Characterization of calretinin I–II as an EF-hand, Ca^{2+} , H^{+} -sensing domain

MAŁGORZATA PALCZEWSKA,^{1,2} GYULA BATTÁ,³ PATRICK GROVES,^{1,4}
SARA LINSE,⁵ AND JACEK KUŹNICKI^{1,6}

¹Department of Molecular and Cellular Neurobiology, Nencki Institute of Experimental Biology, Warsaw, Poland

²Centro Nacional de Biotecnología CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain

³Research Group for Antibiotics of the Hungarian Academy of Sciences and Department of Pharmaceutical Chemistry, University of Debrecen, Debrecen, Hungary

⁴Centro de Investigaciones Biológicas, Madrid, Spain

⁵Department of Biophysical Chemistry, University of Lund, Lund, Sweden

⁶International Institute of Molecular and Cell Biology in Warsaw, Warsaw, Poland

(RECEIVED January 18, 2005; FINAL REVISION March 29, 2005; ACCEPTED April 11, 2005)

Abstract

Calretinin, a neuronal protein with well-defined calcium-binding properties, has a poorly defined function. The pH dependent properties of calretinin (CR), the N-terminal (CR I–II), and C-terminal (CR III–VI) domains were investigated. A drop in pH within the intracellular range (from pH 7.5 to pH 6.5) leads to an increased hydrophobicity of calcium-bound CR and its domains as reported by fluorescence spectroscopy with the hydrophobic probe 2-(p-toluidino)-6-naphthalenesulfonic acid (TNS). The TNS data for the N- and C-terminal domains of CR are additive, providing further support for their independence within the full-length protein. Our work concentrated on CR I–II, which was found to have hydrophobic properties similar to calmodulin at lower pH. The elution of CR I–II from a phenyl-Sepharose column was consistent with the TNS data. The pH-dependent structural changes were further localized to residues 13–28 and 44–51 using nuclear magnetic resonance spectroscopy chemical shift analysis, and there appear to be no large changes in secondary structure. Protonation of His12 and/or His27 side chains, coupled with calcium chelation, appears to lead to the organization of a hydrophobic pocket in the N-terminal domain. CR may sense and respond to calcium, proton, and other signals, contributing to conflicting data on the proteins role as a calcium sensor or calcium buffer.

Keywords: calcium; calretinin; calbindin D28k; EF-hand; ischemia; pH response

Changes in intracellular pH outside a tightly regulated and narrow physiological range can lead to disturbances in cell homeostasis. Acidification can lead to apoptosis (Matsuyama and Reed 2000) or Alzheimer disease (Kourie 2001), while alkalization increases the activity of

stress-activated proteins (Shrode et al. 1997). Proteins generally contain a large number of ionizable residues, but protonation of just one or a few residues normally does not significantly affect the tertiary or secondary structure. However, some proteins contain structural units that sense and translate small changes in intracellular pH into signals leading to cell survival or death. These pH-responsive elements rely on groups with pK_a values in the range 6–8, which includes histidine side chains, N-terminal amino groups, and side-chain carboxylates with upshifted pK_a values.

Reprint requests to: Patrick Groves, Centro de Investigaciones Biológicas, Calle Ramiro de Maeztu 9, 28040 Madrid, Spain; e-mail: pdgroves@cib.csic.es; fax: +34-915627518.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.051369805>.

pH-responsive elements have been reported in calcium-binding proteins inferred in disease states related to increased calcium and/or proton concentrations. Annexin VI has a well-defined calcium-dependent interaction with membranes at pH 7.2 (Bandorowicz-Pikuła et al. 1996). However, lower pH promotes a significant structural change, leading to the exposure of new hydrophobic surfaces and the integration into membranes with ion-channel formation (Golczak et al. 2001). These latter properties have been linked to Annexin VI function in endo/exocytosis (Golczak et al. 2001). A second example is calcium-binding brain nitric oxide synthase (NOS) in *Carassius auratus* (Conte 2003). The NOS activity of this protein is lowered 14-fold between pH 7.2 and pH 6.8 (Conte 2003), and this can be compensated by increased calcium concentrations. A third example is calbindin D28k (CB) for which a pH-responsive element has been identified in the N-terminal half of the protein (Berggård et al. 2000a). CB activates myo-inositol monophosphatase (IMPase) and is a more potent activator at reduced pH (Berggård et al. 2002b). Increased CB protein levels protect against ischemic insult, either when induced before an ischemic event (Yenari et al. 2001) or when induced within a short period after an ischemic event (Phillips et al. 2001). As CB also has the potential to be nitrosylated (Tao et al. 2002) or phosphorylated (Gagnon and Welsh 1997), it is possible that CB has a multifactor response to ischemia.

Calretinin (CR) is a calcium-binding protein homologous to CB (59% identical), calbindin-32 (42% identical), and secretagogin (38% identical). These EF-hand proteins are characterized by six helix-loop-helix EF-hand motifs with distinct, predominantly neuronal localizations (Celio 1996; Wagner et al. 2000; Gartner et al. 2001). Despite the relationship, the domain organizations of CR and CB appear to be different (Linse et al. 1997; Berggård et al. 2000b; Palczewska et al. 2003). CB contains a single domain of six EF-hands, while CR consists of two independent domains (consisting of EF-hand motifs I and II, residues 1–100, termed CR I–II; and EF-hand motifs III–VI, residues 100–271, termed CR III–VI) (Palczewska et al. 2003). CR interacts with cytoskeleton components in a calcium-dependent manner (Marilley and Schwaller 2000). Biochemical experiments have indicated that CR possesses calcium-dependent hydrophobic surfaces but, in contrast to calmodulin (Zhang et al. 1995), calcium-free (apo) CR also displays a significant hydrophobic surface (Kuznicki et al. 1994; Schwaller et al. 1997). Immunohistochemical studies indicate that CR-containing cells are protected against cell death in several neurodegenerative diseases (Fonseca and Soriano 1995; Liang et al. 1996) and ischemia (Kawai et al. 1995; Andsberg et al. 2001). However, other studies suggest CR-containing cells are more vulnerable to ischemia (Freund and Magloczky 1993; Hsu et al. 1994; Yamada et al. 1995; Arabadzisz

and Freund 1999). CR may also act as a calcium buffer (Schwaller et al. 1997; Billing-Marczak and Kuznicki 1999) and its biological role may vary over cell type.

In the present work we investigate the structural modulation of CR by changes in pH over the physiological range. pH effects on hydrophobic surfaces are found for calcium-bound CR. Isolated CR domains are used to localize a major calcium-dependent, pH-responsive element to CR I–II and nuclear magnetic resonance spectroscopy (NMR) data identifies the regions of the domain that might be involved in the process.

Results

Fluorescence spectroscopy

Fluorescence spectroscopy was used to probe the pH dependence of hydrophobic surfaces of CR. Spectra of TNS (2-(p-toluidino)-6-naphthalenesulfonic acid) in the presence of apo or calcium-loaded CR are shown in Figure 1A (pH 8) and B (pH 6). The data indicate a pH-dependent modulation of TNS binding to calcium-bound CR, but not to the apo form. Calmodulin, which displays a significant hydrophobic surface only for the calcium-bound state, was used as a reference (Fig. 1C). Figure 1D provides a wider picture of the pH-dependent TNS fluorescence of CR. The TNS fluorescence of apo CR is constant across the physiological pH range, whereas a distinct elevation of TNS fluorescence is observed for Ca-bound CR below pH 6.5. Spectra of TNS in the presence of CR I–II or CR III–VI were used to localize the pH-induced effect on hydrophobicity (Fig. 1E,F). In the absence of calcium, neither CR fragment displays any significant pH dependence of TNS fluorescence, although the apo CR III–VI fragment displays a significant hydrophobic surface over the examined pH range. However, in the presence of calcium, both fragments yield an increase in TNS fluorescence intensity when the pH is reduced from 7 to 6. For calcium-bound CR III–VI (Fig. 1F), the TNS data reflect a significant hydrophobic surface already at pH 8, and the TNS intensity is enhanced by 52% when changing from pH 8 to 6. However, for calcium-bound CR I–II, pH appears to act as a switch (Fig. 1E), with little TNS fluorescence at pH 8 and a fivefold increase when pH is reduced to 6. The calcium-bound CR I–II domain hence appears to undergo a transformation from a “closed” to “open” state when the pH is lowered. The sum of TNS signals for CR I–II and CR III–VI (Fig. 1G) shows a similar behavior as intact CR, within experimental error.

Phenyl-Sepharose chromatography

The hydrophobicity of CR I–II was studied using phenyl-Sepharose chromatography (Fig. 2). Elution of the

Figure 1. TNS fluorescence spectroscopy. (A,B) Spectra of TNS in the presence of 1 μ M CR and 1 mM CaCl₂ (solid line) or 1 mM EGTA (dotted line). (A) pH 8.0; (B) pH 6.0. (C) Spectra of TNS in the presence of 1 μ M calmodulin and 1 mM CaCl₂ (solid line) or 1 mM EGTA (dotted line) at pH 7. (D,E) TNS fluorescence intensity (at 430 nm) vs. pH in the presence (filled symbols) or absence (open symbols) of calcium. (D) CR; (E) CR I-II; (F) CR III-VI; (G) the adduct of CR I-II and CR III-VI data. Triplicate measurements shown as means with 10% estimated errors (15% for added data). Fluorescence intensity is measured in counts per second (cps).

protein at lower NaCl concentrations is interpreted as stronger hydrophobic interactions with the column matrix. Apo CR I-II elutes from the column at 0 M NaCl (Fig. 2A), at pH 7–8. In contrast, elution of calcium-bound CR I-II depends on pH, with earlier elution (0.2 M NaCl) at pH 8.0 than at pH 7.0 (0 M NaCl) (Fig. 2B). This indicates a weaker hydrophobic interaction with the column matrix at pH 8.0. These data confirm that CR I-II displays a hydrophobic surface, regardless of metal-bound status, with a pH-dependency also confirmed for the calcium-bound form. Larger hydrophobic surfaces, indicated below pH 7.0 by the TNS data, cannot be distinguished using phenyl-Sepharose chromatography because the fragment already elutes at 0 M NaCl at pH 7. The C-terminal domain of calmodulin, TR₂C, was used as a control: TR₂C eluted at 1 M NaCl in the absence

of calcium and at 0 M NaCl in the presence of calcium (data not shown).

NMR spectroscopy

The assignment of calcium-bound CR I-II at pH 6.7 and 7.7 (BMRB entry 5156), were compared for an initial localization of the pH-dependent hydrophobic properties of CR I-II (Fig. 3). These data are derived from a full array of 3D heteronuclear experiments with confirmed backbone assignments. Segments of the N terminus and linker between the EF-hands (spanning residues 1–11 and 53–63) are largely unassigned at pH 7.7 and were excluded from the analysis. The largest chemical shift differences between pH 6.7 and 7.7 are observed at the N- and C-terminal parts of the EF-hand motifs. The first

Figure 2. Hydrophobic interaction chromatography (phenyl-Sepharose column) of CR I–II in 1 mM EGTA (A) or 1 mM CaCl_2 (B), at pH 7.0 (light gray), pH 7.5 (gray), and pH 8.0 (black). The conductivity is shown as a dotted line.

EF-hand motif (consisting of residues 17–53) is more strongly affected by pH than the second motif (residues 66–92), with the most significant effects in helix A. This might be expected as the two histidine residues of CR I–II are found before (His 12) and in (His 27) helix A. Ten of 12 ^1H chemical shift differences ≥ 0.1 ppm, 11 of 15 ^{13}C chemical shift differences ≥ 0.5 ppm, and four of six ^{15}N chemical shift differences ≥ 0.5 ppm originate in the N-loop and first EF-hand region (residues 13–53) of CR I–II (Fig. 3B–D). The predicted dihedral angle analysis using SHIFTOR revealed no change in secondary structure of CR I–II between pH 6.7 and 7.7.

A titration of CR I–II from pH 8.5 to 5.2 was followed by Heteronuclear Single-Quantum Correlation spectroscopy (HSQC) (Fig. 4). The assigned HSQC spectrum of CR I–II at pH 7.7 is shown in Figure 4A. A selected region from the overlay of HSQC spectra at pH 8.5–5.2 is shown in Figure 4B to illustrate the pH-dependent chemical shift

of Ala18. A total of 77 peaks in these HSQC spectra could be traced over at least three pH values between pH 8.5 and 5.2. Figure 4, C and D, summarizes the ^1H N and ^{15}N H chemical shift as a function of pH for the 32 amide groups that display the largest changes. The traces are color-coded to indicate the degree of pH-dependent chemical shift. The Gly34 and Gly81 signals (see Fig. 4A) display chemical shifts characteristic of calcium-bound EF-hands over the full pH range. Many other residues in the calcium-binding loops (e.g., Glu37, Ile83, and Met85) are nearly constant, supporting that the domain retains a calcium-saturated state during the titration. Figure 5A shows the normalized chemical shift for nine ^1H N and six ^{15}N H resonance shifts with pH induced effects over 0.1

Figure 3. Comparison of the pH 7.7 and 6.7 assignments of calcium-bound CR I–II (Palczewska et al. 2001). (A) Secondary structure elements: N-terminal segment (NL), linker (L), and C-terminal linker (CL), the four helices, HA through HD, and Ca-site loops CaI and CaII. (B–D) Absolute chemical shift differences ($\Delta\delta$) in ppm for $\text{H}\alpha$ (circles), $\text{H}\beta$ (triangles), and HN (squares) (B); $\text{C}\alpha$ (circles), $\text{C}\beta$ (triangles), and CO (squares) (C); and NH resonances (D). Note that the residues in the loop regions (spanning residues 1–11, 53–64, and 98–100) are largely unassigned at pH 7.7 leading to gaps in B–D.

Figure 4. pH-dependent NMR analyses of calcium-bound CR I-II. (A) Assigned ¹H, ¹⁵N-HSQC at pH 7.7. Unassigned side-chain NH₂ correlations are marked by horizontal lines in the *top right*-hand corner of the spectrum. (B) Effect of pH on the position of peaks found in the boxed region of A; overlay of spectra collected during a pH titration between pH 8.5 and 5.2. (C) Plot of HN chemical shift as a function of pH. Resonances that shift >0.20 ppm are colored red; resonances that shift between 0.10 and 0.20 ppm are colored green; resonances that shift between 0.05 and 0.10 ppm are colored blue; and selected resonances that shift <0.05 ppm are colored black. Note that 39 traces with changes <0.05 ppm are omitted for clarity. (D) Plot of NH chemical shift as a function of pH. Resonances that shift >1.0 ppm are colored red, resonances that shift between 0.5 and 1.0 ppm are colored green, resonances that shift between 0.25 and 0.5 ppm are colored blue, and selected resonances that shift <0.25 ppm are colored black. Note that 38 traces with changes <0.25 ppm are omitted for clarity.

and 0.5 ppm, respectively. The cutoffs were chosen to be larger than the experimental error yet allowing resonances in EF-hand II to be followed. The titration seems to report on a protonation event with a midpoint of pH 7.2 ± 0.2, and suggest that a single event is registered throughout the whole molecule. This could involve one or more groups that titrate with the same pK_a value. The majority of

shifted resonances reside in the first half of the protein, close to the two histidine residues (His12, His27) and the region between them (Fig. 5B). A second affected region is found in helix B (spanning residues 44–51). The short helix D is mildly affected around the Pro93Thr94 C-capping motif. Chemical shift effects at the lower pH end of the titration are seen for the Ile36HN, Glu37HN,

Figure 5. pH-dependent NMR analyses of calcium-bound CR I–II. (A) Normalized ^1HN and ^{15}NH chemical shift as a function of pH for all resonances with $\Delta\delta$ over 0.1 ppm for ^1HN and 0.5 ppm for ^{15}NH . L13HN, E15HN, E15NH, and T17HN are omitted, as they could not be followed over the full pH range. (B) Summary of categorized chemical shift changes plotted against residue number. Shifts of <0.05 ppm (^1H) or <0.25 ppm (^{15}N), —; shifts of 0.05 – 0.10 ppm (^1H) or 0.25 – 0.5 ppm (^{15}N), light gray squares; shifts of 0.10 – 0.20 ppm (^1H) or 0.5 – 1.0 ppm (^{15}N), dark gray squares; shifts of >0.20 ppm (^1H) or >1.0 ppm (^{15}N), black squares.

Glu84HN, and Ala86HN resonances. This is probably related to the protonation of carboxylate groups in the EF-hand loops that may have pKa values elevated to around 6 (Kesvatera et al. 2001). The pH-dependent changes in NMR shifts were reversible.

Discussion

The hydrophobic surface of CR has been previously investigated at fixed pH using hydrophobic interaction chromatography (Kuźnicki et al. 1995) and TNS fluorescence (Schwaller et al. 1997). Calcium modulated the exposed hydrophobic surfaces of the protein. Here we further investigate hydrophobic properties as a function of pH for intact CR as well as its independent domains, CR I–II and CR III–VI (Palczewska et al. 2003). Conditions were chosen so that the proteins would be maintained in a calcium-free or bound state at all pH values, taking into account the pH-dependent Ca^{2+} -affinities for EGTA (Bers 1982) and the proteins (Stevens and Rogers 1997). Combinations of 10 μM calcium and 1 mM EDTA provided data for CR I–II in the pH range 6.0–7.3 in separate experiments that are consistent with the data in Figure 1E.

Detailed studies on calbindin D9k over a range of pH show that affinity of its calcium-binding sites are lowered

10-fold between pH 7 and pH 6 (Kesvatera et al. 2001). CR, similarly to calbindin D9k and Calb, has a submicromolar affinity for calcium at neutral pH (Kuźnicki et al. 1995; Stevens and Rogers 1997; Kesvatera et al. 2001; Berggård et al. 2002a). While we would expect CR I–II (and CR) to retain its submicromolar affinity for calcium in the pH-sensitive range (pH 6.7–7.7) as suggested by NMR experiments (Figs. 3–5), it is possible that any existing pH-dependent function will be lost under more extreme conditions. In this context, our biochemical data might explain the opposing conclusions relating the effects of calretinin on ischemia (Freund and Magloczky 1993; Hsu et al. 1994; Kawai et al. 1995; Yamada et al. 1995; Arabadzisz and Freund 1999; Andsberg et al. 2001). The calcium, proton effects of CR I–II might provide a spatial function, being activated in regions of the cell that experience high concentrations of calcium and protons. The complex role and response of calretinin in the formation of local calcium effects has been recently studied (Dargan et al. 2004). Also, it is known that the pH at the surface of phosphatidylcholine micelles can be lowered by 1.6 pH units (van der Goot 1991).

Both CR I–II and CR III–VI induce higher TNS fluorescence, as pH is lowered from 7 to 6, suggesting that they become more hydrophobic. However, at pH 7, the much lower TNS intensity in the presence of CR I–II than

CR III–VI may indicate a weaker interaction with TNS and/or smaller hydrophobic surface of the N-terminal domain at neutral pH. It therefore appears that a drop in pH serves as a switch for Ca-bound CR I–II, while for Ca-bound CR III–VI it only modulates an already significant hydrophobic surface.

The sensitivity of calcium-bound CR I–II to pH appears to depend on the employed experimental method. NMR provides direct residue-by-residue measurements, whereas detection by octyl-Sepharose chromatography and TNS fluorescence, important to link the pH effects to changes in hydrophobic surfaces, rely on the binding of nonnative ligands to the protein. Therefore, the pH sensitivity of calcium-bound CR I–II for its targets or function is more likely to occur around physiological pH 7.2 as reported by the NMR titration (Figs. 4, 5), confirmed to be between pH 6.7 and pH 7.7 by NMR assignments on a separate sample (Fig. 3), as well as supported by the pH-response reported by octyl-Sepharose chromatography data (Fig. 2) rather than around pH 6 as reported by TNS fluorescence (Fig. 1). A similar behavior was reported for Annexin VI: The proteins' pH-dependent hydrophobic surface is significant enough to allow integration into asolectin membranes and formation of ion channels at pH 5.3 but TNS detects the hydrophobic surface only at pH 4.5 (Golczak et al. 2001). At the same time, Calb has a contrasting behavior with its pH-dependent ANS-binding properties mirroring its IMPase activating potential (Berggård et al. 2000a, 2002b). This may be explained by the fact that ANS has a slightly different chemical structure to TNS; however, it is interesting that IMPase and caspase-3 (Bellido et al. 2000; Berggård et al. 2002b) have been shown to be Calb targets that do not bind CR.

In the present work, we have compared the pH 6.7 and 7.7 assignments (Fig. 3), and these data have been complemented with a ¹⁵N¹H-HSQC titration between pH 8.5 and 5.2 (Figs. 4, 5). The titration data (Fig. 4) are consistent with the assignment (Fig. 3) in that the absolute changes in chemical shifts for each residue are similar for both sets of data. Both data sets suggest local changes close to the histidine residues with the second EF-hand of CR I–II relatively unaffected by pH. The most affected region comprises the last four residues (12–16) of the N-terminal loop and a large fraction of helix A (residues 17–29) (Figs. 3, 5B). This region includes two His residues (His12 and His27) and the N-terminal capping motif of helix A (Thr17–Gln20). A second affected region is found in the middle of helix B (spanning residues 44–51). Significantly smaller pH induced shift changes are observed in EF-hand II. However, the residues of EF-hand II that do experience pH-induced chemical shift changes are most likely to be close in space to His12 or His27. Based on 3D structures of other EF-hand pair domains, we may

anticipate that EF-hands I and II pack against one another in an anti-parallel fashion with the two calcium loops next to each other. The N termini of helices A and C would be at the opposite end of the molecule in proximity to the C termini of helices B and D. The possible involvement of residues in the structurally unassigned linker regions cannot be addressed. The NMR data reveal no pH induced changes in secondary structure.

Figure 5A indicates a common transition midpoint of pH 7.2 for the resonances displaying the largest shift changes. This suggests that the domain undergoes a concerted response to a single protonation event, or to parallel events involving groups with closely similar pK_a values. Although elevated pK_a values of glutamates up to 6.6 have been observed in the calcium-binding region of calbindin D9k (Kesvatera et al. 2001), His12 and His27 are the residues that are most likely to have pK_a values close to 7.2. The occurrence of large chemical shift effects close to both histidines suggests that they both become protonated during the titration.

The hydrophobic properties of CR are quite different from those of the calcium sensor protein calmodulin. In the absence of calcium, calmodulin is often referred to as “closed,” as it displays no hydrophobic surface. Calcium binding involves a significant rearrangement of the hydrophobic core leading to an “open” state that is hydrophobic and interacts with cellular targets, or with hydrophobic probes (Fig. 1C). In this terminology, intact CR would be referred to as more or less open under all conditions. However, both calcium binding and pH variation for the calcium bound state modulate the hydrophobic surface, and may be a means to regulate the interaction between CR and its targets. Within CR I–II, only very modest pH-induced chemical shift changes are observed for side chain (Hβ and Cβ) resonances of expected hydrophobic core residues (W25 and F28) in CR I–II (Fig. 3). This suggests that the pH-induced transition leading to increased hydrophobic surface does not involve any significant structural rearrangements of the hydrophobic core. We can conclude that the open, low pH Ca-bound CR I–II domain is likely to be different to the open structure of Ca-bound calmodulin in which the hydrophobic core is largely exposed and rearranged (Finn and Forsen 1995). The open, Ca-bound structures of recoverin (Flaherty et al. 1993) and dimeric S100s (Drohat et al. 1998) are known to be distinct to calmodulin with the retention of a hydrophobic core. Therefore, the low pH Ca-bound structure of CR I–II may be more related to the calcium switches observed in recoverin or S100 proteins than in calmodulin.

To summarize, the NMR measurements support a model by which protonation of His12 and/or His27

leads to a structural rearrangement that primarily involves residues at the junction of N-terminal loop and helix A (residues 15–20) and the middle of helix B (residues 44–51). The rearrangement produces a hydrophobic cavity, presumably at the base of the EF-hand domain, without any large-scale rearrangements of the hydrophobic core.

Materials and methods

Protein expression and purification

CR, CR I–II, and CR III–VI were produced as GST fusion proteins from an *Escherichia coli* expression system, cleaved with thrombin and further purified by ion-exchange chromatography, according to Kuźnicki et al. (1995). ^{15}N -labeled CR I–II was prepared from a *Pichia pastoris* expression system according to Palczewska et al. (2001).

Fluorescence spectroscopy

2-(p-Toluidino)-6-naphthalenesulfonic acid (TNS) fluorescence emission spectra were collected for CR in the presence of 1 mM EGTA or 0.1 mM CaCl_2 on a Fluorolog 3 spectrometer (Jobin Yvon-Spex) at a temperature of 20°C (Neslab RTE-300 temperature control unit). Parameters were $\lambda_{\text{ex}} = 328 \text{ nm}$; λ_{em} scanned between 390 and 510 nm; and slits of 5 and 1.4 nm. The buffer was 50 mM Tris, 100 mM KCl (pH 6–8), supplemented with 30 μM TNS (Sigma). Sample volumes of 0.5 mL and protein concentrations (CR, CR I–II, CR III–VI, and calmodulin, as reference) of 1 μM were used. The spectra of TNS, with a low intensity maximum of 488 nm, were unaffected by pH, EGTA, or CaCl_2 additions. Spectra of TNS solutions were subtracted from the same TNS solutions containing protein. Experiments were performed in triplicate.

Phenyl-Sepharose chromatography

CR I–II (50 μL at 1 mg/mL supplemented with 1 M NaCl, 2 mM Tris [pH 7.0, 7.5, or 8.0]) was applied to a 1 mL phenyl-Sepharose (high-substitution) column (Amersham-Pharmacia). Data were collected using a BioLogic FPLC system (BioRad). The column was eluted with a decreasing NaCl gradient (1 M–0 M) over 10 min in 2 mM Tris buffer (pH 7.0, 7.5, or 8.0). Following 10 min elution with 0 M NaCl buffer, the elution conditions were changed to H_2O (10 min). All samples and buffers were supplemented with either 1 mM EGTA or 1 mM CaCl_2 . Control experiments were performed with 50 μg TR₂C (calmodulin residues 77–149) at pH 7.0. Absorbance (280 nm) and conductivity were simultaneously monitored. All runs were performed in triplicate.

NMR spectroscopy

The chemical shift difference ($\Delta\delta$) between the pH 7.7 and 6.7 was calculated for HN, HA, HB, CA, CB, CO, and NH resonances using published assignments of calcium-bound CR I–II (Palczewska et al. 2001) (BMRB entry 5156). These assignments were obtained for 1 mM ^{13}C , ^{15}N -labeled CR I–II in 50 mM Tris- d_{11} with 25 mM NaCl, 10 mM CaCl_2 , 0.03% NaN_3 ,

and 5% D_2O . The Web version of SHIFTOR (H. Zhang and D. Wishart, <http://redpoll.pharmacy.ualberta.ca/shiftor/>) was used to calculate Φ and Ψ angles from the assignment data.

A pH titration of 1 mM ^{15}N -labeled CR I–II in 50 mM Tris- d_{11} and 50 mM acetic acid- d_4 with 25 mM NaCl, 10 mM CaCl_2 , 0.03% NaN_3 , and 5% D_2O was followed by HSQC. Spectra were recorded at pH 5.2, 6.1, 7.0, 7.5, and 8.5 analyzed in SPARKY (T.D. Goddard and D.G. Kneller, University of California Regents, University of California, San Francisco) to transfer peak assignments through the pH titration.

Acknowledgments

We thank Barbara Zarzycka (Warsaw) for technical assistance and Eva Thulin (Lund) for the TR₂C sample. Dr. Bert Heise helped with obtaining NMR data at the NMR facilities in Jena. Funding was provided by the International Center for Genetic Engineering and Biotechnology (ICGEB) grants CRP/Pol97-01(t1) to J.K. and CRP/Hun97-01(t1) to G.B.; Hungarian Scientific Research Fund (OTKA) grant no. T 042567 to G.B.; the Polish State Committee for Scientific Research (KBN) grant no. 6 P04B 01015 to P.G.; and the Swedish Research Council grant no. 621-2002-5111 to S.L. Part of the NMR studies were carried out in the Institute of Molecular Biotechnology, Jena, Germany, under contract no. HPRI-CT-1. M.P. carried out part of the research in Lund thanks to a short-term Fellowship from the Federation of European Biochemical Societies.

References

- Andersberg, G., Kokaia, Z., and Lindvall, O. 2001. Upregulation of p75 neurotrophin receptor after stroke in mice does not contribute to differential vulnerability of striatal neurons. *Exp. Neurol.* **169**: 351–363.
- Arabadzisz, D. and Freund, T.F. 1999. Changes in excitatory and inhibitory circuits of the rat hippocampus 12–14 months after complete forebrain ischemia. *Neuroscience* **92**: 27–45.
- Bandorowicz-Pikuła, J., Sikorski, A.F., Białkowska, K., and Sobota, A. 1996. Interaction of annexins IV and VI with phosphatidylserine in the presence of Ca^{2+} : Monolayer and proteolytic study. *Mol. Membr. Biol.* **13**: 241–250.
- Bellido, T., Huening, M., Raval-Pandya, M., Manolagas, S.C., and Christakos, S. 2000. Calbindin-D28k is expressed in osteoblastic cells and suppresses their apoptosis by inhibiting caspase-3 activity. *J. Biol. Chem.* **275**: 26328–26332.
- Berggård, T., Silow, M., Thulin, E., and Linse, S. 2000a. Ca^{2+} - and H^{+} -dependent conformational changes of calbindin D(28k). *Biochemistry* **39**: 6864–6873.
- Berggård, T., Thulin, E., Åkerfeldt, K.S., and Linse, S. 2000b. Fragment complementation of calbindin D28k. *Protein Sci.* **9**: 1094–1208.
- Berggård, T., Miron, S., Onnerfjord, P., Thulin, E., Åkerfeldt, K.S., Enghild, J.J., Akke, M., and Linse, S. 2002a. Calbindin D28k exhibits properties characteristic of a Ca^{2+} sensor. *J. Biol. Chem.* **277**: 16662–16672.
- Berggård, T., Szczepankiewicz, O., Thulin, E., and Linse, S. 2002b. Myo-inositol monophosphatase is an activated target of calbindin D28k. *J. Biol. Chem.* **277**: 41954–41959.
- Bers, D.M. 1982. A simple method for the accurate determination of free $[\text{Ca}]$ in Ca -EGTA solutions. *Am. J. Physiol.* **242**: C404–C408.
- Billing-Marczak, K. and Kuźnicki, J. 1999. Calretinin—Sensor or buffer—Function still unclear. *Pol. J. Pharmacol.* **51**: 173–178.
- Celio, M.R. 1996. *Guidebook to the calcium-binding proteins*. Sambrook & Tooze with Oxford University Press, Oxford, UK.
- Conte, A. 2003. Physiologic pH changes modulate calcium ion dependence of brain nitric oxide synthase in *Carassius auratus*. *Biochim. Biophys. Acta* **1619**: 29–38.
- Dargan, S.L., Schwaller, B., and Parker, I. 2004. Spatiotemporal patterning of IP₃-mediated Ca^{2+} signals in *Xenopus* oocytes by Ca^{2+} -binding proteins. *J. Physiol.* **556**: 447–461.

- Drohat, A.C., Baldissieri, D.M., Rustandi, R.R., and Weber, D.J. 1998. Solution structure of calcium-bound rat S100B($\beta\beta$) as determined by nuclear magnetic resonance spectroscopy. *Biochemistry* **37**: 2729–2740.
- Finn, B. and Forsen, S. 1995. The evolving model of calmodulin structure, function and activation. *Structure* **3**: 7–11.
- Flaherty, K.M., Zozulya, S., Stryer, L., and McKay, D.B. 1993. Three-dimensional structure of recoverin, a calcium sensor in vision. *Cell* **75**: 709–716.
- Fonseca, M. and Soriano, E. 1995. Calretinin-immunoreactive neurons in the normal human temporal cortex and in Alzheimer's disease. *Brain Res.* **691**: 83–91.
- Freund, T.F. and Magloczky, Z. 1993. Early degeneration of calretinin-containing neurons in the rat hippocampus after ischemia. *Neuroscience* **56**: 581–596.
- Gagnon, A. and Welsh, J. 1997. Modulation and phosphorylation of calbindin-D28K correlates with protein kinase C activation. *Biochem. Cell Biol.* **75**: 33–40.
- Gartner, W., Lang, W., Leutmetzer, F., Domanovits, H., Waldhausl, W., and Wagner, L. 2001. Cerebral expression and serum detectability of secretagogin, a recently cloned EF-hand Ca(2+)-binding protein. *Cereb. Cortex* **11**: 1161–1169.
- Golczak, M., Kicinska, A., Bendorowicz-Pikuła, J., Buchet, R., Szweczyk, A., and Pikuła, S. 2001. Acidic pH-induced folding of annexin VI is a prerequisite for its insertion into lipid bilayers and formation of ion channels by the protein molecules. *FASEB J.* **15**: 1083–1085.
- Hsu, M., Sik, A., Gallyas, F., Horvath, Z., and Buzsaki, G. 1994. Short-term and long-term changes in the posts ischemic hippocampus. *Ann. N. Y. Acad. Sci.* **743**: 121–139.
- Kawai, K., Nowak Jr., T.S., and Klatzo, I. 1995. Loss of parvalbumin immunoreactivity defines selectively vulnerable thalamic reticular nucleus neurons following cardiac arrest in the rat. *Acta Neuropathol. (Berl.)* **89**: 262–269.
- Kesvatera, T., Jonsson, B., Telling, A., Tougu, V., Vija, H., Thulin, E., and Linse, S. 2001. Calbindin D(9k): A protein optimized for calcium binding at neutral pH. *Biochemistry* **40**: 15334–15340.
- Kourie, J.I. 2001. Mechanisms of amyloid β protein-induced modification in ion transport systems: Implications for neurodegenerative diseases. *Cell Mol. Neurobiol.* **21**: 173–213.
- Kuźnicki, J., Winsky, L., and Jacobowitz, D.M. 1994. Ca(2+)-dependent and independent interactions of calretinin with hydrophobic resins. *Biochem. Mol. Biol. Int.* **33**: 713–721.
- Kuźnicki, J., Strauss, K.I., and Jacobowitz, D.M. 1995. Conformational changes and calcium binding by calretinin and its recombinant fragments containing different sets of EF hand motifs. *Biochemistry* **34**: 15389–15394.
- Liang, C.L., Sinton, C.M., and German, D.C. 1996. Midbrain dopaminergic neurons in the mouse: Co-localization with Calbindin-D_{28K} and calretinin. *Neuroscience* **75**: 523–533.
- Linse, S., Thulin, E., Gifford, L.K., Radzewsky, D., Hagan, J., Wilk, R.R., and Åkerfeldt, K.S. 1997. Domain organization of calbindin D_{28k} as determined from the association of six synthetic EF-hand fragments. *Protein. Sci.* **6**: 2385–2396.
- Marilley, D. and Schwaller, B. 2000. Association between the calcium-binding protein calretinin and cytoskeletal components in the human colon adenocarcinoma cell line WiDr. *Exp. Cell Res.* **259**: 12–22.
- Matsuyama, S. and Reed, J.C. 2000. Mitochondria-dependent apoptosis and cellular pH regulation. *Cell Death Differ.* **7**: 1155–1165.
- Palczewska, M., Groves, P., Ambrus, A., Kaleta, A., Kövér, K.E., Batta, G., and Kuźnicki, J. 2001. Structural and biochemical characterization of neuronal calretinin domain I-II (residues 1–100); Comparison to homologous calbindin D28k domain I-II (residues 1–93). *Eur. J. Biochem.* **268**: 6229–6237.
- Palczewska, M., Groves, P., Batta, G., Heise, B., and Kuźnicki, J. 2003. Calretinin and calbindin D28k have different domain organizations. *Protein Sci.* **12**: 180–184.
- Phillips, R.G., Monje, M.L., Giulii, L.C., Meier, T.J., Yenari, M.A., Kunis, D., and Sapolsky, R.M. 2001. Gene therapy effectiveness differs for neuronal survival and behavioral performance. *Gene Ther.* **8**: 579–585.
- Schwaller, B., Durussel, I., Jermann, D., Herrmann, B., and Cox, J. 1997. Comparison of the Ca²⁺-binding properties of human recombinant calretinin-22k and calretinin. *J. Biol. Chem.* **272**: 29663–29671.
- Shrode, L.D., Rubie, E.A., Woodgett, J.R., and Grinstein, S. 1997. Cytosolic alkalization increases stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) activity and p38 mitogen-activated protein kinase activity by a calcium-independent mechanism. *J. Biol. Chem.* **272**: 13653–13659.
- Stevens, J. and Rogers, J.H. 1997. Chick calretinin: Purification, composition, and metal binding activity of native and recombinant forms. *Protein Expr. Purif.* **9**: 171–181.
- Tao, L., Murphy, M.E., and English, A.M. 2002. S-nitrosation of Ca(2+)-loaded and Ca(2+)-free recombinant calbindin D28k from human brain. *Biochemistry* **41**: 6185–6192.
- van der Goot, F.G., Gonzalez-Manas, J.M., Lakey, J.H., and Pattus, F. 1991. A “molten-globule” membrane-insertion intermediate of the pore-forming domain of colicin A. *Nature* **354**: 408–410.
- Wagner, L., Oliynyk, O., Gartner, W., Nowotny, P., Groeger, M., Kaserer, K., Waldhausl, W., and Pasternack, M.S. 2000. Cloning and expression of secretagogin, a novel neuroendocrine- and pancreatic islet of Langerhans-specific Ca²⁺-binding protein. *J. Biol. Chem.* **275**: 24740–24751.
- Yamada, K., Goto, S., Oyama, T., Yoshikawa, M., Nagahiro, S., and Ushio, Y. 1995. Striatal cells containing the Ca(2+)-binding protein calretinin (protein 10) in ischemia-induced neuronal injury. *Acta Neuropathol. (Berl.)* **89**: 172–177.
- Yenari, M.A., Minami, M., Sun, G.H., Meier, T.J., Kunis, D.M., McLaughlin, J.R., Ho, D.Y., Sapolsky, R.M., and Steinberg, G.K. 2001. Calbindin d28k overexpression protects striatal neurons from transient focal cerebral ischemia. *Stroke* **32**: 1028–1035.
- Zhang, M., Tanakaw, T., and Ikura, M. 1995. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat. Struct. Biol.* **2**: 758–767.