The Mode of Action of Centrin

BINDING OF Ca\textsuperscript{2+} AND A PEPTIDE FRAGMENT OF Kar1p TO THE C-TERMINAL DOMAIN

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Centrin is an EF-hand calcium-binding protein closely related to the prototypical calcium sensor protein calmodulin. It is found in microtubule-organizing centers of organisms ranging from algae and yeast to man. In vitro, the C-terminal domain of centrin binds to the yeast centrosomal protein Kar1p in a calcium-dependent manner, whereas the N-terminal domain does not show any appreciable affinity for Kar1p. To obtain deeper insights into the structural basis for centrin's function, we have characterized the affinities of the C-terminal domain of Chlamydomonas reinhardtii centrin for calcium and for a peptide fragment of Kar1p using CD, fluorescence, and NMR spectroscopy. Calcium binding site IV in C. reinhardtii centrin was found to bind Ca\textsuperscript{2+} 100-fold more strongly than site III. In the absence of Ca\textsuperscript{2+}, the protein occupies a mixture of closed conformations. Binding of a single ion in site IV is sufficient to radically alter the conformational equilibrium, promoting occupancy of an open conformation. However, an exchange between closed and open conformations remains even at saturating levels of Ca\textsuperscript{2+}. The population of the open conformation is substantially stabilized by the presence of the target peptide Kar1p-(239–257) to a point where a single ion bound in site IV is sufficient to completely shift the conformational equilibrium to the open conformation. This is reflected in the enhancement of the Ca\textsuperscript{2+} affinity in this site by more than an order of magnitude. These data confirm the direct coupling of the Ca\textsuperscript{2+} binding-induced shift in the equilibrium between the closed and open conformations to the binding of the peptide. Combined with the common localization of the two proteins in the microtubule organizing center, our results suggest that centrin is constitutively bound to Kar1p through its C-terminal domain and that centrin's calcium sensor activities are mediated by the N-terminal domain.

The microtubule-based cytoskeleton in eukaryotic cells is regulated by a large multi-protein assembly termed the microtubule organizing center (MTOC), which organizes the number, direction, and polarity of the microtubules. In higher eukaryotes, the centrosome serves as the MTOC; in lower organisms, equivalent organelles exist, e.g., the basal body in algae and the spindle pole body in yeast. Despite compositional and structural heterogeneity, the MTOCs in all eukaryotes contain conserved protein components (Fig. 1). One of these conserved proteins is centrin (also known as caltractin), an EF-hand calcium-binding protein that has been identified in a wide range of species (1, 2). Genetic studies show that centrin is essential to the cell cycle-dependent duplication and segregation of the MTOCs (3, 4). In Chlamydomonas reinhardtii, centrin-based fiber contraction also plays a fundamental role in microtubule severing at the time of flagellar excision (5).

Centrin is closely related to the archetypal EF-hand calcium sensor protein calmodulin (CaM), which also plays a role in centrosome function. Both proteins are comprised of two structurally independent globular domains connected by a flexible linker, each domain containing two helix-loop-helix calcium binding motifs (Fig. 2). However, centrin and CaM have markedly different calcium binding properties. CaM has four high affinity calcium binding sites with dissociation constants (K\textsubscript{d}) ranging from 1 to 10 \(\mu\text{M}\) under physiological salt concentrations (6), well within the range for effective response to intracellular Ca\textsuperscript{2+} signals. In contrast, most centrins have one or more non-functional calcium binding sites. For example, mutational studies and predictions based on the amino acid sequences of the calcium binding loops both suggest that site II and site III in yeast centrin (cdc31p) are defective in binding calcium (7). Similarly, the C-terminal domain of C. reinhardtii centrin (CRC-C) has substantially lower affinity for calcium than that observed for typical EF-hand calcium sensors such as CaM (8). The reduced affinity of CRC-C is ascribed to the replacement of a highly conserved glutamate with an aspartate at position 12 in calcium binding site III.

Kar1p is one of the few known targets reported for centrin (9, 10). Like cdc31p, Kar1p is an essential component of the spindle pole body in yeast (Fig. 1). Biggins and Rose proposed that one of the functions of Kar1p was to localize cdc31p to the spindle pole body (9). The putative cdc31p-binding domain of Kar1p, Kar1p-(239–257), was identified by Schiebel and co-workers (10). It was further demonstrated that the corresponding 19-residue synthetic peptide (here designated K19) binds to cdc31p in a Ca\textsuperscript{2+}-dependent manner in vitro (7). Remarkably, mutational and biophysical studies strongly suggest that the

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interaction of cdc31p with Kar1p is mediated solely by its C-terminal domain, even though the macroscopic binding affinity for Ca\(^{2+}\) of this domain measured in vitro is outside the range for a normal Ca\(^{2+}\) sensor (7, 11). The structure of the (Ca\(^{2+}\))\(_2\)-CRC-C-K\(_{19}\) complex has recently been determined (12) and reveals that CRC-C occupies an open conformation similar to that adopted by the CaM domains in their Ca\(^{2+}\)-activated state (Fig. 2). That study also revealed how subtle differences in the sequence of residues at the target binding surface translate into the different target selectivities of centrin and calmodulin. In this report, we describe investigations of the calcium and peptide binding properties of CRC-C using CD, fluorescence, and NMR spectroscopy. Our results reveal biochemical features in centrin that enable binding to Kar1p and provide insights into the molecular mechanism of centrin functions in the cell.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The recombinant *C. reinhardtii* centrin C-terminal domain was expressed and purified as described elsewhere (8). The 77-residue construct used in this study consists of residues Gly-95 through Phe-169 plus a remaining Gly-Ser sequence at the N terminus after removal of the His\(_6\) tag. Protein concentration was determined using the Advanced protein assay (Cytoskeleton Inc., Denver, CO).

Preparation of apo CRC-C followed previously published procedures involving extensive Chelex treatment (8). For the calcium titrations, appropriate buffers were also treated with Chelex 100 to minimize calcium concentration. Concentration of residual calcium was estimated to be <1 µM by atomic absorption (Garlabath Laboratories Inc., Knoxville, TN).

**Peptide Synthesis and Purification—**K\(_{19}\), the 19-residue peptide (KKRELIESWHILLFHPK) comprising the essential yeast centrin binding site in Kar1p (239–257) (10), was synthesized using solid phase Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. It was subsequently purified by reverse phase high performance liquid chromatography using a Vydac C18 column (Vydac, Hesperia, CA) on a Waters 600 system (Waters, Milford, MA). The purity of the peptide was established to be >95% by mass spectroscopy. The concentration of the peptide was determined from \(A_{200}\) measurements using a molar extinction coefficient of 5700 M\(^{-1}\) cm\(^{-1}\).

**Circular Dichroism Spectroscopy**—CD measurements were conducted on a Jasco J-810 (Jasco Inc., Easton, MD) spectropolarimeter at 293 K with constant stirring. Samples contained 3 µM CRC-C in 150 mM KCl and 25 mM Tris at pH 7. For the calcium titration, small aliquots of a 9 mM CaCl\(_2\) solution were added to achieve calcium-to-protein molar ratios between 0.5:1 and 50:1. To verify the apo state of the protein, 300 µM EDTA was added to the calcium-free sample, and no discernible change in the CD spectra was observed. Subsequent titrations started with fresh apo protein samples free of EDTA. All spectra were the average of two scans over the range 190–260 nm with a step size of 0.2 nm and a bandwidth of 1 nm. The titration was repeated in the presence of a ~1.2-fold molar excess of the K\(_{19}\) peptide.

**Fluorescence Spectroscopy**—All fluorescence experiments were performed on a Spex Fluorolog 1681 fluorometer (Spex Industries Inc., Edison, NJ) at 298 K, with all slit widths set to 2.0 nm. Excitation wavelengths were 374 and 285 nm for 8-anilino-1-naphthalenesulfonic acid (ANS) and K\(_{19}\), respectively. Fluorescence emission spectra were recorded with a scan range over the range 450–550 nm for ANS or 300–400 nm for K\(_{19}\).

For the conformational characterizations by fluorescence, small aliquots of appropriate dilutions of a 1.0 M CaCl\(_2\) standard solution were added to a sample containing either 40 µM ANS plus 4 µM decalcified CRC-C or 6 µM K\(_{19}\) plus 5 µM decalcified CRC-C, both in 150 mM KCl and 25 mM Tris at pH 7.1. For the ANS binding measurements, a calcium titration was performed, demonstrating that Ca\(^{2+}\) alone has no effect on ANS fluorescence.

For measurement of K\(_{19}\) binding to CRC-C, small aliquots of appropriate dilutions of a 1 mM apo CRC-C stock solution in a buffer containing 150 mM KCl and 25 mM Tris at pH 7.1 were added to a 5 µM K\(_{19}\) peptide solution under identical buffer conditions and incubated with 1 mM EDTA, 5 µM Ca\(^{2+}\), or 5 mM Ca\(^{2+}\). The spectra were corrected for background fluorescence by subtracting out the buffer. In each titration, 20 data points were collected over a protein-to-peptide molar ratio of 0–10:1, except in the presence of 1 mM EDTA, in which case 15 data points were collected over protein-to-peptide molar ratios of 0–50:1. The peptide binding constants were obtained by fitting the fluorescence intensity at 320 nm to a single site binding model using the program Caligator (13).

This fitting was carried out with an initial guess for K\(_{19}\) and then the best fit to the data was obtained using this guessed value. The analysis was repeated with a wide range of initial guesses for K\(_{19}\) and invariably converged. The uncertainties in the K\(_{19}\) values are taken from the S.D. values of the multiple fits. To estimate the accuracy of the fits, theoretical binding curves were generated by simulations with different parameters adjusted. These simulations showed that reasonable estimates could be obtained when K\(_{19}\) values were within 10-fold of the protein concentration, albeit with diminishing precision as the difference between the protein concentration and K\(_{19}\) increases. High S.D. values indicate accuracy to only one significant figure and permit only an estimate of the upper or lower bound to be made.

**NMR Spectroscopy**—All NMR data were acquired at 298 K on a Bruker Avance 600 spectrometer equipped with triple-resonance probe-head and triple-axis pulsed field gradient accessories. 1H and 15N chemical shift assignments for Ca\(^{2+}\)-bound CRC-C in the presence of K\(_{19}\) were reported previously (12). 1H and 13N chemical shift assignments for Ca\(^{2+}\)-bound CRC-C in the absence of the peptide were obtained from HNCA, CBCA(CO)NH, and CBCANH experiments (14) acquired on a ~1 mM CRC-C sample uniformly enriched in 15N and 13C in a buffer containing 20 mM CaCl\(_2\) and 25 mM Tris-d\(_{11}\) at pH 7. Some resonances were broadened beyond detection as a result of conformational exchange between closed and open conformations. Consequently, only 62 residues of 77 (~80%) could be assigned.

The samples used in the two calcium titrations initially contained ~200 µM 15N-enriched CRC-C in a buffer containing 100 mM KCl and 25 mM Tris-d\(_{11}\), at pH 7.1, either in the absence or presence of a 1.2-fold molar excess of the K\(_{19}\) peptide. Small aliquots of a 1 µM CaCl\(_2\) standard solution were added to the protein solution, and two-dimensional 1H and 13C in a buffer containing 20 mM CaCl\(_2\) and 25 mM Tris-d\(_{11}\), at pH 7. Some resonances were broadened beyond detection as a result of conformational exchange between closed and open conformations. Consequently, only 62 residues of 77 (~80%) could be assigned.
RESULTS

The affinity of CRC-C for Ca$^{2+}$ was investigated previously using the calcium-sensitive electrode method and one-dimensional proton NMR (8). The calcium electrode technique is limited in that it provides only the macroscopic calcium binding constants without yielding insight into the microscopic binding events. Although one-dimensional NMR can probe microscopic binding events, it is limited by the very small number of resonances that remain fully resolved during the entire course of a titration.

To gain greater insight into Ca$^{2+}$ binding and the associated effects on the conformational properties of CRC-C, CD, fluorescence, and two-dimensional $^{15}$N-$^1$H HSQC NMR were used to monitor calcium titrations of CRC-C. The two-dimensional NMR approach proved to be particularly powerful, because it enables a large number of site-specific spectral probes to be monitored simultaneously during the course of the titration. However, it is important to note that NMR is limited by sensitivity constraints to protein concentrations in the range of $\sim$10 $\mu$M and above and, thus, is not capable of providing accurate dissociation constants in the low micromolar range or less.

To complement the studies on the free protein and obtain greater mechanistic insight into how centrin functions in the cell, the same biophysical approaches were used to investigate the binding of the K$_{19}$ peptide fragment of Kar1p, one of the known centrin binding partners in the MTOC.

Analysis of Changes in Structure from Circular Dichroism Spectroscopy—Circular dichroism was used to characterize the distribution of secondary structural elements in CRC-C and determine whether any changes occurred upon binding Ca$^{2+}$ and/or K$_{19}$. The far-UV light CD spectra of CRC-C at various [Ca$^{2+}$]/[CRC-C] ratios are highly similar in shape, with two minima at 208 and 222 nm, typical of a protein rich in $\alpha$-helix (Fig. 3A). An increase in molar ellipticity (monitored at 208 nm) is observed upon the addition of calcium, reaching a maximum of $\sim$15%. Similar observations have been made for cdc31p and other EF-hand Ca$^{2+}$-binding proteins (7, 15, 16). In the case of the well studied Ca$^{2+}$ sensors CaM and troponin C, three-dimensional structures determined in the absence and presence of Ca$^{2+}$ show that this effect is due to reorganization of the disposition of the helices within the EF-hand domains. For other proteins, particularly for those that are poorly structured...
in the apo state, the increased molar ellipticity is attributed to an increase in helical content associated with stabilization of the structure.

The CD profile of mixtures of CRC-C and the peptide are similar to those of the free protein, indicating the predominance of helical secondary structure (Fig. 3B). There is a significant increase in molar ellipticity as the peptide is titrated into the protein solution, both in the absence and presence of Ca\textsuperscript{2+}. Because the free peptide occupies a predominantly random coil conformation, as suggested by its CD spectrum (Fig. 3B), the higher overall value of CD molar ellipticity observed in the presence of CRC-C is attributable to induced helicity in K\textsubscript{19}. The change in the CD spectrum elicited by adding Ca\textsuperscript{2+} to the mixture of CRC-C and the K\textsubscript{19} peptide is similar to that observed upon the addition of Ca\textsuperscript{2+} to the protein alone, i.e. the overall increase in the molar ellipticity through the course of the titration is comparable in magnitude. However, saturation of the change in molar ellipticity was achieved at a significantly lower [Ca\textsuperscript{2+}]/[CRC-C] ratio in the protein-peptide mixture, suggesting that CRC-C has a higher affinity for Ca\textsuperscript{2+} in the presence of the peptide.

**Ca\textsuperscript{2+} Binding-induced Changes in the Conformation of CRC-C**—Calcium-induced exposure of hydrophobic surfaces has been shown to be a key step in signal transduction by EF-hand Ca\textsuperscript{2+} sensor proteins. A useful probe of such conformational changes is the hydrophobic fluorophore ANS (17, 18), because its fluorescence is altered when it binds to hydrophobic patches on the accessible surface of proteins. As can be seen in Fig. 4A, the fluorescence emission spectrum of ANS in the presence of apo CRC-C is almost identical to that of ANS by itself, indicating that apo CRC-C does not bind ANS. The addition of calcium leads to increases in the fluorescence intensity and concomitant blue shifts in the maximum wavelength of emission (\(\lambda_{\text{max}}\)), indicating the exposure of hydrophobic patches on the molecular surface of CRC-C. It is of note that saturation of the ANS fluorescence change is not reached at 10 molar eq of Ca\textsuperscript{2+}, or even when the titration is carried out to 100 molar eq (additional data not shown). Moreover, the overall change in fluorescence intensity is considerably smaller than that reported for the corresponding titration carried out on CaM (17, 19). These observations suggest that CRC-C may not adopt a fully open conformation or, more likely, that there is an equilibrium distribution between closed and open conformations even at such high levels of Ca\textsuperscript{2+}.

**Binding of K\textsubscript{19} and Changes in the Conformation of CRC-C**—The K\textsubscript{19} peptide contains a tryptophan residue, which is found to be buried in a deep hydrophobic pocket in the structure of the (Ca\textsuperscript{2+})\textsubscript{2}-CRC-C-K\textsubscript{19} complex (12). This intrinsic fluorophore provides a convenient probe for monitoring concomitant binding of the peptide with calcium-induced conformational changes in CRC-C. The Trp fluorescence emission spectra of K\textsubscript{19} in the presence of CRC-C and various levels of Ca\textsuperscript{2+} are compared in Fig. 4B. The addition of apo CRC-C leads to only very slight changes in the fluorescence spectrum of K\textsubscript{19}, indicating that CRC-C does not bind the peptide with any appreciable affinity in the absence of calcium. Upon the addition of 1 eq of calcium, the fluorescence spectrum displays a substantial increase in intensity and a large blue shift (\(-30 \text{ nm in } \lambda_{\text{max}}\)), indicative of strong interaction between the K\textsubscript{19} peptide and CRC-C. Interestingly, the addition of calcium beyond 1 eq leads to little further change in the fluorescence spectrum, suggesting that the binding of a single Ca\textsuperscript{2+} ion is sufficient to activate CRC-C.

Changes in Trp fluorescence can be quantified and fit to a standard binding curve to determine the affinity of K\textsubscript{19} for CRC-C. The normalized Trp fluorescence intensity versus added CRC-C at various Ca\textsuperscript{2+} levels is plotted in Fig. 5. The best fits of these data to a simple one-site binding model yielded \(K_d\) values of 5.5 \(\pm 0.8 \times 10^{-7} \text{ M}\) in the presence of 1 eq of calcium and 1.2 \(\pm 0.2 \times 10^{-7} \text{ M}\) in the presence of saturating calcium. Interestingly, these are similar to \(K_d\) values reported for yeast centrin in the presence of calcium (7). In the presence of 1 mM EDTA, the titration is far from completion even with a final protein-to-peptide ratio of 50:1; therefore only a lower bound estimate of \(10^{-4} \text{ M}\) can be obtained for \(K_d\). The critical point from this study is the enhancement of the peptide binding affinity by three orders of magnitude or more, which clearly shows that calcium activates CRC-C for the binding of a target. The observation that the affinity of K\textsubscript{19} for CRC-C with one calcium ion bound is nearly as strong as that for the fully calcium-saturated protein indicates that the filling of a single calcium site in CRC-C is sufficient to activate the protein and trigger target binding.

**Ca\textsuperscript{2+} Affinities of CRC-C and the CRC-C-K\textsubscript{19} Complex**—NMR spectroscopy can be used to determine Ca\textsuperscript{2+} dissociation constants by monitoring the change in NMR signals upon the addition of Ca\textsuperscript{2+}. The binding parameters can be derived by fitting plots of NMR chemical shift versus added Ca\textsuperscript{2+} to standard binding equations. Two Ca\textsuperscript{2+} titrations were performed using uniformly \(^{15}\text{N}\)-labeled CRC-C, one in the absence and one in the presence of K\textsubscript{19}. The observed resonances at each calcium concentration were assigned by tracking their movement.
all the way to the calcium-saturated state of CRC-C, for which almost complete backbone resonance assignments were made (see "Experimental Procedures").

Previous studies of CRC have shown that in the absence of Ca$^{2+}$ the NMR spectrum is characteristic of a poorly folded, conformationally heterogeneous protein (8). As for the apo-protein, the CRC-C spectrum at low levels of calcium loading was characterized by a large number of resonances broadened below the noise level. Consequently, it was not possible to monitor all CRC-C signals at all levels of calcium loading. Excluding peaks that did not display appreciable chemical shift changes over the course of the titration, 32 usable titration curves from 22 distinct residues distributed throughout the protein were obtained. In the presence of the K$_{19}$ peptide, the spectral quality of CRC-C at low calcium levels was significantly higher, and a similar analysis of the data generated a total of 49 usable titration curves from 35 distinct residues distributed throughout the protein.

Normalized chemical shift changes of selected residues as a function of the molar ratio of Ca$^{2+}$ to protein are displayed in Fig. 6. The observation of clearly biphasic curves in both titrations reflects the presence of two discrete Ca$^{2+}$ binding events. Fitting the titration data to a two-site model yielded the calcium dissociation constants $K_{d_1} = 2 \pm 2 \times 10^{-5}$ M and $K_{d_2} = 3 \pm 2 \times 10^{-3}$ M in the absence of the K$_{19}$ peptide and $K_{d_1} = 1 \pm 1 \times 10^{-6}$ M and $K_{d_2} = 2 \pm 2 \times 10^{-4}$ M in the presence of K$_{19}$. The very high uncertainties reflect the complexity of fitting the data from a system that has two binding constants separated by at least a factor of 100 and the fact that accurate and precise measurements of binding constants require experiments with protein concentrations in a comparable range. Hence, these results should be viewed as guidelines for interpreting the Ca$^{2+}$ affinities.

Despite the clear limitations in the values of the binding constants, the NMR data reliably show that the two binding constants differ by 100-fold or more in both the absence and presence of K$_{19}$, consistent with a model of two independent sequential binding sites. In this model, the macroscopic $K_d$ values can be regarded as the microscopic binding constants for each individual site (20). The weaker affinity in each case is assigned to site III based on the presence of Asp-122 in place of the highly conserved glutamate residue in position 12 of the canonical EF-hand calcium binding loop. The coupling of the calcium and peptide binding events in CRC-C is reflected in the enhancement of the calcium affinity in each site by the presence of K$_{19}$.

Conformational Heterogeneity of CRC-C—In addition to providing insights into Ca$^{2+}$ binding, the NMR titration data were also extremely useful for characterizing the conformational states of CRC-C at various levels of Ca$^{2+}$ loading in the absence and presence of K$_{19}$. Fig. 7 compares the two-dimensional $^{15}$N-$^1$H HSQC spectra of CRC-C at selected levels of calcium loading where the apo, (Ca$^{2+}$)$_2$, or (Ca$^{2+}$)$_3$ state, respectively, predominates. The amount of Ca$^{2+}$ required to saturate only the high affinity site IV or both sites was calculated on the basis of the binding constants reported above. In the absence of K$_{19}$ (Fig. 7, upper panels), the spectrum of CRC-C with no Ca$^{2+}$ added (Fig. 7A) shows only a limited number (≈25) of resolved peaks, all distributed within a narrow range in the $^1$H dimension (≈7.5–9 ppm). The rest of the resonances are broadened below the noise level. The spectrum of CRC-C in the presence of ≈3 equivalents of Ca$^{2+}$, where the (Ca$^{2+}$)$_3$ state with one ion in site IV is predominant, shows increased dispersion of signals...
and a reduction in line widths (Fig. 7B). Because of the weak affinity of calcium site III ($K_d > 10^{-7}$ M), saturation of this site requires a large excess of Ca$^{2+}$ (~50 mM for 95% saturation). It is estimated that even at ~80 equivalents of Ca$^{2+}$, site III will be only ~85% occupied. The HSQC spectrum of CRC-C under these conditions has excellent dispersion of signals and narrow line widths, except for a small number of residues (Fig. 7C).

The absence of certain NMR signals for CRC-C at sub-stoichiometric levels of Ca$^{2+}$ is attributed to a conformational exchange between the closed and open conformations in the intermediate regime on the NMR time scale (8). The observation of conformational heterogeneity at low levels of Ca$^{2+}$ loading is not unusual for EF-hand protein domains and is believed to contribute favorably to the free energy of binding (21–24). As for other EF-hand domains, the conformational exchange in CRC-C is largely quenched upon saturation with Ca$^{2+}$. However, even at these high levels of Ca$^{2+}$, residues from helix F and the Ca$^{2+}$ site III in CRC-C still exhibit considerably broader signals than the rest of the protein.

The presence of the K$_{19}$ peptide significantly alters the Ca$^{2+}$ binding properties of CRC-C and the conformational dynamics throughout the entire CRC-C domain. This is clearly evident in comparing the NMR spectra of CRC-C in the presence of 3 eq of Ca$^{2+}$ (Fig. 7B) to the spectrum in the presence of 1 eq of Ca$^{2+}$ plus the K$_{19}$ peptide (Fig. 7E). In fact, a full complement of resonances is observed for the complex, even at very low calcium loading levels, and there is no significant line broadening. This observation implies that the presence of the peptide alters the equilibrium between the closed and open conformations. Remarkably, the $^{15}$N-$^1$H HSQC spectrum of CRC-C in the presence of 1 eq of Ca$^{2+}$ and K$_{19}$ (Fig. 7E) greatly resembles that under Ca$^{2+}$-saturating conditions (Fig. 7F), indicating that the protein occupies an open conformation similar to that determined for the (Ca$^{2+}$)$_2$ state in the presence of K$_{19}$ (12). Moreover, Ca$^{2+}$-induced $^1$H chemical shift changes mapped onto the sequence (Fig. 8) show that changes arising during filling of the first Ca$^{2+}$ site (open bars) are far greater than those for filling the second site (filled bars). These observations imply that binding of the first ion into site IV of CRC-C is sufficient to activate the domain, inducing an open conformation that is coupled to the binding of target protein.

**Effect of the Non-conserved Asp at Position 12 in Binding Site III**—There are two primary consequences associated with the substitution of the highly conserved glutamate at position 12 with an aspartate residue (Asp-122) in calcium site III. First, the Glu side chain provides a bidentate ligand, and substitution by Asp alters the ligand-to-metal distance and therefore reduces the affinity of site III significantly. For example, Glu to Gln mutations in CaM and calbindin D$_{9k}$ have been shown to decrease the calcium affinity by $10^4$ (25, 26). The weak affinity ($K_d > 10^{-5}$ M) of site III in CRC-C is fully consistent with these observations.

Second, the Glu residue is critical to the formation of a network of hydrogen bonds among residues within the Ca$^{2+}$ binding loop in EF-hands, which contribute significantly to the overall stability of the open conformation (27). The bidentate Glu forms hydrogen bonds with the backbone amide protons at positions 2 and 9. These hydrogen bonds are reflected in characteristically downfield-shifted $^1$H and $^{15}$N resonances when Ca$^{2+}$-bound (28). In CRC-C, the backbone resonances of Arg-148 at position 2 and Asp-155 at position 9 in the canonical calcium site IV are highly shifted downfield in both dimensions (Fig. 7C), consistent with hydrogen bonding to the side chain of Glu-158. In contrast, the corresponding Asp-112 at position 2 in site III is broadened beyond detection at all calcium levels, and the corresponding Thr-119 at position 9 is only observed in the presence of a large excess of calcium, both indicating that the corresponding hydrogen bonds are less well formed.

Additional insight is provided by the Ca$^{2+}$-induced chemical shift changes in the protein-peptide complex (Fig. 8). Interest-
Binding of Ca\(^{2+}\) and K\(_{19}\) Peptide to Centrin C-terminal Domain

**FIG. 8.** The calcium-induced spectral response of CRC-C in the presence of the K\(_{19}\) peptide. Chemical shift changes for the amide protons of CRC-C are plotted against the residue numbers. Chemical shift changes <0.005 ppm are given a nominal value of 0.005 ppm. Residues whose resonances are absent in the presence of saturating calcium are omitted. The chemical shift differences between spectra acquired with 0 and 1 eq of added Ca\(^{2+}\) are shown as empty bars, and chemical shift differences between 1 and ~20 eq of added Ca\(^{2+}\) are shown as filled bars.

**DISCUSSION**

**Equilibrium between Conformational States of CRC-C**—Important information has been obtained in regard to how the structure of CRC-C is modulated under different conditions of Ca\(^{2+}\). In the absence of Ca\(^{2+}\) ions, the CD spectrum indicates extensive helical content, and the ANS fluorescence assay suggests that the protein occupies a closed conformation with no significantly exposed hydrophobic patches. The corresponding \(^{15}\)N-H HSQC spectrum has many peaks broadened beyond detection, indicating the protein occupies two or more conformational states in the absence of Ca\(^{2+}\). The addition of Ca\(^{2+}\) initiates an exchange between the heterogeneous closed conformations and a more well-defined open conformation.

At intermediate levels of Ca\(^{2+}\), evidence of conformational heterogeneity in the NMR assays clearly exists, consistent with alterations in the ratio of the open and closed conformations in proportion to the amount of Ca\(^{2+}\) present. Under saturating Ca\(^{2+}\) conditions, the open conformation predominates, although there is still some evidence of conformational exchange reflected in the broadened NMR signals for some residues in helix F and Ca\(^{2+}\) site III. Studies of dynamic interchange between the closed and open conformations have been reported for wild-type and mutant CaM (29, 30). The consideration of an equilibrium distribution of closed and open conformations is important for understanding interactions with partner proteins and the mechanism of action of centrin in the cell.

**Energetic Coupling between Ca\(^{2+}\) Binding and Peptide Binding**—Energetic coupling between calcium binding and target interaction has been widely reported for CaM and other EF-hand calcium sensor proteins (31–33). The free energy coupling (\(\Delta \Delta G\)) provides a quantitative measure of the enhancement in the Ca\(^{2+}\) affinity of CRC-C by the presence of the K\(_{19}\) peptide, or equivalently, the enhancement in the affinity of CRC-C for K\(_{19}\) when calcium-bound, as illustrated in Scheme 1 (34). \(\Delta \Delta G\) is defined in Equation 1,

\[
\Delta \Delta G = \Delta G_4 - \Delta G_2 = -RT \ln \frac{K_{ap}}{K_{ap}^*} = -16.7 \pm 0.4 \text{ kJ} \cdot \text{mol}^{-1}
\]  

where \(R\) is the gas constant, \(T\) is the temperature in Kelvin (298 K), and \(K_{ap}\) (Ca\(^{2+}\)) and \(K_{ap}^*\) are the peptide binding constants of calcium-saturated and apo CRC-C, respectively. In theory, the free energy coupling can also be calculated from Equation 2,

\[
\Delta \Delta G = \Delta G_4 - \Delta G_2 = -RT \ln \frac{K_{ap}^*}{K_{ap} K_{ap}^*} = -13.7 \pm 0.4 \text{ kJ} \cdot \text{mol}^{-1}
\]  

where \(K_{ap}(i = 1 \text{ or } 2)\) and \(K_{ap}^*(i = 1 \text{ or } 2)\) are the calcium binding constants of CRC-C in the absence and presence of the K\(_{19}\) peptide, respectively. However, the \(\Delta \Delta G\) value generated by this approach would be of far lower precision because of the considerably larger errors in the calcium binding constants.

The >100-fold difference in the calcium affinity of sites III and IV in CRC-C (Table I) means that calcium is loaded into the two sites in a sequential manner. This property allows the dissection of the contribution of each individual calcium binding event to the enhancement of the peptide affinity of CRC-C. Our results show that the presence of the K\(_{19}\) peptide has profound effects on the calcium affinity of CRC-C. Because of limitations in determining the \(K_d\) values, it was only possible to establish that the Ca\(^{2+}\) affinity in site IV is enhanced by the presence of K\(_{19}\) by a factor \(>10\). However, because binding of Ca\(^{2+}\) and K\(_{19}\) form a closed thermodynamic cycle, estimates of the Ca\(^{2+}\) affinity can be obtained indirectly by determining the increase in the affinity of CRC-C for the peptide due to the presence of Ca\(^{2+}\). The observed enhancement of the peptide affinity in the presence of calcium (\(K_{ap}^* \approx 1.2 \pm 0.2 \times 10^{-7} \text{ M}\)) over apo CRC-C (\(K_d > 10^{-4} \text{ M}\)) is roughly three orders of magnitude. This implies the \(K_d\) for calcium in site IV in the presence of K\(_{19}\) should be well into the nanomolar range. The effect of the peptide on the calcium affinity in site III is considerably smaller (on the order of 10) because of the fundamental defect in its ability to coordinate a Ca\(^{2+}\) ion.

It is important to consider the potential influence of Mg\(^{2+}\), which is present in high concentration (mM) in the cell. Although the effect of Mg\(^{2+}\) has not been directly evaluated here, previous studies from our laboratory have shown that the reduction in Ca\(^{2+}\) affinity in the presence of 5 mM Mg\(^{2+}\) is very modest (a factor of 3–4) (35), in part due to general electrostatic screening. This implies that our results can be effectively correlated to the physiological regulation of centrin.

**K\(_{19}\) Stabilizes the Open Conformation of CRC-C**—The recent structure of (Ca\(^{2+}\))\(_2\)-CRC-C in complex with K\(_{19}\) reveals that the protein occupies an open conformation similar to CaM domains in their calcium-activated state (12). Combined with the current results, the enhanced calcium affinity of CRC-C in the presence of K\(_{19}\) can be attributed to stabilization of the open conformation. This proposal is consistent with the observation of favorable energetic coupling between calcium and peptide binding by CRC-C. The effect can be explained structurally by the peptide covering the hydrophobic surface exposed in the open conformation. Therefore, the destabilizing factor of hydrophobic exposure that must be overcome to open the domain is greatly reduced by the concomitant binding of the peptide.
Table 1 Calcium and peptide binding constants of CRC-C and the CRC-C-K19 complex

| Protein       | $K_d$ $^{1}$ | $K_d$ $^{2}$ |
|---------------|--------------|--------------|
| CRC-C         | $2 \pm 2 \times 10^{-5}$ | $3 \pm 2 \times 10^{-3}$ |
| CRC-C-K19     | $1 \pm 1 \times 10^{-6}$ | $2 \pm 2 \times 10^{-4}$ |

$^a$ The initial concentration of K19 is 5 μM. Buffer also contains 150 mM KCl and 25 mM Tris at pH 7.1.

The impact of the peptide goes beyond the calcium binding properties of CRC-C. Comparison of the two-dimensional $^{15}$N-$^1$H HSQC spectra of CRC-C in the absence and presence of the peptide reveals that peaks in the latter case are considerably sharper, indicating that the conformational exchange between the closed and open conformations observed for CRC-C in the absence of the K19 peptide has been effectively quenched by binding to the peptide. This is readily understood by envisaging the peptide wedging into the hydrophobic cleft on the molecular surface of CRC-C, effectively locking it into the open conformation.

In the presence of K19, the binding of one calcium ion is sufficient to drive CRC-C into the open conformation with concomitant binding of the peptide. This is reflected in the similarity of the $^{15}$N-$^1$H HSQC spectra of (Ca$^{2+}$)$_2$-CRC-C-K19 and (Ca$^{2+}$)$_2$-CRC-C-K19, which suggests that the conformation of CRC-C is the same in the two states. A similar conclusion can be drawn from the observation of nearly identical K19 Trp fluorescence spectra in the presence of (Ca$^{2+}$)$_2$-CRC-C or (Ca$^{2+}$)$_2$-CRC-C.

In fact, our NMR results revealed that even at Ca$^{2+}$ levels substantially below 1:1 stoichiometry, CRC-C in the presence of K19 structurally resembles the Ca$^{2+}$-saturated state. This observation is fully consistent with the CD data, in which 90% of the total change in the CD of the complex is achieved at 0.5 eq of added calcium. To explain how a trace amount of Ca$^{2+}$ suffices to open most of the CRC-C molecules, it is important to consider that two exchange processes are occurring in solution, namely one involving a chemical exchange in CRC-C between the apo and (Ca$^{2+}$)$_2$ state and the other an exchange between the peptide-bound and peptide-free states. NMR analysis at sub-stoichiometric levels of peptide revealed that two sets of signals are present in the $^{15}$N-$^1$H HSQC spectrum of Ca$^{2+}$-saturated CRC-C, whereas at sub-stoichiometric levels of Ca$^{2+}$ only one set of signals are observed in the $^{15}$N-$^1$H HSQC spectrum of CRC-C. These two observations imply that the K19 off-rate is much slower than the Ca$^{2+}$ off-rate. Consequently, a single Ca$^{2+}$ ion can transiently activate a CRC-C molecule long enough for a K19 peptide molecule to bind and then come off and repeat the process with a different CRC-C molecule. Because of the slower off-rate for the peptide, the K19-CRC-C complex stays intact for a much longer time than the Ca$^{2+}$ ion stays bound.

Is Centrin Constitutively Bound to Kar1p$^a$—Kar1p is a permanent component of the half-bridge (10), a specialized region of the nuclear envelope attached to one side of the spindle pole body. Based on the high affinity of CRC-C for calcium in the presence of the K19 peptide ($K_d$ well into the nM range), we propose that Kar1p and centrin are constitutively bound to each other even at the basal level of Ca$^{2+}$ in the cell. This binding would also make centrin a permanent component of the half-bridge as well, which is consistent with the observation of the colocalization of centrin (cdc31p) and Kar1p to the half-bridge of the spindle pole body in yeast (9). In theory, evidence in support of our hypothesis could be obtained if a very careful titration is done under conditions where the concentrations of centrin and K19 are sufficiently low and relevant amounts of calcium are added. Such experiments are not accessible by the methods used in this study. Fluorescence anisotropy might be applicable, as has been reported for examining calmodulin-peptide interactions with dissociation constants in the high nanomolar range (36).

The interaction of centrin with Kar1p is mediated by the C-terminal domain, which leaves the N-terminal domain of centrin to function as a Ca$^{2+}$ sensor that interacts with other target proteins. A model in which the C-terminal domain serves as the “structural” domain and the N-terminal domain functions as a regular calcium sensor is reminiscent of troponin C. In skeletal muscle, the C-terminal domain of troponin C is constitutively bound to the N-terminal domain of troponin I, whereas the N-terminal domain of troponin C interacts with the C-terminal domain of troponin I in a calcium-dependent manner (37, 38). Recently, such extended modes of binding have been observed for calmodulin in its interactions with ion channels and receptors (39, 40). Remarkably, genetic evidence shows that deletion of the high affinity cdc31p binding site in Kar1p does not suppress cell growth at low temperatures (7). Therefore the major role of Kar1p may be to recruit cdc31p to the half-bridge (9). Schiebel and co-workers proposed that at least one other protein, Xp, also interacts with cdc31p (7). Clearly, the identification of one or more binding partners to the centrin N-terminal domain is an urgent priority.

Conclusions—Our detailed analysis of the Ca$^{2+}$ binding and conformational properties of CRC-C provides an example of the importance of an in-depth characterization by biophysical approaches to understanding key aspects of protein function. Clearly, for Ca$^{2+}$ sensor proteins it is critical to consider the calcium affinity in the context of its target. The issue of relative abundance of sensor and target is also highly relevant in this respect. Studies of CaM have shown that factors other than calcium binding, such as phosphorylation, may also play a role in the target regulation mechanism (41). Interestingly, Salisbury and co-workers have recently observed an increase in phosphorylation of centrin at the G/M phase of the cell cycle (42). These authors proposed that phosphorylation of centrin signals the separation of centrosomes at prophase. It was further shown that phosphorylation occurs at a highly conserved protein kinase A phosphorylation site (Ser-167 in CRC) near the very C terminus of centrin. Our recent structure of the (Ca$^{2+}$)$_2$-CRC-C/K19 complex shows that the C terminus of centrin is in a critical region for interaction with Kar1p. Therefore, phosphorylation of Ser-167 could perturb the local hydrophobic packing and subsequently lead to the release of Kar1p. This would provide a means to regulate the proposed constitutive interaction between centrin and Kar1p, an intriguing possibility that would place centrin directly at the intersection between Ca$^{2+}$ and kinase signaling.

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