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Calcium-Induced Folding of a Beta Roll Motif Requires C-Terminal Entropic Stabilization

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Beta roll motifs are associated with several proteins secreted by the type 1 secretion system (T1SS). Located just upstream of the C-terminal T1SS secretion signal, they are believed to act as calcium-induced switches that prevent folding before secretion. *Bordetella pertussis* adenylate cyclase (CyaA) toxin has five blocks of beta roll motifs (or repeats-in-toxin motifs) separated by linkers. The block V motif on its own has been reported to be non-responsive to calcium. Only when the N- and C-terminal linkers, or flanking groups, were fused did the motif bind calcium and fold. In an effort to understand the requirements for beta roll folding, we have truncated the N- and C-terminal flanks at several locations to determine the minimal essential sequences. Calcium-responsive beta roll folding occurred even in the absence of the natural N-terminal flank. The natural C-terminal flank could not be truncated without decreased calcium affinity and only partially truncated before losing calcium-responsiveness. Globular protein fusion at the C-terminus likewise enabled calcium-induced folding but fusions solely at the N-terminus failed. This demonstrates that calcium-induced folding is an inherent property of the beta roll motif rather than the flanking groups. Given the disparate nature of the observed functional flanking groups, C-terminal fusions appear to confer calcium-responsiveness to the beta roll motif via a non-specific mechanism, suggesting that entropic stabilization of the unstructured C-terminus can enable beta roll folding. Increased calcium affinity was observed when the natural C-terminal flank was used to enable calcium-induced folding, pointing to its cooperative participation in beta roll formation. This work indicates that a general principle of C-terminal entropic stabilization can enable stimulus-responsive repeat protein folding, while the C-terminal flank has a specific role in tuning calcium-responsive beta roll formation. These observations are in stark contrast to what has been reported for other repeat proteins.

Keywords: beta roll; repeat folding; capping domains; calcium-induced folding; protein switch

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Introduction

Modular repeat proteins are ubiquitous in nature and often mediate protein–protein interactions. Leucine-rich repeats, ankyrin repeat proteins and tetratricopeptide repeat proteins (TRPs) are among the best characterized repeat proteins.[1–3] Each repeat protein is characterized by a consensus repeating unit, defining the positions within the repeat that are relatively invariable. These units define the structural elements of the specific repeat protein fold, as well as variable positions amenable

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Abbreviations used: RTX, repeats in toxin; T1SS, type 1 secretion system; CyaA, adenylate cyclase; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; MBP, maltose; FRET, Förster resonance energy transfer; bis-ANS, 4, 4′-bis(1-anilinonaphthalene 8-sulfonate); PEG, polyethylene glycol; OMP, outer membrane protein.

to substitution.[4] These structural elements are stabilized via local short-range interactions, as opposed to globular proteins that often exhibit many cryptic long-range interactions along the folding pathway.[2,5] A common feature for repeat proteins is tertiary stabilization by the formation of a hydrophobic core.[4] To stabilize this hydrophobic core, specialized flanking or capping groups are needed on both N- and C- termini to protect the hydrophobic core from solvation.[3,6] Optimization of the capping groups has resulted in stable proteins with well defined tertiary structure.[7,8]

Bacterial type 1 secretion systems (T1SS) are important in the transport of large proteins from the cytoplasm directly to the extracellular environment, without exposing the protein to the periplasm.[9] Proteins secreted through T1SS are unique in that the secretion signal is located in the 60 most C-terminal amino acids and is not cleaved after transport.[10] Directly upstream of the secretion signal is typically a so-called repeats-in-toxin (RTX) domain. Recent work by Chenal *et al.*[11] has demonstrated that the RTX domain of adenylate cyclase (CyaA) toxin from *Bordetella pertussis*, the causative agent of whooping cough, is an intrinsically disordered protein at physiological intracellular concentrations of calcium and becomes structured at the higher concentrations of calcium more typically found in the extracellular environment. The outer membrane protein of the T1SS has a channel 30 Å in diameter,[12] and it has been suggested that CyaA (>200 kDa) must be secreted in an unfolded conformation.[9,11] The prevailing theory on the T1SS is that the entire RTX domain remains unfolded in the cytoplasm and throughout the secretion process, and folds upon exiting into the calcium-rich extracellular environment.[10,13] Only after this calcium-triggered RTX folding event can the catalytic domain of CyaA fold into its active toxic form.[14]

RTX domains are common to several families of proteins that use the T1SS, including extracellular proteases,[15] extracellular lipases,[16,17] epimerases[18] and hemolytic toxins.[19] The RTX domain is composed of tandem repeating nonamers (9mers) of the sequence GXXGXDXUX where U is a large aliphatic amino acid (L, F or I) residue, and X can be any residue.[13,15] The number of RTX repeats in a given protein ranges from 5 to 45. These tandem repeats are often broken up into blocks of between 5 and 12 repeats, separated from one another by linker regions.[19] The RTX blocks, including flanking and repeat sequences, can fold independently of the surrounding blocks and are referred to here as RTX-motifs. Crystallographic data from an alkaline protease (PDB 1SAT)[20] and an extracellular lipase (PDB 2Z8X)[16] indicate the calcium-bound RTX-motif is composed of turns and beta sheets forming a parallel beta helix termed a beta roll. The highly conserved aspartic acid is required for calcium binding,[21] and without calcium the beta roll structure is not formed.[19] When bound to calcium, the RTX-repeats are folded, with GXXGXD forming a turn and XUX forming a beta strand. Two consecutive RTX-repeats form one full repeat of the beta roll structure, with amino acid U forming a hydrophobic core.[20]

The entire CyaA RTX domain has been reported to undergo calcium-responsive structure formation.[11,14] Individual examination of the block V RTX-motif of CyaA was shown to fold with both the entire natural N- and C-terminal flanking sequences, but not in their absence.[19,22] Earlier, we reported the restoration of the calcium-responsive behavior of the otherwise unresponsive block V RTX-repeats by the fusion of the protein Förster resonance energy transfer (FRET)-pair, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP).[22] In this study, we were interested in understanding more about the underlying mechanism of RTX-repeat folding and how that might impact our understanding of stimulus-responsive repeat protein folding. To that end, we examined the minimal natural flanking sequence needed for calcium-responsive structure formation of block V RTX-repeats. Additionally, we examined the effect of non-native fusion partners, such as maltose-binding protein (MBP) and fluorescent proteins (CFP/YFP), on the calcium-responsive structure formation of the otherwise calcium-insensitive block V RTX-repeats. Last, we show that the molecular crowding agent polyethylene glycol (PEG) can restore calcium-responsive folding as well. Taken together, our results point to a specific role for the natural C-terminal flank in tuning calcium-responsive beta roll formation, while a more general underlying entropy-dependent mechanism at the C-terminus might be responsible for enabling calcium-responsive beta roll folding.

Results

The CyaA toxin of *B. pertussis* contains a large C-terminal domain comprising five RTX-repeats, separated by linkers, or flanking residues. In this work, we examined the block V RTX-repeats shown in Fig. 1a. The block V RTX-repeats span amino acids 1529–1612 (numbering based on the CyaA; Fig. 1b). The natural N-terminal flanking residues span residues 1488–1528, and the natural C-terminal flank spans residues 1613–1680. The structure of the N- and C-terminal flanks is not known, so we have depicted them as straight-line extensions attached to the termini of the RTX-motif (Fig. 1c). In the latter part of this work, non-native fusions were made to the RTX-repeats.

Natural flanking residues enable calcium-induced RTX folding

Far-UV CD spectra and 4,4′-bis(1-anilinonaphthalene 8-sulfonate) (bis-ANS) binding experiments shown in Fig. 2a and b indicate that the RTX-repeats on their own do not undergo calcium-induced secondary structure changes. Both calcium and calcium-free CD spectra show a negative peak at approximately 198 nm, indicative of unstructured residues. A subtle negative peak at approximately

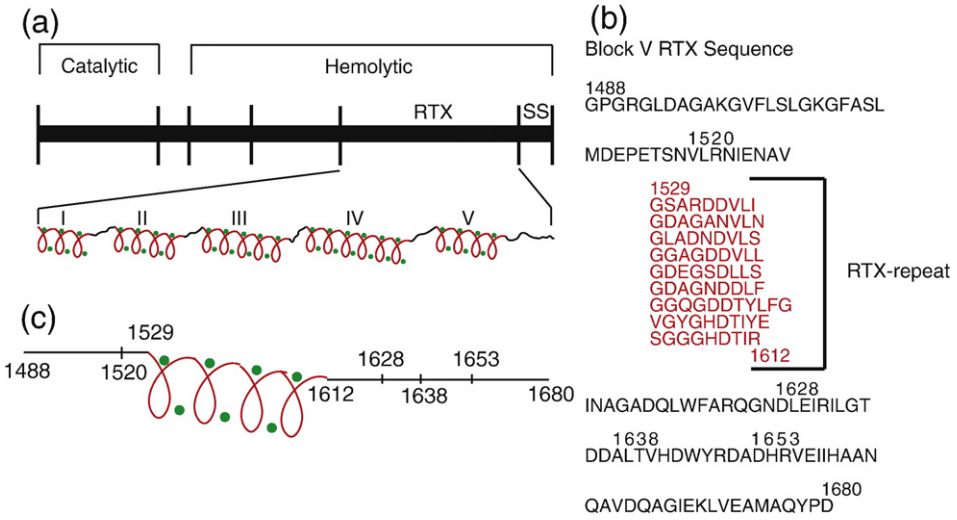

Fig. 1. Structural organization of CyaA. (a) CyaA is a large protein with an N-terminal catalytic domain and a C-terminal hemolytic domain. A large portion of the hemolytic domain is composed of RTX-repeats that are organized into five blocks (I–V) separated by linkers, or flanking sequences. SS denotes the location of the secretion signal. (b) The primary amino acid sequence of the block V RTX-motif, highlighting the RTX-repeats and their flanking sequences. The numbers indicate the amino acid position in the parent CyaA. (c) A representation of the block V RTX-motif. The straight line segments represent the N- and C-terminal flanks. Hatch marks along the lines denote positions of truncation with the corresponding CyaA residue number.

215 nm is also observed, indicating a small amount of residual secondary structure. However, when the amino acids flanking the RTX-repeats are included, the far-UV CD spectrum (Fig. 2c) shows a shift of the negative peak to approximately 218 nm and the appearance of the positive peak around 190 nm

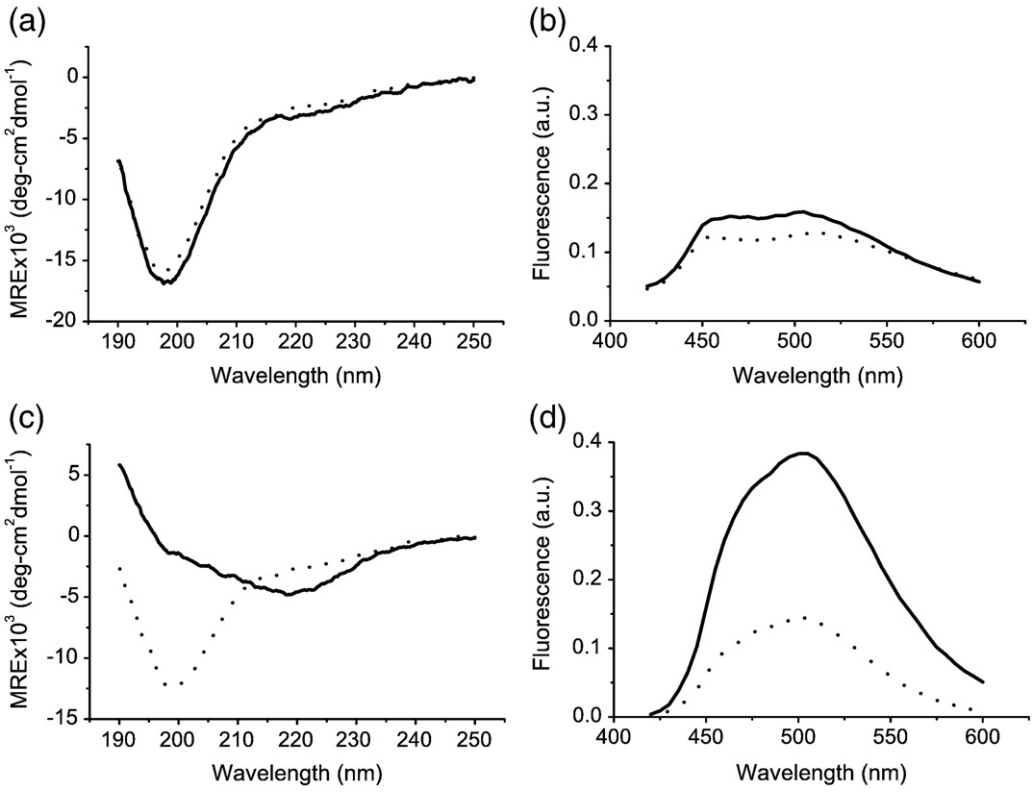

Fig. 2. Flanking sequences enable calcium-induced folding of RTX-repeats into a beta roll. CD spectra of unflanked (a) RTX 1529-1612 and flanked (c) RTX 1488-1680 in the presence (—) and in the absence (- - -) of 10 mM calcium, demonstrate flanking residues are needed for calcium-induced RTX folding. These results are consistent with the bis-ANS binding results for the same two constructs (b and d, respectively). CD data are presented as mean residue ellipticity (MRE).

when calcium is introduced. This spectrum is indicative of increased beta sheet secondary structure and is similar to the results reported by Bauche *et al*.[19] Deconvolution of these spectra also indicates the gain of beta sheet structure and the loss of unstructured residues (see Supplementary Data Table S1). Similarly, Fig. 2d shows increased bis-ANS fluorescence with the addition of calcium, consistent with bis-ANS binding to organized hydrophobic regions on the protein that form either on the surface or within the resulting beta roll structure.

C-terminal flanking residues control calcium-induced RTX folding

Given the important role of the natural flanking amino acids for the calcium-induced folding of the RTX-repeats, studying the effect of truncating the flanking groups could help determine the important elements responsible for facilitating the disordered-to-ordered transition described earlier. Crystallographic data are not available for the CyaA RTX domain, so truncations were made (based on secondary structure predictions)[23] in the N-terminal flank at position 1521, and in the C-terminal flank at 1628, 1638 and 1653 (numbering based on the CyaA protein sequence). Far-UV CD spectra of the block V RTX-repeats with truncated flanking groups were analyzed in the presence (10 mM $CaCl_2$) and in the absence of calcium. Fig. 3a shows that when both full-length N-terminal and C-terminal flanking groups are present (RTX 1488-1680), a calcium-induced conformational change is observed in the CD spectra. When the entire N-terminal flanking group was intact, two mutants with truncated C-terminal flanking groups (RTX 1488-1653 and RTX 1488-1638) still enabled calcium-induced folding (Fig. 3b and c). These spectra had similar characteristic changes indicative of increased beta sheet content. The RTX-repeats fused to the C-terminal flanking group truncated at position 1628 (Fig. 3d; RTX 1488-1628) or removed entirely (Fig. 3e; RTX 1488-1612) no longer exhibited calcium-responsive behavior. When the entire C-terminal flanking group was preserved, any truncation of the N-terminal flank resulted in maintenance of the calcium-induced folding of the RTX-repeats (Fig. 3e and h; RTX 1520-1680; RTX 1529-1680). When the C-terminal flank was truncated, truncation of the N-terminal flank no longer allowed calcium-responsive folding (Fig. 3g; RTX 1520-1653). Deconvolution of these spectra indicated similar changes in beta sheet content (see Supplementary Data Table S1) for fully flanked RTX-repeats as well as truncations. This suggests strongly that the RTX-repeats form beta roll structures, consistent with crystal structure data from RTX-repeat-containing proteins.[15,20]

The calcium-responsive constructs with the largest overall truncations (Fig. 3c and h) were further analyzed by calcium titration. All of the calcium-

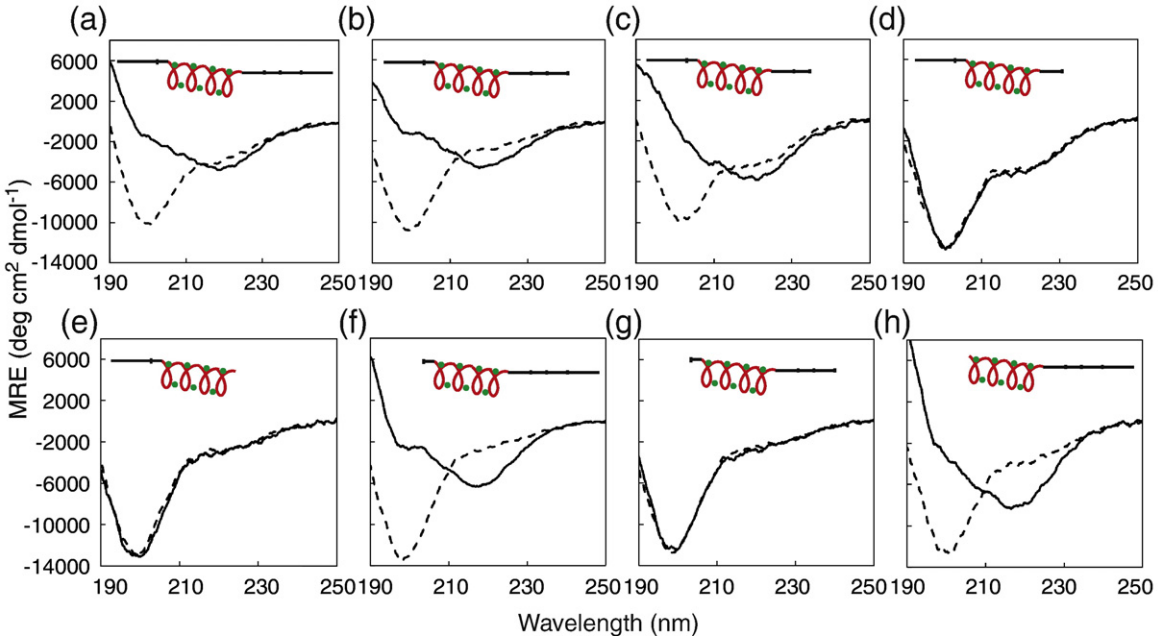

Fig. 3. CD spectra of RTX-repeats flank mutants. Purified mutants were analyzed by CD spectroscopy, in the presence (—) and in the absence (- - -) of 10 mM calcium. Where conformational change was induced by 10 mM calcium, the CD spectra do not overlap each other. RTX 1488-1680 (a), RTX 1488-1653 (b), RTX 1488-1638 (c), RTX 1520-1680 (f) and RTX 1529-1680 (h) all had different CD spectra in the presence of calcium. RTX 1488-1628 (d), and RTX 1488-1612 (e) and RTX 1520-1653 (g) had similar CD spectra regardless of the presence of calcium. The calcium-responsive constructs with fewest flanking amino acids were RTX 1488-1638 and RTX 1529-1680. CD data are presented as mean residue ellipticity (MRE). The RTX representations are shown on each spectra to aid the reader in identifying the truncation made. Flanking regions are shown as linear segments because the structure is unknown.

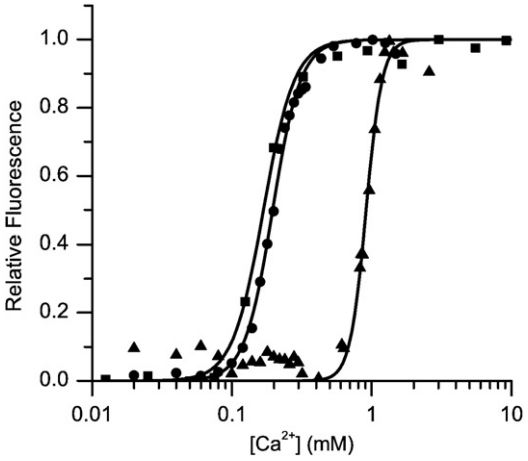

Fig. 4. Calcium titration of RTX-motif flank mutants. Purified mutants of RTX 1488-1680 (●), RTX 1529-1680 (■), and RTX 1488-1638 (▲) all demonstrated calcium-induced changes in intrinsic tryptophan fluorescence consistent with conformational change. The curves refer to data fit to the Hill equation and parameters are given in Table 1.

responsive RTX-motif truncations possess tryptophan residues in the C-terminal flank, so calcium titration experiments could be done by measuring changes in intrinsic tryptophan fluorescence. An increase in fluorescence is consistent with tryptophan residues moving into a more hydrophobic environment. Fig. 4 indicates that the N-terminally truncated RTX-motif (RTX 1529-1680) possessed similar calcium-binding affinity and binding cooperativity when compared to the fully flanked RTX-motif (RTX 1488-1680). Urea denaturation data for these two constructs showed they had similar stability (Supplementary Data Fig. S1). However, the mutant with a partial truncation to the C-terminal flank (RTX 1488-1638) had diminished affinity for calcium as assessed by a fit to the Hill equation (Table 1).

Fusion proteins enable calcium-induced RTX folding without native flanking residues

Truncation experiments on the natural flanking groups have shown that the natural C-terminal flank can enhance calcium affinity and at least part of the flank is necessary for calcium-induced beta roll formation. The RTX-repeats (RTX 1529-1612) and residues 1613-1638 of the C-terminal flank are common to all calcium-responsive constructs, suggesting calcium-responsiveness is either an intrinsic property of the RTX-repeats or specific to the immediate C-terminal flanking residues. Given that the RTX folding is tolerant of truncation of either the natural C-terminal or N-terminal flank, the question remained of whether this effect was specific to native CyaA flanking regions or if other structures could be used. Initially, CFP and YFP were fused to the N- and C- termini of the RTX-motif, and calcium-induced folding was studied by FRET between the fluorescent proteins. For this system, an increase in FRET efficiency is indicative of a decrease in CFP-YFP chromophore separation caused by RTX folding.[22] As shown in Fig. 5, the fluorescent proteins were able to confer calcium-induced folding to the otherwise calcium-insensitive RTX-repeats (1529-1612). The change in FRET efficiency upon addition of 10 mM calcium was ~0.36, similar to the change in FRET efficiency observed for fluorescent proteins attached to the ends of an RTX-motif possessing both natural N- and C-terminal flanking residues. Since the calcium-induced change in FRET efficiency is nearly identical, it seems likely that the change in FRET efficiency is reporting the folding of the RTX-repeats into a beta roll structure rather than changes in chromophore orientation.

The fusion protein signal dominates the CD spectra, so the conformation of these constructs cannot be observed reliably using this technique (data not shown). Likewise, the RTX-repeats (RTX 1529-1612) lack tryptophan residues, preventing the use of intrinsic tryptophan fluorescence. In order to verify the FRET results, we used bis-ANS binding to

Table 1. Summary of Calcium Binding Affinities and Hill Coefficients for RTX-Repeats with Natural Flanking Sequences

	K_D (μM)	n_H	R^2
Tryptophan Fluorescence			
RTX 1488-1680	263±4	5.97±0.5	0.992
RTX 1529-1680	194±2	4.69±0.2	0.997
RTX 1488-1638	923±9	8.92±0.8	0.993
FRET			
RTX 1488-1680	189±13	2.33±0.3	0.989
RTX 1529-1612	2035±110	1.16±0.1	0.999

Errors indicated are standard error values based on nonlinear regression.

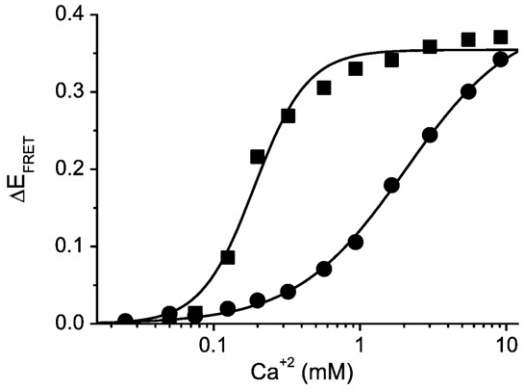

Fig. 5. Non-native capping groups enable RTX-repeats folding. A calcium-induced increase in FRET efficiency is observed when RTX 1529-1612 (●), which does not undergo calcium-responsive folding in 10 mM Ca^{2+}, is fused between CFP and YFP (CFP-RTX-YFP). For comparison, RTX 1488-1680 (■) is fused between CFP and YFP (CFP-N-RTX-C-YFP) and a calcium-induced increase in FRET efficiency is also observed. The E_{FRET} at zero Ca^{2+} has been subtracted from each datum point to give ΔE_{FRET}.

monitor calcium-induced changes in organized surface hydrophobicity of the RTX-repeats. For convenience, RTX 1488-1680 (Fig. 2c) is now referred to as N-RTX-C because it contains both N- and C-terminal flanks. Likewise, RTX 1488-1612 is now referred to as N-RTX and RTX 1529-1680 as RTX-C. The use of the notation RTX refers only to the RTX-repeats themselves (RTX 1529-1612). As mentioned above, bis-ANS binding to bi-terminally flanked RTX (N-RTX-C) increased in response to calcium (Fig. 6c). A similar increase in bis-ANS binding was observed for RTX-C (Fig. 6b). However, no increase in bis-ANS binding was observed when the RTX-repeats were fused only to the natural N-terminal flank N-RTX (Fig. 6a), again suggesting it did not undergo calcium-induced folding. The unflanked RTX had little change in bis-ANS binding in the presence or in the absence of calcium (Fig. 2b).

The FRET construct described earlier was made with G67S mutations in each fluorescent protein (CFP*-RTX-YFP*) to prevent fluorophore formation that would interfere with the bis-ANS measurements.[24] Similar to the natural flanking groups, the fusion of fluorescent protein mutants to both termini of the RTX-repeats (CFP*-RTX-YFP*) enabled a calcium-induced increase in bis-ANS binding (Fig. 6f). Also similar to the natural flanking sequences, fusion of a fluorescent protein mutant to the C-terminus (RTX-YFP*) likewise enabled a calcium-induced increase in bis-ANS binding (Fig. 6e), while N-terminal fusion (CFP*-RTX) had no such effect (Fig. 6d). In order to determine the

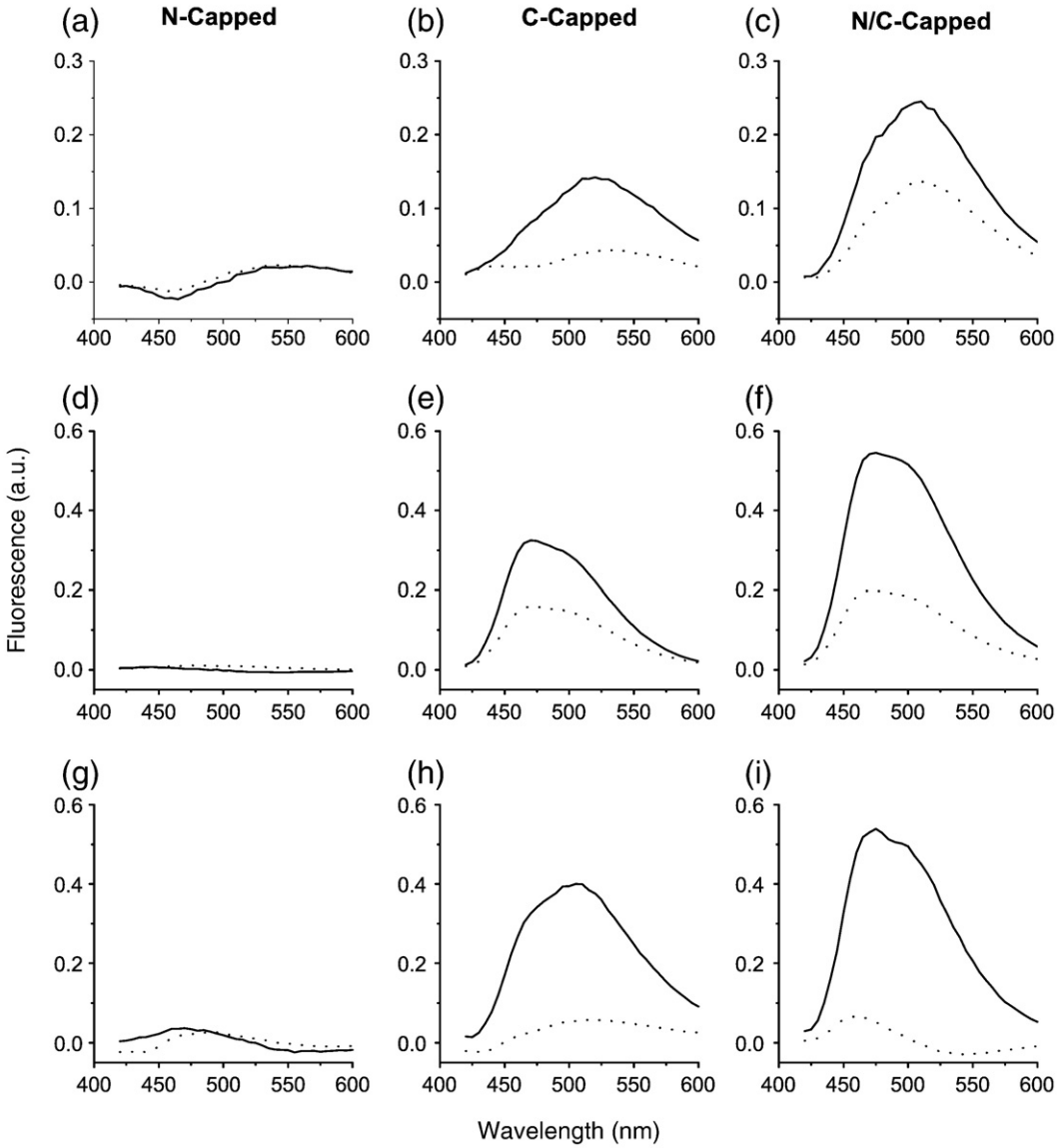

Fig. 6. C-terminal fusions enable calcium-induced folding of RTX-repeats. RTX 1529-1612 was fused to its natural flanking sequences (a–c), to CFP/YFP (d–f), and to MBP (g–i) on its N-terminus (a, d and g), its C-terminus (b, e and h), and on both termini (c, f and i). Fusions to the N-terminus resulted in no difference in the bis-ANS binding in the presence (—) or in the absence (- - -) of 10 mM calcium, while calcium-responsive bis-ANS binding occurred when fusions were made to the C-terminus or to both termini.

generality of this finding, we examined the calcium-induced folding of the RTX-repeats with fusions to MBP. Bi-terminal fusion, MBP-RTX-MBP, and C-terminal fusion, RTX-MBP, exhibited increased bis-ANS binding in the presence of calcium and N-terminal fusion MBP-RTX was once again insensitive to calcium (Fig. 6g–i).

To complement the bis-ANS binding data, we performed terbium titration luminescence resonance energy transfer (LRET) experiments. While often used as a calcium analog, terbium affinity differs from that of calcium and does not, on its own, provide evidence of calcium binding. However, in combination with the CD, bis-ANS and FRET results, the terbium experiments offer further support for calcium-induced structural changes. Fig. 7a–c show terbium titration curves that are consistent with the results of the bis-ANS experiments. Fig. 7a shows that RTX-C and N-RTX-C had similar terbium LRET titration curves. By comparison, the N-RTX titration curve had a lower maximum. The terbium binding curves for RTX-C and N-RTX-C are nearly identical, showing that the contribution of the natural N-terminal flank is small. In Fig. 7b and c, the terbium binding signal for the fusion proteins alone was subtracted from the total fluorescence resulting in titration curves showing the terbium binding resulting solely from the RTX-repeats. In both cases, the titration curve for the bi-terminal and C-terminal fusion converged into similar curves, indicating again that N-terminal fusion contributes little to terbium binding. The consistent demonstration that fusion to the C-terminus of the RTX is critical for calcium-induced beta roll formation suggests a polarity in folding, favoring initiation at the C-terminal end.

PEG restores calcium-induced RTX folding

PEG is often used as a molecular crowding agent to reduce the conformational freedom of proteins.[25,26] We have shown that RTX (RTX 1529-1612) is unable to bind calcium without C-terminal fusion. CD spectra of RTX (Fig. 8) with 100 mM $CaCl_2$ (broad broken line) were identical with the CD spectra of RTX without $CaCl_2$ (continuous line). Addition of PEG 8000 (final concentration 50% (w/v) dotted line) without calcium caused a change in the CD spectra; however, it does not obtain characteristics of a folded beta roll (Fig. 2c). In the presence of both 50% PEG and 100 mM $CaCl_2$ (narrow broken line) the CD spectra obtains features similar to the folded beta roll.

Discussion

A variety of biophysical methods were used to investigate the calcium-induced folding of the block V RTX-repeats of CyaA. It has been shown that the block V RTX-repeats do not fold in response to calcium unless fused to its natural flanking residues, 1488–1528 on the N-terminus and 1613–1680 on the C-terminus.[19] Several truncations were made to the natural flanking groups until calcium-responsive behavior was lost as determined by far-UV CD spectroscopy. Intrinsic tryptophan fluorescence measurements during calcium titrations revealed that alteration of the C-terminal flank could be detrimental to the calcium-induced RTX folding; however, truncation of the entire N-terminal flank had no significant impact. The molecular crowding agent PEG could be used to confer calcium-responsive folding to the otherwise calcium-insensitive RTX. Bis-ANS and terbium fluorescence experiments were used where CD spectroscopy and tryptophan fluorescence could not be applied to show that C-terminal fusions of the RTX-repeats to well-folded globular proteins (YFP* or MBP) likewise conferred calcium-responsive folding; however, N-terminal fusions had little effect. These results taken together show that RTX

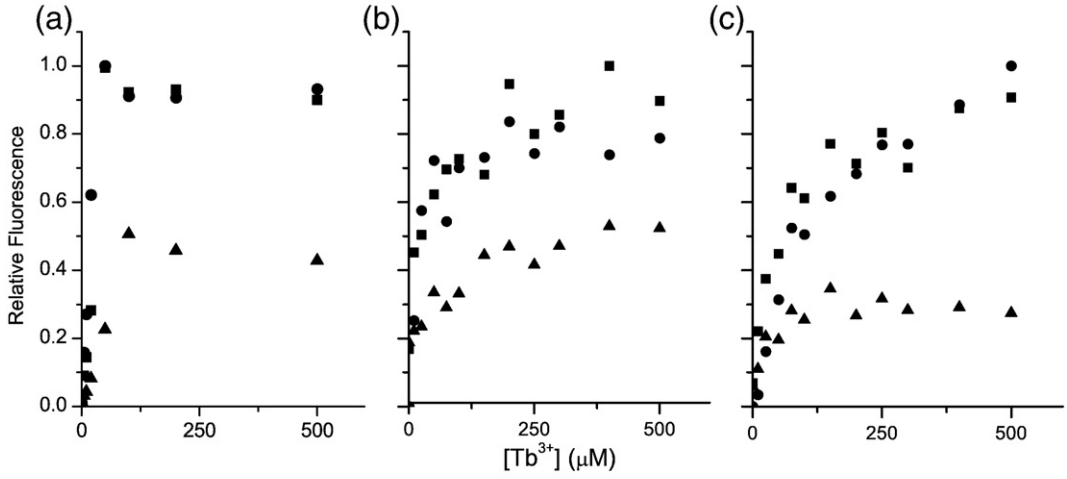

Fig. 7. C-terminal fusions enhance terbium binding of RTX-repeats. Tb^{3+} titration experiments revealed that C-terminal fusions (■) of (a) natural flanking residues, (b) CFP*/YFP* and (c) MBP all had similar Tb^{3+} binding affinity to concomitant N- and C- terminal fusions (●). N-terminal fusions (▲) had lower Tb^{3+} fluorescence. The terbium binding of the fused proteins on their own was subtracted and the resulting data were normalized.

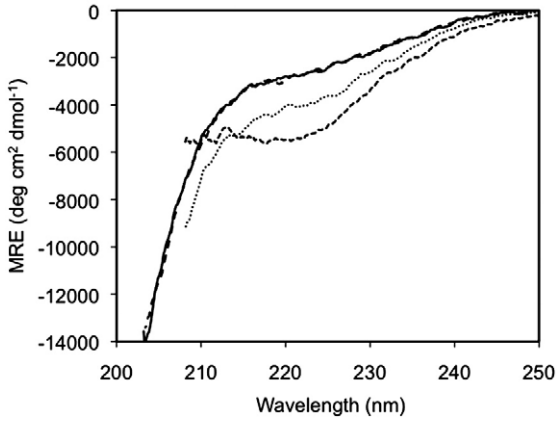

Fig. 8. PEG enables calcium-responsive RTX folding. CD spectra of unflanked RTX without calcium or PEG (continuous line) and RTX with 100 mM $CaCl_2$ (broad broken line) had spectra indicative of unstructured protein. RTX in 50% PEG 8000 (dotted line) had a CD spectrum different from that of either PEG-free sample. Addition of 100 mM $CaCl_2$ and 50% PEG 8000 (narrow broken line) resulted in CD spectra indicative of beta sheet formation.

folding is an intrinsic property of repeats attenuated by the C-terminal flank, suggesting folding of the RTX-repeats is highly dependent on the presence of a C-terminal flank.

To investigate the role of native flanking residues in permitting calcium-induced RTX folding, we made several truncations of the flanks and measured changes in CD spectra to determine if calcium-induced folding was compromised by the truncation. The two calcium-responsive mutants with the largest truncations were a partial C-terminal truncation (RTX 1488-1638; Fig. 3c), and a complete N-terminal truncation (RTX 1529-1680; Fig. 3h). RTX 1488-1638 and RTX 1529-1680 both enabled calcium-responsive RTX conformational change, but truncation of the C-terminal flank reduced the calcium affinity (Fig. 4). Further truncation ablated calcium-responsive folding. This change in affinity suggests a specific role for the natural C-terminal flank for enhancing the calcium-binding affinity. This result is not unexpected, as the cooperative folding of the RTX-repeats and natural flanking group have co-evolved. Furthermore, our data indicate RTX 1488-1638 was calcium-responsive where RTX 1520-1653 was not, likely resulting from truncating the C-terminal flanking sequence at a position that no longer enables the correct structural elements to form properly between positions 1612 and 1638. The N-terminal flank does not play an important role in folding, as observed in native flank truncation and non-native flank experiments.

Like the RTX-motif, the native C-terminal flanking group is rich in aspartic acid residues and is highly charged, suggesting it is relatively unstructured without calcium.[27,28] In fact, when compared with the other flanking groups separating RTX-blocks in CyaA, this is the only flanking group rich in aspartic acid (Fig. S2). It is possible that the native C-terminal flanking sequence either participates in calcium-induced RTX folding in a concerted manner or the flanking group itself may bind calcium. Radioactive calcium-binding studies indicate the block V RTX-motif (with native bi-terminal flanking sequences) bound ~7 calcium ions, the number expected based on nine RTX-repeats;[19] however, this still does not definitively rule out C-terminal flanking sequence calcium binding. Crystal structures of folded beta rolls show that calcium is bound between adjacent turns along the beta helix axis.[15,16] These structures also show that the C-terminal flanking groups "cap off" the beta roll by half coordinating the calcium atoms exposed after the terminal turn and seal off the hydrophobic core. Further studies of the CyaA block V C-terminal flanking group are needed to fully elucidate its role in RTX folding and export through T1SS.

Our study of the truncation of the native flanking groups indicated increased calcium affinity was possible with the full C-terminal flanking group, but how specific is the requirement for the natural C-terminal flank? We showed that partial truncation of the C-terminal flank (RTX 1488-1638) still allows calcium to bind and cause RTX folding, albeit with compromised calcium affinity. Furthermore, we showed that the FRET pair CFP and YFP can enable RTX folding. This suggests non-native flanks can enable calcium-responsive RTX folding. Our results demonstrate that both MBP and YFP* enable calcium-responsive RTX folding (Figs. 4–6) when fused to the C-terminus as we observed with the natural flank, but not to the N-terminus. The varied structures of the fusion proteins used (α/β MBP, β-sheet YFP, and native flank) suggest other well-folded globular proteins could likewise enable calcium responsiveness. This is consistent with the successful use of serralysin RTX-repeats as a calcium-switch to control the spatial separation of bi-terminally fused β-galactosidase,[29] while others have reported difficulty folding unflanked serralysin RTX-repeats.[30,31] While we cannot rule out the possibility that the RTX is binding calcium and folding into some non-native structure when fused to MBP or YFP*, we have ruled out multimer formation through size-exclusion chromatography (Fig. S3), confirming the presence of only monomers in calcium-free and calcium-containing buffers. Furthermore, changes in bis-ANS and terbium binding were similar for native and non-native flanks, suggesting they all undergo similar conformational change. Direct observation of decreased RTX flexibility upon fusion using a technique such as NMR would further bolster this explanation.

Flanking or capping domains are known to be important for the folding and stability of leucine-rich repeat proteins,[3,32] which are dominated by short-range interactions between adjacent repeats, in contrast to globular proteins where long-range interactions can have a profound effect on the folding pathway.[5] These short-range interactions are not strong enough to overcome the high entropic

penalty associated with folding and, as such, require capping groups on both ends to lower the energy barrier and nucleate folding.[33] Our results indicate the capping effect appears to be important for the calcium-induced folding of the beta roll as well. It is important to note that for RTX-repeats the unfolded state is really an intrinsically disordered calcium-free state (not a denatured state), and the folded state is the calcium-bound beta roll. Without a flanking group, the RTX-repeats are unable to fold into the calcium-bound beta roll structure. Since PEG, the native flanking sequence, and two unrelated proteins (YFP* and MBP) all enable calcium-induced RTX folding, it appears beta roll formation does not require any enthalpic interaction (salt bridges or hydrogen bonds) with specific residues or elements of secondary structure found in the flanks. Thus, the calcium-induced folding mechanism suggests RTX-repeats can fold via an entropically driven mechanism. One possible explanation for this effect is a result of the RTX-repeats intrinsic disorder in calcium-poor environments.[11] This intrinsic disorder means that the RTX-repeats are flexible and have greater entropy than the calcium-bound beta roll. Since RTX without C-terminal flanking groups cannot undergo calcium-responsive RTX folding, the enthalpic contribution of calcium binding to RTX folding combined with the entropic contribution of the liberation of water from hydrophobic hydration[34] is not large enough to overcome the entropic cost associated with the reduction in RTX flexibility associated with beta roll formation ($\Delta G > 0$). If the entropy loss associated with beta roll formation could be reduced, it would be possible for calcium to induce RTX folding. We refer to this entropy reduction as entropic stabilization. This entropy-driven stabilization is consistent with a previous study of a synthetic beta roll motif formed by eight RTX-repeats and no capping group. Only in the presence of 25% PEG 8000 and calcium did the RTX-repeats undergo calcium-induced conformational change.[30] PEG is known to enhance molecular crowding and therefore reduce conformational freedom, which decreases the entropy of the protein.[25,26,35] Furthermore, molecular crowding has been shown to reduce the flexibility of some intrinsically disordered proteins,[36–38] pointing to the importance of entropic stabilization as a mechanism for folding RTX-repeat proteins. We have confirmed this earlier finding by showing that PEG induced calcium-dependent structure formation for the block V RTX-repeats without flanking groups.

In repeat proteins, the capping group position can have a profound effect on the folding pathway, helping to nucleate the folding of the rest of the repeats.[5] It is tempting to suggest that the flanking groups might act in a similar manner for the calcium-induced folding of the beta roll domain. Hill coefficients for cooperative calcium binding of the fully flanked RTX-motif were greater for tryptophan fluorescence titration data than for FRET data (Table 1). The tryptophan titration data report conformational change near the C-terminus and the FRET data report conformational change across the entire RTX-motif, which demonstrates greater binding cooperativity toward the C-terminus when compared with the average cooperativity across the entire block V RTX-motif. This difference in distribution of cooperativity indicates once again the importance of the C-terminal repeats, and suggests directionality in beta roll folding from C- to N-terminus. If the calcium-induced folding of the RTX-repeats is controlled principally by the C-terminal repeats, then entropic stabilization of only the C-terminal end, and not the N-terminus, would enable the RTX folding behavior we observe (Figs. 4–7).

The untethered C-terminus of the calcium-free intrinsically disordered RTX would be highly flexible and thus has relatively high entropy. A folding nucleus would have to form at the C-terminal end to allow calcium-induced RTX folding. To form this ordered conformation, the RTX-repeats need to overcome this entropic barrier, specifically near the C-terminus. Fusion to inherently structured proteins would reduce the C-terminal mobility and the entropy of folding nucleus, thereby reducing the entropic penalty for folding. Several pieces of evidence exist that support the hypothesis of folding via entropic stabilization. Friebel *et al*. studied the effect of tethering on the folding pathways of a β-barrel protein.[39] They found that tethering does affect the folding mechanism. Tethering at either terminus caused a reduction in the flexibility of the protein, reducing its entropically driven exploration of space needed to make important long-range contacts. They concluded that tethering could be used to expedite contact formation proximal to the tether. Furthermore, our hypothesis is supported by studies on constrained and linear peptides from phage display experiments.[40] Chen and co-workers found that constrained peptides had different ligand binding affinity compared to linear peptides due to differences in flexibility.

While our results indicate a non-specific mechanism that can enable RTX folding is possible, potentially via entropic stabilization of the C-terminal RTX-repeats, the data for RTX folding with the natural C-terminal flanking sequence indicate a more specific mechanism is physiologically relevant. With increased affinity and greater positive cooperativity at the C-terminus, the natural flanking sequence appears to tune the calcium responsiveness of the RTX. In T1SS, the RTX-motif is believed to remain unfolded at intracellular concentrations of calcium (submicromolar), which helps the protein remain unstructured until secreted into the extracellular environment.[14,41] Once in the extracellular environment, the RTX-motif is exposed to millimolar concentrations of calcium, and the secreted protein folds into its active form.[11,14] Recently, it has been suggested that the calcium-induced RTX-motif structure formation can act, in a sense, as a ratchet, owing to its large size relative to the outer membrane protein (OMP).[11] Since the CyaA protein (and other RTX proteins) are secreted from their C-terminal end, folding of the RTX-motifs in the C to N direction is

consistent and would offer the advantage of rapid RTX structural formation once exited from the OMP. N-to-C folding, on the other hand, would require the entire RTX-motif and N-terminal flank to be secreted in an unstructured form before folding could occur. While mechanistically there is no reason N-to-C folding should be impossible to achieve, the evolution of proteins exported through the T1SS would seem to favor C to N folding. In all cases where non-native flanks were used to enable calcium-responsive RTX conformational change, the affinity and cooperativity were diminished (Figs. 4–6; and data from reference 23). Such changes in both affinity and cooperativity indicate a specific role of the natural C-terminal flank, increasing both the calcium binding affinity and enhancing positive calcium-binding cooperativity.

In summary, we have shown that the block V RTX-motif folds into a beta roll domain in a calcium-responsive manner that depends on the entropic stabilization of the C-terminal end. This is in stark contrast to the stabilization seen in other repeat proteins, where specific capping groups are required on both ends of the protein. The responsive nature of the RTX-motif would seem to require less capping compared to other non-responsive repeat proteins. Our results are consistent with the prevailing T1SS theory that proteins are secreted from C to N-terminus and folding also likely occurs from C to N-terminus, allowing the folded beta roll motifs to act as a molecular ratchet that prevents the diffusion of CyaA back into the OMP. The block V RTX-repeats are an interesting example of how coupled binding and folding can be harnessed as a molecular switch that controls an important biological process. The inherent molecular switching properties of the beta roll scaffold may be useful for controlling future orthogonally engineered protein functions.[42,43]

Materials and Methods

Materials

Plasmid pDLE9-CyaA[19] was a generous gift from Dr Daniel Ladant (Institut Pasteur, Paris, France). The MBP expression and purification kit was purchased from New England Biolabs (Ipswich, MA). Unless stated otherwise, all enzymes were purchased from New England Biolabs and all chemicals were from Sigma-Aldrich (St. Louis, MO). IPTG was purchased from Promega (Madison, WI).

Cloning and purification of RTX-motif mutants

The pDLE9-CyaA plasmid was transformed into 5α *Escherichia coli* (New England Biolabs). RTX-motifs with truncated flanking regions were created using various combinations of oligonucleotide primers (outlined in the Supplementary Data). PCR primers contained unique restriction sites AvaI and HindIII for subsequent cloning into the pMAL-c4e expression plasmid (New England Biolabs). Expression was performed in 5α *E. coli*. Cultures were grown in LB medium with 100 mg/mL ampicillin to $A_{600}=0.5$. Expression was induced with 0.3 mM IPTG and cultures were grown for 3 h. Cells were harvested by centrifugation at 6000*g* for 10 min, sonicated and clarified by centrifugation at 15,000*g* for 30 min. Clarified lysate was diluted fivefold and applied to a gravity flow column containing amylose resin (New England Biolabs) according to the manufacturer's instructions. MBP fusions were eluted using 10 mM maltose. Eluted samples were then digested with 60 ng of enterokinase (New England Biolabs) per liter of culture for at least 36 h at 4 °C. After buffer exchange into 20 mM Bis-Tris, pH 6.0, 25 mM NaCl, ion-exchange chromatography was done with a 16/10 Q FF column (GE Healthcare, Piscataway, NJ) on an ÄKTA FPLC (GE Healthcare, Piscataway, NJ). Uncleaved MBP-fusions and cleaved beta rolls were coeluted at concentrations of NaCl>200 mM. This mixture was further purified on a Superdex 200 preparation grade gel-filtration column (GE Healthcare) equilibrated with 50 mM Tris, pH 8, 200 mM NaCl or repurified on an amylose column. RTX proteins were at least 99% pure as determined by SDS-PAGE. Samples were desalted and concentrations were determined using absorption of light at 280 nm and calculated extinction coefficients.[44]

pET/C-CyaA$^{1488\text{-}1680}$-Y (CBY) was constructed as described.[22] To examine bis-ANS binding, a mutation was made to a residue essential for fluorophore formation in both the CFP and YFP. Plasmid pET/C-CyaA$^{1488\text{-}1680}$-Y was digested with NdeI and BsrGI to remove the CFP cassette and a linker was inserted to preserve both the frame and the restrictions sites (see Supplementary Data). QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis was performed using oligonucleotides described in the Supplementary Data. This mutation changed an essential glycine at position 67[24] to serine, maintaining the native beta barrel structure without forming the fluorophore. This plasmid, pET/CyaA$^{1488\text{-}1680}$-Y* was used for the expression of CyaA$^{1488\text{-}1680}$-YFP* (where * denotes a non-fluorescent mutant). Separately, plasmid pSET-CFP (Invitrogen, Carlsbad, CA) was mutated with oligonucleotides (see Supplementary Data) to produce pSET-CFP*. The mutated CFP* was PCR amplified with NdeI and BsrGI and cloned back into similarly digested pET/CyaA$^{1488\text{-}1680}$-Y* to make pET/C*-CyaA$^{1488\text{-}1680}$-Y*. Lastly, pET/C*-CyaA$^{1488\text{-}1680}$ was made by inserting a His_6 Tag and stop codon into a unique XhoI site immediately downstream of the RTX-repeats (see Supplementary Data). All mutants were purified as described.[22]

RTX-MBP was created by ligating the RTX sequence to the N-terminus of MBP into pMAL-c4e using the NdeI restriction site. The RTX-MBP was subsequently cloned into the pMAL-c4e using the AvaI and HindIII restriction sites to create MBP-RTX-MBP. (All oligonucleotides are described in the Supplementary Data).

CD spectroscopy

Experiments were performed with a Jasco (Easton, MD) J-815 CD spectrometer with Peltier junction temperature control. Sample concentrations of 1–3 μM were analyzed in a 0.01 cm pathlength quartz cuvette at 25 °C. Baseline spectra of 50 mM Tris, pH 8.0, containing either 0 or 10 mM $CaCl_2$ was subtracted from the sample spectra. Spectral deconvolution was performed using CDPro software.[45] Each spectrum was deconvoluted using three different methods (SELCON3, CONTINLL and CDSSTR), in each case with four reference sets. The secondary structure data are presented as the average of the 12 deconvolutions with their respective standard deviation. The alpha helix and beta sheet content is given as the sum of both ordered and disordered helix and sheet.

Tryptophan fluorescence spectroscopy

Fluorescence measurements were performed with a Jasco J-815 CD spectrometer with Peltier junction temperature control and FMO-427S monochromator. Samples in 50 mM Tris, pH 8.0, were analyzed in a 1 cm pathlength quartz cuvette at 25 °C. Changes in intrinsic tryptophan fluorescence were monitored by measuring the emission spectrum between 300 nm and 420 nm (excitation at 294 nm) and are reported at the 340 nm emission peak maximum. Fluorescence measurements were done at least five times. Calcium chloride was used to titrate the samples to determine the effect of calcium-induced structural changes. Calcium titration data were fit to the Hill equation using Origin 8.0 (OriginLab, Northampton, MA).

bis-ANS binding fluorescence spectroscopy

The 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) binding to surface-exposed hydrophobic patches enabled the monitoring of structural changes in RTX-motifs.[46] This technique was applied mainly to constructs lacking the natural C-terminal flanking region that contains the conformationally sensitive tryptophan residues. The applicability of the bis-ANS binding experiment was verified by comparison of results from tryptophan-containing constructs. Changes in bis-ANS binding were monitored by measuring the emission spectrum between 420 nm and 600 nm (excitation at 390 nm). The 250 nM protein samples in 50 mM Tris (pH 8.0) were equilibrated with either 0 or 10 mM calcium (and 10 mM maltose for the MBP fusions) at 25 °C before introduction of 1 μg/mL bis-ANS. The signal from bis-ANS binding to the fusion protein (either CFP* or MBP) was subtracted from the fluorescence spectra. Fluorescence measurements were done at least five times.

Urea denaturation CD spectroscopy

CD spectra were collected for samples in a 0.01 cm pathlength quartz cuvette with various concentrations of urea and either 0 or 10 mM $CaCl_2$. Denaturation was monitored by measuring CD spectra between 200 nm and 250 nm. Data are presented for measurements at 220 nm. Reversibility was confirmed by dilution of denatured samples and measuring the resulting CD spectra. Data were fit to a single unfolding transition with linear baselines, though unfolding intermediates cannot be ruled out.

Förster resonance energy transfer (FRET) efficiency experiments

FRET experiments were done by exciting CFP donor fluorophore at 420 nm and measuring YFP acceptor emission at 527 nm. Experiments were done with a SpectraMax M2 (Molecular Devices, Sunnyvale, CA) spectrophotometer. Titrations were done with 1 μM protein at 25°C. Origin 8.0 software was used to fit the data to the Hill equation. FRET efficiency analysis was done as described.[22]

Terbium Förster resonance energy transfer

Terbium has been widely used to probe calcium-binding sites, owing to its similar ionic radius and favorable spectral properties.[47–49] Terbium binding can be measured by Förster resonance energy transfer (FRET) from excited tyrosine residues in close proximity to a bound terbium ion.[47–49] Changes in terbium binding were monitored by measuring the emission at 545 nm (excitation at 282 nm) with a SpectraMax M2 spectrophotometer. Protein samples (1 μM) in 20 mM Pipes (pH 6.8), 120 mM NaCl, and 10 mM KCl were equilibrated with various concentrations of terbium chloride in 96-well plates (Costar, Lowell, MA) for 30 min at 25°C. Fluorescence measurements were done at least five times.

PEG CD spectroscopy

CD spectra were collected for samples in a 0.1 cm pathlength quartz cuvette. Both 50% PEG8000 and 100 mM $CaCl_2$ were needed to observe beta roll-like CD spectra. Buffer baseline spectra were subtracted from the data. Data collected in the presence of PEG absorbed light too strongly to produce reliable data below 208 nm, preventing deconvolution of the CD spectra.

Analytical size-exclusion chromatography

Protein samples with or without 10 mM $CaCl_2$ were injected (0.5 ml) into a Superdex 200 16/60 size-exclusion chromatography column (GE Healthcare) and eluted in 50 mM Tris–HCl, 200 mM NaCl at pH 8.0 using an ÄKTA FPLC chromatography system (GE Healthcare).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.04.056

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