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Post-translational acylation controls the folding and functions of the CyaA RTX toxin

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Running title: Acylation and calcium control CyaA folding

23 **List of abbreviations**

24 AC, N-terminal Adenylate cyclase Catalytic domain; cAMP, cyclic Adenosine
25 MonoPhosphate; CyaA, adenylate cyclase; HDX-MS, Hydrogen/Deuterium eXchange
26 followed by Mass Spectrometry; HR, Hydrophobic Region; PTM, Post-Translational
27 Modifications; pro-CyaA, non-acylated adenylate cyclase; RD, RTX Domain; RTX motif,
28 Repeat in ToXin motif; T1SS, Type 1 Secretion System; TR, Translocation Region;
29

ABSTRACT

CyaA is synthesized as a pro-toxin, pro-CyaA, and converted into its cytotoxic form upon acylation of two lysines. After secretion, CyaA invades eukaryotic cells and produces cAMP, leading to host defense subversion. To gain further insights into the effect of acylation, we compared the functional and structural properties of pro-CyaA and CyaA proteins. HDX-MS results show that the refolding process of both proteins upon progressive urea removal is initiated by calcium binding to the C-terminal RTX domain. We further identified a critical hydrophobic segment, distal from the acylation region, that folds at higher urea concentration in CyaA than in pro-CyaA. Once refolded into monomers, CyaA is more compact and stable than pro-CyaA, due to a complex set of interactions between domains. Our HDX-MS data provides direct evidence that the presence of acyl chains in CyaA induces a significant stabilization of the apolar segments of the hydrophobic domain and of most of the acylation region. We propose a refolding model dependent on calcium and driven by local and distal acylation-dependent interactions within CyaA. Therefore, CyaA acylation is not only critical for cell intoxication, but also for protein refolding into its active conformation.

Public Summary

The contributions of post-translational modifications on the folding and activity of proteins are still poorly understood. The adenylate cyclase toxin, CyaA, is a major virulence factor of *Bordetella pertussis*, the causative agent of whooping cough. CyaA is produced as an inactive pro-toxin, which is post-translationally acylated in the bacterial cytosol to yield the active CyaA toxin, able to intoxicate human cells and induces cell death. Yet, the link between this post-translational modification, the structure and the cytotoxic activities of CyaA remains elusive. Here, using Hydrogen/Deuterium eXchange followed by Mass Spectrometry (HDX-MS) and a combination of biophysical approaches, we demonstrate that acylation contributes to the proper folding of CyaA into a compact and functional state. Our data sheds light on the complex relationship between post-translational modifications, structural disorder and protein folding. Coupling calcium-binding and acylation-driven folding is likely pertinent for other Repeat-In-Toxin cytolysins produced by many Gram-negative bacterial pathogens.

INTRODUCTION

Post-translational modifications (PTM) are involved in the regulation of many cellular processes and play a critical role in protein localization, structure, stability and function (1, 2). Post-translational addition of acyl chains to bacterial toxins from the Repeat-In-Toxin (RTX) family has been shown to be essential to their cytolytic activities. RTX toxins are calcium-dependent virulence factors produced by various Gram-negative bacteria (3, 4). These cytotoxins are synthesized as non-active precursors that require “activation” by selective post-translational acylation carried out by dedicated acyltransferases that use an acyl-acyl carrier protein (acyl-ACP) as fatty acid donor (5-9). Once acylated, the RTX toxins are secreted *via* dedicated type 1 secretion systems (T1SS) and exert their virulence on a variety of target cells (10). Most are cytolysins endowed with a pore-forming activity that alter the membrane integrity of their target cells (3, 4, 11) while few others have additional cytotoxic activities (see below). How the acyl chains regulate the biological activities of these RTX cytolysins has remained elusive thus far.

Here we address this question by exploring the effects of post-translational acylation on the structure and functions of the adenylate cyclase toxin (CyaA) from *Bordetella pertussis*, the causative agent of whooping cough (12). In addition to its pore-forming activity, CyaA possesses an extra domain endowed with adenylate cyclase activity. This AC domain is delivered into the cytoplasm of target cells (13, 14) where it is activated by calmodulin (15, 16), to produce high levels of cAMP, subverting host immunity (10, 17, 18). CyaA is a large, multi-domain protein of 1706 amino acid residues (Figure 1), with an N-terminal adenylate cyclase catalytic domain, AC, (residues 1-364) (16), while the C-terminal hemolysin region (residues 365-1706) contains several domains responsible for AC membrane translocation and pore-forming activity (19-24). The translocation region, TR, (residues 365-527) is involved in AC translocation across target cell membranes (24), membrane permeabilization (25) and exhibits features similar to membrane-active peptides (24, 26, 27). The hydrophobic region, HR, corresponds to residues 528 to 710 while the acylation region, AR, extends from residues 711 through 1005. CyaA is synthesized as an inactive precursor, pro-CyaA, that is converted into its active form upon specific acylation of two lysine residues (Lys 860 and Lys 983) by CyaC, an acyltransferase that catalyzes the transfer of an acyl chain from an acyl-ACP (acyl-carrier protein) to the epsilon-amino groups of pro-CyaA lysines (7, 28-30). Finally, the C-terminal 701 residues (1006-1706) correspond to the cell receptor-binding domain, RD, which harbors ~40 copies of a glycine and aspartate rich nona-peptide repeat, characteristic of the RTX bacterial cytolysins. In the absence of calcium, these RTX motifs are intrinsically disordered, but undergo a disorder-to-order transition upon calcium binding (4, 31-42). The RTX motifs themselves constitute the primary sites of calcium binding within the protein (3, 31, 43, 44).

It is well known that acylation is critically required for the pore-forming activity of CyaA as well as its ability to invade cells to produce high levels of cAMP (*i.e.*, cell intoxication) (45, 46). Here, we have analyzed the structural and hydrodynamic properties of both pro-CyaA

101 and CyaA monomers to provide insights into their folding process, and to identify the
102 structural basis of the acylation-dependent gain of functions. Our biophysical data show that
103 the acylated CyaA protein adopts a more compact and stable monomeric state than pro-CyaA,
104 while Hydrogen/Deuterium eXchange followed by Mass Spectrometry (HDX-MS) studies
105 provided direct evidence of long-range interactions between the hydrophobic and acylation
106 regions. HDX-MS results further indicate that these interactions are strongly favored in the
107 presence of the acyl chains. We propose that these interactions may directly contribute to the
108 folding of the acylated CyaA protein into a compact and functional monomeric state. Our data
109 indicates that calcium-binding and post-translational acylation play a key role in the folding
110 of CyaA into its cytotoxic form.

MATERIALS AND METHODS

Overexpression and purification of pro-CyaA and CyaA in urea and production of the monomeric species

Buffer A was composed of 20 mM Hepes, 150 mM NaCl, pH 7.4. Production and purification of pro-CyaA and CyaA, and refolding of both species into holo-monomers was performed as previously described (41, 47). The pro-CyaA urea stock solution was loaded at 2 μ M (compared to 5 μ M for CyaA) on a size exclusion chromatography column (HiLoad 26/600 Superdex 200 pg, average particle size: 34 μ m; column dimensions: 26*600 mm or Superdex 200 Increase 10/300 GL, average particle size: 8.6 μ m; column dimensions: 10*300 mm or Agilent BioSEC-5, particle size: 5 μ m, pore size: 300Å, column dimensions: 21.2*300 mm) to refold the protein into a monomeric species. The monomer-to-multimer ratio increases as the particle size decreases.

Identification of the post-translational acylation of pro-CyaA and CyaA by mass spectrometry

Protein digestion. CyaA (9 μ M) and pro-CyaA (7 μ M) in 6 M urea buffer were firstly digested with Lys-C (#V1671, Promega) at a Lys-C/toxin ratio of 1/50 (w/w), at 37°C for 3h. To ensure optimal trypsin activity, the urea concentration was decreased below 2 M with addition of 2 volumes of buffer A, then a second digestion was performed with Trypsin (Sequencing Grade Modified Trypsin #V5111, Promega) at a enzyme/toxin ratio of 1/50 (w/w) at 37°C for 1h. Digestion was stopped by adding formic acid to 2% final concentration. Digested peptides were desalted and concentrated with C18 Spin Columns Pierce™ (ThermoFisher Scientific - 89870) according to manufactures instructions. Peptides were vacuum centrifuged and then lyophilized. Peptides were reconstituted in 2% ACN / 0.1% FA. **LC-MS/MS analysis.** A Proxeon EASY-nLC 1000 nanochromatographic system (Thermo Fisher Scientific, Bremen) was coupled on-line to a LTQ-Orbitrap Velos Mass Spectrometer (Thermo Fisher Scientific, Bremen). For each sample, 0.5 μ g of peptides was directly loaded onto a 15 cm home-made C4 column (5 μ m particles, 300 Å pore size, ReproSil-Pur 300 C4, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted by a one step gradient from 3.0% to 80% solvent B (80 % ACN, 0.1 % FA) in 50min, at a flow rate of 250 nL/min. MS data was acquired using Xcalibur software with a survey scan (100-1800 m/z) analyzed into the Orbitrap mass analyzer at a resolution setting of 60,000 followed by 15 CID fragmentations analyzed in the linear ion trap. The AGC* target for MS and MS/MS scans were set to 1E6 and 5E3 respectively. The isolation width was set to 2.5 m/z and the normalized collision energy was set to 35. Selected ions were dynamically excluded for 15 sec.

Data analysis. All raw data was analyzed using MaxQuant software version 1.5.1.2 (48) and the Andromeda search engine (49). Based on the protein expression system, data was searched against the complete *E. coli* strain K12 UniProt database (4,331 proteins, downloaded 2015-10-08), and CyaA protein sequence. The digestion mode was set to trypsin, and a maximum of two missed cleavages were allowed. Carbamidomethylation of cysteine

was specified as a fixed modification. Variable modifications considered were N-terminal acetylation, oxidation of methionines and N-acylation of lysines (Myristoyl-4H, Myristoleylation, Myristoylation, Palmitoleylation, Palmitoylation). The minimum peptide length was fixed to 5 amino acids and the required false discovery rate was set to 1% at the peptide spectrum match (PSM) and protein level. The main search peptide tolerance was set to 4.5 ppm and to 0.5 Da for the MS/MS match tolerance. Second peptides were enabled to identify co-fragmentation events. Results of PTM identification are shown in Supplemental Figures S2A-C.

HDX-MS of pro-CyaA and CyaA conformation and dynamics

We used Hydrogen/Deuterium eXchange followed by Mass Spectrometry (HDX-MS) to characterize the refolding process of pro-CyaA and CyaA equilibrated in 6, 3 or 1.6 M urea, as well as the monomeric species in the complete absence of denaturant. Two experimental conditions were analyzed; *A*: to monitor the effect of post-translational acylation on the refolding process of full-length pro-CyaA and CyaA at 6 M, 3 M and 1.6 M urea, and *B*: to determine the effect of acylation on the full-length pro-CyaA and CyaA monomers. To achieve this, several proteins and conditions were analyzed; firstly, pro-CyaA and CyaA each in the presence of 6, 3 and 1.6 M urea containing either 2 mM CaCl₂ or 2 mM EDTA, and secondly, fully-folded pro-CyaA and CyaA monomers in buffer A, complemented with 2 mM CaCl₂.

HDX-MS sample preparation. Prior to addition of the deuterated buffer (100% D₂O in 20 mM Hepes, 150 mM NaCl, pH 7.4, supplemented or not with 6 M, 3M or 1.6 M urea), all solutions were equilibrated for 1 h at 20°C. For experiment A, labeling was initiated by diluting each sample five-fold with the deuterated buffer and incubated for *t* = 0.16, 1, 10, 60, and 240 min at 20°C (final protein concentration of 0.3 μM during labeling); experiment B was performed using a final protein concentration of 1 μM and identical labeling conditions for *t* = 0.16, 0.5, 1, 5, 10, 30, 60, 120, 240, 480 and 1440 min, respectively.

The 80% excess of deuterium reached during labeling ensured unidirectional exchange. Aliquots of 3-10 pmol of protein were withdrawn at each experimental time point and quenched upon mixing of the deuterated sample with ice-cold 0.5% formic acid solution to achieve a final pH of 2.5. For experiment A (in the presence of urea), the quench was performed by mixing 14 mL of labeled sample with 56 mL of ice cold 0.5% formic acid (ratio 1/5: v/v, final H₂O/D₂O ratio = 20%/80%). For experiment B (monomeric species), the quench was performed by mixing 30 mL of labeled sample with 30 mL of ice cold 0.5% formic acid (ratio 1/2: v/v, final H₂O/D₂O ratio = 50%/50%). Quenched samples were immediately snap-frozen in liquid nitrogen and stored at -80°C for approximately 12 h. Undeuterated controls were treated using an identical procedure. Triplicate independent technical analyses were performed for each time point and condition for all HDX-MS analyses.

HDX-MS data acquisition. Prior to MS analysis, samples were rapidly thawed. To minimise back exchange, the LC solvent line, injection valve, and sample loop were maintained at 0°C. To achieve this, local HDX analyses were performed with the aid of a

192 cooled HDX Manager (Waters Corporation, Milford, MA). Samples were digested using an
193 in-house prepared cartridge of immobilized pepsin beads (Thermo Scientific, Rockford, IL),
194 for 2 min at 70 μ L/min and 20°C. Peptic peptides were rapidly desalted and concentrated
195 using a Vanguard C18 pre-column (1.7 μ m, 2.1 x 5 mm; Waters Corporation), and separated
196 using an ACQUITY UPLC™ BEH C18 column (1.7 μ m, 1 x 100 mm). Pro-CyaA and CyaA
197 peptides were separated over a 10 min gradient of 5-40% ACN at 38 μ L/min and at 0°C. The
198 LC flow was directed to a Synapt™ G2-Si HDMS™ mass spectrometer (Waters), which was
199 equipped with ESI and lock-mass correction using Glu-Fibrinogen peptide. Mass spectra were
200 acquired in positive-ion mode over the m/z range of 50–1800 using a data-independent
201 acquisition scheme (MS^E) whereby exact mass information is collected at both low and high
202 collisional energies for collisional induced dissociation. Unlabeled pro-CyaA and CyaA
203 samples were digested in triplicate to build a peptide coverage map.

204 *HDX-MS data processing.* Peptide identification was *via* the Protein Lynx Global
205 Server (PLGS) 3.0 (Waters Corporation). Oxidation of methionines and carbamylation of N-
206 terminal and lysine residues were set as variable modifications. The sequence coverage map
207 (Supplemental Figures S3 and S4) was plotted using DynamX 3.0 HDX software (Waters).
208 For the native proteins, pepsin digestion yielded 232 unique peptides identified from their
209 accurate masses and product ion spectra. A total of 141 peptides were brought forward for
210 HDX data analysis, corresponding to a sequence coverage of 91.0%. For the proteins in urea,
211 172 peptides, covering 85.7% of the protein sequence, were selected for final analysis. The
212 reduced sequence coverage witnessed here was due to the experimental design necessitating a
213 lower protein load on column at the 1.6 M urea concentration (down to 3 pmol, in contrast to
214 10 pmol for all other states), resulting in a knock-on decrease in MS sensitivity. D₂O uptake at
215 the peptide level was extracted and visualized in uptake charts, difference plots, and heat
216 maps, performed in both DynamX and MEMHDX (50). Statistical analysis of HDX-MS
217 results was performed with MEMHDX (Wald test, false discovery rate sets to 1%).

218 *Urea unfolding of pro-CyaA and CyaA monomers*

219 A volume of 2.5 μ L of CyaA or pro-CyaA monomers stored in 20 mM Hepes, 150
220 mM NaCl, 2 mM CaCl₂, pH 7.5 was diluted in 20 mM Hepes, 150 mM NaCl, 2 mM CaCl₂,
221 containing urea at various concentrations to obtain a final concentration of toxin of 50 nM. A
222 total of 22 tubes were prepared, with a urea gradient from 0 to 6 M. Once diluted, the toxin
223 was incubated at room temperature for 1 h. Emission spectra of the protein were acquired on a
224 Jasco FP 8200 spectrofluorimeter (Jasco, Tokyo, Japan) using a 3 x 3 mm path length quartz
225 cell (105.251.QS, Hellma Analytics). The excitation wavelength was set to 280 nm, both
226 emission and excitation slits were fixed at 5 nm, and fluorescence emission spectra were
227 recorded from 300 to 400 nm. Spectra were acquired at 25°C. The ratio of fluorescence
228 intensities at 340 and 380 nm were plotted against the urea concentration.

229 *ANS binding to CyaA and pro-CyaA monomers*

230 Freshly prepared ANS was added to pro-CyaA or CyaA monomers to reach final

concentrations of 5 and 0.5 μM , respectively (molar ratio of ANS:toxin was 10:1). Excitation was fixed at 360 nm and emission spectra were recorded between 400 and 600 nm on a Jasco FP 8200 spectrofluorimeter (Jasco, Tokyo, Japan) using either a 3 x 3 mm path length quartz cell (105.251.QS, Hellma Analytics) or a 10 μL drop cell. All spectra were acquired at 25°C.

Analytical ultracentrifugation (AUC) analysis of pro-CyaA and CyaA monomers

Sedimentation velocity experiments were performed on a Beckman XL-A analytical ultracentrifuge (Beckman Coulter) in a AN60-Ti rotor at 20 °C. All samples were filtered on 0.2 μm filters before experiments. Detection of the protein concentration as a function of radial position and time was performed by optical density measurements at a wavelength of 280 nm. For sample analysis, 400 μL of pro-CyaA or CyaA at 1 μM was loaded into a 1.2 mm-thick two-channel epoxy centerpiece and spun at 20,000 rpm. Data were analyzed with SEDFIT software using a continuous size distribution c(s) model (15, 41, 51).

Hemolysis of erythrocytes

The hemolytic activity of pro-CyaA and CyaA was determined on sheep erythrocytes as previously described (47). Briefly, sheep erythrocytes were washed several times with buffer A and resuspended at 5×10^8 cells/mL. The hemolytic activity of pro-CyaA and CyaA was measured after overnight incubation of erythrocytes at 37 °C by quantifying the amount of hemoglobin released at 540 nm (and of intracellular content release at 405 nm).

Pro-CyaA and CyaA translocation and cAMP production in erythrocytes

The invasive activity of CyaA was determined by measuring the intracellular cAMP accumulation as previously described (47). Briefly, erythrocyte suspensions (5×10^8 cells/mL in buffer A) were incubated with 5.6 nM CyaA ($1 \mu\text{g} \cdot \text{mL}^{-1}$) at 37 °C for 20 min. The cells were washed and lysed as described (41) and then the intracellular cAMP content was determined by a homemade competitive ELISA immunoassay (52) using a rabbit anti-cAMP antiserum (1818-II) and a goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma, Life Science). The alkaline phosphatase activities were revealed with 4-Nitrophenyl phosphate disodium salt hexahydrate (PA substrate from Sigma, Life Science) and cAMP concentrations were calculated from a standard curve established with known concentrations of cAMP diluted in buffer A.

Data availability

All data generated and analyzed during this study are included in this published article and its supplemental information files.

RESULTS

Production and MS characterization of CyaA and pro-CyaA

Pro-CyaA and CyaA were overexpressed in *E. coli* - the latter by co-expression of pro-CyaA and CyaC (53-55) – and purified to homogeneity using established methods (47). To identify the acyl chains incorporated in CyaA, the pro-CyaA and CyaA proteins were digested by Lys-C and trypsin and analyzed by MS. Two peptides (858-872 and 972-984) containing the K860 and K983 residues were identified. Both residues were primarily palmitoleylated (C16:1) in CyaA (see Supplemental Figures S1A-C). Palmitoylation (C16:0) was also identified on both K860 and K983 residues. Traces of myristoylation (C14:0) were observed on K983. As expected, no acylation was detected on K860 or K983 in pro-CyaA. In summary, CyaA is primarily post-translationally modified with C16 acyl chains on both lysines, while pro-CyaA is devoid of the PTM.

Refolding and functional activities of CyaA and pro-CyaA

Due to their size and hydrophobic character, both pro-CyaA and CyaA proteins are prone to aggregation. They are traditionally purified in the presence of chaotropic agents (usually urea) and stored in an unfolded state in denaturing conditions (*i.e.*, 6-8 M urea). Upon refolding by urea dilution, mixtures of multimeric and monomeric species are commonly obtained, precluding further biochemical and biophysical characterization (41). Yet, we have recently achieved to refold both pro-CyaA and CyaA into monomeric states (see Supplemental Figure S2) that remain stable in the absence of urea (41, 47), thus allowing us to investigate the relationship between the structure and function of these two species. The method is based on molecular confinement to prevent intermolecular interactions between hydrophobic regions of proteins upon their refolding. Refolding is performed on a size-exclusion chromatography column used as molecular confinement media comprising a matrix characterized by small size particles (see methods for details and Supplemental Figure S2). In addition to the monomeric species, this refolding procedure also leads to the formation of a heterogeneous population of CyaA multimers. We have previously observed that beside molecular confinement, refolding is critically dependent on the presence of calcium and toxin acylation (41, 47). However, we succeeded to refold pro-CyaA into a monomeric protein by optimizing the procedure, essentially by decreasing the initial protein concentration, although the recovery yield was low as compared to that of acylated CyaA (see Supplemental Figure S2 and methods for details).

The biological activities of the refolded pro-CyaA and CyaA species were assessed on sheep erythrocytes by monitoring hemolytic activity and the ability of the toxins to invade cells (Supplemental Table S1). As expected, the monomeric CyaA species induced hemolysis and was able to bind to erythrocytes and translocate into the cytosol in a calcium-dependent manner, in agreement with prior studies (25, 47, 55, 56). In contrast, the multimeric CyaA species exhibited strongly reduced hemolytic activity and was not able to translocate into erythrocytes while the pro-CyaA species were totally lacking hemolytic and cell invasion capacities (Supplemental Table S1) in agreement with previous studies (53, 57). This

confirms that the monomeric, acylated CyaA is the physiological competent state of the toxin, able to invade eukaryotic target cells.

Compactness and solvent-exposed hydrophobic regions in CyaA and pro-CyaA

We then characterized the biophysical and hydrodynamic properties of pro-CyaA and CyaA. We first determined the compactness and stability of both proteins using macroscopic approaches, and then characterized the proteins at medium resolution by HDX-MS. The solvent-exposed hydrophobic surfaces of pro-CyaA and CyaA monomers were probed using ANS fluorescence (58) in buffer A, complemented with 2 mM CaCl₂. While a slight increase in ANS fluorescence intensity was observed in the presence of CyaA monomers, a marked increase (approximately 3-fold) was found with the pro-CyaA monomers, suggesting that these latter expose more hydrophobic regions to the solvent than the former (Figure 2A). In other words, the hydrophobic regions in CyaA monomers are less accessible to ANS than those in the pro-CyaA species, suggesting that acylation favors the shielding of hydrophobic regions within the protein. We then investigated the compaction state of CyaA and pro-CyaA by sedimentation velocity analysis using AUC. Both species remain essentially monomeric, with only a few aggregates observed on the time scale of the experiment (Figure 2B). The analysis provided a sedimentation coefficient of 7.4 and 7.1 S, respectively, for CyaA and pro-CyaA monomers (Figure 2B and Supplemental Table S2), suggesting that pro-CyaA monomers are slightly less compact than CyaA monomers. We further compared the stability of pro-CyaA and CyaA monomers by following their urea induced unfolding by intrinsic tryptophan fluorescence. The denaturation data also indicate that CyaA is more stable than pro-CyaA at urea concentrations above 2 M (Figure 2C) as highlighted by the plot of the maximum wavelength differences between the two proteins (Figure 2D). Taken together, these results indicate that pro-CyaA monomers expose more hydrophobic regions to the solvent (Figure 2A), and are less compact (Figure 2B) and stable (Figures 2C-D) than CyaA monomers.

HDX-MS analysis of the pro-CyaA and CyaA refolding process

To further characterize the refolding process of pro-CyaA and CyaA, we used HDX-MS to probe the structure and dynamics of both full-length proteins equilibrated in 6, 3 or 1.6 M urea, as well as the monomeric species in the complete absence of denaturant. Figure 3 displays a global view of the relative deuterium fractional uptake of CyaA equilibrated in 6, 3 or 1.6 M urea in the presence of 2 mM CaCl₂. The relative fractional exchanges of three independent replicates were calculated for each peptide at each time point and plotted as a function of peptide position (CyaA peptide maps are shown in figures S3 and S4). In 6 M urea (Figure 3B), the protein is totally devoid of dynamic HDX-MS events, confirming that CyaA is completely denatured. In 3 M urea (Figure 3C), a short segment of the hydrophobic region (658-719 in HR) and a large part of the RTX domain (RD, from Block II to the C-terminal extremity of CyaA) exhibit dynamic HDX-MS behavior, while in 1.6 M urea (Figure 3D), part of the catalytic domain (the T25 region of AC), AR and most of HR also has dynamic HDX-MS activity. These results suggest that these regions of CyaA sequentially acquire

structural elements upon progressive urea removal. This sequential folding of CyaA is further highlighted in Supplemental Figure S5. To identify the contributions of calcium on CyaA refolding, similar HDX-MS experiments were repeated in the absence of calcium (Supplemental Figure S6). In these conditions, the entire CyaA polypeptide remains solvent accessible and unfolded at all urea concentrations, even down to 1.6 M. The uptake difference plots between CyaA samples in the presence of calcium or EDTA at various urea concentrations are compared in Figure 4 (and Supplemental Figures S7-S9 for a detailed presentation of the HDX-MS data). These HDX-MS results show that the presence of calcium is critical for the acquisition of structural elements in CyaA at 3 M and 1.6 M urea. This further indicates that calcium binding to the RTX motifs is critical not only to induce folding of the C-terminal RD domain (see the effect of calcium versus EDTA at 3 M urea in Figure 4C), but also for the structuration of all of the upstream N-terminal domains of CyaA (see the effect of calcium versus EDTA at 1.6 M urea in Figure 4D).

Similar HDX-MS experiments were also performed on pro-CyaA (Supplemental Figure S10) and revealed a comparable sequential refolding process with RD acquiring structures at intermediate urea concentrations (3 M), followed by the refolding of AC and HR regions at lower urea concentrations (1.6 M) (Supplemental Figures S10 and S11). As in CyaA, several regions of pro-CyaA display non-dynamic HDX-MS throughout, indicating that these regions remain largely unstructured, even at low urea concentrations. These include a large stretch of amino acids from the C-terminal end of the catalytic domain (T18 fragment), across the translocation region, TR, and through the N-terminal part of HR, *i.e.*, from residues 345 to 570. Similarly, residues 821 through 981 of the AR domain show non-dynamic HDX-MS behavior throughout. Significant differences do exist between the acylated and non-acylated proteins, however, as illustrated in Figure 5, which shows the difference in deuterium uptake between pro-CyaA and CyaA at both 3 M and 1.6 M urea. In 3 M urea (Figure 5B), the differences are confined to a segment of the HR between amino acids 658 and 719. In 1.6 M urea (Figure 5C), further minor structural disparities between pro-CyaA and CyaA are evidenced, extending upstream from the HR to the TR region, through to the N-terminal part of AC, and, conversely, downstream, to the AR region. It therefore seems that the acyl chains at K860 and K983 of CyaA are critically involved in the folding of the segment encompassing residues 658 to 719 (Figures 5B-C). We propose that the hydrophobic effects between the acyl chains and HR restrict the conformational landscape of CyaA, thus favoring a faster refolding towards the folded state, as compared to pro-CyaA. The present data suggests that the segment covering residues circa 660-710 of the HR can act as a hydrophobic folding nucleus, even in the presence of 3 M urea if K860 and K983 are acylated (Figures 3 and 5C). In pro-CyaA, this hydrophobic collapse occur at lower urea concentrations due to the lack of acyl chains (Figure S11), and consequently, the partially folded hydrophobic regions may remain exposed to the solvent, thereby favoring the aggregation of pro-CyaA into multimeric species.

The relative fractional uptake maps of monomeric pro-CyaA and CyaA species (both in the absence of urea) are displayed in Figure 6. Extensive dynamic HDX-MS activity, indicative of secondary structural elements, is observed throughout both proteins. Yet, segments that connect individual sub-domains, such as at the boundary of the AC/TR, HR/AR, and AR/RD domains, appear to be more accessible than the adjacent domains. Equivalent HDX-MS patterns are observed between several domains of pro-CyaA and CyaA monomers as illustrated in the uptake difference plot (Figure 6D), in particular the N-terminal parts of both AC and TR, and the blocks III to V of RD. These data suggest that the folding of these regions is not affected by CyaA acylation. The C-terminal part of TR (circa 454-520) and the N-terminal part of RD (mainly blocks I and II) are weakly but significantly stabilized by the presence of the acyl chains (Figure 6D).

The strongest differences in HDX-MS activity between the two forms of CyaA are found in the hydrophobic and acylation regions (Figures 6D-E). Firstly, peptides from the AR region (i.e. residues between amino acids 798 and 958), appear to be more solvent accessible in pro-CyaA monomer than in the monomeric CyaA toxin (Figures 6B and 6C, respectively). Secondly, significant changes in HDX-MS patterns are observed in the hydrophobic region of CyaA, most notably at peptides 539-546, 571-582, 615-622, 658-672, 682-696 and 686-696 (highlighted in Figures 6D, 6E and S12), corresponding to the four hydrophobic segments of HR (Supplemental Figures S12 and S13). Interestingly, a significant difference in HDX-MS activity is observed in all experimental conditions between pro-CyaA and CyaA for the hydrophobic segment between amino acids 660 and 710 (Figures 5B-C, 6 and S13). This confirms that these hydrophobic segments may indeed play an essential role in the folding process of the acylated toxin, from the initial structural elements observed at 3 M urea (Figures 3 and 5) to the native state of the monomeric CyaA (Figure 6). The HDX-MS data provides direct evidence that CyaA acylation favors a hydrophobic collapse of these regions that may constitute the apolar core of the protein structure.

DISCUSSION

Acylation is a requisite PTM for the cytotoxicity of RTX toxins, but the precise contribution of acyl chains to the structure and function of these cytolysins still remains largely unknown. We previously suggested that acylation may play a crucial role in the refolding of the *B. pertussis* adenylate cyclase toxin, CyaA, a prominent member of the RTX toxin family, into a monomeric and functional species (41, 47). To gain further insights into the effect of CyaA acylation, we directly compared the biophysical and functional properties of the monomeric acylated and non-acylated forms (CyaA and pro-CyaA, respectively) of this toxin.

MS analysis indicates that CyaA produced in *E. coli* in the presence of the CyaC acyltransferase is mainly modified by C16 acyl chains on K860 and K983, and, as expected, pro-CyaA is not. AUC experiments demonstrate that CyaA monomers are more compact than their pro-CyaA counterparts, while ANS binding studies show that hydrophobic regions are

less solvent-exposed in CyaA than in pro-CyaA. This indicates that the presence of acyl chains in CyaA may favor a more compact structural state. Furthermore, urea-induced unfolding experiments suggest that the acylated toxin is more stable than pro-CyaA. All together, our AUC and fluorescence data shows that several hydrophobic regions may be more solvent-exposed in pro-CyaA than CyaA, leading to a less compact and stable protein.

We exploited HDX-MS to unravel the folding process of both pro-CyaA and CyaA by analyzing their structural dynamics at different urea concentrations. These studies lead to two important conclusions. Firstly, we show that in both forms of the protein, *i.e.*, independently of the acylation-status, the C-terminal RTX-containing domain RD is the first region to acquire structure when the refolding is carried out in the presence of calcium (Figures 3, 4 and S5-11). More importantly, we found that in the absence of calcium the entire protein remains essentially unstructured even after lowering urea concentrations down to 1.6 M urea, as indicated by the absence of any significant solvent-protection across the entire CyaA polypeptide chain (Figures S6-S9 and 4D). In marked contrast, in the presence of calcium, most CyaA domains do contain structural elements at 1.6 M urea and in the absence of urea, as evidenced by HDX-MS (Figures 3D, 4D, 6C, S5A and S9). Our previous studies already established that the RTX domain of CyaA, RD, (residues 1006-1706) is intrinsically disordered in the calcium-free apo-state and folded in the calcium-bound holo-state (4, 31-39, 41, 42, 59). A likely scenario is that, in the absence of calcium, the intrinsically disordered nature of RD within the full-length protein may result in a huge entropic penalty that prevents folding of the other CyaA domains. This data has direct implications for CyaA secretion. We previously suggested that in the low calcium environment of the bacterial cytosol, RD adopts disordered conformations favorable for transport through the type 1 secretion machinery, and folds upon binding calcium in the extracellular, calcium-rich environment (4, 32, 34, 39, 41, 42). Our present data now extends this model to the full-length CyaA toxin. Indeed, our HDX-MS results clearly indicate that in the low calcium environment of the bacterial cytoplasm, it is not only the RD domain, but the CyaA polypeptide chain as a whole, that preferentially adopts unstructured conformations that fosters protein secretion through the type 1 secretion channel. Upon secretion, as the protein reaches the calcium-rich extracellular medium, RD binds calcium, folds, and may then act as a scaffold for the refolding of the upstream domains of CyaA that are progressively exiting the T1SS machinery in a C- to- N-terminal vectorial secretion process (39-41).

The second major finding of this study is that acylation plays a critical role in the folding of CyaA into a functional cytotoxic state. HDX-MS revealed dramatic differences in deuterium uptake between the acylated and non-acylated CyaA, mainly located in the hydrophobic and acylation regions (Figures 5, 6, S5, S11 and S12). More specifically, we identified a segment (circa residues 660-710) within the hydrophobic region and distal from the acylation sites, which appears to play a central role in toxin refolding (Figures 5B-C). We propose that in CyaA, this region may directly interact and collapse with the distant acyl chains, in a process driven by hydrophobic effects. The formation of these structural elements

may orient the initial CyaA folding pathway and favor toxin refolding into the monomeric state. In contrast, pro-CyaA may have to explore a broader conformational landscape due to the absence of the initial hydrophobic collapse between residues 660-710 and the acyl chains. Thus, from a kinetic point of view, the acyl chains may favor a faster folding of CyaA as compared to pro-CyaA. This could also explain the higher propensity of pro-CyaA to aggregate, and consequently the lower yield of pro-CyaA monomers compared to CyaA during the refolding process (Supplemental Figure S2).

We propose the following calcium- and acylation-dependent refolding scheme for CyaA: as CyaA exits from the T1SS, calcium-binding triggers refolding of the C-terminal RTX domain. As previously proposed, the calcium-loaded, folded RTX domain might act as a scaffold for the folding of other CyaA domains, i.e., AR and HR (Figures 3, 4 and S7-S9). The acyl chains attached to K860 and K983 foster a hydrophobic collapse within the apolar segment spanning residues 660-710 (Figures 5 and S13), which acts as a nucleus for the folding into the native state (Figure 6D) of both HR (residues 528-710) and AR (711-1005). Finally, TR and AC are secreted and, interestingly, their folding appears minimally affected by the acylation status. This is in agreement with the fact that the isolated AC and AC-TR polypeptides can autonomously fold in solution (15, 16, 24). Taken together, the calcium-induced folding, coupled with hydrophobic effects between distal regions containing apolar segments and acyl chains, is likely shared with other multi-domain proteins. This is probably the case of other large RTX cytotoxins like CyaA, which are secreted in an unfolded state and refold in the host environment to exert their virulence (3, 4).

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AUTHOR CONTRIBUTIONS

A.C. designed the project. D.O.B., T.D., M.M., S.B., and A.C. designed the experiments. D.O.B., S.E.C., A.V., M.D., T.D., S.B., and A.C. performed the experiments. D.O.B., S.E.C., T.D., M.M., S.B., D.L. and A.C. analyzed the data. D.O.B., S.E.C., S.B., D.L., and A.C. wrote the manuscript. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

The Supplemental Information file contains 13 Supplemental Figures and 2 Supplemental Tables.

FIGURE TITLES AND LEGENDS

Figure 1: Schematic representation of the multi-domain CyaA toxin (1706 amino acid residues). The toxin contains the following domains: a N-terminal adenylate cyclase catalytic domain (AC, residues 1-364), a translocation region (TR, residues 365-527), a hydrophobic region (HR, residues 528 to 710), an acylation region (AR, residues 711-1005 with acylated Lys 860 and Lys 983 marked with yellow stars), and a C-terminal RTX domain (RD, 1006-1706), composed of five consecutive blocks.

Figure 2: Macroscopic characterization of CyaA and pro-CyaA monomeric species.

Panel A shows the emission fluorescence spectra of ANS in the absence (black) and in the presence of CyaA (blue) and pro-CyaA (red) monomers. Panel B shows the distribution of sedimentation coefficients $c(s)$ of CyaA (blue) and pro-CyaA (red) monomers measured by analytical ultracentrifugation (AUC). The AUC data are presented in Supplemental Table S2. Panel C shows the urea-induced unfolding of CyaA (blue) and pro-CyaA (red) monomers followed by tryptophan fluorescence (s.d.: ± 1 nm). The variation of maximum wavelength emission (λ_{max}) between CyaA and pro-CyaA is highlighted in panel D. Buffer: buffer A, complemented with 2 mM $CaCl_2$. These experiments were performed in triplicate.

Figure 3: HDX-MS analysis of CyaA refolding. The structural organization of each domain is displayed in Panel A and fits to the peptide maps shown in panels B, C and D. The 5 blocks of RD are sized to fit to the peptide maps shown in panels B, C and D. The HDX-MS behavior of CyaA in various concentrations of urea and in the presence of 2 mM calcium is displayed in Panels B to D. At 6 M urea (B), no dynamic HDX-MS behavior is observed throughout the protein. At 3 M urea (C), only RD and a small section of HR display dynamic activity. At 1.6 M urea (D), the AC, HR and RD domains exhibit dynamic HDX-MS events. Each dot corresponds to the average value of three independent replicates.

Figure 4: Deuterium uptake differences of CyaA in urea in the presence and absence of calcium.

The structural organization of each domain is displayed in Panel A and fits to the peptide maps shown in panels B, C and D. The fractional uptake difference plots were calculated at each urea concentration by subtracting the uptake measured in the absence (2 mM EDTA) and the presence of calcium (2 mM $CaCl_2$) for each peptide and at each time point (Panels B-D). At 6 M urea, no difference of HDX-MS behavior is observed throughout the protein. At 3 M urea (C), only RD displays significant positive difference of deuterium uptake, indicative of a calcium-induced reduction of solvent accessibility, i.e., protein folding in the presence of calcium. At 1.6 M urea (D), the AC, AR and RD domains show a positive difference of deuterium uptake, indicating that the presence of calcium induces the folding of these domains. Each dot corresponds to the average value of three independent replicates.

Figure 5: Unique differences in HDX-MS behavior between pro-CyaA and CyaA in the presence of calcium at 3 and 1.6 M urea. The structural organization of each domain is displayed in Panel A and fits to the peptide maps shown in panels B and C. Panels B-C display the uptake difference plot between pro-CyaA and CyaA proteins at 3 M and 1.6 M urea concentrations, respectively. Only a difference in HR was observed (highlighted in red boxes). Each dot corresponds to the average value of three independent replicates. Panel C shows peptides from HR with the greatest deuterium uptake differences between the two proteins. Errors bars (SD) are within the size of the dots.

Figure 6: HDX-MS analysis of monomeric pro-CyaA and CyaA species in solution. The structural organization of each domain is displayed in Panel A and fits to the peptide maps shown in panels B, C and D. The HDX-MS behaviors of full-length pro-CyaA and CyaA monomers measured in the presence of 2 mM calcium are given in Panels B and C. Dynamic HDX-MS are observed throughout both proteins and over the time course of the reaction. The uptake difference plot between both pro-CyaA and CyaA monomers is shown in Panel D. Large differences in HDX-MS activity between the two proteins were observed in HR and AR (red boxes). Smaller changes were found in T18, TR and Blocks I and II of RD. Each dot corresponds to the average value of three independent replicates. Panel E shows selected peptides from HR and AR. Errors bars (SD) are within the size of the dots.

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Figure 1

figure 2

Figure 2

figure 3

figure 4

figure 5

figure 6