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**RTX Calcium Binding Motifs Are Intrinsically Disordered in the Absence of Calcium**

**IMPLICATION FOR PROTEIN SECRETION**

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The Repeat in Toxin (RTX) motif is a tandemly repeated calcium-binding nonapeptide sequence present in proteins that are secreted by the type I secretion system (T1SS) of Gram-negative bacteria. Here, we have characterized the structural and hydrodynamic properties of the RTX Repeat Domain (RD) of the CyaA toxin from *Bordetella pertussis*. This 701-amino acid long domain contains about 40 RTX motifs. We showed that, in the absence of calcium, RD was natively disordered, weakly stable, and highly hydrated. Calcium binding induced compaction and dehydration of RD, along with the formation of stable secondary and tertiary structures. The calcium-induced conformational switch between unfolded conformations of apo-RD and stable structures of holo-RD is likely to be a key property for the biological function of the CyaA toxin: in the low calcium environment of the bacterial cytosol, the intrinsically disordered character of the protein may facilitate its secretion through the secretion machinery. In the extracellular medium, calcium binding can then trigger the folding of the polypeptide into its functional state. The intrinsic disorder of RTX-containing proteins in the absence of calcium may thus be directly involved in the efficient secretion of proteins through T1SS.

The type I secretion system (T1SS)3 is one of the major export machineries that are used by Gram-negative bacteria to secrete proteins into their external medium (1, 2). This machin-
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through the T1SS, without being per se part of the secretion signal (13–15). It has been suggested that the RTX motifs may serve as a flexible hinge between the secretion signal, and the upstream domains of the substrate. Besides, it is generally assumed that the newly synthesized polypeptide chain has to adopt a loosely folded conformation compatible with the passage through the narrow channel of the T1SS (6). Given the low calcium concentration within the bacterial cytosol, it has been hypothesized that, inside bacteria, the RTX sequences could adopt an unfolded conformation favorable for the secretion of the protein substrate (1, 2, 5).

To provide direct experimental evidences for this model, we have examined the conformational flexibility of the RTX motifs from the adenylate cyclase toxin (CyaA) of B. pertussis, the causative agent of whooping cough (16, 17). CyaA, one of the major virulence factors of B. pertussis, is secreted by a dedicated T1SS, made of the CyaB, CyaD, and CyaE proteins, and is able to invade eukaryotic target cells, where upon activation by calmodulin, it produces supraphysiological levels of cAMP. CyaA is a 1706-amino acid long protein that contains in its C-terminal residues 1006–1706) about 40 RTX motifs organized in five successive blocks, each consisting of 8–10 RTX motifs, separated by linkers of variable length (from 23–49 residues). Here, we have characterized the conformational, thermodynamic, and hydrodynamic properties of the apo (Ca<sup>2+</sup>-free) and holo (Ca<sup>2+</sup>-bound) forms of the CyaA RTX domain (RD) by various biophysical approaches. We showed that at low calcium concentrations, similar to those prevailing in the extracellular medium, the protein exhibits a stable and compact β-sheet conformation. The biological relevance of these findings for the secretion by the T1SS of CyaA and, more generally, of RTX-containing protein substrates will be discussed.

EXPERIMENTAL PROCEDURES

Materials—Hepes-d18 (D18, 98%, DLM-3786–0) was purchased from Cambridge Isotope Laboratories. D<sub>2</sub>O (D215B), NaOD, and DCl were from Euriso-top (C.E.A. Saclay, Gif-Sur-Yvette, France). Experiments were done in 5 mM Hepes, 150 mM NaCl, pH 7.3 (buffer A), at 37 °C unless stated otherwise.

Protein Preparation—The RD of CyaA corresponds to residues 1006–1706 of CyaA. The RD protein was overproduced in E. coli and purified by Ca<sup>2+</sup>-dependent phenyl-Sepharose chromatography as described previously (10). After hydrophobic interaction chromatography, RD was further purified by size exclusion chromatography on Sephacryl S300 (20 mM Hepes, pH 7.3, 150 mM NaCl) and by ion exchange chromatography (IEC) on Q Sepharose. Elution with 20 mM Hepes, pH 7.3, 500 mM NaCl at room temperature. The IEC elution buffer was finally exchanged against 5 mM Hepes, pH 7.3 on a G25SF column. The protein solution was stored at −20 °C or dialyzed against 10 mM NH₄HCO₃ and lyophilized. We checked by CD, fluorescence, and trypsin partial digestion that the lyophilization process did not affect the calcium-induced conformational changes of RD. Protein batches were analyzed by SDS-PAGE, N-terminal sequencing, and by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS model PCS 4000, Ciphergen). A molar mass of 72,621 g mol⁻¹, and a molar extinction coefficient of 72000 M⁻¹cm⁻¹ were computed from the amino acid sequence of RD.

Circular Dichroism Spectroscopy—CD spectra were recorded on an Aviv circular dichroism spectrometer model 215, equipped with a water-cooled Peltier unit. CD measurements were carried out in a 1-mm path length Suprasil cell (111.QS, Hellma) at a scan rate of 0.5 nm/sec (step: 0.5 nm and integration time: 1 s) with a time constant of 100 ms and a bandwidth of 1 nm. Four scans were averaged to obtain each spectrum. The spectrum of buffer A was subtracted to all spectra. The CD units used are the mean residue ellipticity (MRE), expressed in degrees square centimeter per decimol of residue ((deg cm<sup>2</sup>/dmol-res) and calculated from the relationship in Equation 1,

\[
[\theta]_r = (100\theta_m)/(C/N),
\]

(Eq. 1)

where \(\theta_m\) is the measured ellipticity in degrees, C is the concentration in moles per liter, l is the path length of the cell in centimeter, and N is the number of residues. The value 100 arises from the conversion of the concentration in mole per liter to decimole per cubic centimeter.

Fluorescence Spectroscopy—Thermal-induced denaturation of RD at various calcium concentrations was followed by fluorescence spectroscopy. Measurements were performed with an FP-6200 spectrofluorimeter (Jasco) in a Peltier-thermostatted cell holder, using a 1-cm path length quartz cell (111.QS, 110.QS) with constant stirring. The tryptophan emission spectra of RD at a concentration of 1 μM at different calcium concentrations (from 0 to 2 mM) were recorded from 300 to 400 nm (excitation at 292 nm at a scan rate of 125 nm min⁻¹) as a function of temperature, ranging from 10 to 98 °C, with a temperature increment of 1 °C between each spectrum. A bandwidth of 5 nm was used for both excitation and emission beams. The ratio of fluorescence intensities (rFI) at 360 nm and 320 nm (rFI<sub>360</sub>/rFI<sub>320</sub>) was used for both excitation and emission beams. The ratio of fluorescence intensities (rFI) at 360 nm and 320 nm (rFI<sub>360/320</sub>) was used to determine the fraction of unfolded protein rFI<sub>U</sub> using the relation: 

\[
f_{U} = \left( \frac{N}{N + U} \right) (1 + \left( \frac{U}{N} \right))^{-1} = (1 + K_T)^{-1}
\]

(Eq. 2)

where U is the concentration of RD in the thermal-unfolded state and \(K_T\) the equilibrium constant at the given temperature: 

\[K_T = \frac{U}{N}\]

The equilibrium constant \(K_T\) is related to the free energy \(\Delta G\) according to 

\[K = \exp(-\Delta G/RT)\]

and in the vicinity of the half-melting point \(T_{1/2}\), the fraction of native RD, \(r_{N}\), is related to the Van’t Hoff enthalpy \(\Delta H_{1/2}\), according to Equation 3,

\[r_N = \left(1 + \exp\left(\frac{\Delta H_{1/2}}{RT} \times \left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right)\right)^{-1}
\]

(Eq. 3)

Fittings were done with Kaleidagraph (Synergy Software, Reading, PA).
Nuclear Magnetic Resonance Spectroscopy—Purified RD in 20 mM Heps, 2 mM EDTA was buffer-exchanged against 10 mM ammonium bicarbonate (NH₄HCO₃) on prepacked G25SF desalting columns at room temperature and freeze-dried. Two cycles of resuspension of the lyophilized protein in D₂O and freeze-drying were performed to exchange amide protons and to reduce H₂O concentration. Buffers and guanidine hydrochloride (GdnHCl) were D₂O-exchanged twice by repeated freeze-drying and resuspension. Apo-RD samples were prepared by dissolving lyophilized RD in 5 mM Hepes-d18, 150 mM NaCl, pH 7.5, prepared in D₂O (99.99%), supplemented or not with 5.3 mM GdnHCl. Holo-RD samples were prepared from apo-RD samples by addition of 2 mM CaCl₂. Protein concentrations ranged between 55 and 75 μM.

NMR experiments were conducted at 37°C on an Inova 600 (Varian Inc., Palo Alto) spectrometer with a 14.1 Tesla magnetic field, and equipped with a cryoprobe. Spectra were recorded, processed and analyzed using Vnmr 6.1C (Varian). Water signal was suppressed by low-power irradiation during the 2 s recovery delay or using the jump-return (21) or double pulse-field gradient stimulated-echo (22) schemes. Proton spectra were acquired with 4096 complex points and a sweep width of 12 ppm. Transverse relaxation times (T₂) were obtained from standard spin-echo experiments that used short (≤4 ms) variable τ relaxation delays (π/2-τ-π/2) to safely neglect J-coupling evolution.Bulk T₂ values were estimated by fitting the data of the aliphatic region (below 3.2 ppm) to a single exponential decay.

One-dimensional saturation transfer experiments were performed by selective saturation (1.7 ppm) of the aromatic region using trains of 90° gaussian pulses centered at 7.1 ppm. Thirty-two scans were accumulated for each saturation time, which varied between 0 and 1.7 s. An identical set of experiments with off-resonance saturation was used as reference. The nuclear Overhauser effect (nOe) between aromatic and aliphatic protons was calculated from the intensities of the aromatic region (upfield of 2.5 ppm) of the on- (Iₗ) and off- (Iᵦ) resonance experiments as follows: nOe = [Iₗ - Iᵦ]/Iᵦ.

Intrinsic Viscosity and Molecular Mass Measurements with SEC-TDA—Size exclusion chromatography (SEC) experiments were done on a Superdex 200 column (GE Healthcare) controlled by a GPcmax module and connected on-line to a triple detector array (TDA) model 302 (Viscotek Ltd., Houston, Basingstoke, UK). The oven of the TDA contained (i) a static light scattering cell with two photodiode detectors, at 7° for low angle (LALS) and at 90° for right angle laser light scattering (RALS), (ii) a deflection refractometer, (iii) a photometer, and (iv) a differential viscometer.

The general procedures described by Viscotek were followed. All solutions were filtered on 0.2-μm filters and allowed to equilibrate at 10°C. SEC was performed at 10°C, and detection in the TDA oven was done at 20°C. All experimental sequences contained injections of bovine serum albumin (2 mg/ml, various volumes) and apo-RD or holo-RD (at least four injections of different volumes). Bovine serum albumin injections were used for TDA internal constants calibration. The refractive index increments, dn/dc, were experimentally determined and were similar for both states (dn/dc: 0.184). Buffer A (5 mM Heps, 150 mM NaCl, pH 7.3) was used for apo-RD and buffer A supplemented with 2 mM CaCl₂ for holo-RD. All data were acquired using the Omniscx software.

Protein concentration was determined using both the photometer and the deflection refractometer. The RALS and LALS data, in combination with the concentration, provided the molecular mass M. Intrinsic viscosity [η] was calculated using the differential viscometer, which is made of a balanced four-capillary bridge, an original adaptation of the electrical Wheatstone bridge to fluid materials (23). Both molecular mass and intrinsic viscosity were calculated with the Omniscx software.

Analytical Ultracentrifugation—Sedimentation equilibrium and velocity experiments were performed at 20°C on a Beckman XL-A analytical ultracentrifuge (Beckman Coulter) equipped with an AN60-Ti rotor. The samples were centrifuged at 13,000 × g for 10 min prior to 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. Buffer A supplemented with either 2 mM CaCl₂ or 2 mM EDTA was used for holo-RD or apo-RD, respectively. The buffer viscosity η and density ρ, calculated with Sednterp 1.09 were η = 1.016 cP and 1.00456 g/ml. For sedimentation equilibrium experiments, protein samples (40 μl, 2.5 μM) were loaded in a 1.2-mm thick six channels epon centerpiece. Apo-RD samples were centrifuged successively 3 h at rotor speeds of 10,000 rpm, 2 h at 12,000 rpm, and then 2 h at 18,000 rpm. Holo-RD samples were centrifuged 3 h at 18,000 rpm, 2 h at 22,000 rpm, and 2 h at 26,000 rpm. Data were recorded for each speed after controlling that the sedimentation/diffusion equilibrium had been effectively reached. The baseline was measured at 42,000 rpm after 2 h. Radial distributions were analyzed by global fitting of the three speeds using the one species model of the Ultrascan 9.5 software. Partial specific volumes were obtained by fixing the molecular mass to the computed mass of the monomer.

For sedimentation velocity experiments, the protein samples (300 μl at 2.5, 7, 15, and 25 μM) were loaded in 1.2-mm thick epon double sector cells and spun at 35,000 rpm. Sedimentation velocity profiles were monitored at 3-min intervals. Data were analyzed with the Sedfit 11.3 software using a continuous size distribution c(s) model with invariant diffusion coefficient D (24).

Quasi-elastic Light Scattering—QELS experiments were performed on a DynaPro MS800 (Wyatt), using the Protein Solution Dynamics software version 6.2.05. The laser power was 100%, and its incident light wavelength was λ_QELS = 824.7 nm. Buffers and samples were filtered on 0.2-μm filters prior to acquisition. Protein concentration was routinely 5 μM. A microcuvette of dimensions 3 × 8.5 mm (105.251 QS) was loaded with 100 μl of RD. Samples were thermally equilibrated for 10 min in the cell compartment at 20°C. Acquisition time was 10 s, with an interval time of 1 s. At least 30 acquisitions were averaged to produce a data collection. A set of three independent data collections was obtained for each experimental condition. The data were processed with SEDFIT 9.2 and analyzed using: (i) Continuous Hydrodynamic Radius Distribution and (ii) Stokes Radii models. The maximum entropy and the Tikhonov-Phillips 2nd derivative regularization procedures
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were used with a confidence level (F-ratio) of 0.55. The hydrodynamic radius, \( R_D \), was calculated from the Stokes-Einstein equation \( R_D = \left( k_B T / 6 \pi \eta D_T \right)^{1/2} \), where \( k_B \) is the Boltzmann’s constant, \( T \) the temperature, and \( \eta \) the viscosity of the solvent. The frictional ratio from quasi-elastic light scattering is given by \( f/f_0 = R_D/R_S \), where \( R_S \) stand for the radius of an anhydrous sphere of the same mass as RD.

**Protein Shape and Hydration**—The viscosity increment \( \nu \) (also called the Simha-Einstein hydrodynamic function) is related to the axial ratio \( a/b \) of an ellipsoid of revolution and can be calculated from the Einstein’s viscosity relation: \( M[\eta] = \nu V_H N_A \), where \( V_H \) is the hydrodynamic volume defined by \( V_H = 4\pi R_D^3/3 \) and \( N_A \) the Avogadro number. Hydration is calculated from the intrinsic viscosity relation described below. The intrinsic viscosity in a defined solvent depends on the shape, the hydration, the molecular volume of the protein, and electroviscous effects. Its expression is the product of the viscosity increment \( \nu \) and the swollen volume \( V_s \) according to \( \eta = \nu \rho V_s + \delta \rho \), from which the hydration parameter \( \delta \) is extracted: \( \delta = [(\eta/\nu) - \nu] \rho \). The swollen volume, \( V_s \) is the sum of the partial specific volume \( \nu \) of the protein (volume occupied by one gram of protein) and the time-averaged apparent hydration \( \delta \) of the protein (volume of water per gram of protein). The hydration parameter of the protein includes: (i) the water molecules bound to the protein and (ii) the water molecules dragged by the diffusion of the protein. The molecular mass \( M \) was measured by SELDI and static light scattering. The partial specific volume \( \nu \) was determined by equilibrium AUC and the translational diffusion coefficient \( D_t \) by velocity AUC and QELS (see above). The viscosity increment provides the axial ratio \( a/b \), using the inversion formulae of the hydrodynamic function \( \nu \) using the parameters for polynomial fit described by Harding and Cölfen (25). The semi axes \( a \) and \( b \), with \( a > b \), describe the shape of the ellipsoid of revolution. The values of \( a \) and \( b \) semi axes for prolate \((a, b, b)\) and oblate \((a, a, b)\) conformations were determined using Ultrascan.

**RESULTS**

**Calcium-induced Conformational Changes of RD**—The RD protein (amino acids 1006–1706 of CyaA; molecular mass of 72.6 kDa) was overexpressed as a soluble polypeptide in *E. coli* and purified to homogeneity (10, 26). The far-UV CD spectrum of the apo-state of RD (apo-RD, in the absence of calcium) was typical of a predominantly unfolded protein, as shown by the strong negative \( n_0 - \pi^a \) band around 200 nm (Fig. 1). Yet, the weak negative \( n_0 - \pi^a \) band, appearing as a shoulder around 220 nm, indicated the presence of some residual secondary structure elements in apo-RD. A very similar spectrum was observed for the protein equilibrated in the presence of 5 M GdnHCl. Upon addition of 2 mM calcium to apo-RD, secondary structures were formed, as revealed by the concomitant intensity decrease of the \( \pi^a - \pi^a \) band and the increase of the \( n_0 - \pi^a \) band in the holo-RD spectrum, as previously reported (10, 26). The structures of holo-RD were readily denatured upon addition of 5 M GdnHCl, leading to a spectrum similar to that of the apo-state.

Proton nuclear magnetic resonance (NMR) was used to further characterize the calcium-induced conformational changes of RD. The one-dimensional spectrum of apo-RD in the presence of the denaturant GdnHCl (5.3 M) showed no evident secondary chemical shifts, with all peaks appearing at the expected frequencies for an unfolded protein, the so-called “random-coil” chemical shifts (Fig. 2A). The low chemical-shift dispersion was consistent with a protein with no stable secondary and tertiary structures. The spectrum of the apo-form under native conditions also showed very poor chemical shift dispersion, and resembled that of the protein denatured in GdnHCl, but with somewhat narrower lines. Noticeably, the bands of the apo-RD spectrum were strikingly narrow for a 701-residue long protein, suggesting that apo-RD had a highly dynamic structure in which most of its side-chains could freely reorient in solution. These observations suggested that apo-RD was mainly natively disordered.

Yet, its NMR spectrum contained few signals that were well dispersed and that appeared at chemical shifts characteristic of a well-ordered conformation with stable tertiary structure (see Fig. 2B). The apo-RD spectrum indeed displayed some downfield shifted H\( \alpha \) resonances (\( \geq 4.8 \) ppm) indicative of \( \beta \)-sheet structures, upfield shifted signals in the methyl region (\( \leq 0.79 \) ppm), as well as several dispersed peaks in the aromatic region (7.7–6.5 ppm) at non-random coil frequencies indicating the presence of stable hydrophobic interactions. From integration of the downfield part of the H\( \alpha \) region and comparison with the whole H\( \alpha \) envelope integration (excluding the residual water signal at 4.62 ppm), we could roughly estimate that apo-RD contained at least \( \sim 7 \% \) of \( \beta \)-sheets. Similarly, integration of the upfield signals in the methyl region (\( \leq 0.79 \) ppm) of the apo-RD spectrum suggested that ca. 17 methyls (i.e. 6% of the 286 Ile/Leu/Val methyls) were implicated in a stable hydrophobic environment that likely involved aromatic residues.

The NMR spectrum of calcium-bound RD was drastically different. First, it exhibited an increase of the envelope of upfield-shifted H\( \alpha \) protons, providing direct evidence that holo-RD was more structured, with a \( \beta \)-sheet content (calculated as above) of at least 30–35%. Second, the signals became
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Calcium-induced Stability Changes of RD

Solvent-exposed Hydrophobic Clusters of RD Probed by ANS Binding—ANS (1-anilino-8-naphthalene sulfonate) fluorescence enhancement was used to probe the presence of organized hydrophobic clusters accessible to the solvent. As shown in Fig. 3, addition of RD, either in the apo- or in the holo-form, only weakly affected the fluorescence intensity and \( \lambda_{\text{max}} \) of ANS, indicating that apo-RD and holo-RD did not expose organized hydrophobic regions to the solvent. Hence, although RD contains many hydrophobic residues mostly exposed to the solvent in the apo-state, they were not organized into hydrophobic surfaces or clusters large enough to bind the ANS probe.

Calcium-induced Stability Changes of RD—Thermal-induced unfolding of RD was followed by intrinsic fluorescence of tryptophan at various calcium concentrations. The ratio of fluorescence intensity was detected upon binding to apo-RD or holo-RD.

The absolute value of the nOe obtained by integration of the aliphatic region (upfield of 2.5 ppm) is shown as a function of the irradiation time (\( t \)) of the aromatic resonances. Curves were fitted by an isolated spin-pair relaxation model: \[ \text{nOe} = \frac{1}{\rho} \left(1 - \exp(-\rho t)\right) \], where \( \rho \) (s\(^{-1}\)) represents the cross-relaxation rate and \( \rho \) (s\(^{-1}\)) the auto-relaxation rate.

In summary, CD and NMR data indicate that the apo-form of RD was largely unfolded with a weak secondary and tertiary structure content, while calcium binding triggered the folding of the protein into a compact and \( \beta \)-rich (>30–35\%) conformation.

Very broad as expected for a compact protein of 72.6 kDa. The line broadening of the \(^1\)H spectrum reflected a major change in the dynamic behavior of the protein upon calcium binding. Indeed, the value of the bulk transverse relaxation time \( T_2 \) (which depends on the overall molecular tumbling and on the internal dynamics of the molecule) of the aliphatic region (\( \leq 3.2 \text{ ppm} \)) of holo-RD (~5 ms) was very short compared with the \( T_2 \) value of the apo-form (~29 ms). As both states were monomeric (see below), the short \( T_2 \) value (characteristic of slow dynamic processes) of holo-RD mainly reflected the compactness of the folded protein, while the \( T_2 \) value of apo-RD most likely resulted from the fast internal motions within the unstructured conformations of the \( \text{Ca}^{2+} \)-free polypeptide. The high \( \beta \)-sheet content of holo-RD, as evidenced by the envelope of upfield-shifted H\( \alpha \) signals, also strongly suggested that the observed line-broadening resulted from a calcium-induced folding-compaction process.

Saturation transfer experiments were performed to further probe the compactness of the different states of RD. The well-isolated envelope of aromatic signals (7.9–6.3 ppm) was selectively saturated for different lengths of time and the intensity of the aliphatic region was followed. Saturation can be transferred by cross-relaxation (nuclear Overhauser effect) to aliphatic protons located at short distances from the aromatic rings. The cross relaxation rate (\( \rho \)) is distance-dependent and can thus be used to evaluate the relative compactness of a protein in different states. As shown in Fig. 2C, The nOe build-up curves obtained for apo-RD in 5.3 M GdnHCl showed practically no transfer of saturation, as expected for the disordered conformations adopted by the protein in these conditions. Apo-RD in the absence of denaturant showed also very limited nOes, albeit significantly higher than that of RD in GdnHCl. In contrast, holo-RD showed a very efficient transfer of saturation: the apparent cross-relaxation rate of the holo-form (\( \rho = 3.1 \pm 0.1 \text{ s}^{-1} \)) was respectively, 14- and 100-fold higher than that of apo-RD or that of apo-RD in the presence of GdnHCl. Therefore, the holo state was much more compact than the apo-form.

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orescence intensities at 360 and 320 nm (rFI360/320) was used to follow the unfolding of RD (Fig. 4A). The van’t Hoff free enthalpy ($\Delta H_v$) and temperature of half-melting ($T_m$) were then determined. As shown in Fig. 4B, both $T_m$ and $\Delta H_v$ increased with calcium concentrations to reach a plateau above 1.5–2 mM. We obtained similar results from thermal-induced denaturation of RD followed by far- and near-UV CD (data not shown). All together these data indicated that apo-RD was unstable, as expected for a disordered state, while holo-RD was strongly stabilized by calcium binding.

**Calcium-induced Hydrodynamic Changes of RD—**The hydrodynamic behavior of apo- and holo-RD was explored using SEC on a Superdex 200 column coupled on-line to a Triple Detector Array (TDA, Viscotek). The equipment included a deflection refractometer and a UV-photometer for concentration measurements, two static light scattering detectors for molecular mass determination and a differential pressure transducer for intrinsic viscosity measurement. As shown in Fig. 5A, holo-RD eluted at a retention volume ($R_v$) of 13.8 ml. This $R_v$ value was close to that expected for a globular protein of 73 kDa ($R_v$ ~ 15 ml). In marked contrast, apo-RD eluted at a retention volume of 10.4 ml, a value corresponding to a globular protein of ~600 kDa. In both cases, the molecular mass determined on-line by static light scattering corresponded to that of the monomeric RD protein (Fig. 5B and Table 1). Hence, the low retention volume of apo-RD was not due to oligomerization. However, the differential pressure transducer intensities for apo-RD and holo-RD were dramatically different (Fig. 5B). From these data, an intrinsic viscosity [$\eta$] of 5.5 ml·g$^{-1}$ was
In agreement with these data, quasi-elastic light scattering (QELS) analysis showed that upon calcium binding, RD was strongly compacted with its hydrodynamic radius ($R_H$) decreasing from about 7 nm to about 3 nm (Table 1).

From the hydrodynamic parameters measured by TDA, AUC, and QELS, we calculated the hydration $\theta$ and the viscosity increment $\nu$ of apo-RD and holo-RD (see “Experimental Procedures” for details). Apo-RD was found to be highly solvated, with a hydration of $\sim 10$ ml$^2$g$^{-1}$, in marked contrast to holo-RD ($\delta \sim 0.4$ ml$^2$g$^{-1}$), which was hydrated like standard folded proteins. The high hydration of apo-RD may explain its low retention volume on SEC, while the change of the viscosity increment $\nu$ between the apo and holo states may reflect a compaction and elongation of RD upon calcium binding (Table 1). Taken together, our results indicate that apo-RD was intrinsically disordered and strongly hydrated, and that calcium binding triggered a massive dehydration of the polypeptide chain that folded into a compact and stable state.

### DISCUSSION

Complementary biophysical approaches were used to describe the conformational, thermodynamic, and hydrodynamic properties of the CyaA RTX domain, RD, in both calcium free (apo) and calcium-bound (holo) states. Static light scattering and equilibrium analytical ultracentrifugation experiments showed that RD was monomeric both in the apo- and holo-state. CD and NMR studies indicated that apo-RD was largely unfolded, with more than 90% of the polypeptide in disordered conformations, in good agreement with previous experiments showing that apo-RD was highly susceptible to proteolysis (26). Furthermore, ANS binding experiments did not reveal the presence of any solvent-exposed hydrophobic clusters in apo-RD, indicating that even hydrophobic residues adopted disordered conformations. Moreover, thermal-induced denaturation experiments revealed a high instability of the apo-state, as compared with the holo-state. Finally, hydrodynamic analysis indicated that apo-RD was highly hydrated. Its retention volume on SEC was remarkably low for a monomeric protein of 73 kDa, corresponding to that of a globular protein of 600 kDa with an $R_H$ close to 7 nm. This unusual behavior resulted mainly from the natively disordered and highly hydrated state of the Ca$^{2+}$-free protein.

### TABLE 1

#### Hydrodynamic parameters of RD at 20 °C

The solvent density and viscosity, partial specific volume and hydration were calculated to be 1.00456 g ml$^{-1}$, 0.01016 poise, 0.7152 ml g$^{-1}$, and 0.432 ml g$^{-1}$, respectively, using the Sednterp software.

| Parameters                      | Units  | Apo-RD ± S.D.       | Holo-RD ± S.D.      |
|---------------------------------|--------|---------------------|---------------------|
| Mass spectrometry molecular mass| g/mol  | 72672 ± 76          | 72638 ± 68          |
| AUC Retention volume            | ml     | 10.4 ± 0.1          | 13.8 ± 0.1          |
| Intrinsic viscosity             | ml/g   | 35.1 ± 1.7          | 5.5 ± 0.3           |
| Molecular mass                  | kg/mol | 73.6 ± 1.6          | 73.2 ± 1.4          |
| AUC equilibrium Partial specific volume | ml/g | 0.788 ± 0.001       | 0.724 ± 0.001       |
| AUC velocity Sedimentation coefficient | S | 2.53 ± 0.24         | 4.54 ± 0.53         |
| Trans. diffusion coefficient    | cm$^2$/s | 3.31E-07 ± 1.90E-08 | 6.69E-07 ± 1.20E-08 |
| Frictional ratio                |        | 2.29 ± 0.12         | 1.17 ± 0.07         |
| Anhydrous radius, $R_o$         | nm     | 2.83                | 2.75                |
| Hydrodynamic radius             | nm     | 6.48 ± 0.35         | 3.21 ± 0.18         |
| QELS Trans. diffusion coefficient | cm$^2$/s | 2.98E-07 ± 1.20E-08 | 6.92E-07 ± 2.20E-08 |
| Hydrodynamic radius             | nm     | 7.2 ± 0.3           | 3.1 ± 0.1           |
| Frictional ratio                |        | 2.54                | 1.13                |
| Hydrodynamic function           |        |                     |                     |
| Viscosity increment             | g/g    | 3.2 ± 0.5           | 5.1 ± 0.3           |
| Hydration                       |        | 10.3 ± 1.6          | 0.36 ± 0.05         |
| Semi axes (a/b)                 |        |                     |                     |
| Prolate ellipsoid               | nanometers | 2.4 ± 0.5           | 4.4 ± 0.2           |
| a                               | nm     | 12.1 ± 1.2          | 8.4 ± 0.3           |
| b                               | nm     | 5.2 ± 0.7           | 1.9 ± 0.1           |
| Oblate ellipsoid                |        |                     |                     |
| a                               | nm     | 2.6 ± 0.7           | 5.6 ± 0.4           |
| b                               | nm     | 9.3 ± 0.4           | 5.5 ± 0.1           |
| Partial specific volume         |        | 0.53 ± 0.1          | 1.0 ± 0.1           |

Intrinsically Disordered RTX Motifs

The one-dimensional NMR spectrum revealed a high content of $\beta$-sheets in holo-RD, in agreement with the previously published parallel $\beta$-roll structures of the RTX-motifs of the *Serratia marcescens* and *Erwinia chrysanthemi* metalloproteases and lipase (11, 12).
Intrinsically Disordered RTX Motifs

All together, our results demonstrate that the RTX domain from *B. pertussis* CyaA adopts natively disordered conformations at submicromolar calcium concentrations, corresponding to that found in the bacterial cytosol, whereas upon exposure to millimolar calcium concentrations, the RD domain acquires a stable tertiary fold rich in β-structures. The calcium-induced conformational switch between the unfolded conformations of the apo-state and the stable structure of holo-RD is likely to be a key property for the biological function of the CyaA toxin. This could allow for a tight coupling between the export of the protein out of bacteria and its folding into a biologically active conformation. For CyaA, the calcium-bound folded state is indeed critical for the interaction of the secreted toxin with the eukaryotic target cells. Prior studies have shown that the RTX domain is directly involved in the interaction of CyaA with the CD11b/CD18 integrin receptor and that this interaction is strictly dependent upon the presence of calcium (27). Besides, calcium is also required for the pore-forming activities of CyaA as well as for the translocation of the CyaA catalytic domain across the plasma membrane of target cells (16, 17).

If the calcium-bound, folded state of the RTX domain of CyaA is the functionally relevant one for the cytoxic activities of the toxin, the natively disordered conformational ensemble of the calcium-free RTX domain is likely to be equally important for the earlier steps of CyaA biogenesis. This unstructured conformation may be adopted by the newly synthesized CyaA polypeptide in the low calcium environment of the bacterial cytosol and may be favorable for the secretion of the protein through the narrow T1SS machinery. The requirement of an unfolded structure for secretion through the T1SS has been experimentally demonstrated for the protein HasA, a hemophore produced by *S. marcescens*, eventhough HasA is one of the few T1SS substrate that has no RTX sequences (28, 29). Moreover, the efficiency of secretion of artificial substrates made by fusion of various passenger proteins to a T1SS secretion signal (such as the the HlyA C-terminal sequence) was found to be inversely correlated with the overall structural stability of the passenger proteins. Hence a loosely folded polypeptide appears to be a prerequisite for an efficient secretion through a T1SS machinery (1, 2).

The results presented here indicate that apo-RD exhibits all the characteristic features of a so-called intrinsically disordered protein (18–20). Intrinsic disorder in proteins is implicated in many biological functions, such as cell signaling and homeostasis regulation. The transition from order to disorder (or reverse) is becoming increasingly recognized as being a fundamental property of many proteins. In many instance the order/disorder transition is coupled to the selective binding of ligands or protein partners. This is indeed the case for the RTX motifs for which the transition from a disordered to a folded and stable state is triggered by calcium binding, a process that occurs concomitantly with the protein secretion to the external medium.

Our present data therefore suggest an important role for intrinsically disordered polypeptides like the RTX motifs in secretion-refolding processes. The presence of short disordered sequences in proteins that are translocated across membranes has been previously documented, in particular in the case of proteins imported into mitochondria or proteins exported to the endoplasmic reticulum (30). In these examples, however, the disordered sequences are restricted to an N-terminal pre-sequence that is recognized by the specific translocation apparatus. These sequences are usually proteolytically removed before the translocated proteins are folded into their active forms. Bacterial flagellar proteins also contain a disordered N-terminal segment of about 60 residues that serves as a secretory signal, but in this case, the segment will fold upon final assembly of the monomer into the flagellar structure at the tip of the filament. Compared with these disordered domains, the RTX domain of CyaA is more than one order of magnitude larger (701 residues) and represents about 40% of the full-length secreted toxin. To our knowledge, this is the largest experimentally characterized disordered protein domain for which the intrinsic disorder is associated with a secretory process. It is remarkable that such a large polypeptide of 700 residues remains perfectly soluble in a disordered state. Indeed, aggregation is one of the most frequent problems encountered by partially unstructured proteins, and is at the origin of many pathologies. Several physico-chemical characteristics of the RTX domain may contribute to prevent protein aggregation in the calcium-free state: (i) the electrostatic repulsion between the highly negatively charged polypeptides (pI = 3.8); (ii) the absence of defined hydrophobic patches in the unstructured apo-state; and (iii) the high hydration of apo-RD (although the exact hydration status in the highly crowded intracellular environment is difficult to estimate).

Besides providing a conformational flexibility essential for the passage through the T1SS, the RTX motifs may also contribute to the secretory process at two additional levels: (i) the negatively charged RTX sequences may be particularly favorable to harness the electrostatic field across the inner membrane as an energy source for the polypeptide export. It is noteworthy that, like CyaA, most T1SS substrates are very acidic proteins with pI in the range of 3.9–4.5; (ii) the spontaneous folding of the RTX domain into a compact and highly stable structure in the presence of calcium may also facilitate the export of the protein according to a molecular ratchet mechanism. Indeed, the calcium-induced folding of individual RTX modules as they reach the external medium, or even in the lumen of the T1SS channel, could prevent any backtracking of the polypeptide chain, thus facilitating a uni-directional movement of the protein toward the outside of the bacterium.

Finally, it is possible that the RTX domain, which is presumably the first part of the protein to be released into the external medium, may also serve as a nucleus for the folding of the remaining part of the polypeptide chain. Interestingly, Pimenta et al. (31) have reported that point mutations in the T1SS apparatus partially affected the secretion of HlyA but, more importantly, dramatically decreased its folding into a helically active molecule. These data suggest an intricate connection between the secretion of this T1SS substrate and its folding into a biologically active form. Further biophysical and structural characterizations of these RTX containing proteins will be necessary to unravel the complexity of the relationships between the secretion, the folding, and the cytoxic activities of this important class of pathogenic factors.
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