Calcium-induced Folding of Intrinsically Disordered Repeat-in-Toxin (RTX) Motifs via Changes of Protein Charges and Oligomerization States*5

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Ligand-induced disorder-to-order transition plays a key role in the biological functions of many proteins that contain intrinsically disordered regions. This trait is exhibited by so-called RTX (repeat-in-toxin) motifs found in many virulence factors secreted by numerous Gram-negative pathogenic bacteria: RTX proteins are natively disordered in the absence of calcium but fold upon calcium binding. The adenylate cyclase toxin (CyaA) produced by Bordetella pertussis, the causative agent of whooping cough, contains ~40 RTX motifs organized in five successive blocks separated by non-RTX flanking regions. This RTX domain mediates toxin binding to its eukaryotic cell receptor. We previously showed that the last block of the RTX domain, block V, which is critical for CyaA toxicity, exhibits the hallmark of intrinsically disordered proteins in the absence of calcium. Moreover, the C-terminal flanking region of CyaA block V is required for its calcium-induced folding. Here, we describe a comprehensive analysis of the hydrodynamic and electrophoretic properties of several block V RTX polypeptides that differ in the presence and/or length of the flanking regions. Our results indicate that the length of the C-terminal flanking region not only controls the calcium-induced folding but also the calcium-induced multimerization of the RTX polypeptides. Moreover, we showed that calcium binding is accompanied by a strong reduction of the net charge of the RTX polypeptides. These data indicate that the disorder-to-order transition in RTX proteins is controlled by a calcium-induced change of the polypeptide charges and stabilized by multimerization.

The past decade has seen a fundamental reappraisal of the protein structure-to-function paradigm because it became evident that a significant fraction of polypeptide sequences code for proteins, commonly designated as natively or intrinsically disordered proteins, that are lacking ordered structures under physiological conditions (1–8). Numerous intrinsically disordered proteins are nevertheless able to acquire ordered conformations upon binding to specific ligands, and in most cases, these disorder-to-order transitions are linked to key functional properties of these proteins. Elucidation of the biochemical factors and physicochemical parameters that control the transition between these different states of the polypeptides is therefore essential to understand the biological functions of intrinsically disordered proteins.

We have recently begun to explore the biophysical mechanisms at work in this process in a newly described class of intrinsically disordered protein that contains so-called RTX repeated motifs and in which the disorder-to-order transition is triggered by the binding of calcium ions (9–11). These motifs are found in many virulence factors secreted by numerous Gram-negative pathogenic bacteria (12, 13). The original name (repeat-in-toxin) was first coined to group four virulence factors secreted by Escherichia coli, Bordetella pertussis, Pasteurella hemolytica, and Actinobacillus sp., that contain a series of tandemly repeated RTX sequences at their C-terminal end. Since then, many other RTX proteins have been identified in more than 250 bacterial strains (14), and they cover a vast array of biological activities, many of them involved in host-pathogen interactions (13, 14). In most cases, the RTX sequences are located at the C-terminal end of the polypeptides, appended to functional domains endowed with specific biochemical activities such as pore-forming cytolytic activity, proteases, lipases, etc. (13, 14). Despite this functional diversity, most known RTX proteins are thought to be secreted by a dedicated type I secretion system and to require calcium to exert their biological activities.

The prototype sequence of the RTX motif is GGXG(N/D)DX(U)X, where X represents any amino acid, and (U) represents any large hydrophobic residue such as Ile, Leu, Val, Phe, and Tyr (13). The number of RTX motifs in RTX proteins may vary from 5 to more than 50. Structural data available on some RTX-containing proteins showed that these sequences fold in the presence of calcium into an idiosyncratic parallel β-helix structure, where the first six residues, GGXGAD, of each RTX motif form a calcium-binding turn and the last three residues XUX make a short β-strand (15, 16). The assembly of consecutive turns and β-strands builds up a right-handed parallel β-helix that is stabilized by calcium binding. In contrast, in the
absence of calcium, these RTX sequences appeared to be largely disordered as revealed in particular by our biophysical studies of the adenylate cyclase (CyaA) toxin from *B. pertussis*, the causative agent of the whooping cough (17, 18). CyaA contains ~40 RTX motifs grouped in five consecutive blocks (named blocks I–V) separated by non-RTX-flanking regions. This RTX-containing domain (RD)\(^4\) is located at the C-terminal moiety of CyaA (residues 1006–1706) and is implicated in the calcium-dependent binding of the toxin to a specific receptor, the CD11b/CD18 integrin, at the surface of the host target cells. We indeed showed that RD is intrinsically disordered in the absence of calcium but acquires a stable β-rich fold upon calcium binding (9).

It has been proposed that the calcium-induced disorder-to-order transition in RTX motifs may be essential for the secretion of the corresponding polypeptides by their dedicated type I secretion system. The disordered conformations of RTX motifs in the low calcium environment of the bacterial cytosol may facilitate the protein secretion through the type I secretion system machinery, whereas in the external medium, calcium binding could trigger the folding of the proteins into their active conformations for host-pathogen interactions (9, 19–21).

To gain further insights into the mechanisms of calcium-induced folding of the CyaA RTX domain, we further characterized a region of RD that encompasses the last block (block V) of RTX motifs and that was found to be essential for the cytotoxic activity of CyaA (22–24). We demonstrated that the RTX motifs *per se* were not able to fold in the presence of calcium and that the polypeptide segments at the C termini of the tandemly repeated RTX motifs were essential for the calcium-induced folding (10), yet the molecular basis allowing this C-terminal flanking region to induce the folding of the RTX motifs remained elusive.

Here, we have investigated the electrokinetic and hydrodynamic properties of several RTX polypeptides derived from block V of RD by a combination of complementary biophysical approaches. Our results indicate that the C terminus–flanking region is not only involved in the calcium-induced folding but can also modulate the multimerization of the RTX polypeptides.

Moreover, we showed that calcium binding induced a strong reduction of the mean net charges of the RTX polypeptides. This indicated that the disorder-to-order transition of RTX proteins could be controlled by calcium-induced changes in protein valence.

**RESULTS**

The polypeptides studied in this work have been described previously (10) and are schematized in Fig. 1. They all encompass the core of the last block (block V) of RTX motifs (residues 1530–1630, noted R) from CyaA but differ in the presence of either the N-terminal (residues 1487–1529, noted N) or the C-terminal (C) flanking region, the latter being further subdivided into short C\(_N\) (residues 1631–1652) and long C\(_L\) (residues 1631–1680) regions. These polypeptides were produced and purified as described previously (see “Experimental Procedures” and Ref. 10 for details).

**EXPERIMENTAL PROCEDURES**

*Reagents*—The experiments were done at 25 °C in 20 mM Hepes, 20 mM NaCl, pH 7.4 (Buffer A). All reagents were of the highest purity grade.

*Polypeptides Production and Purification*—RTX polypeptide production and purification have been described elsewhere (10). Protein preparation is further described in the supplemental materials.
and supplemental Fig. S1). Accordingly, the addition of calcium did not change the hydrodynamic radii of NR and R (Table 1), whereas it triggered a large decrease of the $R_H$ of NR and R, indicative of a strong compaction of their hydrodynamic volumes upon acquisition of secondary and tertiary structures.

Strikingly, the $R_H$ of holo-RS was not significantly different from that of the apo-form, although the CD spectra clearly indicated that RCS acquired secondary and tertiary structures in the absence of calcium (Table 1 and supplemental Fig. S1). Unfortunately, we could not measure the $R_H$ of holo-NRCS, as a significant fraction of the protein aggregated in these conditions, precluding an accurate analysis of the QELS data. In contrast, the QELS data of apo- and holo-RS were robust and good enough to accurately determine their translational diffusion coefficients that were also found (see below) to be in agreement with AUC data. It was therefore intriguing that the intrinsically disordered apo-RS state could fold in the presence of calcium with AUC data. It was therefore intriguing that the intrinsically disordered apo-RS state could fold in the presence of calcium (supplemental Fig. S1) without a concomitant decrease of its hydrodynamic radius (Table 1).

To better describe the calcium-induced folding reaction of RCS, we further investigated its hydrodynamic properties by SEC-TDA, by analytical ultracentrifugation (AUC) in velocity mode, by fluorescence anisotropy, and by electrophoretic mobility.

The SEC-TDA experiments were performed on a Superdex 200 column at 25 °C. The chromatographs of RCS in the apo-state and in the holo-state (in the presence of 5 mM calcium) are shown in Fig. 2A. The retention volume of RCS in the apo-form was similar in the absence and in the presence of calcium (Fig. 2A and supplemental Table S1), suggesting that both states exhibited similar hydrodynamic volumes, in agreement with the QELS data. The right angle light scattering signals (RALS, green profiles), combined with the protein concentration determined by the deflection refractometer (black profiles), provided on-line measurements of the molecular mass of each eluting species. As shown in Fig. 2B, the molecular mass determined for the apo-state of RCS was 15.1 kDa, as expected for a monomeric species (Fig. 2B, green dashed trace), whereas the molecular mass of the holo-state (Fig. 2B, green solid trace) was 42 kDa. Hence, these data indicated that RCS formed a trimer upon calcium binding (supplemental Table S1). To further estimate the equilibrium between the monomer and the trimer, holo-RCS was loaded on the SEC-TDA at three different concentrations (70, 35, and 7 μM). Irrespective of the protein concentration, holo-RCS eluted as a single peak exhibiting a molecular mass of 42 ± 2 kDa (data not shown). No other oligomeric states (i.e. monomer, dimer, tetramer, etc.) could be detected, suggesting that the trimeric state was the most stable form of holo-RCS, if not the only possible one. To rule out any possible artifact, we analyzed in parallel the RCS protein by SEC-TDA, and as shown in Fig. 2 (C and D), we found that, in agreement with our previous results (10), RCS is monomeric in both states, and its $R_H$ decreased dramatically upon calcium binding (Table 1). Finally, the SEC-TDA experiments also provided the intrinsic viscosity (measured by on-line differential pressure transducers) of each eluting species. As shown in Fig. 2B, the intrinsic viscosity of RCS strongly decreased from 14.7 to 6.4 ml g$^{-1}$ upon calcium binding (Fig. 2B, red traces, and supplemental Table S1). This change of intrinsic viscosity indicates that RCS underwent a change of hydration and/or shape upon calcium binding (see below).

### Hydrodynamic Parameters of RTX Proteins

We then measured the sedimentation coefficients of the apo- and holo-states of RCS by AUC (i) to independently confirm the results obtained by QELS and SEC-TDA and (ii) to further determine the respective contribution of the viscosity increment and hydration parameters in the intrinsic viscosity values of the different species. Velocity experiments provided sedimentation...
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coefficients of 1.46 and 3.65 S for apo-RC$_S$ and holo-RC$_S$, respectively (Fig. 3 and supplemental Table S1). These data, together with those acquired by QELS and SEC-TDA, provided independent measurements of $S$, $R_H$, and molecular mass (supplemental Table S1) that are related to each other according to the Svedberg equation $S = M(1 - \rho_d)/(6\pi\eta N_A R_H)$. All of the three experimental parameters, $S$, $R_H$, and $M$, were found to be remarkably consistent for both the apo- and the holo-states of RC$_S$. Indeed, when two of these experimentally determined parameters, e.g. $R_H$ and $M$, were used to calculate (using the Svedberg equation) the third one ($S$), the theoretical value obtained was very close to the one experimentally measured. This further confirmed the robustness and accuracy of the measured hydrodynamic parameters. Altogether, the hydrodynamic analysis indicated that apo-RC$_S$ was monomeric with a $R_H$ of 3.1 nm, whereas holo-RC$_S$ folded into a stable and compact trimeric conformation exhibiting a $R_H$ of 3.1 nm.

From the intrinsic viscosity of both apo- and holo-RC$_S$, we then determined the respective contribution of the shape factor and the time-averaged apparent hydration. The method to estimate these parameters has been described elsewhere (9, 10, 25, 26). Briefly, the molecular mass, the intrinsic viscosity, and the $R_H$ (or alternatively the sedimentation coefficient; see “Experimental Procedures”) are required to calculate the shape factor and the protein hydration using the Einstein viscosity relation. The viscosity increment, $\eta$, is a hydrodynamic function (i.e. a shape factor) from which the values of the semi-axes a/b of an ellipsoid can be inferred (27). The time-averaged apparent hydration, $\delta$, defined as the mass of solvent bound to and dragged by the protein along its Brownian motion, is related to the exclusion volume of the protein, i.e. the volume defined by the shearing plane between the protein and the bulk solvent.

As shown in supplemental Table S1, the viscosity increment values, and hence the shape of apo-RC$_S$ and holo-RC$_S$, were similar. Therefore, the difference between the intrinsic viscosity of apo-RC$_S$ (14.7 ml/g) and holo-RC$_S$ (6.4 ml/g) was mainly due to a difference of hydration (see “Experimental Procedures”). Indeed, apo-RC$_S$ was characterized by a high time-averaged apparent hydration, close to 3 g/g (supplemental Table S1), a value much higher than the theoretical one (0.43 g/g) computed from the protein sequence using the Sednterp software. RC$_S$ underwent a strong dehydration upon calcium binding, although its hydration value (1 g/g) still remained higher than the computed one (supplemental Table S1). The calcium-induced dehydration of RC$_S$ was also evidenced by the changes of the frictional ratio (supplemental Table S1). The frictional ratio $f/f_0$ (defined in Table 1 as the ratio of radii, $f/f_0 = R_H/R_0$) is related, through the Perrin equation (see “Experimental Procedures”), to the hydrodynamic shape function (the Perrin factor) and the time-averaged apparent hydration, $\delta$. Interestingly, the decrease of the frictional ratio from 1.8 for apo-RC$_S$ to 1.35 for holo-RC$_S$ (supplemental Table S1) was essentially assigned to a change of hydration, whereas the shape function was not affected. Similar observations have been reported for the other RTX proteins (Table 1 and Ref. 10), indicating that the strong calcium-dependent dehydration may be a general feature of these polypeptides.

**RTX Protein Tryptophan Fluorescence Anisotropy Studies—**

We further analyzed the properties of RC$_S$ and RC$_C$ by steady-state tryptophan fluorescence anisotropy, a sensitive tool to explore the flexibility of a fluorescent probe. As shown in Fig. 4A, the anisotropy values of apo-RC$_C$ and holo-RC$_C$ were similar and quite low yet higher than that of l-Trp used as standard. The low anisotropy value of apo-RC$_C$ was not unexpected because the polypeptide adopted intrinsically disordered conformations in which the tryptophans may be freely exposed. However, the fact that the anisotropy of RC$_C$ did not increase upon calcium binding was more surprising, because the tryptophan probes experienced steric constraints from the surrounding amino-acids in the folded holo-RC$_C$ state. We hypothesized that the effects of local constraints on the tryptophan anisotropy may be compensated by the lower hydrodynamic radius, allowing a faster motion (related to the rotational diffusion coefficient of the protein) of holo-RC$_C$, as compared with apo-RC$_C$. In other words, the effects of the local constraints on RC$_C$ anisotropy (expected increase) may be counterbalanced by the faster rotation of the protein (decreasing anisotropy), thus explaining the absence of a global anisotropy change between the apo- and holo-states. Alternatively, this effect may be due to a nonuniform folding of holo-RC$_C$: a tryptophan-containing region may remain locally partially unfolded even in the presence of calcium. This scenario is possible...
because one of the RC₈ tryptophans is located in the C-terminal region.

In contrast, the anisotropy of RC₈ was modulated by calcium binding as shown in Fig. 4B. In the apo-state, the anisotropy of RC₈ was low likely because of the absence of constraints on the tryptophan residues in this unfolded state, as noted above for apo-RC₁. At variance with RC₁, calcium binding triggered a significant increase of the anisotropy of RC₈. This increase of anisotropy of holo-RC₈ may reflect the restriction of the tryptophan side chain flexibility as a result of structural constraints within the folded holo-RC₈. However, because the hydrodynamic volumes of the trimeric holo-RC₈ (Rᵥ, 3.1 nm) and of the monomeric apo-RC₈ (Rᵥ, 2.9 nm) were similar, the macromolecular dimension of RC₈ did not change and therefore should not contribute to any variation of anisotropy. Hence, only the tryptophan side chain flexibility (related to the local tertiary contacts) ultimately contributed to the anisotropy changes of RC₈. Altogether, these data suggest that the anisotropy of RC₁ was not modified upon calcium binding due to the compensating effects of local and global changes, whereas that of RC₈ increased upon calcium binding due to a unique effect of the folding-induced constraints on the tryptophans.

Calcium-induced Changes of the Electrophoretic Mobilities of RTX Proteins—We recently proposed that electrostatic interactions could play a critical role in the folding and stability of RTX proteins (11). To provide a direct experimental evidence of a charge effect on the unfolded-to-folded transition of the RTX proteins, we characterized the electrophoretic mobility (μₑ), i.e. the velocities in a given electric field, of RC₁ and RC₈ in both the apo- and holo-states. Combined with the above-determined hydrodynamic properties, these data allowed us to estimate the mean net charge (i.e. the protein valence) of the polypeptides in the apo- and holo-states. The number of calcium ions bound per protein was then inferred, assuming that the difference in charges between the apo- and the holo-state should correspond to the number of charges brought by the calcium ions bound to the protein in the holo-state.

The approach used to convert the electrophoretic mobility into a number of charges is based on the study of Basak and Ladisch (28) (see “Experimental Procedures”). These authors empirically determined, for a large set of proteins, a relationship between the electrophoretic mobility of a protein (μₑ), the number of charges (Nₑ), its molecular mass (Mₑ), and its frictional ratio (f₀/fₐ) (see the equation under “supplemental materials”). Briefly, this relation indicates that the electrophoretic mobility is proportional to the number of charges and inversely correlated to both the frictional ratio and the molecular mass of the protein. Because we already determined the molecular masses and the frictional ratios of RC₁ and RC₈ in both of their apo- and holo-states, the measurement of the electrophoretic mobility values should allow us to estimate the number of charges of each species.

The electrophoretic mobility values measured in fast field reversal mode for RC₁ and RC₈ are shown in Fig. 5. These electrophoretic mobility values, together with the molecular masses and frictional ratios determined previously, are also reported in Table 2. As expected for proteins binding several calcium ions (two charges per cation), large variations of the electrophoretic mobility were evidenced between the apo- and the holo-state of each protein. Upon calcium binding, the electrophoretic mobility of RC₁ changed from −1.75 to −1.06 μm/cm·V⁻¹·s⁻¹ (Fig. 5A). This corresponded to a number of charges of −23.6 for apo-RC₁ and −9.1 for holo-RC₁ (Table 2). The charge difference between the holo- and apo-forms (ΔNₑ = −9.1 − (−23.6) = 14.5) provided an estimation of the number of charges neutralized by the binding of the divalent cation, implicating that approximately seven calcium ions were bound to holo-RC₁ (NₑCa²⁺ = ½ΔNₑ = 7.25; Table 2). The electrophoretic mobility of RC₈ decreased from −1.35 to −0.98 μm/cm·V⁻¹·s⁻¹ upon calcium binding (Fig. 5B). From these values, we estimated a number of charges of −14.9 for the monomeric apo-state and of −15.5 for the trimeric holo-state (Table 2), this latter value corresponding to an averaged charge of −5.2 (i.e. −15.5/3) per monomer of holo-RC₈. Therefore the charge difference between holo-RC₈ and apo-RC₈, ΔNₑ, was 9.7 (ΔNₑ = −5.2 − (−15.4)) suggesting that approximately five calcium ions were bound per polypeptide in the trimeric holo-RC₈ (NₑCa²⁺ = ½ΔNₑ = 4.86 in Table 2).

**DISCUSSION**

Previous studies have shown that RTX-containing polypeptides are remarkable models to explore the biophysical principles underlying the ligand-induced disorder-to-order transition in proteins (9–11, 24, 29–31). We indeed showed that a
We examined more thoroughly the two polypeptides, RCS and repeats but differing in their N- or C-terminal flanking regions. RTX polypeptides, all containing the RTX core (R) of block V of the CyaA toxin, is natively disordered (yet fully soluble) in the absence of calcium but with a significant difference in affinity for RCS.

To address these questions, we have performed here a comprehensive analysis of the hydrodynamic properties of several RTX polypeptides, all containing the RTX core (R) of block V of peptides but differing in their N- or C-terminal flanking regions. We examined more thoroughly the two polypeptides, RCS and RTX motifs. Indeed, Iwaki et al. (29) showed that a synthetic polypeptide made of several consensus RTX motifs was able to form a β-helix in the presence of calcium and polyethylene glycol but only upon polymerization, suggesting that intermolecular interactions were indeed essential to stabilize the putative β-roll structure of these synthetic RTX peptides (29).

Our present results strongly support the idea that a β-helix structure made of RTX motifs is thermodynamically weakly stable per se. In the RCS polypeptide, the short C-terminal flanking region may not be sufficient to stabilize the β-helix fold within a monomeric species, whereas the thermodynamic contribution of intermolecular interactions within a trimer of RCS may provide additional energy to maintain a β-helix conformation. In other words, by restricting the degrees of freedom of the polypeptides, the trimeric state may provide a significant entropic contribution to stabilize the β-helix structure. This may explain why we could not detect any monomeric or dimeric holo-RC5 by SEC-TDA even at the lowest protein concentration tested. The holo-state may only exist as a trimer, which provides the favorable environment to stabilize the calcium-loaded structure. We propose that monomeric RCS mainly populates the intrinsically disordered state. In the presence of calcium, monomers may experience transient exchanges between calcium-loaded partially folded and unfolded states. Upon diffusion/collision, intermolecular interactions between partially folded RCS could take place and lead to the formation of a stable trimeric complex. At variance, in RC1, the long C terminus-flanking region may provide enough intramolecular contacts to stabilize the holo-protein in a monomeric form.

The propensity of the RTX β-helices to be stabilized via intermolecular interactions may be pertinent as well for the folding of the whole RD domain that contains five blocks of RTX motifs. Indeed, Iwaki et al. (23) initially reported that the C-terminal region of RD encompassing the RTX block V could functionally complement in vitro a truncated CyaA (lacking the last 75 residues) to restore its cytotoxic activities. Later, Bejerano et al. (22) showed that this C-terminal region of RD could associate in a calcium-dependent manner with the trun-
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Our present study also highlighted some important biophysical properties of the RTX polypeptides regarding, in particular, their calcium-induced folding process, which is pertinent for the disorder-order equilibrium of proteins in general. We first compared and described quantitatively the hydrodynamic properties of the apo- and holo-states of both RCL and RCS by using the expression that relates the hydrodynamic volume to the molecular mass, the partial specific volume, the time-averaged hydration, and the buffer density.

\[ V_d N_A = M (\bar{v} + (\bar{\mu}/\bar{\rho})) \]  

(Eq. 1)

As previously reported (10), apo- and holo-RCL exhibited a large difference in their retention volumes on SEC. This variation resulted from a decrease of both the hydrodynamic volume \( V_d \) in the left part of the equation) and the hydration \( \bar{\rho} \) in the right part of the equation) of the protein upon calcium binding. RCL yet remained a monomer in both the apo- and the holo-state. The response of RCS to calcium binding was found here to be drastically different. RCS also experienced a large reduction of its hydration upon calcium binding, but at variance with RCL, it was essentially compensated by an increase in molecular mass, from monomer to trimer, whereas the overall hydrodynamic volume remained unchanged. A plot of the hydrodynamic radii of the RTX polypeptides as a function of their respective molecular masses, according to Uversky (37), revealed that all of the apo-forms lie between premolten globule conformations and natively unfolded coils, whereas the holo-forms adopt compact conformations (Fig. 6). Also, whereas NRCs, RCL, and RD proteins experienced a calcium-induced decrease of their hydrodynamic radius (i.e. change along the y axis to reach the region of compact proteins), RCS responded to calcium by a change of its apparent molecular mass (horizontal shift on the x axis).

Finally, we attempted to further define the biophysical principles that might control the calcium-induced folding of RTX proteins. We suggested recently that electrostatic interactions might play an important role in the disorder-to-order transition of RD (11). We proposed that, in the apo-form, electrostatic repulsions between the charged Asp residues of the RTX motifs might force the polypeptide chain to adopt expanded and disordered conformations. Calcium binding to the RTX motifs could screen the Asp negative charges, thus allowing the polypeptide chain to collapse and fold into a compact structure.

To directly test this model, we experimentally measured here the changes of the mean net charge induced by the binding of calcium to the RTX proteins. Electrophoretic mobility measurements, in conjunction with AUC, QELS, and SEC-TDA (see “Experimental Procedures” for details), allowed us to estimate the mean net charge of both the apo- and the holo-state of RCS and RCL. From the mean net charge values, we calculated that the RCL polypeptide binds ~5 calcium ions/monomer on average. These results are in good agreement with our prior calcium binding measurements (10, 24). Interestingly, the somewhat lower number of calcium-binding sites in RCS may indicate that in the trimeric form of holo-RCS, the packing constraints may distort or prevent the folding of certain RTX motifs (as revealed by the lowest n-\(\pi\) band intensity of RCS as compared with RCL in supplemental Fig. S1). The large changes in the mean net charge of the RTX polypeptides upon calcium binding are interesting to compare in terms of a charge-hydrophobicity phase plot (38). As shown in Fig. 7, the apo-proteins range in the intrinsically disordered region of the charge-hydrophobicity phase space, whereas the strong decrease of the mean net charge upon calcium binding shifts the holo-proteins well below the boundary between unfolded and folded proteins (Fig. 7). To our knowledge, these are the first experimental measurements of ligand-induced changes of polypeptide charges associated with a disorder-to-order transition.

Concluding Remarks—We previously proposed that the intrinsically disordered state of the CyaA RTX motifs in the bacterial cytoplasm may favor the secretion of the toxin by the type I secretion system (9, 10), whereas the calcium-induced folding of the RD domain in the extracellular milieu is critical for the CyaA cytotoxic activity. We showed here that RTX blocks could fold and stabilize upon multimerization, a
cooperative process that might contribute to improve the secretion efficiency. We further showed that the apo-forms of RTX block V polypeptides adopt a premolten globule state that switches to a native state in the presence of calcium. This transition appeared finely tuned from a thermodynamic point of view to respond to calcium binding in physiological conditions. Moreover, we showed that the high number of calcium bound per protein allows switching from an intrinsically disordered, highly charged apo-form to a folded holo-state with a reduced mean net charge. Our data suggest that the folding of the RTX proteins is controlled by a charge change effect caused by the variation of physiological concentrations of calcium. The simplicity of this calcium-induced, charge-driven disorder-to-ordered transition may favor a robust and chaperone-independent oligomerization of the proteins.

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