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Conformational Changes and Calcium Binding by Calretinin and Its Recombinant Fragments Containing Different Sets of EF Hand Motifs

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ABSTRACT: Four recombinant fragments, representing different sets of EF-hand motifs of rat calretinin (CR) (I−II, I−III, III−VI, IV−VI), were prepared, and their Ca^{2+}-induced conformational changes were compared with those of full-length recombinant CR. All fragments were able to bind calcium ions as shown by $^{45}Ca^{2+}$ overlay method on nitrocellulose and fluorescence measurements. The intrinsic tryptophan fluorescence intensity (F_I) of apo-CR reversibly increased about 3-fold upon addition of calcium, indicating a change of conformation. The F_I of fragments I−II (Trp 25) and I−III (Trp 25 and 116) increased about 1.4-fold on calcium binding, but that of fragment III−VI (Trp 116) increased 3.5-fold. Calcium titration of CR monitored by Trp fluorescence intensity showed that recombinant CR and some fragments bound Ca^{2+} with high affinity (K_d below 0.4 μM) and with high cooperativity. An apparent Hill coefficient for Ca^{2+}-induced fluorescence changes in CR was about 3.7. CR bound to organomercurial-agarose independent of Ca^{2+} concentrations and could be eluted with 2-mercaptoethanol or DTT, indicating that Cys 101 and 266 did not form cystine. The fluorescence intensities of cysteine-linked fluorescent probes 5-iodoacetamidofluorescein and *N*-(1-pyreneiodoacetamide) were increased approximately 1.3-fold upon calcium binding by CR. These data indicate that CR binds Ca^{2+} with high affinity and cooperativity and that this binding induces a change of conformation that involves the interaction of different parts of the molecule. Taken together, our results suggest that CR works as an on/off switch within a narrow range of free Ca^{2+} by interacting with as yet unidentified targets.

Calretinin (CR)[1] is a 31.5 kDa member of the EF-hand superfamily of calcium binding proteins which includes calbindin D28k, calmodulin, troponin C, parvalbumin, and S100 proteins [reviewed in Persechini et al. (1989), Baimbridge et al. (1992), and Andressen et al. (1993)]. CR is homologous to calbindin D28k, sharing 58% identical residues (Rogers, 1987; Parmentier & Lefort, 1991; Strauss & Jacobowitz, 1993). Both proteins contain six putative EF-hand motifs, each of which consists of a helix−loop−helix structure and represents a potential calcium binding site [reviewed in Kretsinger (1987) and Heizmann and Hunziker (1991)]. CR is present in a subpopulation of brain neurons (Rogers, 1987; Winsky et al., 1989, 1992; Résibois & Rogers 1992), but its function is not known. The intracellular distribution of CR (Winsky & Kuźnicki, 1995) and its ability to bind calcium ions would be consistent with some role in neurotransmission. This role could involve interactions with other proteins, modulating calcium diffusion, or the short-term buffering of local calcium concentrations in cytosol. A

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis (oxyethylenenitrilo)tetraacetic acid; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; CR, calretinin; DTT, dithiothreitol; F_I, fluorescence intensity; IAF, 5-iodoacetamidofluorescein; IPTG; isopropyl β-D-thiogalactopyranoside; pyrene, *N*-(1-pyreneiodoacetamide); MOPS, 3-(*N*-morpholino)propanesulfonic acid.

calcium buffering role has been suggested for calbindin D28k and parvalbumin [reviewed in Miller, (1991), Baimbridge et al. (1993), and Iacopino et al. (1994)]. However, it is unclear whether these proteins have a neuroprotective role against Ca^{2+} overload [reviewed in Andressen et al. (1993)]. Among the recent studies in which the possible neuroprotective role of CR was analyzed in tissue culture, few reports support such a conclusion (Lukas & Jones, 1994; Isaacs et al., 1996). Other studies indicate that CR per se is not protective, although it may act together with other yet unknown factors (Möckel & Fischer, 1994; Mouatt-Prigent et al., 1994), or that no protection is rendered by the presence of CR in contrast to the presence of parvalbumin (Elliot & Snider, 1995). On the basis of the low evolutionary rate of CR and the strong sequence conservation with calbindin D28k in EF-hand regions and sequences that follow the even-numbered EF-hands, it has been suggested that CR may do more than simply bind calcium for the purpose of lowering cytosolic concentrations (Parmentier & Lefort, 1991; Heizmann & Hunziker, 1991; Cheung et al., 1993). To understand if CR is a Ca^{2+} buffer or a Ca^{2+} sensor interacting with specific targets, we analyzed its responses to Ca^{2+} binding with the use of spectroscopic techniques, since such responses may be an indication of the protein function (Skelton et al., 1994). The results show that CR significantly changes its conformation upon Ca^{2+} binding, which allows us to speculate that it may play a role in Ca^{2+}-mediated signaling.

EXPERIMENTAL PROCEDURES

Cloning of the Recombinant Rat CR Fragments. Four inserts were created using the polymerase chain reaction (PCR, Gene Amp PCR System 9600, Perkin-Elmer/Cetus) with the full-length CR coding region (pGEXCR; Strauss et al., 1994) as template and custom primer pairs (Operon, California) as follows: Fragment I−II, CR826F (1−18) and CR281R (281−299), aa, GS(CR 1−100)NSS; fragment I−III, CR826F (1−18) and CR407R (407−425), aa, GS-(CR 1−142)NSS; fragment III−VI, CR297F (297−314) and CR826R (799−816), aa, GS(CR 100−271); fragment IV−VI, CR433F (433−450) and CR826R (799−816), aa, GS-(CR 145−271).

The numbers in parentheses designate the position in the rat CR coding sequence, each "F" primer contained the nucleotides TAC CCG GGG ATC C upstream of the 18-mer CR sequence, and each "R" primer contained the nucleotides GAA GCT TGA ATT C upstream of the complementary 18-mer CR sequence. This allowed the products to be subsequently cut with restriction enzymes *Bam*HI and *Eco*RI (Boehringer-Mannheim) for directional subcloning into a similarly cut pGEX-1, pGEX-2, or pGEX-3 plasmid (Pharmacia) so that the coding regions remained in frame with the glutathione-*S*-transferase sequence of these vectors. Positive clones were identified by restriction analyses and Sanger sequencing (Sequenase 2.0 US Biochemical) of double-stranded plasmids using pGEX primers. Positive clones were also verified by Western immunoblot analysis and $^{45}Ca^{2+}$ binding of bacterial cell lysates on nitrocellulose both before and after IPTG (Sigma) induction for recombinant protein production.

Production and Purification of Recombinant Rat CR and Its Fragments. Recombinant CR and its fragments were produced as glutathione-*S*-transferase fusion proteins in *Escherichia coli* and purified as published earlier (Strauss et al., 1994) with modifications. The bacterial suspensions were frozen, thawed, sonicated briefly, and centrifuged at 10 000*g* for 5 min. Pellets from this spin were re-extracted twice in the same way, except they were not frozen before the third extraction. The supernatants were filtered (0.45 μm) and applied (0.6 mL/min) to a prepacked 2 mL glutathione-Sepharose 4B column (Pharmacia) equilibrated with PBS and 1% Triton X-100. The column was washed with 10 volumes of PBS and 1% Triton X-100 to remove unbound proteins and 10 volumes of buffer A (50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) prior to the addition of thrombin. Approximately 2 volumes of 10 μg/mL thrombin (Sigma) in buffer A was applied to the column. The flow was stopped, and the column was incubated overnight at 4 °C. Cleaved recombinant CR or fragments (shown above) were eluted with buffer A. CR containing fractions were equilibrated with buffer B (50 mM Tris, pH 8.0, 20 mM NaCl, 1 mM EGTA) on PD-10 Sephadex G-25 columns (Pharmacia). The samples were then applied to a DEAE-cellulose column (2 mL DE52, Whatman) equilibrated with buffer B, and the columns were washed with 20 volumes of buffer B. Recombinant CR or fragments were eluted with buffer C (50 mM Tris, pH 8.0, 20 mM NaCl, 2 mM $CaCl_2$), concentrated (Centricon 10, Millipore), and desalted on PD-10 columns. The purity of all preparations was estimated using SDS−PAGE. The gels were stained with silver or Coomassie brilliant blue. Protein concentration was measured using a Bradford procedure (Bio-Rad) with bovine serum albumin as a standard. The proteins were stored at −80 °C.

Ca^{2+}-Dependent Measurements of UV Spectra and Fluorescence Intensity. The UV spectrum of CR (0.8 mg/mL) was performed using a Shimadzu UV-160U Spectrophotometer. Fluorescence intensity of CR (0.08 mg/mL) in 50 mM Hepes pH 7.4 was measured in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA at 37 °C using Perkin-Elmer LS50B Luminescence Spectrometer. IAF-CR and pyrene-CR were analyzed at 0.07 mg/mL. The titration of fluorescence intensity of CR and its fragments with increasing concentrations of free Ca^{2+} was performed in the presence of Ca^{2+}/EGTA mixture, 100 mM KCl, and 10 mM MOPS, pH 7.2, at 20 °C (Calcium Calibration Buffer Kit I, Molecular Probes). Solutions of concentrated CR and its fragments were desalted on PD-10 columns (Pharmacia) equilibrated with 100 mM KCl and 10 mM MOPS, pH 7.2, creating the stocks of CR and recombinant fragments. The CR (or fragments) stocks were diluted at least 100-fold to a final concentration of 1 μM each in solution A and B. Solution A ("Zero Free Calcium Buffer") contained 10 mM EGTA, 100 mM KCl, 10 mM MOPS, pH 7.2, while solution B ("High Calcium") contained 39.8 μM free calcium buffer (10 mM Ca^{2+}/EGTA, 100 mM KCl, 10 mM MOPS, pH 7.2). The pH of both solutions was carefully monitored and the initial fluorescence measurements were performed using 2 mL of CR (or fragments) in solution A. Subsequent measurements were performed using 2 mL of the mixtures obtained by removal of a volume of CR (or fragments) in solution A and replacing the same volume of CR (or fragments) in solution B. The free Ca^{2+} concentration was calculated as shown in the protocol provided by Molecular Probes and described by Tsien and Pozzan (1989). Calcium

Table 1: Molecular Mass and Amino Acid Composition of CR Fragments

EF hand domains	amino acids[a]	molecular mass (Da) determined by MS: theoretical	molecular mass (Da) determined by MS: experimental
CR	GS-1−271	31 549	31 548
fragment I−II	GS-1−100-NSS	11 539	11 541
fragment I−III	GS-1−142-NSS	17 110	17 109
fragment III−VI	GS-100−271	20 169	20 169
fragment IV−VI	GS-145−271	14 961	ND

[a] Numbers refer to the sequence of rat calretinin; MS, mass spectrometry; G, Gly; S, Ser; N, Asn represent amino acid residues that are present due to the molecular biology manipulations.

titration data were fitted with the Hill equation: $F = F_0 + \Delta F(K_a[Ca^{2+}])^n/(1 + (K_a[Ca^{2+}])^n)$ using a least-squares method. F_0 and ΔF are the initial fluorescence and maximal change in fluorescence, respectively, K_a is the apparent binding constant, and n is the Hill coefficient. The fluorescence intensities for the various titrations curves were normalized by taking the fitted values for F_0 and ΔF as 0 and 1, respectively. The solid line in Figure 4 represents the best fit with the values for K_a and n given below (± standard error). The data were fitted and plotted using a Macintosh computer and the program Kaleidagraph (Synergy Software, Reading, PA).

Labeling of Cysteine Residues with Fluorescent Probes. CR was labeled with 10-fold excess of 5-iodoacetamidofluorescein or *N*-(1−pyreneiodoacetamide) (Molecular Probes) (in dimethylformamide) in a buffer solution containing 150 mM NaCl, 50 mM Tris, pH 8.0, and either 1 mM EGTA or 1 mM $CaCl_2$ for 16 h at 4 °C. Solutions were desalted on NAP-5 column (Pharmacia) equilibrated with 10 mM MOPS and 150 mM KCl, pH 7.2, and then concentrated using CentriCell 10 000 N.M.W.L. (Polysciences, Inc., Warrington, PA). The gel was photographed using Transilluminator (UVP) and NP4 camera (Polaroid).

Analysis of Ca^{2+}-Dependent Hydrophobicity. The ability of CR and its fragments to bind to octyl-agarose resin in the presence of calcium and the elution from the resin in low calcium buffers was analyzed as described (Kuźnicki et al., 1994).

PAGE, $^{45}Ca^{2+}$-Overlay Assay. SDS−PAGE was performed on 15% precast gels (Bio-Rad) in Tris-glycine buffer, pH 8.3. The Ca^{2+}-binding of CR fragments was analyzed on nitrocellulose filters (BA85, Schleicher & Schuell) as described by Maruyama et al. (1984).

RESULTS

Characterization of CR Fragments. Four inserts representing portions of rat CR DNA (described in Experimental Procedures) were created using PCR and cloned into pGEX expression vectors. The polypeptides were expressed in *E. coli* and purified to homogeneity. The molecular mass of these CR fragments, determined by mass spectrometry, was consistent with that from the predicted amino acid sequence (Table 1).

All fragments were able to bind calcium ions as shown using $^{45}Ca^{2+}$ overlay method (Figure 1A). The extent of binding by the fragments appeared similar to that observed for recombinant CR.

Changes of the UV Spectrum and Intrinsic Fluorescence Intensity of CR and Its Fragments as a Result of Ca^{2+} Binding. The UV absorption spectrum of Ca^{2+}-bound CR had a peak at 279.2, and apo-CR had a peak at 277.6 (not shown). The difference spectrum revealed a negative absorbance at 250−280 nm and positive absorbance between 280 and 310 nm (Figure 2), indicating that a conformational change may have occurred around aromatic residues.

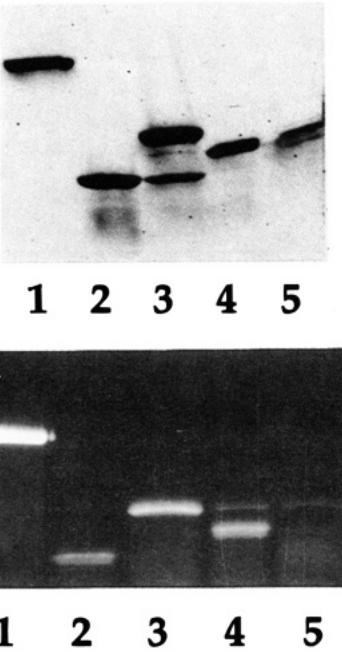

FIGURE 1: Properties of CR recombinant fragments. Lane 1, recombinant CR (31.5 kDa); lane 2, fragment I−II (11.5 kDa); lane 3, fragment III−VI (20 kDa); lane 4, fragment I−III (17 kDa); lane 5, fragment IV−VI (15 kDa). (A, top) Ca^{2+} binding to CR fragments using the $^{45}Ca^{2+}$-overlay assay. Proteins were separated by SDS/15% PAGE, blotted onto nitrocellulose, stained with Ponceau Red (not shown), destained, and incubated with $^{45}Ca^{2+}$. The preparation of fragment III−VI contained a degradation product, absent from another preparation below (panel B). (B, bottom) Labeling of cysteine residues in CR and its fragments with IAF. Proteins were labeled with IAF, desalted, concentrated and separated by SDS/15% PAGE. Fragment IV−VI was degraded during labeling.

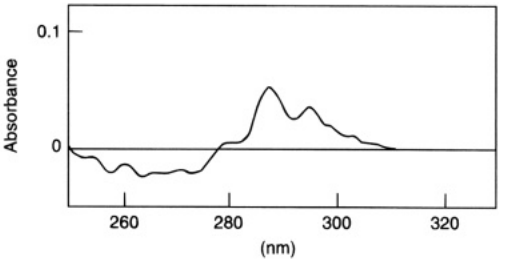

FIGURE 2: Ca^{2+}-induced UV difference spectrum of recombinant CR. Spectra of CR (0.8 mg/mL) were taken at room temperature in the presence of 50 mM HEPES, pH 7.4, and either 0.5 mM EGTA or 0.1 mM $CaCl_2$. The spectrum obtained in the presence of Ca^{2+} was subtracted from that obtained in the presence of EGTA.

CR contains two tryptophan residues, Trp 25 and Trp 116, which are located within the predicted α-helical regions of the EF-hand motifs I and III, respectively. Excitation at 280 nm resulted in a fluorescence emission spectrum with peak intensity at 334 nm (Figure 3). The intrinsic tryptophan fluorescence intensity decreased about 3-fold when EGTA was added and returned to the initial level when the concentrations of extra added calcium exceeded that of EGTA (Figure 3). These changes of fluorescence intensity indicate that reversible conformational changes take place

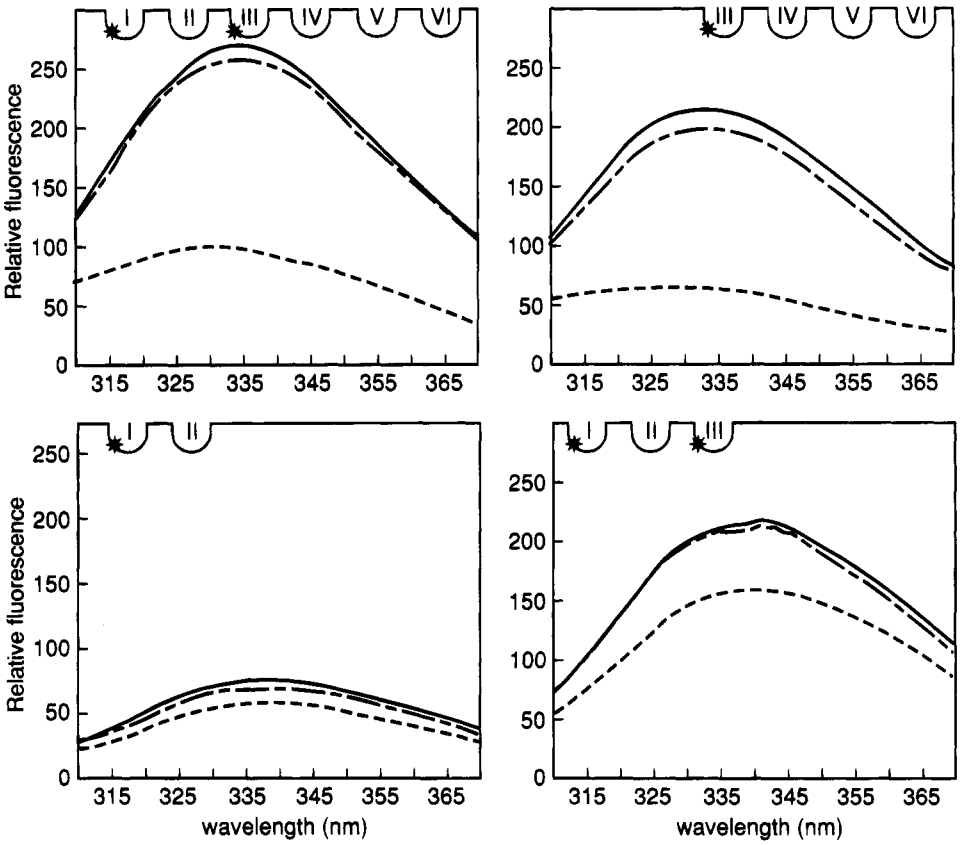

FIGURE 3: Tryptophan fluorescence spectra of CR (1 μM) and its fragments (1 μM) at 20 °C. Initial spectra were taken in 10 mM MOPS, 100 mM KCl, and 0.1 mM $CaCl_2$, pH 7.2 (—), and then EGTA was added to 0.5 mM final concentration (- - -), and finally $CaCl_2$ to 1.1 mM final concentration (- — -). CR and fragments are represented by schematics at the top of each spectra. An asterisk (*) shows the location of Trp.

upon Ca^{2+}-binding and that tryptophan residue(s) are in a different environment in Ca^{2+}-bound and Ca^{2+}-free conformation.

Recombinant fragments of CR were analyzed to establish which tryptophan residue(s) effect the observed fluorescence changes. Tryptophan fluorescence intensity increased 3.5-fold upon addition of calcium for fragment III—VI (Trp 116), and 1.4-fold for fragment I—II (Trp 25) (Figure 3). This suggests that conformational changes around Trp 116 is primarily responsible for the observed changes of fluorescence in the intact CR. Interestingly, fragment I—III, which represents the N-terminal half and contains both Trp 116 and Trp 25, increases fluorescence intensity only 1.4—fold upon Ca^{2+} binding. These fluorescence observations could be explained if EF-hand motif III is lacking its pairing partner (e.g., EF-hand motif IV) to form an active calcium binding domain. Examination of fragment IV—VI did not reveal significant changes in fluorescence intensity; this fragment contains no Trp and Tyr fluorescence intensity did not change detectably.

Since the intrinsic tryptophan fluorescence intensity of CR is sensitive to calcium binding, it could be used to estimate Ca^{2+}-binding parameters of CR and the fragments. The initial measurements were performed in the presence of EGTA, and subsequent titration was performed using Ca^{2+}/EGTA buffers with known free Ca^{2+} concentration (Figure 4). Fitting the Ca^{2+}-titration data with the Hill equation, (e.g., as shown in Figure 4) provides the same information as the Hill plot. The parameters of the best fit are in Table 2. The data indicate that CR binds Ca^{2+} with $K_d \simeq 0.4$ μM, and the

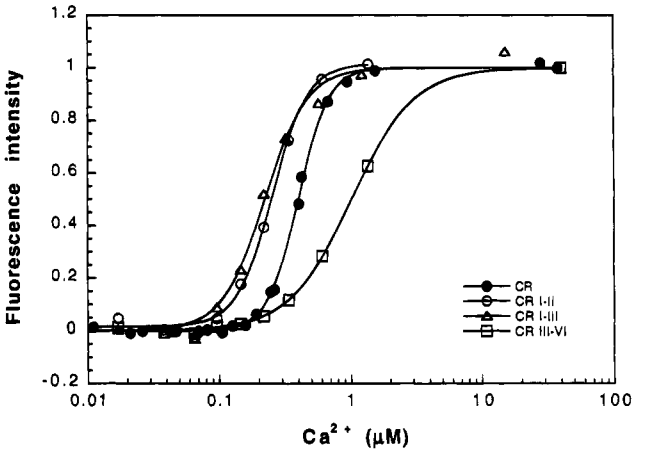

FIGURE 4: Changes of Trp fluorescence intensity of CR and its fragments as a function of free [Ca^{2+}]. CR, closed circles; fragment I—II, open circles; fragment I—III, triangles; fragment III—VI, squares. Ca^{2+}/EGTA solutions were obtained from Molecular Probes as a Calcium Calibration Kit. Protein concentrations were 1 μM. Ca^{2+}-binding parameters were calculated using Hill's equation as described in Experimental Procedures and are shown in Table 2.

fragments representing the N-terminal portion of the molecule bind Ca^{2+} with $K_d \simeq 0.2$ μM, whereas the fragment representing the C-terminal portion (III—VI) binds with $K_d \simeq 1$ μM. The analysis of data by Hill's equation indicates that CR and its fragments bind Ca^{2+} with high cooperativity (Table 2).

Reactivity of Cysteine Residues of CR. CR has two cysteine residues: one located in the region connecting EF

Table 2: Ca^{2+}-Binding Constants and Octyl-Agarose Affinity of CR and its Fragments

EF-hand domains of CR and fragments	$K_a \times 10^{-6}$ (M^{-1})	n	elution with EGTA from octyl-agarose
CR	2.5 (±0.03)	3.7 (±0.1)	+
I−II	4.0 (±0.15)	3.2 (±0.3)	−[a]
I−III	4.5 (±0.31)	2.7 (±0.4)	−
III−VI	1.0 (±0.02)	1.8 (±0.07)	−
IV−VI	ND	ND	−
II−VI[b]	ND	ND	+
II−V[b]	ND	ND	−

[a] Only partial binding occurs. [b] Tryptic peptides described by Kuźnicki et al. (1995); K_a, apparent binding constant; n, Hill coefficient calculated from data shown in Figure 4; ND, not determined.

hand motif II and III (Cys 101) and another located near the C-terminal end of the molecule (Cys 266). It was found that both native and recombinant CR could bind to organo-mercurial-agarose (a resin capable of binding reduced thiols) and was eluted with reducing agents such as DTT or 2-mercaptoethanol. These observations indicate that CR thiols do not form a disulfide bridge. Only minor differences in binding and elution of CR were found in the presence and absence of calcium (not shown). The reduced state of the cysteine residues was confirmed by labeling CR with the fluorescent probes *N*-(1-pyreneiodoacetamide) (not shown) and 5-iodoacetamidofluorescein (Figure 1B). The efficiency of labeling was the same for apo- and Ca^{2+}-bound CR (not shown). The optical properties of the probes were sensitive to Ca^{2+}-binding. When labeled IAF-CR was excited at 491 nm the emission at 515 nm was increased 1.3-fold upon calcium binding and was reversible. Similarly when pyrene-CR was excited at 343 nm, the emission at 393 nm was increased about 1.3-fold (not shown). No shift in the emission or excitation spectra was observed. All four recombinant fragments of CR could be labeled with IAF indicating that both thiols are accessible in the fragments (Figure 1B), although fragment IV−VI (Cys 266) was degraded during the treatment. The fluorescence intensity of the IAF attached to recombinant fragments I−III (Cys 101) and III−VI (Cys 101 and 266) decreased 10%, and that attached to fragment I−II (Cys 101) increased 10% upon Ca^{2+} binding.

Ca^{2+}-Dependent Hydrophobicity of CR and Its Fragments. Intact CR has been shown to exhibit complex interactions with hydrophobic matrices. CR binds to hydrophobic resins such as octyl-agarose in the presence of calcium and is only partly released when the calcium concentration is lowered by EGTA (Kuźnicki et al., 1994). All fragments except fragment I−II bound to octyl-agarose in the presence of calcium (Table 2). Upon addition of EGTA none of the recombinant fragments were eluted from octyl-agarose, but all were eluted with SDS. These results indicate that none of the recombinant fragments has Ca^{2+}-dependent hydrophobic properties in contrast to the intact protein or tryptic fragment C1 (II−VI EF hand motifs) (Table 2).

DISCUSSION

Cell-type specific distribution of CR in the adult nervous system is well documented (Rogers, 1987; Winsky et al., 1989; Arai et al., 1991; Jacobowitz & Winsky, 1991; Résibois & Rogers, 1992), but little is known about the biochemical properties and function of CR. CR is a small, predominantly cytosolic protein that binds up to five Ca^{2+} per molecule (Cheung et al., 1993). One may expect that CR's physiological role is related to its Ca^{2+} binding properties, as it is commonly held that the nature of the response to Ca^{2+}-binding is correlated with function of the protein (da Silva & Reinach, 1991; Skelton et al., 1994). An intracellular buffer, such as parvalbumin or calbindin D_{9k} need only bind Ca^{2+} efficiently. In contrast, calmodulin requires conformational changes and a subsequent exposure of a hydrophobic region upon Ca^{2+} binding for activity (Klee, 1991). In fact, limited conformational changes and lack of hydrophobic exposure may be necessary for selectivity between "sensor" proteins and "buffer" proteins (Skelton et al., 1994). Therefore, information on the nature of the response of CR to Ca^{2+} may help to discriminate between possible Ca^{2+}-buffer or Ca^{2+}-sensor activities of this protein.

CR and its fragments demonstrated high affinity for Ca^{2+}, in the sub-micromolar range. Fragment IV−VI, consisting of the C-terminal half of the molecule, had lower affinity for Ca^{2+} than both fragments containing the N-terminal EF-hand motifs. This might be due to the fact that CR EF hand motif VI appears to be defective in Ca^{2+} binding. We found changes in the UV spectrum (between 250−300 nm) of CR upon Ca^{2+} binding, indicating a change in the environment surrounding aromatic residues in Ca^{2+}-free and Ca^{2+}-bound conformations. The strong Ca^{2+}-dependent change in the 250−270 nm region indicates a structural change around Tyr and Phe residues. The large (3-fold) and reversible increase of Trp fluorescence intensity observed upon Ca^{2+} binding by CR also indicates that a conformational change occurs upon ion binding. The use of recombinant fragments of CR allowed us to determine that Trp 116, located in EF-hand motif III, is primarily responsible for the observed changes of fluorescence intensity. Since fragment I−III, containing Trp 116, but lacking the C-terminal half of the molecule, does not change fluorescence intensity as does fragment III−VI or the entire molecule, we suggest that the EF-hand motif III forms a globular domain with EF-hand motif IV in the intact molecule. Since such domain is a basic structural/functional unit in the EF-hand family of calcium binding proteins, the fragments of CR containing an odd number of EF-hands (such as fragment I−III) are expected to have structurally and biochemically dysfunctional sites.

Hill plot analyses of the Ca^{2+}-dependent changes of fluorescence intensity (Figure 4) indicated that CR and its recombinant fragments bound Ca^{2+} with high affinity and high cooperativity. An apparent Hill coefficient for the entire CR was approximately 3.7 which indicates that there is a cooperativity among the Ca^{2+}-binding sites (Grabarek & Gergely, 1983). It is not clear if this cooperativity is due to intramolecular or intermolecular interactions. The fact that fragment I−II has a greater Hill coefficient than its number of Ca^{2+}-binding sites suggests that in addition to intramolecular cooperativity there may be dimer formation in the presence of Ca^{2+}. This might be attributed to the common phenomenon of increased hydrophobicity upon Ca^{2+} binding by calcium binding proteins.

We showed earlier that the hydrophobicity (Kuźnicki et al., 1994), pattern of tryptic digestion (Kuźnicki et al., 1995), intracellular distribution (Winsky & Kuźnicki, 1995), and antibody recognition (Winsky & Kuźnicki, 1996) of CR are Ca^{2+}-dependent. The results of this work extend our earlier

observations and confirm the conclusion that CR has different conformations in the Ca^{2+}-free and Ca^{2+}-bound states. This in turn implies the existence of some as yet unidentified targets of CR. We suggest that such target(s) may exist in membranes, since CR shows a Ca^{2+}-dependent interaction with synaptic and microsomal membranes (Winsky & Kuźnicki, 1995). Such an interaction could be achieved via Ca^{2+}-induced exposure of a hydrophobic patch, the existence of which was suggested in our earlier work (Kuźnicki et al., 1994). The results reported here suggest that CR is a Ca^{2+}-sensor that works as an on/off switch within the narrow and physiologically relevant range of free Ca^{2+} concentrations, 0.2−0.8 μM.

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