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Characterization of Calcium Binding to Brain Spectrin*

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Brain spectrin α and β chains bind $^{45}Ca^{2+}$, as shown by the calcium overlay method. Flow dialysis measurements revealed eight high affinity binding sites/tetramer that comprise two binding components (determined by nonlinear regression analysis). The first component has one or two sites (k_d = 2–30 × 10^{-8} M), depending on the ionic strength of the binding buffer, with the remaining high affinity sites in the second component (k_d = 1–3 × 10^{-6} M). In addition, there is a variable, low affinity binding component (n = 100–400, k_d = 1–2 × 10^{-4} M). Magnesium inhibits calcium binding to the low affinity sites with a K_I = 1.21 mM.

Proteolytic fragments from trypsin or chymotrypsin digests of brain spectrin bind $^{45}Ca^{2+}$ if they include α domain IV, α domain III, or the amino-terminal half of the β chain (but more than 25 kDa from the amino-terminal). These data suggest that calcium ions bind with high affinity to the putative EF-hands in α domain IV and to one site in the amino-terminal half of the β chain that is associated with α domain IV in the native dimer. This localization is consistent with a direct calcium modulation of the spectrin-actin-protein 4.1 interaction. In addition, there appears to be one high affinity site near the hypersensitive region of α brain spectrin. All four proposed binding sites occur near probable calmodulin-binding or calcium-dependent protease cleavage sites.

Brain spectrin (fodrin) is a major cytoskeletal protein underlying the plasma membrane that is involved in establishing and stabilizing regional membrane specializations (Willard, 1977; Lazarides and Nelson, 1983; Marchesi, 1985; Srinivasan *et al.*, 1988; Goodman *et al.*, 1988; Bennett, 1990). Spectrin $\alpha\beta$ heterodimers self-associate head-to-head into stable tetramers, and ankyrin binds to the 15th repeat unit of β spectrin (Kennedy *et al.*, 1991) and to integral proteins, joining fodrin to the membrane (Bennett, 1990). The fodrin tetramer binds protein 4.1 and actin near each end (Tyler *et al.*, 1980), linking the spectrin tetramers into a hexagonal lattice (Liu *et al.*, 1987). Brain spectrin also is linked to the plasma membrane by proteins other than ankyrin because brain spectrin binds with high affinity to brain membranes that have been depleted of spectrin and ankyrin (Steiner and Bennett, 1988).

Calcium ions are reported to influence membrane skeleton functions in a variety of cells (Baudry *et al.*, 1981; Aunis and Bader, 1988; Lee *et al.*, 1988). Brain spectrin functions may be modulated by calcium in at least three ways: 1) direct binding of calcium to spectrin putative EF-hands (Wasenius *et al.*, 1989; Dubreuil *et al.*, 1991), 2) calmodulin control of spectrin-protein interactions (Tsukita *et al.*, 1983; Steiner *et al.*, 1989; Tanaka *et al.*, 1991), and 3) calcium-dependent protease cleavage of spectrin to change its physiological properties (Siman *et al.*, 1987; Ivy *et al.*, 1988; Seubert *et al.*, 1988; Harris *et al.*, 1989; Harris and Morrow, 1990; DiStasi *et al.*, 1991; Hu and Bennett, 1991).

Calcium ions play a critical role in exocytotic events such as neurotransmitter release, spectrin is a component of brain synaptic junctions (Carlin *et al.*, 1983; Koenig and Repasky, 1985), and a role for spectrin in exocytosis has been proposed (Perrin and Aunis, 1985; Perrin *et al.*, 1987). However, measurements of calcium binding to brain spectrin have not been reported. The purpose of the present work was to measure the calcium-binding ability of brain spectrin and to locate, at least in a preliminary way, the high affinity binding sites. We show that both the α and β chains of brain spectrin bind calcium and suggest that the high affinity sites occur on the actin-binding ends of the tetramers and near the proposed calmodulin-binding and calcium-dependent protease I cleavage site.

EXPERIMENTAL PROCEDURES

Materials—Fresh horse brains or blood were obtained from Beltex Corp., Fort Worth, TX. Fresh cow brains or blood were obtained from Estes Packing Co., Fort Worth, TX. Acrylamide, bisacrylamide, and glycerol were purchased from Hoefer Scientific Instruments (San Francisco, CA). SDS[1]-polyacrylamide gel electrophoresis molecular weight standards were from Bio-Rad. Sepharose CL-4B came from Pharmacia LKB Biotechnology Inc. Aquacide I was purchased from Calbiochem. Phenylmethylsulfonyl fluoride, trypsin, chymotrypsin, and other chemicals were obtained from Sigma.

Preparation of Brain Spectrin—Brain spectrin was isolated by a combination of the methods of Cheney *et al.* (1986) and Bennett *et al.* (1986). When necessary, further purification was obtained by DE53 chromatography as described by Davis and Bennett (1983). The brain spectrin was pooled, concentrated against Aquacide I, dialyzed against short term storage buffer (20 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.3 mM NaN_3, pH 8.0) and stored at 4 °C. For long term storage, protein was dialyzed into a buffer containing 50 mM NaCl, 25 mM dithiothreitol, 10 mM HEPES, 1 mM EDTA, 1 mM NaN_3, 0.05% Tween 20, 10% sucrose, 5 μg/ml leupeptin, pH 7.4, and stored at −70 °C.

Polyacrylamide Gel Electrophoresis and Blotting—Routine SDS-polyacrylamide gel electrophoresis was performed using the discontinuous system of Laemmli (1970) with a 4% stacking gel and a 4–20% linear gradient resolving gel using glycerol to stabilize the gradient.

For polyacrylamide gel electrophoresis of native proteins, the slab gel method of Morrow and Haigh (1983) was employed using 3-mm-thick, 2–4% linear gradients. Native gels were run at 4 °C with 4 watts of constant power.

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Proteins were transferred to nitrocellulose or Immobilon P membranes in the Hoefer TE 70 Semiphor semidry transfer apparatus at room temperature or in a tank blotting apparatus at 4 °C. The blotting buffer was 20 mM Tris, 150 mM glycine, 10% methanol, pH 9.5.

^{45}Ca Autoradiography—Calcium binding to proteins on membranes was performed by the method of Maruyama *et al.* (1984) or as modified by Gültekin and Heermann (1988), except that the magnesium chloride concentration was varied from 1 to 5 mM and the ^{45}Ca concentration varied from 0.2 to 2.0 μM. Dried blots were exposed to X-Omat film for 2 days at −70 °C.

Flow Dialysis—Brain spectrin was prepared by dialysis against 1000 volumes of the desired buffer, usually 20 mM HEPES, pH 7.5, containing 8 g/liter Chelex 100 for at least 72 h at 4 °C. Each batch of protein was checked by autoradiography for the presence of $^{45}Ca^{2+}$ binding contaminants. All flow-through buffers were Chelex-treated by passage through a 2.5 × 12-cm Chelex 100 column and were stored in acid-washed plastic bottles.

Flow dialysis was performed in two cells as described by Feldmann (1978) that were modifications of the original apparatus described by Colowick and Womack (1969). In the smaller cell, the upper sample volume was 600 μl, and the lower spiral volume was 30 μl. In the larger cell, the upper sample volume was 3 ml, whereas the lower spiral volume was 600 μl. The lower spiral flow-through rate was 1 ml/min, produced by a constant flow peristaltic pump. Samples were collected at 1-min intervals for the large apparatus and at 0.25-min intervals for the small apparatus. Divalent cation additions were made every six samples, and samples 4–6 were counted for each addition. Three ml of scintillation fluid were added and mixed, and label was measured by liquid scintillation counting. Unless otherwise stated, measurements were made at room temperature.

Two methods of binding were used. In the constant specific activity method, small additions of labeled $^{45}Ca^{2+}$ were made, and binding was determined using loss rates through the membrane as determined by comparison with runs where no protein or where hemoglobin (nonbinding control protein) was present. Because of the high binding capacity of spectrin, it was necessary to use a second method, isotope dilution, to measure the binding at higher calcium concentrations. In this method, 0.5–1.0 μM $^{45}Ca^{2+}$ was added, and subsequent additions contained unlabeled calcium or magnesium. Control runs without protein or with hemoglobin were performed to get estimates of the loss rate across the membrane as unlabeled divalent cation was added. For each membrane used, at least one control run and runs at 2–3 concentrations of protein were performed. Protein concentration was maintained equal to or below 1.5 μM brain spectrin tetramer, and calcium was maintained below 1 mM to prevent concentration-dependent polymerization or aggregation. Total, bound, and free concentrations of ligand were calculated with corrections for loss across the membrane.

Binding parameters were determined by iterative nonlinear regression using the Systat computer program for the IBM PC and the model $B = \sum_i (n_i K_i F/(1 + K_i F))$, where K_i is the affinity constant for class i, n_i is the number of sites for class i, B is the molar concentration of bound ligand, and F is the molar concentration of free ligand (Klotz and Hunston, 1971). Initially, no correction for "nonspecific" binding was made, because it is described by one component of the model as low affinity binding (Mendel and Mendel, 1985) and it is due only to the presence of spectrin. Because of the variability of the low affinity component between experiments, it was subtracted from individual data sets before they were combined for more accurate estimates of the high affinity binding parameters. The original data and points generated from the proposed model were then plotted on a Scatchard plot (Scatchard, 1949) and compared for fit to the model. In some cases, the method of Rosenthal (1967) was used to get a first approximation of binding parameters. Cooperativity was examined by use of a Hill plot.

The interaction of magnesium-binding sites with calcium was examined by using magnesium as the diluting cation and varying the initial concentration of $^{45}Ca^{2+}$. Data were plotted on a Dixon plot to obtain the K_I of Mg^{2+} for Ca^{2+} binding.

Equilibrium Dialysis—Equilibrium dialysis was performed in modified 1.5-ml microcentrifuge tubes. A 100-μl protein sample was placed in the cap chamber, and 900 μl of solution containing label was put in the tube chamber. They were separated by a 6–8000 molecular weight cutoff dialysis membrane. Tubes without protein were included to demonstrate that equilibrium had been reached at various time intervals. Samples were laid with their long axis horizontal and shaken on an orbital shaker for 16 h at room temperature. The free concentration was determined by measurement of the label concentration in the tube chamber. The bound concentration was determined by measuring the total concentration in the cap chamber and subtracting the free concentration. The data were then analyzed as described for flow dialysis.

Trypsin and Chymotrypsin Cleavage—Brain spectrin (1 mg/ml) in digestion buffer (40 mM NaCl, 10 mM $NaPO_4$, pH 8.0) was reacted with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (substrate:enzyme, 100:1 (w/w) or as stated in figure legends) at 0 or 25 °C for 0.5, 3, or 27 h. The reaction was terminated by addition of 0.2 mg/ml soybean trypsin inhibitor.

Chymotrypsin cleavage of brain spectrin was performed as described by Harris and Morrow (1988) at a 250:1 (mol:mol, substrate:enzyme) ratio. For extensive chymotrypsin cleavage, the reaction was allowed to progress for 27 h at 25 °C.

The reaction products were separated on 4–20% linear gradient SDS-polyacrylamide gels and transferred to Immobilon P at 4 °C overnight.

Protein Measurements—The protein concentration for purified brain spectrin was determined spectrophotometrically using $\epsilon^{1\%}_{280\,nm} = 9.7$ and confirmed using the Lowry protein assay (Lowry *et al.*, 1951).

RESULTS

Autoradiography using the $^{45}Ca^{2+}$ overlay method on blots of SDS gels showed that both α and β erythrocyte and brain spectrins bind $^{45}Ca^{2+}$ (Fig. 1). Calcium binding appears to be proportional to Coomassie blue staining, but this is deceptive because, at 0.5 mM Mg^{2+}, calcium binding can occur to both high and low affinity sites (see below). This binding is not due to SDS contamination because proteins such as troponin T and troponin I (Fong *et al.*, 1988) or hemoglobin do not bind calcium under these conditions and binding can be seen

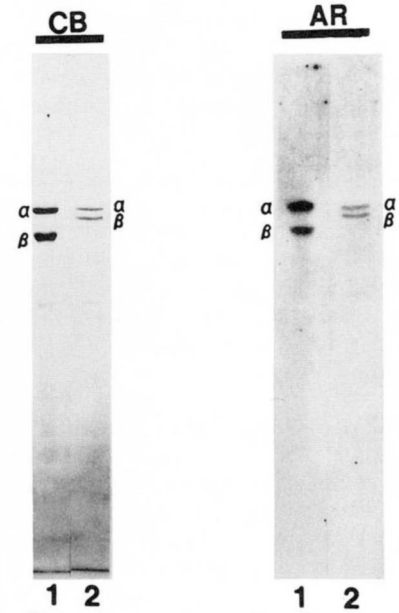

FIG. 1. **$^{45}Ca^{2+}$ binds to both α and β subunits of erythrocyte and brain spectrins.** *CB*, Coomassie Blue stained gels. *AR*, autoradiograms. *Lane 1*, 20 μg of erythrocyte spectrin; *lane 2*, 10 μg of brain spectrin. Erythrocyte spectrin was prepared from fresh cow blood. Brain spectrin was prepared from fresh cow brain gray matter. Spectrin subunits were separated by gel electrophoresis on 4–15% sodium dodecyl sulfate-polyacrylamide slab gels and electrophoretically transferred to nitrocellulose sheets or stained with Coomassie Blue. $^{45}Ca^{2+}$ binding was performed as described by Maruyama *et al.* (1984), with the following modifications. Nitrocellulose sheets were washed in 60 mM KCl, 0.5 mM $MgCl_2$, 10 mM imidazole HCl, pH 7.4, for 1 h with two changes. The sheets were incubated with 0.1 mCi (1.3 μM) $^{45}CaCl_2$ in the same buffer for 30 min with constant shaking. The membranes were washed in water for 15 min with two changes and air-dried flat overnight. Autoradiograms were exposed for 7 days at −20 °C using Kodak XAR-2 film.

TABLE I

Flow dialysis measurement of $^{45}Ca^{2+}$ binding to horse brain spectrin

Buffer was 20 mM HEPES, pH 7.5, and n is expressed per tetramer. Data are the mean ± S.D. or ranges with the number of experiments in parentheses.

Component	Buffer (12)	Buffer + 10 mM KCl (2)	Buffer + 0.1 M KCl (2)
I	$n = 1.2 \pm 0.1$	$n = 1.9 \pm 0.1$	$n = 2.1 \pm 0.3$
	$k_d = 2.0 \pm 0.4 \times 10^{-8}$	$k_d = 2.9 \pm 6.5 \times 10^{-8}$	$k_d = 3.1 \pm 1.3 \times 10^{-7}$
II	$n = 7.1 \pm 0.5$	$n = 6.2 \pm 0.8$	$n = 5.7 \pm 0.5$
	$k_d = 1.1 \pm 0.15 \times 10^{-6}$	$k_d = 1.8 \pm 0.2 \times 10^{-6}$	$k_d = 2.7 \pm 0.6 \times 10^{-6}$
III	$n = 196 \pm 52$	$n = 350 \pm 78$	$n = 120 \pm 42$
	$k_d = 1.2 \pm 0.4 \times 10^{-4}$	$k_d = 1.5 \pm 0.2 \times 10^{-4}$	$k_d = 1.9 \pm 0.4 \times 10^{-4}$

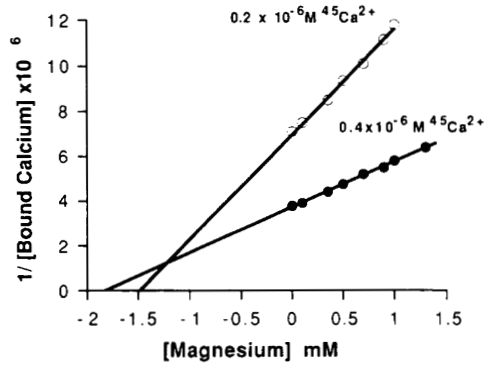

FIG. 2. **Dixon plot of displacement of $^{45}Ca^{2+}$ from horse brain spectrin in 20 mM HEPES, pH 7.5, by titration with $MgCl_2$.**

in autoradiograms of native gel blots[2] where the protein was never exposed to SDS.

Using isotope dilution flow dialysis with best-fit, nonlinear regression analysis of the data, we found that brain spectrin has eight high affinity sites/tetramer that constitute two apparent high affinity binding components, and there is a low affinity binding component (Table I). The number of high affinity sites was confirmed by equilibrium dialysis to be n = 8.1 ± 0.2 sites/tetramer (three experiments). In the presence of 0.01 or 0.1 M KCl, there were still eight high affinity sites/tetramer, but there was a shift of one site from component II to component I, with a small reduction in the affinity of component I sites and increased variability of the parameters (Table I). The increased variability at more physiological KCl concentrations made it difficult to generate precise data. Because these solutions were filtered through a Chelex-resin column prior to use, the shift in affinity and increased variability is not due to calcium or magnesium contamination of the KCl. The third binding component was variable between batches of protein and probably represents ionic interactions due to the highly charged surface of brain spectrin instead of true "binding sites." The estimates for the low affinity component may have been complicated also by the tendency of brain spectrin to aggregate at saturating levels of calcium. The shift of a high affinity site from one component to another with a change in ionic conditions indicates a possible ionic or conformational effect on calcium-binding affinity. Within a given salt condition there did not appear to be cooperativity, as determined by the Hill plot (n_H = 0.95 ± 0.04, r = 0.999 for seven experiments). These data indicate that at near psysiological salt concentrations the brain spectrin tetramer binds two calcium ions with high affinity and six calcium ions with approximately 10-fold lower (but still micromolar) affinity. In these experiments, the $^{45}Ca^{2+}$ detected in the flow-through with hemoglobin did not differ from that seen in the absence of protein.

[2] C. J. Wallis and J. A. Babitch, unpublished data.

When $^{45}Ca^{2+}$ is diluted with unlabeled magnesium, only one binding component is detected, requiring much higher concentrations to displace $^{45}Ca^{2+}$ as compared with unlabeled calcium.[2] By Dixon plot, the magnesium K_I = 1.21 mM for calcium binding (Fig. 2) and is similar to the EC_{50} = 1.3 mM. Concentrations of magnesium greater than 10 mM in the protein buffer completely block calcium binding to brain spectrin, but aggregation may occur under these conditions.

In preliminary experiments, brain spectrin was treated with trypsin or chymotrypsin, and the cleavage products were transferred from SDS gels to Immobilon P membranes. $^{45}Ca^{2+}$ overlay was performed to identify fragments containing high affinity calcium-binding sites. To eliminate binding to the low affinity calcium-magnesium sites, we routinely used 3–5 mM $MgCl_2$ in the $^{45}Ca^{2+}$ overlay buffer. At 0.2 μM $^{45}Ca^{2+}$ and 3 mM $MgCl_2$, all (T126, T91, T80, T54) trypsin cleavage fragments containing the carboxyl end of the α chain (α domain IV) bound calcium (Fig. 3*a*). This domain, near the carboxyl end of the α chain, is the site of the two putative EF-hands. In addition, trypsin cleavage fragments from β domain IV (T74, T52) bound calcium with high affinity, indicating a binding site on the amino-terminal end of the β chain. Finally, a band from α domain III (T27) bound calcium. This is the area of the "hypersensitive site" on the α chain.

Chymotrypsin cleavage under mild conditions yielded two fragments of the α chain (150 and 145 kDa). The smaller carboxyl-terminal fragment bound calcium. The β chain was cleaved to a 210-kDa piece that continues to bind calcium, whereas the small cleavage fragment (25 kDa) from the amino-terminal end of the molecule does not (Fig. 3). Therefore, the β chain-binding site is not at the very end of the β domain IV, but at least 25 kDa from the amino terminal.

To summarize the proteolysis experiments, the four high affinity binding sites/dimer appear to be distributed as follows: 1) two sites in α domain IV at the two proposed EF-hand sites, 2) one site in the area of the hypersensitive site described by Harris and Morrow (1988) with a probable location at the end of repeat 11 or the beginning of repeat 12 (α domain III), and 3) one site in β domain IV at least 25 kDa from the amino terminus.

DISCUSSION

Calcium binding affinity for the first class of sites decreases about 10-fold with increasing ionic strength, similar to effects on calcium binding to calmodulin (Cox, 1988), whereas increasing KCl affects the other two types of sites relatively little (Table I). However, the reported binding affinities for the two high affinity classes are greater than or approximately equal to the calcium affinities of calmodulin and calcium-dependent protease I. This suggests that when intracellular calcium rises in response to neuronal depolarization, calcium ions will bind, at least to the first spectrin class of sites, before binding to proteins such as calmodulin or calcium-dependent protease I.

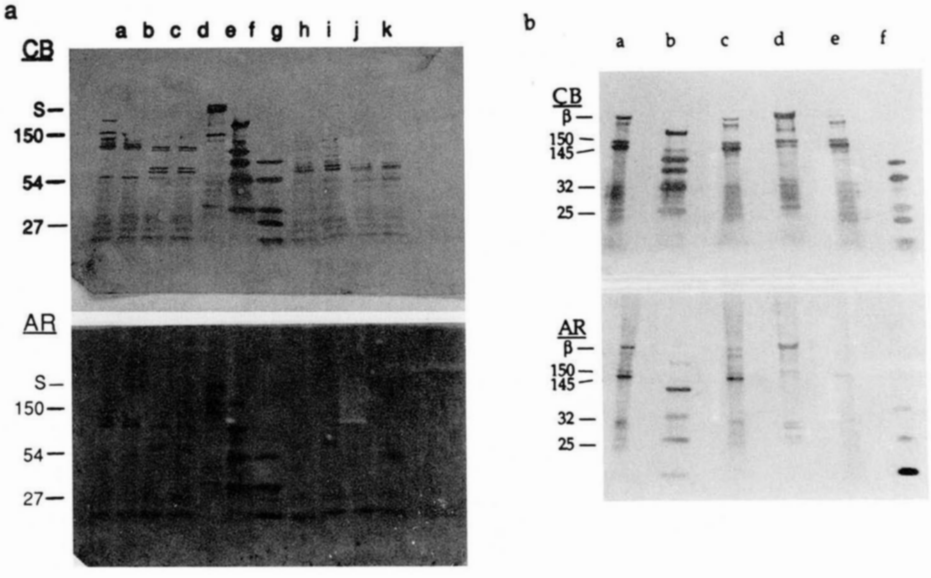

FIG. 3. **Calcium binding to horse brain spectrin fragments produced by digestion with trypsin, chymotrypsin, or endogenous protease.** *a*, trypsin digestions for 30 min (*lanes a, b, h,* and *i*) or 3 h (*lanes c, d, j,* and *k*) at 1:100 (w/w) (*lanes a, c, h,* and *j*) or 1:200 (*lanes b, d, i,* and *k*) enzyme:substrate. *Lane e*, horse brain spectrin. *Lanes f* and *g*, high and low molecular weight markers. *CB*, Coomassie Blue-stained blot; *AR*, autoradiogram produced by incubating the blot in buffer containing 60 mM KCl, 3 mM $MgCl_2$, and 0.2 μM $^{45}CaCl_2$ as described under "Experimental Procedures." Molecular weights of major polypeptides are on the *left side*. *S*, intact spectrin. *b*, chymotrypsin and endogenous protease (calcium-dependent protease I?) digestions for 15 min (*lane a*), 30 min (*lane c*), or 1 h (*lane e*). *Lane d*, endogenous protease digestion after storage. *Lanes b* and *f*, high and low molecular weight markers. β, intact β subunit.

The similarities in the 106–122 amino acid repeats of members of the α-actinin-spectrin-dystrophin superfamily suggest that these polypeptides have similar tertiary structures (Glenny and Glenny, 1984; Davison *et al.*, 1989), with the erythroid and nonerythroid spectrins differing principally in their calmodulin-binding and oligomer domains. Our observing proteolysis fragments with molecular weights similar to those found for other brain spectrins (Burridge *et al.*, 1982; Harris and Morrow, 1988) supports the idea that the tertiary structure forms an exposed region between the repeats so that proteases yield fragments with multiples of the repeat molecular weight, in addition to rapid cleavage at the hypersensitive site. However, our peptide assignments will not be certain until the peptides are at least partially sequenced and the horse brain α- and β-fodrins have been completely sequenced.

This high affinity binding of calcium to brain spectrin suggests that spectrin function may be modified directly by calcium in addition to previously revealed regulation by calcium-dependent interactions with calmodulin or calcium-dependent protease I. The proposed localization of binding sites to α domain IV is consistent with calcium binding to the putative EF-hand sequences (Wasenius *et al.*, 1989; Dubreuil *et al.*, 1991). The additional proposed β chain binding site(s) on the amino-terminal half of the chain indicates that calcium's direct binding to brain spectrin may influence spectrin-actin-protein 4.1 interactions at this end of the spectrin dimer. The conformational consequences of this high affinity calcium binding to brain spectrin are still unknown, but it is possible that calcium stabilizes a tertiary or quaternary structure in this region necessary for successful formation of the spectrin-actin-protein 4.1 ternary complex. This would be consistent with reports that submicromolar calcium concentrations stimulate spectrin-actin-protein 4.1 binding (Tanaka *et al.*, 1991). In addition, it is possible that increased calcium levels may prepare spectrin for the binding of calmodulin by forming hydrophobic patches both on calmodulin and on spectrin. This is suggested by preliminary work which shows small increases in spectrin hydrophobicity with increasing calcium.[2]

The subtle and complicated regulation that may occur because the protease hypersensitive, calmodulin-binding, and calcium-binding sites all occur in the same region of the α chain also deserves mention. In a variety of tissues, it has been shown that an increase in calcium concentration may result in disruption of the cytoskeleton and redistribution of integral membrane proteins such as receptors or ion channels (Levine and Willard, 1983; Perrin and Aunis, 1985; Siman *et al.*, 1985). After redistribution of such proteins (or removal of the stimulus), the cytoskeletal proteins would presumably reassociate as calcium concentration fell to resting levels. Calcium-dependent protease I modification of brain spectrin has been implicated in prolonging and intensifying the effect of calcium in long term potentiation, and this entire process has been proposed as an hypothesis for a molecular basis of memory (Siman *et al.*, 1987). The interaction of calcium with spectrin near the hypersensitive and calmodulin-binding sites may be a necessary part of this process.

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