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

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ABSTRACT: The adenylate cyclase (CyaA) toxin is a major virulence factor of Bordetella pertussis, the causative agent of whooping cough. 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 provide 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. Our data shed 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.—O’Brien, D. P., Cannella, S. E., Voegele, A., Raoux-Barbot, D., Davi, M., Douché, T., Matondo, M., Brier, S., Ladant, D., Chenal, A. Post-translational acylation controls the folding and functions of the CyaA RTX toxin. FASEB J. 33, 10065–10076 (2019). www.fasebj.org

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Post-translational modifications (PTMs) 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 nonactive precursors that require activation by selective post-translational acylation carried out by dedicated acyltransferases that use an acyl–acyl carrier protein 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), whereas few others have additional cytotoxic activities (see, for instance, the CyaA toxin below). How the acyl chains regulate the biologic 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. The N-terminal CyaA catalytic domain (AC) 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, multidomain protein of 1706 amino-acid residues, with AC (residues 1–364) (16), whereas 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) and membrane permeabilization (25) and exhibits features similar to membrane-active peptides (24, 26, 27). The hydrophobic region (HR) corresponds to residues 528–710, whereas the acylation region (AR) extends from residues 711 through 1005. CyaA is synthesized as an inactive precursor, non-acylated CyaA (pro-CyaA), that is converted into its active form upon specific acylation of 2 lysine residues (Lys 860 and Lys 983) by CyaC, an acyltransferase that catalyzes the transfer of an acyl chain from an acyl–acyl-carrier protein to the ε-amino groups of pro-CyaA lysines (7, 28–30). Finally, the C-terminal 701 residues (1006–1706) correspond to the cell receptor–binding domain, RTX 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 invad 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 and CyaA monomers to provide insights into their folding process and to identify the structural basis of the acylation-dependent gain of functions. Our biophysical data show that the acylated CyaA protein adopts a more compact and stable monomeric state than pro-CyaA, whereas hydrogen/deuterium exchange followed by mass spectrometry (HDX-MS) studies provided direct evidence of long-range interactions between the hydrophobic region and the acylation region. HDX-MS results further indicate that these interactions are strongly favored in the presence of the acyl chains. We propose that these interactions may directly contribute to the folding of the acylated CyaA protein into a compact and functional monomeric state. Our data indicate that calcium-binding and post-translational acylation play a key role in the folding of CyaA into its cytotoxic form.

**Figure 1.** Schematic representation of the multidomain CyaA toxin (1706 aa residues). The toxin contains the following domains: AC (residues 1–364), TR (residues 365–527), HR (residues 528–710), AR (residues 711–1005 with acylated Lys 860 and Lys 983 marked with yellow stars), and a C-terminal RD (1006–1706) composed of 5 consecutive blocks.

**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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 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 and 47). The pro-CyaA urea stock solution was loaded at 2 μM (compared with 5 μM for CyaA) on a size exclusion chromatography column (HiLoad 26/600 Superdex 200 pg, average particle size: 34 μm; column dimensions: 26 × 600 mm; Superdex 200 Increase 10/300 GL, average particle size: 8.6 μm; column dimensions: 10 × 300 mm (GE Healthcare; Chicago, IL, USA); or Agilent BioSEC-5, particle size: 5 μm, pore size: 300 Å, 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 MS**

**Protein digestion**

CyaA (9 μM) and pro-CyaA (7 μM) in 6 M urea buffer were first digested with Lys-C (V1671; Promega, Madison, WI, USA) at a Lys-C/toxin ratio of 1/50 (w/w) at 37°C for 3 h. To ensure optimal trypsin activity, the urea concentration was decreased below 2 M with the addition of 2 volumes of buffer A, and then a second digestion was performed with Trypsin (Sequencing Grade Modified Trypsin V5111; Promega) at an enzyme/toxin ratio of 1/50 (w/w) at 37°C for 1 h. Digestion was stopped by adding formic acid to 2% final concentration. Digested peptides were desalted and concentrated with Pierce C18 Spin Columns (90870; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Peptides were vacuum centrifuged and then lyophilized. Peptides were reconstituted in 2% acetonitrile/0.1% formic acid.

**LC–tandem MS analysis**

A Proxeon EASY-nLC 1000 nanochromatographic system (Thermo Fisher Scientific) was coupled online to an LTQ-Orbitrap Velos Mass Spectrometer (Thermo Fisher Scientific). For each sample, 0.5 μg peptides was directly loaded onto a 15-cm home-made C4 column (5 μm particles, 300 Å pore size, ReproSil-Pur 300 C4; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted by a 1-step gradient from 3.0 to 80% solvent B (80% acetonitrile/0.1% formic acid) in 50 min at a flow rate of 250 nl/min. MS data were 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 collision induced dissociation (CID) fragmentations analyzed in the linear ion trap. The Automatic Gain Control (AGC) target for MS and tandem MS scans was set to 1E6 and 5E3, respectively. The isolation width was set to 2.5 m/z, and the normalized collision energy was set to 30.
energy was set to 35. Selected ions were dynamically excluded for 15 s.

Data analysis

All raw data were analyzed using MaxQuant software v.1.5.1.2 (48) and the Andromeda search engine (49). Based on the protein expression system, data were searched against the complete E. coli strain K12 UniProt (https://www.uniprot.org) database (4331 proteins, downloaded 2015-10-08) and CyaA protein sequence. The digestion mode was set to trypsin, and a maximum of 2 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-acetylation of lysines (Myristoylation, Myristoylation, Palmitoylation, Palmitoylation). The minimum peptide length was fixed to 5 aa, and the required false discovery rate was set to 1% at the peptide spectrum match and protein level. The main search peptide tolerance was set to 4.5 ppm and to 0.5 Da for the tandem MS match tolerance. Second peptides were enabled to identify cofragmentation events. Results of PTM identification are shown in Supplemental Fig. S2A–C.

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

We used 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. The following 2 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, 3, 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. First, pro-CyaA and CyaA were analyzed each in the presence of 6, 3, and 1.6 M urea containing either 2 mM CaCl₂ or 2 mM EDTA; and second, fully folded pro-CyaA and CyaA monomers were analyzed in buffer A, complemented with 2 mM CaCl₂.

HDX-MS sample preparation

Prior to the addition of the deuterated buffer (100% D₂O in 20 mM HEPES, 150 mM NaCl, pH 7.4, supplemented or not with 6, 3, 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 5-fold with the deuterated buffer and incubated for 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 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 2.5. For experiment A (in the presence of urea), the quench was performed by mixing 14 μl of labeled sample with 56 μl 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 μl of labeled sample with 30 μl 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 ~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 processing

Prior to MS analysis, samples were rapidly thawed. To minimize back exchange, the liquid chromatography (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 cooled HDX Manager (Waters, Milford, MA, USA). Samples were digested using an in-house prepared cartridge of immobilized pepsin beads (Thermo Fisher Scientific) for 2 min at 70 μl/min and 20°C. Peptic peptides were rapidly desalted and concentrated using a Vanguard C18 precolumn (1.7 μm, 2.1 × 5 mm; Waters) and separated using an Acquity UPLC BEH C18 column (1.7 μm, 1 × 100 mm). Pro-CyaA and CyaA peptides were separated over a 10-min gradient of 5–40% acetonitrile at 38 μl/min and at 0°C. The LC flow was directed to a Synapt G2-Si HDMS mass spectrometer (Waters), which was equipped with electrospray ionization and lock-mass correction using Glu-Fibrinogen peptide. Mass spectra were acquired in positive-ion mode over the m/z range of 50–1800 using a data-independent acquisition scheme (MS³), whereby exact mass information is collected at both low and high collisional energies for collision-induced dissociation. Unlabeled pro-CyaA and CyaA samples were digested in triplicate to build a peptide coverage map.

HDX-MS data acquisition

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

Urea unfolding of pro-CyaA and CyaA monomers

A volume of 2.5 μl of CyaA or pro-CyaA monomers stored in 20 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂, pH 7.5, was diluted in 20 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂ containing urea at various concentrations to obtain a final concentration of toxin of 50 nM. A total of 22 tubes were prepared with a urea gradient from 0 to 6 M. Once diluted, the toxin was incubated at room temperature for 1 h. Emission spectra of the peptide were acquired on a Jasco FP 8200 spectrophotofluorimeter (Jasco, Tokyo, Japan) using a 3 × 3-mm path length quartz cell (105.251; Q5, Helima Analytics, Plainview, NY, USA). The excitation wavelength was set to 290 nm, both emission and excitation slits were fixed at 5 nm, and fluorescence emission spectra were recorded from 300 to 400 nm. Spectra were acquired at 25°C. The ratio of fluorescence intensities at 340 and 380 nm were plotted against the urea concentration.
ANS binding to CyaA and pro-CyaA monomers

Freshly prepared 8-anilino-1-naphthalenesulfonic acid (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) using either a 3 × 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 analysis of pro-CyaA and CyaA monomers

Sedimentation velocity experiments were performed on a Beckman XL-A analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA) 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 2-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).

Hemolytic and cytotoxic activities of the different CyaA species

Toxin binding and translocation into sheep erythrocytes were assayed essentially as previously described in refs. 52 and 53. CyaA activity was measured as previously described in ref. 54; 1 U CyaA activity corresponds to 1 μmol cAMP formed per min at 30°C and pH 8.0. The different CyaA preparations were diluted directly to final concentrations of 5.6–11 nM (1–2 μg/ml) into suspensions of sheep erythrocytes (5 × 108/ml) in buffer A (20 mM HEPES-Na, pH 7.5, 150 mM NaCl) supplemented with either 2 mM CaCl2 or 1 mM EDTA and incubated at 30°C for 20 min. An aliquot was removed to determine the total CyaA activity added to each sample. The cell suspensions were chilled on ice and centrifuged at 4°C, and the pelleted cells were resuspended at 5 × 108 cells/ml. The hemolytic activity of the different CyaA species was determined in the absence of urea (41, 47), thus allowing us to investigate the relationship between the structure and function of these 2 species. The method is based on molecular confinement to prevent intermolecular interactions between HRs of proteins upon their refolding. Refolding is performed on a size-exclusion chromatography column used as molecular confinement medium comprising a matrix characterized by small size particles (see Materials and Methods for details and Supplemental Fig. 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

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 (Fig. 1) were overexpressed in Escherichia coli, the latter by coexpression of pro-CyaA and CyaC (52, 55, 56), 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 (see Supplemental Fig. S1A–C). Two peptides (858–872 and 972–984) containing the K860 and K983 residues were identified. Both residues were primarily palmitoleylated (C16:1) in CyaA (Supplemental Fig. S1A, B). Palmitoylation (C16:0) was also identified on both K860 and K983 residues. Traces of myristoylation (C14:0) were observed on K983. Interestingly, residues K860 and K983 were always detected as modified in the CyaA sample, suggesting that the 2 lysine residues are fully acylated (see Supplemental Fig. S1D). However, we cannot rule out that a small fraction of CyaA remains unmodified, but the abundance of such peptides appears too low to be detected by the latest generation of the LC-MS Orbitrap instrument. Finally, as expected, no acylation was detected on K860 or K983 in pro-CyaA (Supplemental Fig. S1C). In summary, CyaA is primarily post-translationally modified with C16 acyl chains on both lysine residues, whereas pro-CyaA is devoid of PTMs.

Refolding and functional activities of CyaA and pro-CyaA

Because of 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 (Supplemental Fig. 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 2 species. The method is based on molecular confinement to prevent intermolecular interactions between HRs of proteins upon their refolding. Refolding is performed on a size-exclusion chromatography column used as molecular confinement medium comprising a matrix characterized by small size particles (see Materials and Methods for details and Supplemental Fig. 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 compared with that of acylated CyaA (see Supplemental Fig. S2 and Materials and Methods for details).

The biologic 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, 52, 53). In contrast, the multimeric CyaA species exhibited strongly reduced hemolytic activity and was not able to translocate into erythrocytes, whereas the pro-CyaA species were totally lacking hemolytic and cell-invasion capacities (Supplemental Table S1), which is in agreement with previous studies (55, 57). This confirms that the monomeric, acylated CyaA is the physiologic competent state of the toxin that is able to invade eukaryotic target cells.

### Compactness and solvent-exposed HRs 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 CaCl$_2$. Though a slight increase in ANS fluorescence intensity was observed in the presence of CyaA monomers, a marked increase (~3-fold) was found with the pro-CyaA monomers, suggesting that these latter expose more hydrophobic regions to the solvent than the former (Fig. 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 analytical ultracentrifugation (AUC). Both species remain essentially monomeric, with only a few aggregates observed on the time scale of the experiment (Fig. 2B). The analysis provided a sedimentation coefficient of 7.4 and 7.1 S, respectively, for CyaA and pro-CyaA monomers (Fig. 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 (Fig. 2C) as highlighted by the plot of the maximum wavelength differences between the 2 proteins (Fig. 2D). Taken together, these results indicate that pro-CyaA monomers expose more hydrophobic regions to the solvent (Fig. 2A) and are less compact (Fig. 2B) and stable (Fig. 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 CaCl$_2$. The relative fractional exchanges of 3 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 Supplemental Figs. S3 and S4). In 6 M urea (Fig. 3B), the protein is totally devoid of dynamic HDX-MS events, confirming that CyaA is completely denatured. In 3 M urea (Fig. 3C), a short segment of HR (658–719 in HR) and a large part of RD (from Block II to the C-terminal extremity of CyaA) exhibit dynamic HDX-MS behavior, whereas in 1.6 M urea (Fig. 3D), part of the catalytic domain (the T25 region of AC), AR, and most of HR also have 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 Fig. S5. To identify the contributions of calcium on CyaA refolding, similar HDX-MS experiments were repeated in the absence of calcium (Supplemental Fig. 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 Fig. 4 (and Supplemental Figs. 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 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 vs. EDTA at 3 M urea in Fig. 4C) but also for the structuration of all of the upstream N-terminal domains of CyaA (see the effect of calcium vs. EDTA at 1.6 M urea in Fig. 4D).

Similar HDX-MS experiments were also performed on pro-CyaA (Supplemental Fig. 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 Figs. 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 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 nondynamic HDX-MS behavior throughout. However, significant differences do exist between the acylated and nonacylated proteins, as illustrated in Fig. 5, which shows the difference in deuterium uptake between pro-CyaA and CyaA at both 3 and 1.6 M urea. In 3 M urea (Fig. 5B), the differences are confined to a segment of the HR between amino acids 658 and 719. In 1.6 M urea (Fig. 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 658–719 (Fig. 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 toward the folded state compared with pro-CyaA. The present data suggest 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 (Figs. 3 and 5C). In pro-CyaA, this hydrophobic collapse occurs at lower urea concentrations because of the lack of acyl chains (Supplemental Fig. S11), and consequently, the partially folded HRs 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 Fig. 6. Extensive dynamic HDX-MS activity, indicative of secondary structural elements, is observed throughout both proteins. Yet, segments that connect individual subdomains, 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 (Fig. 6D), particularly the N-terminal parts of both AC and TR, and the blocks III–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 (Fig. 6D).

The strongest differences in HDX-MS activity between the 2 forms of CyaA are found in the HRs and ARs (Fig. 6D, E). First, 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 (Fig. 6B, C, respectively). Second, significant changes in HDX-MS patterns are observed in the HR of CyaA, most notably at peptides 539–546, 571–582, 615–622, 658–672, 682–696, and 686–696 (highlighted in Figs. 6D, E and Supplemental Fig. S12), corresponding to...
insights into the effect of CyaA acylation, we directly structure and function of these cytolysins still remains structural elements observed at 3 M urea (Figs. 3 and 5) to maps; the 5 blocks of RD are sized to segment between amino acids 660 and 710 (Figs. 5 HDX-MS activity is observed in all experimental condi-

Figure 3. HDX-MS analysis of CyaA refolding. A) The structural organization of each domain is displayed. B–D) Fits to the peptide maps; the 5 blocks of RD are sized to fit to the peptide maps shown. The HDX-MS behavior of CyaA in various concentrations of urea and in the presence of 2 mM calcium is displayed. 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 3 independent replicates.

the 4 hydrophobic segments of HR (Supplemental Figs. 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 (Figs. 5B, C and 6 and Supplemental Fig. 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 (Figs. 3 and 5) to the native state of the monomeric CyaA (Fig. 6). The HDX-MS data provide 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 cytolsins still remains largely unknown. We previously suggested that acylation may play a crucial role in the refolding of the B. pertussis 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 nonacylated 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, whereas 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. Together, our AUC and fluorescence data show that several hydrophobic segments 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 2 important conclusions. First, 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 (Figs. 3
Figure 4. Deuterium uptake differences of CyaA in urea in the presence and absence of calcium. A) The structural organization of each domain is displayed. B–D) Fits to the peptide maps are shown. 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 CaCl₂) for each peptide and at each time point. C, 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 3 independent replicates.

and 4 and Supplemental Figs. S5–S11). 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 (Fig. 4D and Supplemental Figs. S6–S9). 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 (Figs. 3D, 4D, and 6C and Supplemental Figs. S5A and S9). Our previous studies already established that 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. These data have 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 extend 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 foster 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 TISS 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 CyaA and pro-CyaA, mainly located in the hydrophobic and ARs (Figs. 5 and 6 and Supplemental Figs. S5, S11, and S12). More specifically, we identified a segment (circa residues 660–710) within the HR and distal from the acylation sites, which appears to play a central role in toxin refolding (Fig. 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 because of 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 compared with pro-CyaA. This could also explain the higher propensity of pro-CyaA to aggregate and, consequently, the lower yield of pro-CyaA monomers compared with CyaA during the refolding process (Supplemental Fig. S2).

We propose the following calcium- and acylation-dependent refolding scheme for CyaA: as CyaA exits from the TISS, calcium binding triggers refolding of the C-terminal RD. As previously proposed, the calcium-loaded, folded RD might act as a scaffold for the folding of other CyaA domains (i.e., AR and HR) (Figs. 3 and 4 and Supplemental Figs. S7–S9). The acyl chains attached to K860 and K983 foster a hydrophobic collapse within the apolar segment spanning residues 660–710 (Fig. 5 and Supplemental Fig. S13), which acts as a nucleus for the folding into the native state (Fig. 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 multidomain proteins. This is probably the case of other

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. A) The structural organization of each domain is displayed and fits to the peptide maps. B, C) Peptide maps and the uptake difference plot between pro-CyaA and CyaA proteins at 3 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 3 independent replicates. D) Peptides from HR with the greatest deuterium uptake differences between the 2 proteins. Errors bars (si) are within the size of the dots.
large RTX cytolysins 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. Chenal designed the project; D. P. O’Brien, T. Douché, M. Matondo, S. Brier, and A. Chenal designed the experiments; D. P. O’Brien, S. E. Cannella, A. Voegele, M. Davi, T. Douché, S. Brier, and A. Chenal performed the experiments; D. P. O’Brien, S. E. Cannella, T. Douché, M. Matondo, S. Brier, D. Ladant, and A. Chenal analyzed the data; D. P. O’Brien, S. E. Cannella, S. Brier, D. Ladant, and A. Chenal wrote the manuscript; and all authors discussed the results and commented on the manuscript.
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