding for a given denaturant concentration (ΔG) was calculated using the equation (1):

\[
ΔG = -RT \times lnK
\]  

(1),

where R is the universal gas constant, T is temperature in Kelvin; K is the equilibrium constant, which was calculated from the equation (2):

\[
K = \frac{y_F}{y_U} = \frac{e^{\frac{\lambda_{max} - \lambda_{max}^U}{kT}}}{e^{\frac{\lambda_{max} - \lambda_{max}^F}{kT}}}
\]

(2),

where \(y\) is the observed \(\lambda_{max}\) at different concentrations of denaturant, and \(y_F\) and \(y_U\) are the \(\lambda_{max}\) for the folded (F) and unfolded (U) conformations of the protein, respectively (Pace, 1986). ΔG was plotted as a function of denaturant concentration and a linear extrapolation to zero denaturant concentration was applied to calculate free energy difference of unfolding in the absence of denaturant (ΔGᴴ₂O) using the equation (3) (Greene & Pace, 1974):
\[ \Delta G = \Delta G^{\text{H}_2\text{O}} + m \times [\text{GdnHCl}] \]  

(3),

where \( m \) is a slope term, which determines the change in \( \Delta G \) per unit concentration of denaturant [GdnHCl] or [urea]. The transition midpoint corresponding to denaturant concentration at which 50% of a protein is in the unfolded state \([\text{denaturant}]_{1/2}\) was determined according to the equation (4):

\[ [\text{denaturant}]_{1/2} = \frac{\Delta G^{\text{H}_2\text{O}}}{m} \]  

(4).

Data were fit to equation (5) using Kaleidaggraph software:

\[ L = \frac{\left(L_u + m_u \times [D]\right) + \left(L_f + m_f \times [D]\right)}{1 + e^{-\frac{-\left(\Delta G^{\text{H}_2\text{O}} + m \times [D]\right)}{RT}}} \]  

(5),

where \( L \) – wavelength maximum, \([D]\) – denaturant concentration, \( L_f \) and \( L_u \) – wavelength maximums of folded and unfolded states respectively, \( m_f \) and \( m_u \) are slopes of pre- and post-transition baselines, \( \Delta G^{\text{H}_2\text{O}} \) - free energy change of unfolding in the absence of denaturant, and factor \( m \) is a slope term, which determines the change in \( \Delta G \) per unit concentration of denaturant.

**Fluorescent labeling of Cys residue in the active site of CPDVc.** CPDVc was incubated in the presence of 1 mM TCEP for 1 hour and the reducing agent was removed by passing the protein through Zeba column (Pierce) equilibrated with PBS. 1.5 molar excess of acrylodan or pyrene maleimide (Life Technologies) were added and the labeling proceeded for 4 hours followed by free dye removal by Zeba column. Acrylodan and pyrene fluorescence of 1 \( \mu \)M labeled CPDVc was monitored using multifunctional plate-reader Infinite-M1000 Pro (Tecan). Excitation wavelength was set to 391 nm for acrylodan and 340 nm for pyrene; excitation and emission slits were set to 5 nm. Urea-induced CPDVc unfolding was detected as a shift in acrylodan emission wavelength maximum or a decrease of pyrene fluorescence intensity at 374 nm (Fig. S10).

**CD spectroscopy.** Far- and near-UV CD spectra were obtained using JASCO J-815 CD instrument (JASCO Analytical Instruments) equipped with a Peltier temperature controller. Quartz cuvettes with 0.1 and 1 cm length path were used for far- and near-UV CD, respectively. All proteins were dialyzed against 50 mM sodium phosphate buffer, pH 7.4. The protein concentration was determined by the Edelhoch method (Edelhoch, 1967). The concentrations of the samples were adjusted to 0.5 mg/ml for both far- and near-UV CD.
Each wavelength scan was obtained as an average of three accumulations after background subtraction. Temperature denaturation curves (CD signals at a specific wavelength as a function of temperature) and corresponding dynode voltages were recorded over a range of temperatures at a rate 2°C per minute every 1°C. A slower rate of 1°C/min yielded similar results (data not shown). Apparent Tm values of protein secondary structure unfolding were determined as far-UV CD transition midpoints. Additionally, entire spectra of far- and near-UV CD signals for ACDvc were collected at certain temperatures during ramping between 20 and 80°C, at a scanning speed 100 nm/min, using 1 and 2 nm bandwidths for far- and near-UV CD, respectively. Far-UV CD signals were expressed as the mean residue molar ellipticity:

\[
[\theta] = \frac{\theta \times 100 \times M}{C \times l \times n}
\]  

(6),

where \( \theta \) is the ellipticity (degrees), \( l \) - optical path (cm), \( C \) - concentration (mg/ml), \( M \) - molecular mass, \( n \) - the number of amino acid residues. Near-UV CD signals were expressed as the molar circular dichroism (Kelly & Price, 1997):

\[
\Delta \varepsilon = \frac{[\theta]}{3298.2}
\]  

(7).

**Differential scanning fluorimetry (DSF).** Temperature denaturation curves in the presence of SYPRO Orange dye (Invitrogen) were obtained using CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The proteins were diluted in phosphate buffered saline (pH 7.4) to 10 - 20 μM. The dye was used at 1 x concentration (molar concentration of SYPRO Orange is proprietary information of Invitrogen). Temperature was increased at a rate 2°C per minute and fluorescent signal was recorded every 1°C. DSF fluorescence intensity was plotted as a function of temperature, which generates a two-state transition sigmoidal curve. Therefore, a variation of Boltzmann equation is generally applied to fit the DSF data and to calculate the inflection point of the transition (Tm) (Niesen et al., 2007):

\[
F = F_f + \frac{(F_u - F_f) + e^{\frac{Tm-T}{a}}}{1 + e^{\frac{Tm-T}{a}}}
\]  

(8),

where \( F \) is fluorescence signal, \( F_f \) and \( F_u \) – fluorescence of folded and unfolded states respectively, \( T \) – temperature, \( T_m \) – melting temperature, and factor \( a \) is related to the slope of the transition within \( T_m \). To account for non-flat pre- and post-transition baselines according to Clarke and Fersht (Clarke & Fersht, 1993):

\[
F_f = \alpha_f + \mathbb{E}(\beta_f \times T) \quad \text{and} \quad F_u = \alpha_u + \mathbb{E}(\beta_u \times T)
\]  

(9) and (10).
where $\alpha$ and $\beta_f$ are intercept and slope of pre-transition baseline, $\alpha_u$ and $\beta_u$ are intercept and slope of post-transition baseline. To calculate the DSF $T_m$ values, the DSF data were fit using Kaleidagraph software to the modified Boltzman equation:

$$F = (\alpha_f + \beta_f \times T) + \frac{(\alpha_u + \beta_u \times T) - (\alpha_f + \beta_f \times T)}{1 + e^{-\frac{T_m-T}{\alpha}}}$$

The values of $\alpha_f$, $\beta_f$, $\alpha_u$, and $\beta_u$ were fit from the fluorescence signals at temperatures $T$ in pre- and post-transition regions by least-squares using Microsoft Excel; $a$ – an exponential factor dependent on the slope of the transition at the apparent $T_m$ (Table S2).

**Statistical analysis.** Data were analyzed using Microsoft Excel software. Average values were obtained from 3 independent experiments. Errors represent standard errors of mean values. Statistical significance was determined by two-tailed Student’s t-test ($p<0.05$).

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**Conflict of interest**

The authors declare no conflict of interest.

**REFERENCES**


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**FIGURE LEGENDS**

**Figure 1.** Domain structure of MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub>. (A) Schematic diagrams of MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub> toxins: conserved repeats (A1, A2, B, and C) and effector domains are shown in scale. (B) SDS-PAGE of purified recombinant domains of MARTX toxins. ACD – actin crosslinking domain, ABH – α/β hydrolase, PMT – similar to Pasteurella multocida toxin, RID – Rho-GTPase inactivation domain, CPD – cysteine protease domain. Multi-domain constructs are numbered as follows: 1 - ACD-ABH<sub>Ah</sub>, 2 - MCF-PMT<sub>Ah</sub>, 3 - PMT-CPD<sub>Ah</sub>, 4 - ACD-ABH-MCF-PMT<sub>Ah</sub>, 5 - ACD-ABH-MCF-PMT-CPD<sub>Ah</sub>, 6 - ACD-RID<sub>Vc</sub>, 7 - RID-ABH<sub>Vc</sub>, 8 - ABH-CPD<sub>Vc</sub>, 9 - ACD-RID-ABH<sub>Vc</sub>, 10 - ACD-RID-ABH-CPD<sub>Vc</sub>. Additional minor 40 kDa band in lane 8 is a result of residual activity of CPD<sub>Vc</sub> (as seen on Fig. 1D(3)). Loading – 5 μg of protein per well. (C, D) Activity assays were carried out for ACD- and CPD-containing recombinant constructs to ensure their proper folding. (C) Actin-crosslinking was carried out for 30 min at room temperature in the presence of ACD<sub>Vc</sub> (lane 2), ACD-RID<sub>Vc</sub> (lane 3), ACD-RID-ABH<sub>Vc</sub> (lane 4), ACD-RID-ABH-CPD<sub>Vc</sub> (lane 5) at a molar ratio to actin 1:500. M – MW ladder; lane 1 – actin alone. (D) Protease activity of CPD was verified for CPD<sub>Vc</sub>, ACD-RID-ABH-CPD<sub>Vc</sub>, and ABH-CPD<sub>Vc</sub>. Reactions were initiated by addition of 0.1 mM InsP<sub>6</sub>. 1 – CPD<sub>Vc</sub> was incubated in the presence of its substrate ACD-RID-ABH<sub>Vc</sub>; 2 and 3 – self-cleavage of ACD-RID-ABH-CPD<sub>Vc</sub> and ABH-CPD<sub>Vc</sub>, respectively. Final concentrations of proteins were 0.1 mg/ml for CPD<sub>Vc</sub> and 0.5 mg/ml for all other constructs. Resulting cleavage products are indicated at right based on their molecular weights (MW is shown in parenthesis); 4d (185) - ACD-RID-ABH-CPD<sub>Vc</sub> (185 kDa), 3d (163) - ACD-RID-ABH<sub>Vc</sub> (163 kDa), 2d (126) - ACD-RID<sub>Vc</sub> (126 kDa), 2d (111) - RID-ABH<sub>Vc</sub> (111 kDa).

**Figure 2.** Temperature dependence of ACD activity. (A) Actin crosslinking by ACD<sub>Ah</sub> and ACD<sub>Vc</sub> at different temperatures was monitored by SDS-PAGE. (B) Relative ACD activity

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(26 % of maximum) was plotted as a function of temperature. Error bars represent standard errors of means; n = 3.

**Figure 3.** Chemically induced denaturation of ACD<sub>Vc</sub>. Intrinsic Trp fluorescence during chemically induced unfolding of ACD<sub>Vc</sub> was monitored in the presence of increasing concentrations of GdnHCl (A) or urea (B). λ<sub>max</sub> was plotted against concentration of the denaturants. Inserts show plots of free energy changes of ACD<sub>Vc</sub> unfolding (ΔG) for a given denaturant concentration, [GdnHCl] or [Urea]. Parameters obtained from fitting the data to the equation 5 for GdnHCl denaturation: ΔG<sub>H2O</sub> = 2.75±0.14 kcal/mol (11.5 kJ/mol), m=3.16±0.17 kcal/mol/M, [GdnHCl]<sup>1/2</sup>=0.87 M; for urea denaturation: ΔG<sub>H2O</sub> = 2.72±0.11 kcal/mol (11.4 kJ/mol), m=1.63±0.07 kcal/mol/M, [urea]<sup>1/2</sup>=1.67 M.

**Figure 4.** Thermal unfolding of ACD<sub>Vc</sub> as revealed by CD. Thermal unfolding of secondary and tertiary structures of ACD<sub>Vc</sub> was monitored by far- (A, B) and near-UV (C, D) CD spectroscopy. Entire CD spectra were obtained at different temperatures as indicated on figures (A) and (C). CD signals at a specific wavelength were recorded as functions of temperature: 208 nm for far-UV CD (B) and 290 nm for near-UV CD (D). Dynode voltage (black lines in (B) and (D)) was routinely monitored as a measure of protein aggregation. Note two transition steps for near-UV CD temperature dependence (C, D). Leveling of the ACD<sub>Vc</sub> characteristic minima at 290 and 283 nm reflects rearrangements in the environment of tertiary structure elements at <46°C (#1 in C and D). The drop of signal (#2 in C and D) coincides with precipitation at >46°C as seen by concurrent dramatic increase of dynode voltage (D).

**Figure 5.** Thermal unfolding of the effector domains and CPD from MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub> toxins as monitored by CD. Far-UV CD signals of individual (A, B) or fused (C, D) MARTX domains of *V. cholerae* (A, C) or *A. hydrophila* (B, D) were recorded as functions of temperature. Signals were normalized for each recombinant protein with the highest in the transition region expressed as 1 and the lowest set as 0. Apparent half-transition points are shown with dotted lines.

**Figure 6.** Thermal unfolding of the effector domains and CPD from MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub> toxins as monitored by DSF. DSF signals of individual (A, B) or fused (C, D) MARTX domains of *V. cholerae* (A, C) or *A. hydrophila* (B, D) were recorded as functions of temperature and normalized for each recombinant protein with the highest signal in the transition region expressed as 1 and the lowest signal set as 0. Half-transition points are shown with dotted lines.
Figure 7. Destabilization of CPD$_{Vc}$ by fusion with the adjacent domain and by Cys-labeling. (A) Trp fluorescence of CPD$_{Vc}$ was monitored during denaturation of CPD$_{Vc}$ (closed circles) or ABH-CPD$_{Vc}$ (open circles) with urea. Parameters obtained from fitting the data to equation 5 for CPD$_{Vc}$ in separation: $\Delta G_{H_2O}^f = 5.22 \pm 0.56$ kcal/mol (21.84 kJ/mol), $m = 2.37 \pm 0.35$ kcal/mol/M, $[\text{urea}]_{1/2} = 2.2$ M; for CPD$_{Vc}$ fused to ABH$_{Vc}$: $\Delta G_{H_2O}^f = 3.84 \pm 0.5$ kcal/mol (16.07 kJ/mol), $m = 2.23 \pm 0.27$ kcal/mol/M, $[\text{urea}]_{1/2} = 1.72$ M. (B) CPD$_{Vc}$ was labeled with acrylodan or pyrene maleimide and its unfolding in urea was monitored by changes in the corresponding fluorescence signals. Additionally, Trp fluorescence was recorded for unlabeled or acrylodan-labeled CPD$_{Vc}$. Fraction of unfolded protein was calculated and plotted against urea concentration. Parameters obtained from fitting the data to equation 5 are as followed: 1) unlabeled CPD$_{Vc}$ (Trp fluorescence; closed black circles): $\Delta G_{H_2O}^f = 5.22 \pm 0.56$ kcal/mol (21.84 kJ/mol), $m = 2.37 \pm 0.35$ kcal/mol/M, $[\text{urea}]_{1/2} = 2.2$ M; 2) acrylodan-labeled CPD$_{Vc}$ (Trp fluorescence; open black circles): $\Delta G_{H_2O}^f = 2.78 \pm 0.34$ kcal/mol (11.6 kJ/mol), $m = 2.06 \pm 0.29$ kcal/mol/M, $[\text{urea}]_{1/2} = 1.35$ M; 3) acrylodan-labeled CPD$_{Vc}$ (acrylodan fluorescence; gray triangles): $\Delta G_{H_2O}^f = 3.07 \pm 0.11$ kcal/mol (12.8 kJ/mol), $m = 2.06 \pm 0.08$ kcal/mol/M, $[\text{urea}]_{1/2} = 1.49$ M; 4) pyrene-labeled CPD$_{Vc}$ (pyrene fluorescence; gray squares): $\Delta G_{H_2O}^f = 2.38 \pm 0.12$ kcal/mol (9.96 kJ/mol), $m = 1.71 \pm 0.09$ kcal/mol/M, $[\text{urea}]_{1/2} = 1.39$ M.
<table>
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<tr>
<th>Domains</th>
<th>MW, kDa</th>
<th>Position in the MARTX&lt;sub&gt;Vc&lt;/sub&gt; or MARTX&lt;sub&gt;Ah&lt;/sub&gt; (AAD21057.1 or YP_855898.1)*</th>
<th>Secondary structure T&lt;sub&gt;m&lt;/sub&gt; (°C) by far UV CD (T aggregation)**</th>
<th>Tertiary structure T&lt;sub&gt;m&lt;/sub&gt; (°C) by DSF</th>
<th>ΔT&lt;sub&gt;m&lt;/sub&gt;</th>
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<td>44.3 (n/a)</td>
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<tr>
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<td>24.2</td>
<td>3429-3631</td>
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<td>37.4 (36)</td>
<td>28.2±0.7</td>
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* – NCBI accession numbers for protein sequences of MARTX<sub>Vc</sub> and MARTX<sub>Ah</sub> toxins are shown in parentheses.

** – temperature of protein aggregation as determined by mid-point transition of dynode voltage curve.

T<sub>m</sub> – protein melting temperature as determined by mid-point transition of far-UV CD or DSF fluorescence curves. Aggregation temperatures are given in parenthesis.

ΔT<sub>m</sub> – difference between melting temperatures of secondary and tertiary structures determined by far-UV CD and DSF, respectively.

MW – protein molecular weight (including the N-terminal 6xHis tag).

n/a – not applicable.