Structural Independence of the Two EF-hand Domains of Caltractin*

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Caltractin (centrin) is a member of the calmodulin subfamily of EF-hand Ca\(^{2+}\)-binding proteins that is an essential component of microtubule-organizing centers in many organisms ranging from yeast and algae to humans. The protein contains two homologous EF-hand Ca\(^{2+}\)-binding domains linked by a flexible tether; each domain is capable of binding two Ca\(^{2+}\) ions. In an effort to search for domain-specific functional properties of caltractin, the two isolated domains were subcloned and expressed in *Escherichia coli*. Ca\(^{2+}\) binding affinities and the Ca\(^{2+}\) dependence of biophysical properties of the isolated domains were monitored by UV, CD, and NMR spectroscopy. Comparisons to the corresponding results for the intact protein showed that the two domains function independently of each other in these assays. Titration of a peptide fragment from the yeast Kar1p protein to the isolated domains and intact caltractin shows that the two domains interact in a Ca\(^{2+}\)-dependent manner, with the C-terminal domain binding much more strongly than the N-terminal domain. Measurements of the macroscopic Ca\(^{2+}\) binding constants show that only the N-terminal domain has sufficient apparent Ca\(^{2+}\) affinity in vitro (1–10 \(\mu\)M) to be classified as a traditional calcium sensor in signal transduction pathways. However, investigation of the microscopic Ca\(^{2+}\) binding events in the C-terminal domain by NMR spectroscopy revealed that the observed macroscopic binding constant likely results from binding to two sites with very different affinities, one in the micromolar range and the other in the millimolar range. Thus, the C-terminal domain appears to also be capable of sensing Ca\(^{2+}\) signals but is activated by the binding of a single ion.

Caltractin (also known as centrin) is an EF-hand protein found widely distributed in eukaryotes. This protein is an essential component of the centrosome, which mediates chromosome segregation during mitosis. In lower organisms, the protein is found in analogous structures, e.g. the microtubule-organizing center (MTOC) in algae and the spindle pole body in yeast. Mutations and complete deletions of the caltractin gene have shown that the protein is required for proper cell division (1, 2). Algal caltractin and the caltractin analog in *Saccharomyces cerevisiae* (cdc31p) have been shown to be required for the normal duplication and separation of the MTOC in their respective cells (3–5). The yeast protein has been shown to be essential for cell viability, and temperature-sensitive mutants cause cell cycle arrest (6, 7). In *Chlamydomonas reinhardtii* algae, caltractin has been shown to be a major component of the nucleus basal body connector and of the distal striated fiber that connects the basal bodies of contractile fibers, thereby playing an essential role in flagellar excision (8, 9).

Caltractin is a protein of 169 residues, which shares about 50% sequence identity with the well studied EF-hand protein calmodulin (CaM).1 Like CaM, *C. reinhardtii* caltractin (CRC) is comprised of two globular domains connected by a central linker, and each domain contains a pair of EF-hands that has the potential to bind two Ca\(^{2+}\) ions. However, several characteristics of caltractin are distinct from CaM. For example, there are 40 conserved residues within the EF-hand domains of caltractins that are not shared by calmodulins (10). Unlike CaM, caltractin contains a positively charged N-terminal extension and conserved aromatic residues near both termini. Caltractin is also distinguished from CaM by having two sites with Ca\(^{2+}\) affinities outside the normal range for transducing Ca\(^{2+}\) signals, and UV and CD spectra that do not change upon the addition of calcium (11).

Current evidence suggests that the two domains of caltractin interact with different target proteins. Two caltractin-specific targets, Kar1p and Kic1p, have been identified in yeast; like caltractin, they are essential components of the spindle pole body (12, 13). Mutational studies suggest that the C-terminal domain mediates the Ca\(^{2+}\)-dependent interaction of caltractin with these proteins. It is assumed that other protein targets bind to the N-terminal domain of caltractin, although these have yet to be identified.

*In vitro*, the two domains of CaM behave as independent domains connected by a flexible tether (14, 15). The flexibility of the tether allows the two domains to wrap around and act in concert to tightly bind to a single target (16). Given the strong likelihood of domain-specific functions for caltractin, we have carried out a series of biophysical characterizations of intact caltractin and its two isolated domains. These data provide insights into biochemical characteristics of caltractin that show the independence of its two domains in vitro. The results are

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1 The abbreviations used are: CaM, calmodulin; HSQC, heteronuclear single quantum coherence, CRC, *C. reinhardtii* caltractin; CRC-N, 96 amino acid N-terminal fragment of CRC; CRC-C, 77 amino acid C-terminal fragment of CRC.
consistent with a model in which caltractin serves as a bridging/assembly factor in microtubule-organizing centers (17).

**EXPERIMENTAL PROCEDURES**

**Reagents—**HEPES and Tris buffers, salts, and other chemicals utilized in protein purification were reagent grade. Tris-d$_4$, used in the NMR sample buffer was obtained from Cambridge Isotope Laboratories (Andover, MA). Ultra pure potassium chloride used in calcium titration buffer was from Aldrich. Hirudin and bovine thrombin were purchased from Calbiochem. The 19-residue peptide (KKRELIESKWHRLLFH-(9-fluorenyl) methylcarbonyl) chemistry.

**Subcloning—**Three constructs of CRC were used in this study; namely, the full-length (CRC, 1-169 amino acids) protein, the N-terminal domain (CRC-N, 1-94), and the C-terminal domain (CRC-C, 95-169). CRC-N, and CRC-C were each subcloned into a pRSET-A vector (Invitrogen, Carlsbad, CA) modified to include a thrombin cleavage site for removal of the N-terminal His$_8$ tag and transformed into BL21(DE3) pLyS E. coli cells (Novagen). Cloning procedures resulted in the addition of two extra amino acids (Gly-Ser) to the N-terminal end of each half-fragment.

**Expression—**For each protein preparation, up to 12 colonies from freshly transformed cells were checked by SDS-PAGE for overexpression upon induction of 3 ml of cultures with 0.2 mg isopropyl-1-thio-β-D-galactopyranoside (IPTG). The best overexpressors were used to prepare inocula for larger cultures (2-6 liters). Unlabeled protein samples were obtained from cells grown in 2¥YT at 37°C in the presence of appropriate antibiotics. Uniformly $^{15}$N-labeled CRC-N or CRC-C was obtained by growing freshly transformed cells in minimal M9 medium with $^{15}$NH$_4$Cl (1g/liter) as the sole nitrogen source. To prevent loss of selection, overnight cultures were centrifuged and re-suspended in fresh medium twice, before using them to inoculate large cultures. Cultures were grown to an OD$_{600}$ of 0.6-0.7, induced with IPTG, and grown for 3 more hours before harvesting. Cell cultures reached an OD$_{600}$ of about 6.5 at the time of harvest.

**Decalcification—**Protein E. coli cell pellets were resuspended in a 10-fold excess of sonication buffer (w/v) consisting of 25 mM Tris, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride at pH 8.0. Cells were lysed by sonication and centrifuged at 14,000 rpm for 1 h at 4°C to remove cell debris. The supernatant, titrated with 10 mM imid-azole, was incubated with preequilibrated Ni-nitrilotriacetic acid resin (Qiagen) for 2 h at 4°C on a tilt table. The resin was loaded into filtration columns (Fisher Scientific) and washed with 2 column volumes of wash buffer (25 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0). His-tagged caltractin or its domains were then eluted with a similar buffer that contained 150 mM imidazole. Fractions containing caltractin, identified using the Bio-Rad protein assay and by SDS-PAGE, were pooled and diluted with 25 mM Tris, pH 8.0 to 3x the volume. The ammonium chloride was added to 2.5 molar final concentration at 2°C immediately prior to cleavage of the His$_8$ tag with 0.5 units of bovine thrombin (Sigma) comprising the essential yeast caltractin-binding region (18) was synthesized using solid phase Fmoc (9-fluorenyl)methylcarbonyl) chemistry.

**Isolation and Purification—**Protein samples from cells grown in M9 medium with 15NH$_4$Cl (1g/liter) as the sole nitrogen source. To prevent loss of selection, overnight cultures were centrifuged and re-suspended in fresh medium twice, before using them to inoculate large cultures. Cultures were grown to an OD$_{600}$ of 0.6-0.7, induced with IPTG, and grown for 3 more hours before harvesting. Cell cultures reached an OD$_{600}$ of about 6.5 at the time of harvest.

Titrations were monitored by measuring the concentration of free calcium in solution using a calcium-sensitive electrode (Model 93-20, Orion, Boston, MA) or by following NMR chemical shift changes. For the calcium-sensitive electrode measurements, 4 ml of a 100 μM solution of protein was used per titration. Electrochemical data were analyzed by non-linear curve fitting using the equation and methods described by Weber et al. (11) for binding of calcium ions to the two calcium binding sites within a given domain. In brief, the data was fitted to Equation 1.

\[
\text{Fraction Bound} = \frac{n_1K_{21}[\text{Ca}^{2+}]_{\text{free}}}{1 + K_{21}[\text{Ca}^{2+}]_{\text{free}}} + n_2[\text{Ca}^{2+}]_{\text{free}} + n_3[\text{Ca}^{2+}]_{\text{free}} \]  

(1) Where the binding sites are equivalent, this reduces to Equation 2.

\[
\text{Fraction Bound} = \frac{n_1K_{21}[\text{Ca}^{2+}]_{\text{free}}}{1 + K_{21}[\text{Ca}^{2+}]_{\text{free}} + n_3[\text{Ca}^{2+}]_{\text{free}}} \]  

(2) Where $K_{21}$, $n_1$, $K_{22}$, and $n_2$ reflect the association constants and number of bound calcium ions per mole of protein. The term in $n_3$ is included to account for nonspecific binding, which can become significant upon saturation of the binding sites, especially at high calcium concentrations. When the fit to the second equation was as good as the first, the simpler model was selected. Uncertainties in the protein concentration led to non-ideal stoichiometries of binding derived from curve fitting titration data.

The NMR experiments were conducted by measuring the chemical shift changes in one-dimensional 1H NMR spectra. Ca$^{2+}$ binding parameters from NMR data were derived using the computer program Crvfit (R. Boyko and B. D. Sykes, University of Alberta) as described elsewhere (20).

**Kar1p Binding—**Binding of the 19-residue Kar1p peptide to CRC, CRC-N, and CRC-C was monitored by fluorescence spectroscopy using a Spex Fluorolog fluorimeter (Spex Industries, Inc., Edison, NJ). The excitation wavelength was set at 295 nm to minimize possible interference from the tyrosine residue in CRC and CRC-N. Excitation and emission slit widths were 1.5 mm. Sample buffer consisted of 25 mM Tris, 100 mM KCl, 5 mM CaCl$_2$, pH 8.0. Peptide concentration was determined using a protein extinction coefficient of 5700 M$^{-1}$ cm$^{-1}$ at 278 nm.

Aliquots of a 300 μM protein sample were added to a 3 μM (initial concentration) Kar1p peptide solution, and the corresponding single scan emission spectrum was recorded over the range 300-450 nm. Spectra were corrected for background fluorescence by subtracting the buffer spectrum. About 20 data points were collected per titration over a protein-to-peptide molar ratio of 0-50.

**NMR Spectroscopy—**Bruker AMX 500 MHz, AMX 600 MHz, Avance 600, and DRX 750 MHz spectrometers were used to collect NMR data. The two-dimensional 1H-$^2$H HSQC spectra were acquired in the apo state, calcium-loaded state (10-fold molar excess of calcium), or the calcium-loaded state in the presence of Kar1p peptide (1.2-fold molar excess). The solutions contained 1 mM protein in a buffer containing 20 mM Tris-d$_4$ and 100 mM KCl at pH 7.55 and 30°C. 1H-$^2$H HSQC spectra were recorded with spectral widths of 13.3 ppm (apo) or 16 ppm (Ca$^{2+}$) in the proton dimension and 32 ppm in the nitrogen dimension. 16-48 scans were acquired over 1024-2048 128-512 complex points.

The data were processed using Felix (version 95.0 or 97.0, MSI, San Diego, CA) with zero-filling to 512 points in the $^1$H dimension and 128 complex points in the $^15$N dimension. The 1H dimension was referenced to water at 4.74 ppm, and the center of the $^15$N dimension was indirectly referenced to 11.36 ppm.

**Titration of Ca$^{2+}$** into unlabeled apoCRC-C was monitored by one-dimensional 1H NMR spectroscopy. Sample conditions were 0.5 mM protein in 50 mM HEPES, 75 mM KCl, pH 7.51, and 303 K. These conditions were chosen to match those of calcium-sensitive electrode
measurements reported here and in previous studies (11), so as to allow direct comparison of these results. FIDs were processed using Felix 98 (MSI, San Diego, CA).

RESULTS
To pursue the question of the functional independence of the two domains of caltractin, we subcloned the N- and C-terminal fragments and characterized their biophysical properties. Both the 96 amino acid N-terminal fragment (CRC-N) and 77 amino acid C-terminal fragment (CRC-C) contain a pair of calcium-binding EF-hand motifs that constitute an independent folding unit. New expression vectors were constructed because expression of the two fragments was at levels of weak to none in the system used for the intact protein (11). The genes for the two domains were subcloned into a vector that produces protein with a His$_6$ tag in a 20-residue extension fused to the N terminus of the protein. The tag enables rapid purification by Ni$^{2+}$ affinity chromatography. This system produces milligrams of protein per liter of culture, a prerequisite for high resolution structure determination, which is one of the primary objectives of our research program.

Caltractin Domains Are Structurally Independent—To validate the strategy of studying the two CRC domains independently of each other, UV, CD, and NMR spectroscopy were used to probe the structural features of the two domains as independent modules and in the intact protein. This involved comparing summed spectra of the two domains to the corresponding spectrum of the intact protein obtained under identical conditions in both the absence and presence of Ca$^{2+}$. The results obtained by monitoring each of these spectroscopic probes were consistent with each other; when the spectra of the domains are added together, they correspond closely to the spectrum of intact CRC. The NMR spectra are most powerful in this context because there are such a large number of discrete signals that can be monitored, so these data will be discussed in more detail.

Two-dimensional $^{15}$N,$^1$H HSQC spectra were collected from $^{15}$N-enriched samples in the apo- and Ca$^{2+}$-loaded states for CRC-N (Fig. 1, A and B), CRC-C (Fig. 1, D and E) and intact CRC (Fig. 1, G and H), which shows a single peak for each NH group in each protein. The most obvious difference in these
spectra is the poorer quality of the data for the intact protein, as evidenced by the significantly broader signals. This arises from a combination of the substantially larger molecular weight of the intact protein relative to the fragments, as well as a greater tendency to self-associate and precipitate, particularly in the presence of Ca\(^{2+}\).

NMR spectra of the apo proteins (panels A, D, and G) consist of cross peaks within a narrow range of chemical shifts (7.5–9.0 ppm). Interestingly, although CRC-N is larger than CRC-C by 19 residues, apoCRC-N exhibits narrower line widths and greater spectral dispersion. The lower dispersion for CRC-C is independent of protein concentration; apoCRC-C at 1 mM gives a spectrum nearly identical to that at 0.2 mM (data not shown). Thus, the poorer spectral features of CRC-C are presumably not the result of protein aggregation, and it is likely that apoCRC-N is more structured than apoCRC-C.

HSQC spectra of the Ca\(^{2+}\)-saturated proteins (panels B, E, and H) are significantly different from the corresponding apo-proteins. In general, the dispersion of cross peaks for the calcium-loaded proteins is greater than for the apoproteins, indicating a significant change in the chemical environment and possibly an increase in the amount of ordered structure in the Ca\(^{2+}\)-bound state. The appearance of low field-shifted \(^1\)H resonances at >10 ppm is characteristic of Ca\(^{2+}\)-loading of EF-hand proteins (21). These signals arise from a highly conserved glycine in position 6 of the calcium binding loop that is part of the extensive hydrogen-bonding network formed upon Ca\(^{2+}\) binding (22). Two such signals are clearly observed in the spectrum of CRC-N (Fig. 2). Only one Gly resonance with a chemical shift >10 ppm was observed for CRC-C, and this resonance at 10.8 ppm is assigned to Gly-116 in Ca\(^{2+}\) site III. The peak at 10.0 ppm is presumed to arise from the corresponding residue in position 6 of site IV in which the glycine is substituted by an asparagine (Asn-152).

It is noteworthy that the spectrum of CRC corresponds closely to the sum of those of CRC-N and CRC-C, with the very few differences readily explained by the additional N and C termini resulting from separation of the two domains. Fig. 2 shows an expansion of the low field region from the \(^{15}\)N-\(^1\)H HSQC spectra acquired for the Ca\(^{2+}\)-loaded states, which contains signals from residues in and adjacent to the Ca\(^{2+}\) binding loops that are extremely sensitive to conformation of the protein. The near identity of the chemical shifts in the two panels of this figure indicates that the structures of the fragments are the same as when the two domains are fused to each other. Our total observations provide strong evidence in support of the structural independence of the two domains in the intact protein.

**Caltractin Domains Have Significantly Different Calcium Affinities**—One of the primary motivating factors for studying the two domains of CRC in isolation is to better understand the Ca\(^{2+}\) binding properties of the protein. The Ca\(^{2+}\) binding curve observed for the intact protein by the method of calcium-sensitive electrode was very complex (11). Binding of four Ca\(^{2+}\) ions was observed, and macroscopic binding constants were determined assuming a model of two equivalent higher affinity sites (\(K_a = 8.3 \times 10^3 \text{ M}^{-1}\)) and two equivalent lower affinity sites (\(K_a = 6.2 \times 10^4 \text{ M}^{-1}\)). The data could also be fit to more complex models, but there was not sufficient evidence to distinguish among various models, so the simplest was chosen to represent the data. Based on similarity to the consensus EF-hand loop Ca\(^{2+}\) binding loop sequence, the pair of higher affinity Ca\(^{2+}\) sites was assigned to CRC-N. The pair of lower affinity sites was assigned to CRC-C on the basis of the substitutions relative to the consensus sequence in the Ca\(^{2+}\) binding loops in both site III and site IV (Fig. 3).

In an attempt to better understand the Ca\(^{2+}\) binding properties of CRC, the Ca\(^{2+}\) binding affinity of each domain was determined using the calcium-sensitive electrode method. The analysis of intact CRC was repeated as a control, and the results corresponded very closely to that reported previously. Experiments on the isolated domains revealed that both CRC-N and CRC-C bind two calcium ions (Fig. 4). Using a model in which the calcium binding affinities of both sites of each domain are equivalent, the calculated calcium affinities (\(K_a\)) for CRC-N and CRC-C are 3.4 ± 0.2 \(\times\) 10\(^4\) M\(^{-1}\) and 2.2 ± 0.2 \(\times\) 10\(^5\) M\(^{-1}\), respectively (Table I). Attempts at fitting the data to a model with two different calcium binding affinities for the two sites did not provide a significant improvement, so the simplest model was selected. Note that these calcium binding affinities correspond reasonably well to those measured previously (11). Moreover, these results confirmed our previous proposal that the N-terminal domain contains the higher affinity Ca\(^{2+}\) binding sites and the C-terminal domain the lower affinity sites. The low macroscopic binding affinity determined in this way for CRC-C implies that the C-terminal domain does not sense Ca\(^{2+}\) signals in a physiological environment.

**Interaction of CRC with a Kar1p Peptide Is Mediated by the C-terminal Domain**—Studies of the interaction of the yeast spindle pole body component Kar1p and yeast caltractin...
Comparisons of the spectra for the Ca2+ peptide were carried out to determine whether the interaction of CRC and its isolated domains with the Kar1p peptide were monitored by 15N,1H NMR using 15N-enriched protein could be substituted for the yeast protein and if the yeast caltractin (cdc31p) have identified a minimal Cdc31p-binding region (18). These authors also showed that binding of a Kar1p peptide to yeast caltractin in vitro is calcium-dependent. Studies of the interaction of CRC and its isolated domains with the Kar1p peptide were carried out to determine whether the C. reinhardtii protein could be substituted for the yeast protein and if so, to examine if the two domains bound the peptide with different affinities, as would be predicted on the basis of the results from the analysis of the yeast protein (18).

Interactions of CRC, CRC-C, and CRC-N with the Kar1p peptide were monitored by 15N,1H NMR using 15N-enriched protein and a large excess of peptide (Fig. 1, C, F, and I). Comparisons of the spectra for the Ca2+-loaded states with and without peptide (panels B/C, E/F, H/I) reveal that signals are affected in both domains, and therefore that the peptide interacts at least weakly with both domains. There is a very noticeable change in the apparent quality of the spectrum for intact CRC upon addition of the peptide. This arises from a significant reduction in the resonance line widths of the protein, which we attribute to a reduction in the relative degree of nonspecific aggregation of the protein. Comparison of panels C, F, and H in Fig. 1 show that the spectrum of the CRC-Kar1p peptide complex is nearly equivalent to the sum of the spectra of the Kar1p complexes with CRC-N and CRC-C. As noted above, this implies that contact between the two domains of CRC in the Kar1p complex is minimal.

The peptide binding-induced changes in NMR chemical shifts demonstrate that an interaction between the protein and the peptide occurs but does not indicate the relative strength of these interactions. Because the Kar1p peptide contains one tryptophan and CRC has none, the interaction between the peptide and the protein was monitored by tryptophan fluorescence spectroscopy. Changes in peptide fluorescence at 330 nm as a function of the molar ratio of protein to peptide are plotted in Fig. 5. The titration curves for CRC and CRC-C reveal a rapid increase in the fluorescence signal intensity that indicates strong binding with a 1:1 stoichiometry. The fluorescence response in the CRC-N titration is very much weaker; so weak that not all peptide molecules have protein bound even with a 25-fold excess of protein. Estimates of \( K_D \) from these data are in the nanomolar range for CRC and CRC-C, versus in the high micromolar range for CRC-N. These results imply that the interaction of caltractin with Kar1p is mediated primarily or exclusively by the C-terminal domain, consistent with conclusions drawn from genetic experiments showing that only mutations in the C-terminal domain of yeast caltractin cause reductions in the binding affinity for Kar1p (e.g. Refs. 18 and 23).

The data in Fig. 5 do not preclude the possibility that in intact CRC, both domains may interact with a single peptide molecule. In fact, we believe that weak binding to CRC-N contributes to the vertical offset in the titration curve for CRC versus CRC-C in Fig. 5. Although the intrinsic affinity of the N-terminal domain is very low (as shown by the fluorescence titration of CRC-N), binding of a peptide molecule to the C-terminal domain introduces a very high local concentration of the peptide, which greatly increases the potential for interaction with the N-terminal domain. It is important to note this “linkage effect” may occur in vitro, but is presumably not physiologically relevant because the N- and C-terminal domains of CRC are believed to bind to different target proteins. This point is supported by the fact that only mutations in the C-terminal domain of yeast caltractin affect the binding of yeast CRC to Kar1p in cell-based assays (18, 23).

Detailed NMR Analysis of Ca2+ Binding by CRC-C—Given that CRC-C is the primary mediator of the Ca2+-dependent interaction of CRC with Kar1p, it was somewhat surprising to find that in vitro macroscopic Ca2+ binding constants for this domain were outside the micromolar range that is normally associated with Ca2+ signaling by EF-hand proteins. This apparent anomaly prompted a more in-depth investigation of Ca2+ binding by CRC-C using 1H NMR spectroscopy. The power of applying the NMR approach is that binding events can be monitored by a large number of distinct probes, which can provide detailed information on the microscopic binding events that contribute to the observed Ca2+ affinity. One-dimensional 1H NMR spectra of CRC-C acquired at Ca2+ concentrations in the range 0–7 molar equivalents showed a characteristic increase in dispersion in the presence of Ca2+ (Fig. 6A). Most resonances of apo- and fully Ca2+-loaded CRC-C are sharp, and the line widths are similar. However, peaks are broader in the range 0.25–2 molar equivalents of Ca2+, indicative of an exchange between chemical environments occurring in the NMR intermediate exchange regime (24). This was readily apparent in complementary two-dimensional 15N,1H HSQC experiments because resonance lines broaden sufficiently to cause significant reductions in peak intensity (data not shown).

The response of five different well dispersed peaks in the one-dimensional 1H NMR spectrum are plotted as a function of added Ca2+ in Fig. 6B. The individual \( K_D \) values were first determined using a model assuming two binding sites with the same affinity shown in Reaction 1.

\[
\text{CRC-C} + 2\text{Ca}^{2+} \rightleftharpoons \frac{K_A}{K_D} \text{CRC-C} \cdot \left(\text{Ca}^{2+}\right)_2
\]

For the three best fit curves, the values determined were: \( 1.9 \pm 0.2 \times 10^{-4} \, \text{m}, 5.7 \pm 0.8 \times 10^{-5} \, \text{m}, \) and \( 1.3 \pm 1.0 \times 10^{-5} \, \text{m} \). The average \( K_D \) value for CRC-C based on these values is \( 8.7 \times 10^{-6} \, \text{m} \). A fourth curve shows a steeper ascent and yields a stronger \( K_D \) of \( 4 \pm 7 \times 10^{-6} \, \text{m} \). The cumulative results from attempts to fit these data with a simple model with two Ca2+ sites of equal affinity strongly suggest the need for a more complex model. In fact, the fifth resonance plotted in Fig. 6B displays a clearly biphasic binding...
mode with the chemical shift initially increasing in value, then decreasing in accord with the existence of two discrete Ca$^{2+}$ binding events. Moreover, closer inspection of the plots in Fig. 6B reveals that the signal with the lowest $K_D$ value is consistent with reporting on a single high affinity binding site, whereas the others report on a combination of this site and a second site that has at least an order of magnitude weaker affinity.

The observation of two-phase binding curves (20) strongly indicates Ca$^{2+}$ binding to CRC-C occurs in a stepwise manner as shown in Reaction 2.

\[
\text{CRC-C} + \text{Ca}^{2+} \rightleftharpoons \text{CRC-C-Ca}^{2+} \rightleftharpoons \text{CRC-C-Ca}_{2}^{2+}
\]

**REACTION 2**

An estimate of 5 $\mu$M for the $K_D$ value of the high affinity site can be derived from the monophasic binding curve with the lowest $K_D$. Assuming that the biphasic curve reports separately on the two binding events, the slope of the second phase provides a very crude estimate for the weaker binding site in the range of 3 mM (Table I). However, simulations showed that this combination of binding affinities would result in a two-phased binding curve in our Ca$^{2+}$-sensitive electrode experiments. Because we have some confidence in the $K_D$ estimate from the NMR titration data for the high affinity site, but very low confidence in the $K_D$ estimate for the low affinity site, we carried out simulations with one site fixed at 5 $\mu$M and established that under the conditions of our experiments, an inflection would clearly detectable in our electrode response data only if the affinity in the weaker site was 500 $\mu$M or weaker. Thus, a better estimate of the upper limit for $K_D$ in the weaker binding site is 500 $\mu$M. Regardless of the exact values of $K_D$, the microscopic binding events dissected out by NMR reveal the following observations. (i) Measurement of the macroscopic binding constant of CRC-C is not sufficient to define its ability to be activated by Ca$^{2+}$ signals. (ii) The affinity of site IV in CRC-C is likely to be sufficient to enable this domain to sense typical intracellular Ca$^{2+}$ signals.

**DISCUSSION**

Cloning and expression of isolated domains of CRC have provided useful new reagents to probe the biochemical functions of this protein. Our studies showed that CRC is organized into two structurally independent domains that bind calcium and a peptide fragment from the Kar1p target as independent modules. The macroscopic Ca$^{2+}$ binding constant for CRC-N is in the range of 1–10 $\mu$M as expected for Ca$^{2+}$ sensors, whereas the value for CRC-C is significantly higher. However, we have shown that determination of the macroscopic binding constants of CRC-C is insufficient to truly understand whether or not it serves as a Ca$^{2+}$ sensor, as dissection of the microscopic Ca$^{2+}$

<table>
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<tr>
<th>Protein</th>
<th>Method$^a$</th>
<th>$K_{A1}$$^b$ $\mu$M$^{-1}$</th>
<th>$N_I$</th>
<th>$K_{A2}$$^b$ $\mu$M$^{-1}$</th>
<th>$N_2$</th>
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<td>CRC$^c$</td>
<td>A</td>
<td>3.4 ± 0.2 × 10^5</td>
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<td>2.2 ± 0.2 × 10^4</td>
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<tr>
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<tr>
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<td>2.0</td>
<td>6.2 ± 1.6 × 10^5</td>
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<tr>
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<td></td>
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<tr>
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<td>C</td>
<td>2 × 10^5</td>
<td></td>
<td>3 × 10^5</td>
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</table>

$^a$ Calcium binding affinities were derived from: A, Ca$^{2+}$-sensitive electrode; B, NMR data-fitting function for a single type of binding site ($N$) with a corresponding binding affinity, $K_D$; or C, NMR data-fitting function with two explicit binding sites (with high uncertainty in the value of $K_{A2}$ due to the lack of an experimentally determined zero point).

$^b$ Values determined by Method A were averages of three independent measurements.

$^c$ Taken from Weber et al. (11).
binding events by NMR revealed CRC-C binds Ca\textsuperscript{2+} sequentially, with one-site binding in the 1–10 μM range and a second site with much weaker affinity. Thus, both the N- and C-terminal domains of CRC appear to be able to sense typical intracellular Ca\textsuperscript{2+} signals.

We also showed that *C. reinhardtii* caltractin binds as strongly as yeast caltractin to a Kar1p peptide fragment containing the caltractin-binding region. Fluorescence binding assays showed that the C-terminal domain binds this peptide much more strongly than the N-terminal domain. The results confirm genetics experiments indicating that the substrate affinity of CRC for Kar1p is primarily derived from the C-terminal domain. For example, it has been shown that a chimera containing the N-terminal half of calmodulin and the C-terminal half of yeast caltractin (cdc31p) behaves more like native caltractin with respect to binding to targets (18). A recent study of temperature-sensitive alleles of cdc31p in yeast (23) showed a majority of phenotypes, including G_{1}/M arrest, plasma membrane disruption, protein mislocalization, and binding/activation of Kic1p kinase (another cdc31p substrate), arising from mutations within the C-terminal domain of cdc31p. Some alleles that affect Kic1p binding also affect Kar1p binding. Taken together these findings confirm a central role for the C-terminal domain of caltractin in binding to Kar1p.

As noted above, CRC and CaM share about 50% overall sequence identity. Proteins or domains with such a high degree of sequence identity usually tend to have similar three-dimensional structures. However, we have shown there are several significant differences between CaM and caltractin. Structures of calmodulin with peptide fragments of a few of its target proteins provided the first detailed information about the mode of interaction between EF-hand proteins and their targets (25, 26). In these structures, peptide binding is seen to cause a reorganization of the domains from a flexibly extended dumbbell to a single compact globule binding around the target. The plasticity of the central linker is the key factor enabling the coordinate action of the two domains to envelope a single substrate molecule. In contrast, the mode of target interaction in troponin C is strikingly different in that the two domains extend away from each other and bind two discrete sites on troponin I (27). Our results strongly suggest that CRC utilizes its two domains independently to function, and so, the structural mode of target binding of CRC is likely to be more similar to troponin C than calmodulin. The functional independence of the CRC domains is attained by fine-tuning metal ion affinities and binding surfaces. These factors may also serve as the source of the differences among different caltractin isoforms that are found in higher eukaryotes including humans.

Caltractin appears to have a critical function in the cell. As noted above, cdc31p is essential for cell viability in yeast, and the protein is required for normal duplication and separation of the microtubule-organizing center in several organisms. There is of course an implied relationship to diseases such as cancer, which involve abnormal cell cycling and indeed, high levels of caltractin mRNA have been found in breast carcinoma cell lines (28). Moreover, a human caltractin isoform (centrin2) has recently turned up as part of the xeroderma pigmentosum group C complex, which is involved in nucleotide excision repair of damaged DNA (29). Investigations of downstream signaling pathways will help in elucidating the mechanism of normal and abnormal functions of caltractin. To this end, biophysical and structural investigations of the interaction of caltractin with Kar1p and other targets are ongoing in our laboratory in an effort to obtain an improved understanding of the molecular basis for caltractin function.

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Structural Independence of the Two EF-hand Domains of Caltractin
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