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Electrophoretic mobility shift in native gels indicates calcium-dependent structural changes of neuronal calcium sensor proteins

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ABSTRACT

In proteins of the neuronal calcium sensor (NCS) family, changes in structure as well as function are brought about by the binding of calcium. In this article, we demonstrate that these structural changes, solely due to calcium binding, can be assessed through electrophoresis in native gels. The results demonstrate that the NCS proteins undergo ligand-dependent conformational changes that are detectable in native gels as a gradual decrease in mobility with increasing calcium but not other tested divalent cations such as magnesium, strontium, and barium. Surprisingly, such a gradual change over the entire tested range is exhibited only by the NCS proteins but not by other tested calcium-binding proteins such as calmodulin and S100B, indicating that the change in mobility may be linked to a unique NCS family feature—the calcium–myristoyl switch. Even within the NCS family, the changes in mobility are characteristic of the protein, indicating that the technique is sensitive to the individual features of the protein. Thus, electrophoretic mobility on native gels provides a simple and elegant method to investigate calcium (small ligand)-induced structural changes at least in the superfamily of NCS proteins.

Change in protein function, which is at the heart of cell signaling, is governed by change in its structure. This change may be captured through the simple technique of electrophoresis. The relationship between the structure of a particle and its mobility in the electrical field has been derived mathematically by several investigators beginning with von Smoluchowski, Huckel, and Henry (reviewed in Ref. [1]) and extended to proteins [2–4]; the general agreement is that a protein's mobility is a balance between the electrostatic driving force and the hydrodynamic drag on the protein. Properties that influence these two parameters include the amino acid composition, charge, shape, and mass of the protein as well as the pH and viscosity of the medium. However, mobility in gels containing sodium dodecyl sulfate (SDS) (denaturing conditions) is mostly determined by size [5,6], and such denaturing gels are typically used to assess the addition or removal of larger groups such as phosphate, ubiquitin, and thiols (reviewed in Refs. [7–9]). Electrophoresis under non-denaturing (native) conditions allows the charge, shape, and size of a protein to contribute to its mobility. Therefore, this technique can serve as a tool to investigate changes in conformation of a protein brought about by charge density modification due to its interaction with ligand(s) and/or other protein(s).

The family of neuronal calcium sensor (NCS) proteins governs the cellular response to calcium signals. The members of the NCS family are encoded by 14 distinct genes: frequenin (FRQN), visinin-like protein 1 (VSNL1), hippocalcin-like protein 1 (HPCAL1), hippocalcin-like protein 4 (HPCAL4), neurocalcin delta (NCALD), hippocalcin (HPCA), K-channel interacting proteins 1 through 4 (KChIP1–4), guanylate cyclase activator proteins 1 through 3 (GCAP1–3), and recoverin (RCVRN). They are characterized by a conserved calcium binding motif known as an EF hand and a myristoylated N terminus that allows for translocation from the cytosol to the membrane [10,11]. Many have been characterized biochemically [12–16], and some members of the family have been shown to be involved in memory formation, vision, and DNA transcription [17–22] (reviewed in Ref. [23]). The structure (and thus the function) of these proteins oscillates between the calcium-bound and calcium-free states, and the transition involves the

Abbreviations used: SDS, sodium dodecyl sulfate; NCS, neuronal calcium sensor; FRQN, frequenin; VSNL1, visinin-like protein 1; HPCAL1, hippocalcin-like protein 1; HPCAL4, hippocalcin-like protein 4; NCALD, neurocalcin delta; HPCA, hippocalcin; KChIP1-4, K-channel interacting proteins 1 through 4; GCAP1-3, guanylate cyclase activator proteins 1 through 3; RCVRN, recoverin; CALM1, calmodulin; OVAL, ovalbumin; PAGE, polyacrylamide gel electrophoresis.

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calcium–myristoyl switch (reviewed in Refs. [23–25]). For several NCS proteins, binding of calcium induces changes in structure that may be detected even under denaturing conditions on SDS polyacrylamide gel [13,14,26,27].

How does the binding of calcium affect the mobility of these proteins under native conditions? What information regarding the resultant structural changes in the NCS family members may be gleaned?

In this article, we provide answers to these questions and demonstrate, for the first time, the use of electrophoresis in native gels to assess structural changes solely due to calcium (ligand) binding in several members of the NCS protein family.

Materials and methods

Materials

Fine chemicals, ovalbumin and S100B, were purchased from Sigma–Aldrich. Calmodulin was purchased from Roche. Calcium calibration buffers were purchased from Molecular Probes by Life Technologies. All experiments involving recombinant materials and organisms were carried out in compliance with National Institutes of Health (NIH) guidelines and the institutional biosafety committee.

Methods

Protein purification

pET-21d plasmids encoding NCALD, HPCA, GCAP1, and GCAP2 were generated by inserting appropriate coding regions between NcoI and HindIII sites, so that proteins could be generated without tags. The plasmids were transformed into ER2566 *Escherichia coli* cells that coexpressed yeast *N*-myristoyl transferase. For expression, overnight cultures were inoculated (1%) into fresh LB medium containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml). All bacterial cells were grown in standard LB medium at 37 °C with shaking at 200 rpm. The cells were grown to an optical density (OD) of 0.6, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Myristic acid was added to the cells (final concentration of 50 μg/ml) to generate the myristoylated form of the proteins. To generate the non-myristoylated form, NCALD was expressed in ER2566 cells without the yeast *N*-myristoyl transferase, and myristic acid was not added either. The cells were collected by centrifugation 2.5 h after induction and lysed by sonication, and then the expressed proteins were purified by calcium-dependent hydrophobic interaction chromatography using phenyl sepharose as described previously [14,15,22]. After concentration, the proteins were washed with calcium-depleted 20 mM Tris–HCl (pH 7.5) to remove the bound calcium. Calcium depletion was carried out by incubation with Chelex-100 resin (Bio-Rad Laboratories) that selectively binds calcium using standard procedures [28–30]. Calcium depletion was confirmed through flame ionization photometry.

The concentration of the protein was determined by the Bio-Rad protein assay according to the manufacturer's protocols (Bio-Rad Laboratories). At least three preparations of two different clones were purified and tested.

Protein charge calculations

Protein pI and charges were calculated using the web-based program ProteinCalculator [31]. The accession numbers along with their gene symbols for the proteins referred to are as follows: calmodulin (P62158.2; CALM1), frequenin (AAF01804.1; NCS1), recoverin (NP_002894.1; RCVRN), neurocalcin delta (NP_001035720.1; NCALD), hippocalcin (BAA74456.1; HPCA), visinin-like protein 1 (P62760.2; VSNL1), HPCL1 or VILIP3 (P37235.3; HPCAL1), HPCL4 (Q9UM19.3; HPCAL4), GCAP1 (P43080.3; GUCA1A), GCAP2 (Q9UMX6.4; GUCA1B), GCAP3 (NP_005450.3; GUCA1C), KChIP1 (Q9NZI2.2; KCNIP1), KChIP2 (Q9NS61.3; KCNIP2), KChIP3 (Q9Y2W7.1; KCNIP3), KChIP4 (Q6PIL6.1; KCNIP4), and ovalbumin (AAB59956.1; OVAL).

Native gel electrophoresis

Purified proteins were separated by native discontinuous (12%) polyacrylamide gel electrophoresis (PAGE; pH 8.8) at a constant voltage of 165 V for 2.5 h in running buffer composed of 25 mM Tris base and 190 mM glycine. The gels were 8 cm long, 10 cm wide, and 0.75 mm thick. The gels were discontinuous, with stacking (4% acrylamide in 125 mM Tris–Cl, pH 6.8) and separating (12% acrylamide in 300 mM Tris–Cl, pH 8.8) components. The electrophoresis chamber (standard miniPROTEAN apparatus, Bio-Rad Laboratories) was kept on ice to prevent overheating of the gel. Here, 5 μl (1 μg/μl) of each of the proteins was mixed with 15 μl of buffer containing specific concentrations of the free divalent cation, obtained by mixing EGTA/EDTA (ethyleneglycoltetraacetic acid/ethylenediaminetetraacetic acid) and the divalent cation in appropriate proportions based on the program MaxChelator [32]; for increments of calcium, calcium calibration buffers of increasing calcium concentrations from 0 to 39 μM were used. Here, 5 μl of 6× sample buffer (Laemmli sample buffer without SDS) was added to all samples [5,32].

After electrophoresis, proteins were visualized with Coomassie R250. The relative mobility value for each band was measured as the distance migrated by the band measured from the top of the gel in relation to the total gel length from scanned images using ImageJ. To determine the effect of calcium on a single protein, the mobility value at each calcium concentration was estimated in relation to that obtained at the maximal concentration of calcium (39 μM). Data compiled from several replicates were analyzed and presented as means ± standard errors using Prism 6.0. Statistical significance between indicated groups was determined by two-tailed Student's *t* tests.

SDS–PAGE

All procedures were identical to that described above except that the polyacrylamide gels and the gel buffer contained 0.1% SDS.

Results

As a first step toward investigating the mobility of NCS proteins in native gels, information on their charge and size was compiled along with other proteins used in this study as controls (Table 1). The molecular weights of the NCS family members range from approximately 22 kDa (FRQN) to 31 kDa (KChIP2). The proteins are presented in order of increasing charge at pH 8.8 (that of the gel matrix and the buffer used for electrophoresis). The charges at pH 8.8 range from −25.50 (CALM1) to −9.50 (NCALD and HPCL1). The charges at pH 7.4 (the physiological pH) have also been provided for the proteins along with their isoelectric points for comparison. Four proteins were chosen for our study to represent the range in charges; GCAP1 and NCALD represent the extremes, whereas GCAP2 and HPCA represent the middle of the range. Three proteins were chosen in addition (Table 1; indicated in bold font): (i) calmodulin, an archetypal calcium sensor protein with 4 EF hands like the NCS family; (ii) S100B, which also binds calcium but contains only 2 EF hands; and (iii) ovalbumin, which is not known to bind calcium but is similar in charge to the proteins being studied.

To investigate whether the calcium-induced conformational changes in NCS proteins may be detected by electrophoresis under native conditions, proteins were incubated in the presence (39 μM)