Characterization of Calcium Binding to Spectrins†

Susanne Lundberg,‡ Veli-Pekka Lehto,§ and Lars Backman*,‡

Department of Biochemistry, University of Umeå, S-901 87 Umeå, Sweden, and Department of Pathology, University of Oulo, Kajaanintie 52D, SF-90220 Oulo, Finland

Received December 9, 1991; Revised Manuscript Received February 24, 1992

ABSTRACT: Calcium binding to brain and erythrocyte spectrins was studied at physiological ionic strength by a calcium overlay assay and aqueous two-phase partitioning. When the spectrins were immobilized on nylon membranes by slot blotting, the overlay assay showed that even though both spectrins bound $^{45}Ca^{2+}$, the brain protein displayed much greater affinity for calcium ions than erythrocyte spectrin did. Since the observed binding was weaker than that displayed by calmodulin under similar conditions, the overlay assay results indicated that the binding must be weaker than 1 μM. The phase partition experiments showed that there are at least two sites for calcium on brain spectrin and that calcium binding to one of these sites is reduced significantly by magnesium ions. From the partition isotherm, the dissociation constants were estimated as 50 μM for the Mg^{2+}-independent site and 150 μM for the Mg^{2+}-dependent site. The phase partition results also showed that erythrocyte spectrin bound calcium ions at least 1 order of magnitude weaker. By examining calcium binding to slot-blotted synthetic peptides, we identified two binding sites in brain spectrin. One mapped to the second putative calcium binding site (EF-hand) in α-spectrin and the other to the 36 amino acid residue long insert in domain 11. In addition, a tryptic fragment derived from the C-terminal of erythrocyte α-spectrin, which contained the two postulated EF-hands, also bound calcium. These findings suggest that the calcium signal system may also involve direct binding of calcium to spectrin beside known calcium modulators such as calmodulin and calpain. The results also indicate that the difference between the erythrocyte type of spectrin and the brain type involves not only the 36 amino acid residue insert in the brain type but also the C-terminal part of the α-subunit.

Signal transduction (triggered by ligand–receptor interactions) often elicits a change in the intracellular concentration of calcium, either by mobilization of internal calcium stores or by altered calcium flux across the plasma membrane. Calcium ions can therefore act as intracellular messengers, conveying the external signal to the internal target (Adelstein et al., 1988; Rasmussen, 1989). Transmission of the calcium signal inside the cell may be accomplished by binding to the target protein directly, as in the activation of calpains (calcium-activated neutral proteases) (Melloni & Pontremoli, 1989). The signal may also be transmitted in an indirect manner by binding to and concomitant activation of a regulatory protein, which in turn will regulate the target process.

Several calcium-dependent regulatory proteins have been identified and characterized. The behavior of calmodulin is well-known; this major intracellular receptor for calcium in eukaryotic cells undergoes a gross conformational change upon binding of calcium ions and is thereby activated to regulate a broad range of cellular processes, notably many involving the cytoskeletal framework (Klee, 1988; Vandekerckhove, 1990). However, most of these calcium mediators are more restricted in their actions, and many of them, such as gelsolin (Forscher, 1989; Yin, 1990), villin (Hesterberg & Weber, 1983; Northrop et al., 1986; Janmey & Matsudaira, 1988), and α-actinin (Burridge & Feramisco, 1981; Bennett et al., 1984; Duhaiman & Bamburg, 1984; Blanchard et al., 1989), are controlling the cellular state of actin filaments (and thus also the actin cytoskeleton).

Spectrins are a family of proteins that, in addition to their ability to cross-link and attach actin filaments to the membrane (Marchesi, 1983; Goodman et al., 1988; Bennett, 1989; 1990; Coleman et al., 1989), appear to be regulated by several calcium mediators. Calmodulin associates, in a calcium-dependent manner, with spectrins (Glenney et al., 1982; Berglund et al., 1986; Harris et al., 1988; Leto et al., 1989), though the regulatory consequence of this association is unknown. Spectrins are major substrates for the calpains (Siman et al., 1984; Lynch & Baudry, 1987; Seubert et al., 1987; Harris et al., 1989; Backman et al., 1991; Peterson et al., 1991); it has been suggested that proteolytic degradation of spectrin is an obligatory step in neuronal degeneration (Arai et al., 1991). There might also be direct calcium control as sequencing of several spectrins has revealed the presence of possible calcium binding sites (EF-hands) (Dubreuil et al., 1989; Wasenius et al., 1989; Sahr et al., 1990). As many of these calcium-dependent regulatory proteins are present in most cells, it is obvious that a very complicated regulatory mechanism may be involved.

In order to understand the role of calcium in regulation better, we have characterized the binding of calcium ions to spectrins. We have chosen to compare the binding properties of mammalian erythrocyte spectrin with those of brain spectrin. These two spectrins show many structural similarities; they are both highly elongated (~100 nm) and flexible molecules, comprising two distinct subunits. The α- and β-subunits associate in an antiparallel fashion to form a heterodimer, which in turn forms tetramers by head-to-head associations (Speicher et al., 1982). Sequencing of several spectrins has shown that the subunits of these 2, like of other spectrins, contain homologous, 106 amino acid residue long repetitive segments (Speicher & Marchesi, 1984; Birkenmeier et al., 1985; Cioë et al., 1987; McMahon et al., 1987; Dubreuil et al., 1989; Hong & Doyle, 1989; Wasenius et al., 1989; Moon & McMahon, 1990; Sahr et al., 1990; Winkelmann et al., 1990). Close to the C-terminus of the α-subunit, two putative EF structures have been found (Dubreuil et al., 1989; Was-

† This work was supported by grants to L.B. from the Swedish Natural Science Research Council (K-KU 1517-302) and from Magn. Bergvalls Stiftelse.

* Correspondence should be addressed to this author.
‡ University of Umeå.
§ University of Oulo.

0006-2960/92/0431-5665$03.00/0

enius et al., 1989; Sahr et al., 1990). The C-terminus of the α-subunit also forms, together with the N-terminus of the β-subunit, the actin binding site (Calvert et al., 1980; Cohen & Langley, 1984; Karinch et al., 1990).

There are also several important structural differences. The α-subunit of erythrocyte spectrin is only 50–60% homologous compared to the more than 90% homology displayed by all other α-spectrins (both from different tissues of the same organism and from different organisms) (Wasenius et al., 1989; Bennett, 1990; Sahr et al., 1990). The major difference has been found in the middle of the molecule; brain spectrin contains a 36 amino acid residue insert in segment 11 of the α-subunit that is lacking in the erythrocyte form (Wasenius et al., 1989; Sahr et al., 1990). Since the insert contains a typical calmodulin binding sequence, this may explain the different affinities for calmodulin that have been observed (Glenney et al., 1982; Harris et al., 1988; Leto et al., 1989). Although both spectrins are substrates for calpain, the site of proteolytic cleavage differs; again, the difference is located to the insert which contains a calpain cleavage site. Interestingly, erythrocyte β-spectrin is cleaved close to the C-terminus, a site that appears to be available in brain β-spectrin also, though only in the presence of calmodulin (Harris et al., 1988; Harris & Morrow, 1990; Backman et al., 1991). In addition, tryptic digestion gives distinct peptide maps, and there is limited cross-reactivity (Davis & Bennett, 1983).

From the results obtained, it is clear that the calcium affinities of brain and erythrocyte spectrin differ significantly. We conclude, in accordance with a previous report (Beaven & Gratzer, 1980), that erythrocyte spectrin binds calcium very weakly. Brain spectrin, on the other hand, binds calcium with much higher affinity, and we have been able to distinguish two different sites for calcium, one dependent on and one independent of magnesium. On the basis of binding experiments using synthetic peptides, one site appears to be located close to the C-terminus of the α-subunit, probably at the second predicted EF-hand, whereas the other site is located close to the insert.

Materials and Methods

Protein Purification. Human erythrocyte spectrin was prepared from fresh blood by a procedure based on that of Ohanian and Gratzer (1984). Cells were freed of buffy coat by several washes with isotonic phosphate-buffered saline. These cells were lysed in an equal volume of 24 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[1] pH 7.2, 1 mM β-mercaptoethanol, 1 mM EGTA, 300 mM NaCl, 15% Triton X-100, 0.6 mM PMSF, and 100 μg/mL leupeptin, immediately layered on a 40% sucrose cushion containing, in addition to the lysis buffer, 0.6 M KCl, and centrifuged (48000*g* for 50 min). The pelleted membrane skeletons were twice suspended in ice-cold 20 mM Tris and 0.3 mM PMSF, pH 7.6, and pelleted. The skeletons were then dissociated by adding about an equal volume of 1.2 M sodium bromide, 40 mM Tris, 2 mM EGTA, and 30 mM sodium pyrophosphate, pH 8.0, and incubating the suspension at 30 °C for 20–30 min with occasional agitation. After a brief centrifugation, the material was applied to a column of Sepharose CL4B (2.5 × 150 cm; Pharmacia LKB Technology, Sweden) equilibrated with 0.6 M sodium bromide, 20 mM Tris, 15 mM sodium pyrophosphate, 1 mM EGTA, and 0.02% sodium azide, pH 8.0. The fractions containing spectrin were precipitated by addition of an equal volume of cold, saturated ammonium sulfate containing 1 mM DTT, redissolved in 10 mM Tris, pH 7.5, and dialyzed against the same buffer. The α- and β-subunits were obtained by dissociation of the spectrin dimer in 3 M urea followed by separation on an ion-exchanger as described by Yoshino and Marchesi (1984). Spectrin concentration was determined spectrophotometrically using a specific absorbance of $E^{1\%}_{1cm}$ = 10.7 at 280 nm (Kam et al., 1977).

Brain spectrin was prepared from calf forebrains following the procedure of Bennett et al. (1986) with some minor modifications. After the gel filtration step (Sepharose CL2B, 5 × 90 cm), this method gave brain spectrin that was 60–80% pure (as determined by densitometry of Coomassie blue stained SDS–PAGE gels). The protein was usually stored in gel filtration buffer containing 10% sucrose to reduce aggregation and prior to use dialyzed against 10 mM Tris, 0.4 mM DTT, and 10% sucrose, pH 8.2. Occasionally fodrin was purified further on DEAE-Sepharose as described before (Bennett et al., 1986). Since the ion-exchange step caused large losses in protein, most experiments were done with fodrin taken from the gel filtration step. However, there was no significant difference in the affinity for calcium between the two preparations, indicating that the contaminants did not bind calcium.

Peptides. Synthetic peptides to parts of chicken brain α-spectrin were prepared as follows: the first EF-hand (CB EFI), KFHFDKDKSGRLNHQEFKSF; the second EF-hand (CB EFII), ILDTVDPNRDGHVSLQEYMAF; the N-terminal part of the insert in domain 11 (CB seg11), QAVEHQEVYGMMPRDETD; the C-terminal part of the insert, SKTVSPWKSARMMVHTVATF; and the putative calmodulin binding site in C-terminal, TKRKHQEIRAMRSQLKKIE. These peptides were used directly in the calcium binding assay.

Calcium Binding Assay. To probe for binding of calcium, proteins or fragments were first separated by SDS–PAGE, using the discontinuous buffer system of Laemmli (1970), and then transferred to PVDF membranes by semi-dry or tank (Towbin et al., 1970) electroblotting. Alternatively, proteins, fragments, or synthetic peptides were slot-blotted directly to the membrane.

After transfer, the membrane was incubated in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole, pH 6.8 for 1.5 h, with repeated changes (Maruyama et al., 1984). The membrane was then soaked for 15 min in the same buffer containing 1 mCi/L $^{45}Ca^{2+}$ (New England Nuclear, Sweden). After being washed in water for 5 min, the membrane was dried and autoradiographed at −70 °C.

Two-Phase Partition. An aqueous two-phase system containing 16.25% (w/w) Ficoll 400 and 7.5% (w/w) Dextran T500 (both from Pharmacia LKB Technology), 0.25% (w/w) poly(ethylene glycol) 8000 (Union Carbide), 12.5 mM HEPES, 125 mM KCl, and 1.25 mM $MgCl_2$ (when present), pH 7.0, was thoroughly mixed and left to separate overnight at room temperature. Aliquots of 0.4 mL from each phase were taken and mixed in test tubes. After addition of 0.2 mL of sample (protein and/or calcium solution) to each tube, the mixtures were incubated for 2 h at 20 °C with end-over-end rotation. The phases were then separated by centrifugation (2250*g* for 15 min), and the partition of $^{45}Ca^{2+}$ was determined by counting 0.1 mL of upper and lower phase, respectively, in Lumagel scintillation fluid (Lumac, The Netherlands).

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); kDa, kilodalton(s).

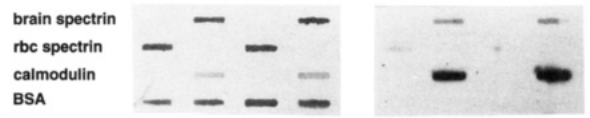

FIGURE 1: Calcium binding to intact spectrins. Brain spectrin (50 and 75 μg), erythrocyte (rbc) spectrin (50 and 75 μg), calmodulin (10 and 25 μg), and bovine serum albumin (BSA) (10 and 50 μg) were immobilized by slot blotting on a PVDF membrane. After several washes in 10 mM imidazole, pH 6.8, 60 mM KCl, and 5 mM $MgCl_2$, the membrane was soaked in the same buffer containing 1 μCi/mL $^{45}Ca^{2+}$ for 15 min. Unbound calcium was removed by a quick rinse in water, and then the membrane was dried and autoradiographed. Calcium binding proteins were identified from the resulting autoradiogram (right panel). The corresponding Coomassie blue stained membrane is shown in the left panel.

Proteolytic Digestion. Trypsin (Sigma), usually dissolved in 10 mM HCl to 1 mg/mL to prevent autoproteolysis, was added to the purified protein at an enzyme:substrate ratio of 1:25 to 1:100. Digestions were performed on ice and stopped either by adding soybean trypsin inhibitor (5-fold molar excess) or by denaturation in SDS–PAGE sample buffer. *Staphylococcus aureus* V8 (1:25; Boehringer Mannheim Scandinavian AB, Sweden) and chymotrypsin (1:25; Sigma) digestions were done similarly, but the reactions were stopped either by adding sample buffer and boiling the samples for 5 min or by adding PMSF.

Results

Probed by a $^{45}Ca^{2+}$ overlay assay, slot-blotted brain spectrin and also erythrocyte spectrin bound calcium as Figure 1 shows. The autoradiograph (Figure 1, right) indicated that more $^{45}Ca^{2+}$ was bound to brain spectrin than to the erythrocyte form. Since similar amounts of both proteins were blotted to the membrane, it follows that the affinity of brain spectrin for calcium was greater than that of erythrocyte spectrin. Calmodulin, which was included as a positive control, gave a much stronger band on the autoradiograph than both spectrins, indicating that brain spectrin bound calcium weaker than calmodulin does. This sets a higher limit on the affinity of brain spectrin for calcium to about 1 μM as the dissociation constants for the high-affinity sites on calmodulin are close to this value (Burger et al., 1984; Beckingham, 1991). The level of unspecific binding was negligible as no binding of calcium to bovine serum albumin could be detected.

To localize the binding site(s), subunits and proteolytic fragments (obtained by trypsin and V8 protease as well as chymotrypsin digestion) were separated by SDS–PAGE and then electroblotted onto PVDF membranes. However, independent of whether semi-dry or tank blotting was used, none of the transferred polypeptides bound detectable amounts of $^{45}Ca^{2+}$. The upper panel in Figure 2 shows that proteolytic digestion did not destroy the binding sites as digests still retained affinity for calcium after slot blotting, the only exception being V8 protease that diminished calcium binding to erythrocyte spectrin. Since heat denaturing in electrophoresis sample buffer, i.e., in the presence of SDS and β-mercaptoethanol, reduced calcium binding considerably (lower panel in Figure 2), the results implied that electrophoresis and/or electrotransfer may have altered the structure of the polypeptides in such a way that the affinity for calcium was reduced or even completely lost.

It is well-known that not all protein loaded on the polyacrylamide gel is recovered on the membrane after the blotting process (Mozdzanowski & Speicher, 1990). This is in particular true for high molecular weight polypeptides such as spectrin subunits. Since proteolysis of brain spectrin resulted in several high molecular weight fragments, in contrast to

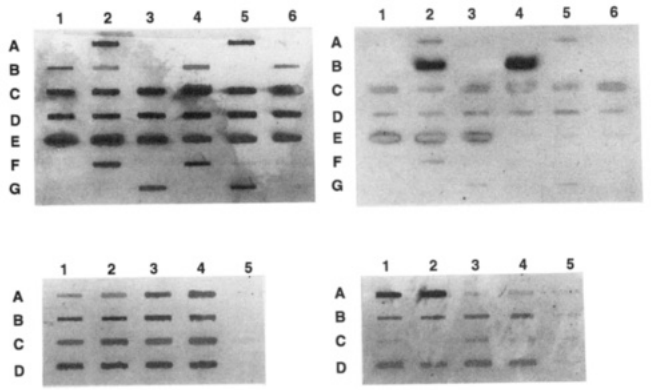

FIGURE 2: Calcium binding to proteolytically digested and heat-denatured spectrins. (Upper panels) Brain and erythrocyte spectrins were hydrolyzed by trypsin (with an enzyme:substrate ratio of 1:25) in 10 mM Tris-HCl (pH 8.2) on ice. Digestion was terminated by adding trypsin inhibitor after 0.5 (partial cleavage) or 13 (complete cleavage) h. Both spectrins were also digested by V8 protease (1:25), except that the reaction was terminated by addition of PMSF. After slot blotting to the PVDF membrane, calcium binding was probed by the calcium overlay assay. Row A, intact brain spectrin (50 μg, columns 2 and 5); row B, soybean trypsin (4 μg, column 1), calmodulin (10 and 25 μg, columns 2 and 4, respectively), V8 protease (4 μg, column 6); row C, partial trypsin-digested brain spectrin (50 and 100 μg, columns 1–3 and 4–6, respectively); row D, partial V8 protease-digested brain spectrin (50 and 100 μg, columns 1–3 and 4–6, respectively); row E, partial trypsin-digested erythrocyte spectrin (50 μg, columns 1–3), partial V8 protease-digested erythrocyte spectrin (50 μg, columns 4–6); row F, completely V8 protease-digested brain spectrin (100 μg, column 2) and erythrocyte spectrin (100 μg, column 4); row G, completely trypsinated brain spectrin (100 μg, column 3) and erythrocyte spectrin (100 μg, column 5). To all empty slots was added only phosphate-buffered saline, pH 7.5. (Lower panels) To determine the effect of SDS denaturing on calcium binding, both brain and erythrocyte spectrins were treated with SDS gel solubilizing buffer and boiled for 5 min. After slot blotting, calcium binding was assayed as before. Row A, calmodulin (25 and 75 μg, columns 1 and 2) and bovine serum albumin (25 μg, columns 3 and 4); row B, native brain spectrin (25 and 75 μg, columns 1 and 2) and native erythrocyte spectrin (25 and 75 μg, columns 3 and 4); row C, SDS-denatured brain spectrin (25 and 75 μg, columns 1 and 2) and SDS-denatured erythrocyte spectrin (25 and 75 μg, columns 3 and 4); row D, trypsinated and SDS-denatured brain spectrin (50 μg, columns 1 and 2) and erythrocyte spectrin (50 μg, columns 3 and 4). Coomassie blue stained membranes (left panels) and autoradiograms (right panels).

erythrocyte spectrin (Morrow et al., 1980), the lack of any observable calcium binding to the separated fragments may to some extent have been dependent on too low protein concentration on the membrane.

In an attempt to increase the amount of material transferred, we trypsinated isolated erythrocyte α- and β-spectrin instead of intact spectrin; thereby it was possible to load larger amounts of each subunit on the gel. After electrophoretic separation and transfer to the membrane, there was a weak but clearly detectable $^{45}Ca^{2+}$ binding to certain fragments of both subunits; binding was mainly associated with the 66- and 40-kDa fragments of α-spectrin and with the 46- and 33-kDa fragments of β-spectrin (not shown). Sequencing of the 40-kDa fragment indicated that the excised Coomassie blue stained band actually comprised two distinct polypeptides and that both localized to the C-terminal part of α-spectrin; one fragment started at residue 1494 and the other at residue 1919.

To characterize calcium binding in terms of binding strength and stoichiometry, we used partition in an aqueous two-phase system. The partition coefficient of calcium in such a two-phase system is defined by

$$K_{Ca} = [Ca^{2+}]^{upper}/[Ca^{2+}]^{lower}$$

where $[Ca^{2+}]^{upper}$ and $[Ca^{2+}]^{lower}$ denote the concentrations in

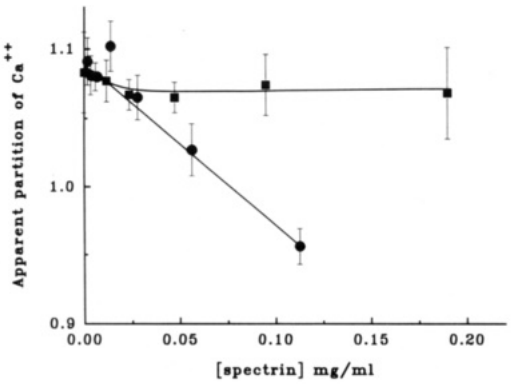

FIGURE 3: Effect of brain and erythrocyte spectrins on the partition of $^{45}Ca^{2+}$. 50 μL of calcium stock was added to each two-phase system to give a final concentration of 0.012 μM. Brain spectrin (●) or erythrocyte spectrin (■) (150 μL) was then added to give the indicated concentrations, and the phase system was incubated. After 2 h of end-over-end mixing, the phases were separated, and the apparent partition of calcium was determined. Phase system: 13% (w/w) Ficoll 400, 6% Dextran T500, 0.2% PEG 8000, 10 mM HEPES, pH 7.0, and 100 mM KCl.

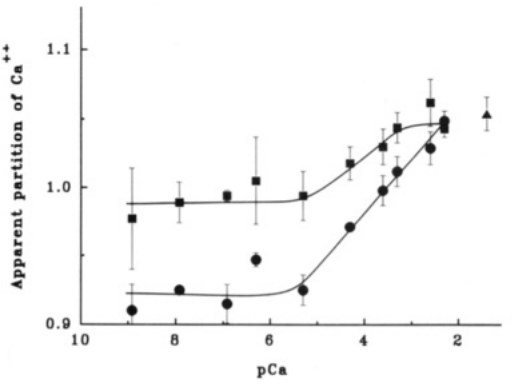

FIGURE 4: Calcium binding to brain spectrin in the presence and absence of Mg^{2+}. 50 μL of calcium stock was added to each two-phase system to give the indicated concentrations. 150 μL of brain spectrin was then added to the magnesium-containing (■) or the magnesium-free (●) phase system to give a final concentration of 0.4 mg/mL. The phase system was then incubated for 2 h (end-over-end mixing) before the phases were separated by centrifugation and the apparent partition of $^{45}Ca^{2+}$ was determined. Phase system: 13% (w/w) Ficoll 400, 6% Dextran T500, 0.2% PEG 8000, 10 mM HEPES, pH 7.0, and 100 mM KCl, with or without 1 mM $MgCl_2$.

the upper and lower phase, respectively. In the presence of a calcium binding protein, a certain fraction of calcium will be bound, and it is therefore more appropriate to describe the partition of calcium in terms of an apparent partition coefficient, defined as (Backman, 1985)

$$K_{Ca}^{app} = \frac{[Ca^{2+}]_{total}^{upper}}{[Ca^{2+}]_{total}^{lower}} = \frac{[Ca^{2+}]_{free}^{upper} + [Ca^{2+}]_{bound}^{upper}}{[Ca^{2+}]_{free}^{lower} + [Ca^{2+}]_{bound}^{lower}}$$

The apparent partition coefficient of calcium will therefore equal the partition coefficient of free calcium in the absence of any binding protein. As the concentration of the calcium binding protein is increased, an increasing fraction of calcium will be bound, and the apparent partition coefficient will approach that of bound calcium.

If instead the concentration of calcium is varied and that of the protein is kept constant, the resulting partition isotherm takes a different shape. In this case, when the concentration of calcium is much higher than that of the protein, nearly all ligand will be free and only an infinitesimal fraction bound; the apparent partition coefficient should be identical with the partition coefficient of free ligand. As the concentration of ligand is decreased, due to the law of mass action, the fraction of bound ligand increases, and consequently, the apparent partition coefficient will be affected. The exact shape of the partition isotherm depends not only on the partition properties of free and bound ligand but also on binding strength and stoichiometry as well as type of binding (i.e., cooperative versus noncooperative). It can, for instance, be shown that the concentration at which the transition in the apparent partition occurs corresponds roughly to the dissociation constant.

In the phase system used, both spectrins were highly concentrated in the lower dextran-rich phase; >95% of both proteins was recovered in the lower phase, independent of the concentration of calcium. The partition of calcium (in the absence of any added protein) was close to unity (K_{Ca} = 1.06) and constant throughout the concentration range studied. In the presence of brain spectrin, the apparent partition coefficient of $^{45}Ca^{2+}$ decreased and displayed a strong dependence on the protein concentration as Figure 3 shows. This partition behavior clearly demonstrated that brain spectrin bound calcium. Contrary to brain spectrin, erythrocyte spectrin did not influence the partition of calcium significantly, not even at very high protein concentrations (up to 1 μM), and therefore the affinity of erythrocyte spectrin for calcium must be much lower, if any, than that of brain spectrin.

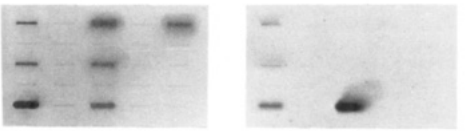

FIGURE 5: Calcium binding to synthetic peptides of chicken brain α-spectrin. Peptides (ca. 1 mg) were dissolved in 10 mM Tris, pH 7.5, and immobilized by slot blotting onto a PVDF membrane. After several washes in 10 mM imidazole, pH 6.8, 60 mM KCl, and 5 mM $MgCl_2$, the membrane was soaked in the same buffer containing 1 μCi/mL $^{45}Ca^{2+}$ for 10 min to determine calcium binding. Unbound calcium was removed by a quick rinse in water, the membrane was dried, and an autoradiogram was prepared (right panel). Finally the membrane was stained by Coomassie blue (left panel). The following peptides were tested: the N-terminal part of the insert in domain 11 (CB seg11, top row, left); the C-terminal part of the insert in domain 11 (top row, middle); the first EF-hand (CB EFI, top row, right); the second EF-hand (CB EFII, middle row, left); the putative calmodulin binding site in the C-terminal (middle row, middle). Both calmodulin (0.5 mg, bottom row, right) and erythrocyte spectrin (0.5 mg, bottom row, left) were included as controls.

In another series of experiments, we wanted to assess the role of magnesium ions on the binding of calcium to brain spectrin and to estimate the binding strength. Therefore, we partitioned calcium, at different concentrations, in the absence and presence of 1 mM Mg^{2+}. Again, as Figure 4 shows, the partition behavior of calcium was typical of a solute undergoing reversible association. However, magnesium ions reduced the decrease in the apparent partition coefficient and shifted the transition of the partition isotherm toward lower concentrations of calcium. Such a reduction in the amount of bound calcium may be caused by a decreased number of binding sites or by a weaker binding (or a combination of the two). From the concentrations at which the transitions in the partition isotherms occur, we estimated the dissociation constants in the absence and presence of 1 mM Mg^{2+} as approximately 50 and 150 μM, respectively. Therefore, it appears that there were (at least) two distinguishable sites for calcium on brain spectrin: one site that is magnesium-independent and another much weaker site that is dependent on magnesium.

When calcium binding to synthetic peptides was probed with the calcium overly assay, we detected $^{45}Ca^{2+}$ binding to a peptide containing the second putative EF-hand but not to a peptide containing the first EF-hand. A peptide to the 36 amino acid residue long insert in domain 11 also bound calcium

(Figure 5). When these peptides were included in the phase system, neither peptide affected the partition of calcium and therefore did not displace calcium from brain spectrin.

Discussion

Our results show that both brain and erythrocyte spectrins have affinity for calcium. Partition experiments in an aqueous two-phase system indicate that there are two nonidentical binding sites in brain spectrin. From the partition isotherm, we estimate the dissociation constants for these sites as about 50 and 150 μM, respectively. Calcium binding to the low-affinity site is inhibited by magnesium, in contrast to the high-affinity site. Although erythrocyte spectrin displayed calcium binding, though less than the brain form, when probed by an overlay assay, we observed no (or insignificant) binding in solution. It is therefore apparent that erythrocyte spectrin has much lower affinity for calcium than brain spectrin has. Due to the minor effect erythrocyte spectrin has on the partition behavior of calcium, the data do not allow a reliable estimate of the dissociation constant. As the detection limit (for this particular system) is around 1 mM, it seems reasonable to assume that the affinity of erythrocyte spectrin for calcium should be in the millimolar range. This is substantiated by Beaven and Gratzer (1980), who, based on equilibrium dialysis data, found that calcium binding to erythrocyte spectrin is weaker than 0.1 mM.

Although calcium binding activity was present in digests of brain spectrin, the lack of calcium binding to any fragment after electrophoretic separation and subsequent electroblotting prevented further identification of the calcium sites. This apparent loss in binding affinity can probably be attributed to the transfer procedure as well as to the incomplete transfer of the high molecular weight fragments present in the brain spectrin digests.

A common feature of all spectrins (at least those that have been sequenced so far) is the presence of two putative EF-hands close to the C-terminal of the α-subunit. However, only one of these sites appears to be functional, as only synthetic peptides corresponding to the second, but not to the first, EF-hand bound calcium. Similarly, Dubreuil et al. (1991) observed calcium binding only to recombinant *Drosophila* α-spectrin polypeptides that included the second EF-hand. The calcium binding activity of erythrocyte spectrin may also occur to the same site as the binding appears to be associated with the C-terminal of the α-subunit. This second EF-hand may therefore constitute a common functional calcium binding site in all spectrins.

Although the 36 amino acid residue insert in segment 11 of brain α-spectrin (Wasenius et al., 1989) does not contain a typical EF-hand structure (Kretsinger et al., 1991), a corresponding synthetic peptide bound calcium. In the overlay assay, this peptide appears to bind more calcium than the peptide from the second EF–hand. Therefore, it is tempting to assume that the stronger and magnesium-independent calcium binding site is associated with the insert and that the low-affinity site is located to the second EF-hand. This could explain the great difference seen in calcium affinity for different spectrins, as the insert is missing in erythrocyte spectrin.

The EF-hands in brain, erythrocyte, and *Drosophila* α-spectrin are all very homologous; of the 16 informational positions in the consensus sequence (Kretsinger et al., 1991), they contain 13 or more conserved amino acid residues (Figure 6). Except for the second EF-hand in erythrocyte α-spectrin, all of these EF-hands have conserved the oxygen-bearing amino acid residues (X, Y, Z, –X, and –Z in the consensus) that are believed to be required for binding the calcium ion (Strynadka & Jones, 1989). The invariant glycine and the hydrophobic residue in the calcium binding loop as well as the hydrophobic residues in the flanking regions are also well conserved. Thus, the primary structure does not uncover any direct reasons why the second but not the first of the EF-hands should bind calcium.

FIGURE 6: EF-hand sequences from brain, erythrocyte, and *Drosophila* spectrins. The two EF-hand sequences (CB EFI and CB EFII) and the N-terminal part of the insert in domain 11 (CB seg11) of chicken brain α-spectrin (Wasenius et al., 1989) were aligned with the two EF-hands in human erythrocyte α-spectrin (HE EFI and HE EFII; Sahr et al., 1990) and *Drosophila* α-spectrin (D EFI and D EFII; Dubreuil et al., 1989). The consensus sequence of EF-hands (Kretsinger et al., 1991) is shown at the top. Highly conserved positions (shaded residues) are indicated: the X, Y, Z, –X, and –Z positions are oxygen-bearing amino acid residues (Asp, Asn, Glu, Gln, Ser, and Thr) required for calcium binding; E = Glu; G = Gly; I = Ile, Val, or Leu; n = Val, Ile, Leu, Met, Phe, Tyr, or Trp; (#) and (*) = any residue. The score represents the number of conserved residues from the consensus sequence. The ability to bind calcium is indicated at the far right. The underlined residues represent the synthetic peptides tested for calcium binding.

Crystallographic studies on know calcium binding proteins, such as calmodulin, troponin C, and parvalbumin (Strynadka & Jones, 1989), have shown that the EF-hand must form a helix–loop–helix structure in order to bind calcium. When the EF-hand sequences in the spectrins were analyzed for any secondary structures, using the program GARNIER from the University of Wisconsin Genetics Computer Group, a very interesting pattern emerged. In all cases, only the second EF-hand was predicted to give a helix–loop–helix structure. The first EF-hand was predicted to give a single helix (erythrocyte and *Drosophila*) or a too short binding loop (brain), that probably would not have affinity for calcium. Although structure prediction analysis should be interpreted cautiously, it gives a plausible explanation to the observed difference in calcium binding between the first and the second EF-hand.

It is possible to envisage several roles that calcium binding to spectrin could serve in a cell. The tail regions of the spectrin tetramer, which contain the EF-hands, are homologous to the ends of the α-actinin homodimer. Calcium binding to the EF-hands may, as in nonmuscle α-actinin (Burridge & Feramisco, 1981; Duhaiman & Bamburg, 1984; Bennett et al., 1984), reduce the actin binding activity. Recently, Fishkind et al. (1987) showed that sea urchin spectrin is under calcium control, as cross-linking of actin by spectrin was inhibited by micromolar concentrations of calcium. They could not, however, conclusively establish whether the diminished gelation activity was due to inhibition of the actin–spectrin interaction or disruption of the dimer–dimer interaction. Even though erythrocyte spectrin also appears to be controlled by calcium (Fowler & Taylor, 1980), this does not probably involve calcium binding to the spectrin. Again, the gelation process was greatly inhibited by calcium concentrations as low as 1 μM. Since the dissociation constant for calcium binding is in the millimolar range, erythrocyte spectrin would be expected to be devoid of bound calcium at such low concentrations.

In nerve cells, the action potential triggered increase in intracellular calcium concentration is short-lived because

binding proteins, sequestering vesicles, and mitochondria take up calcium and pumps in the plasma membrane pump calcium out of the cell (Tsien & Tsien, 1990). In this way, the nerve terminal becomes ready to transmit another signal. As brain spectrin is a major protein in nerve endings (Levine & Willard, 1981), it might be part of such a calcium buffering system. Another possibility is that calcium may induce a conformational change in spectrin that is transmitted along the molecule and thereby could affect more distantly lying parts, as has been suggested for the binding of calmodulin to brain spectrin (Harris & Morrow, 1990).

Since the intracellular concentration of free calcium in a resting cell is below 0.1 μM (Lew et al., 1982), very little calcium would be bound to spectrin in an unstimulated cell. Upon stimulation, the free calcium concentration in the cell may transiently rise 100-fold and locally (i.e., close to the plasma membrane) even higher concentrations may occur (Larsen et al., 1981). In the cell, spectrin is usually concentrated to the plasma membrane (Coleman et al., 1989); therefore, calcium may bind to a significant fraction of the spectrin molecules, despite the relatively weak binding. If this is the case, calcium may control spectrin and the associated cytoskeleton not only indirectly via calcium mediator proteins but also directly by binding to the spectrin.

ACKNOWLEDGMENTS

The technical skill of Mrs. Eva-Maj Hägglöv is greatly appreciated.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

REFERENCES

Adelstein, R. S., Klee, C. B., & Rodbell, M. (1988) *Adv. Second Messenger Phosphoprotein Res. 21*, 1–222.

Arai, A., Vanderklish, P., Kessler, M., Lee, K., & Lynch, G. (1991) *Brain Res. 555*, 276–280.

Backman, L. (1985) in *Partitioning in Aqueous Two-phase Systems: Theory, Methods, Uses and Applications in Biotechnology* (Walter, H., Brooks, D. E., & Fisher, D., Eds.) pp 267–314, Academic Press, Orlando.

Backman, L., Pekrun, A., & Gratzer, W. B. (1991) *J. Biol. Chem. 266*, 3835–3840.

Beaven, G. H., & Gratzer, W. B. (1980) *Biochim. Biophys. Acta 600*, 140–149.

Beckingham, K. (1991) *J. Biol. Chem. 266*, 6027–6030.

Bennett, J. P., Zaner, K. S., & Stossel, T. P. (1984) *Biochemistry 23*, 5081–5086.

Bennett, V. (1989) *Biochim. Biophys. Acta 988*, 107–121.

Bennett, V. (1990) *Curr. Opin. Cell Biol. 2*, 51–56.

Bennett, V., Baines, A. J., & Davis, J. (1986) *Methods Enzymol. 134*, 55–69.

Berglund, Å., Backman, L., & Shanbagh, V. P. (1986) *FEBS Lett. 201*, 306–310.

Birkenmeier, C. S., Bodine, D. M., Repasky, E. A., Helfman, D. M., Hughes, S. H., & Barker, J. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5671–5675.

Blanchard, A., Ohanian, V., & Critchley, D. (1989) *J. Muscle Res. Cell Motil. 10*, 280–289.

Burger, D., Cox, J. A., Comte, M., & Stein, E. A. (1984) *Biochemistry 23*, 1966–1971.

Burridge, K., & Feramisco, J. R. (1981) *Nature (London) 294*, 565–567.

Calvert, R., Bennett, P., & Gratzer, W. (1980) *Eur. J. Biochem. 107*, 355–361.

Cioé, L., Laurila, P., Meo, P., Krebs, K., Goodman, S., & Curtis, P. J. (1987) *Blood 70*, 915–920.

Cohen, C. M., & Langley, R. C., Jr. (1984) *Biochemistry 23*, 4488–4495.

Coleman, T. R., Fishkind, D. J., Mooseker, M. J., & Morrow, J. S. (1989) *Cell Motil. Cytoskel. 12*, 225–242.

Davis, J., & Bennett, V. (1983) *J. Biol. Chem. 258*, 7757–7766.

Dubreuil, R. R., Byers, T. J., Sillman, A. L., Bar-Zvi, D., Goldstein, L. S. B., & Branton, D. (1989) *J. Cell Biol. 109*, 2197–2205.

Dubreuil, R. R., Brandin, E., Sun Reisberg, J. H., Goldstein, L. S. B., & Branton, D. (1991) *J. Biol. Chem. 266*, 7189–7193.

Duhaiman, A. S., & Bamburg, J. R. (1984) *Biochemistry 23*, 1600–1608.

Fishkind, D. J., Bonder, E. M., & Begg, D. A. (1987) *Cell Motil. Cytoskel. 7*, 304–314.

Forscher, P. (1989) *Trends Neurosci. 12*, 468–474.

Fowler, V., & Taylor, D. L. (1980) *J. Cell Biol. 85*, 361–376.

Gardner, K., & Bennett, V. (1987) *Nature (London) 328*, 359–362.

Glenney, J. R., Jr., Glenney, P., & Weber, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4002–4005.

Goodman, S. R., Krebs, K. E., Whitfield, C. F., Riederer, B. M., & Zagon, I. S. (1988) *CRC Crit. Rev. Biochem. 23*, 171–234.

Harris, A. S., & Morrow, J. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3009–3013.

Harris, A. S., Croall, D. E., & Morrow, J. S. (1988) *J. Biol. Chem. 263*, 15754–15761.

Harris, A. S., Croall, D. E., & Morrow, J. S. (1989) *J. Biol. Chem. 264*, 17401–17408.

Hesterberg, L. K., & Weber, K. (1983) *J. Biol. Chem. 258*, 365–369.

Hong, W., & Doyle, D. (1989) *J. Biol. Chem. 264*, 12758–12764.

Janmey, P. A., & Matsudaira, P. T. (1988) *J. Biol. Chem. 263*, 16738–16743.

Kam, Z., Josephs, R., Eisenberg, H., & Gratzer, W. B. (1977) *Biochemistry 16*, 5568–5572.

Karinch, A. M., Zimmer, W. E., & Goodman, S. R. (1990) *J. Biol. Chem. 265*, 11833–11840.

Klee, C. B. (1988) in *Molecular Aspects of Cellular Regulation* (Cohen, P., & Klee, C. B., Eds.) Vol. 5, pp 35–56, Elsevier, Amsterdam.

Kretsinger, R. H., Tolbert, D., Nakayama, S., & Pearson, W. (1991) in *Novel Calcium-Binding Proteins, Fundamentals & Clinical Implications* (Heizmann, C. W., Ed.) pp 17–37, Springer Verlag, Berlin.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Larsen, F. L., Katz, S., Roufogalis, B. D., & Brooks, D. E. (1981) *Nature (London) 294*, 667–668.

Leto, T. L., Pleasic, S., Forget, B. G., Benz, E., Jr., & Marchesi, V. T. (1989) *J. Biol. Chem. 264*, 5826–5830.

Levine, J., & Willard, M. (1981) *J. Cell Biol. 90*, 631–643.

Lew, V. L., Tsie, R. T., Miner, C., & Bookchin, R. M. (1982) *Nature (London) 298*, 478–481.

Lynch, G., & Baudry, M. (1987) *Brain Res. Bull. 18*, 809–816.

Marchesi, V. T. (1983) *Ann. N.Y. Acad. Sci. 416*, 159–165.

Maruyama, K., Mikawa, T., & Ebashi, S. (1984) *J. Biochem. 95*, 511–519.

McMahon, A. P., Giebelhaus, D. H., Champion, J. E., Bailes, J. A., Lacey, S., Carritt, B., Henchman, S. K., & Moon, R. T. (1987) *Differentiation 34*, 68–78.

Melloni, E., & Pontremoli, S. (1989) *Trends Neurosci. 12*, 438–444.
Moon, R. T., & McMahon, A. P. (1990) *J. Biol. Chem. 265*, 4427–4433.
Morrow, J. S., Speicher, D. W., Knowles, W. J., Hsu, C. J., & Marchesi, V. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6592–6596.
Morrow, J. S., Haigh, W. B., Jr., & Marchesi, V. T. (1981) *J. Supramol. Struct. Cell Biochem. 17*, 275–287.
Mozdzanowski, J., & Speicher, D. W. (1990) in *Current Research in Protein Chemistry: Techniques, Structure, & Function* (Villafranca, J. J., Ed.) pp 87–93 Academic Press, Inc., San Diego.
Northrop, J., Weber, A., Mooseker, M. S., Franzini-Armstrong, C., Bishop, M. F., Dubyak, G. R., Tucker, M., & Walsh, T. P. (1986) *J. Biol. Chem. 261*, 9274–9281.
Ohanian, V., & Gratzer, W. B. (1984) *Eur. J. Biochem. 144*, 375–379.
Peterson, C., Vanderklish, P., Seubert, P., Cotman, C., & Lynch, G. (1991) *Neurosci. Lett. 121*, 239–243.
Rasmussen, H. (1989) *Sci. Am. 261*, 44–51.
Sahr, K. E., Laurila, P., Kotula, L., Scarpa, A. L., Coupal, E., Leto, T. L., Linnenbach, A. J., Winkelmann, J. C., Speicher, D. W., Marchesi, V. T., Curtis, P. J., & Forget, B. G. (1990) *J. Biol. Chem. 265*, 4434–4443.
Seubert, P., Baudry, M., Dudek, S., & Lynch, G. (1987) *Synapse 1*, 20–24.
Siman, R., Baudry, M., & Lynch, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3572–3576.
Speicher, D. W., & Marchesi, V. T. (1984) *Nature (London) 311*, 177–180.
Speicher, D. W., Morrow, J. S., Knowles, W. J., & Marchesi, V. T. (1982) *J. Biol. Chem. 257*, 9093–9101.
Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951–998.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tsien, R. W., & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol. 6*, 715–760.
Vandekerckhove, J. (1990) *Curr. Opin. Cell Biol. 2*, 41–50.
Wasenius, V.-M., Saraste, M., Salven, P., Eraemaa, M., Holm, L., & Lehto, V.-P. (1989) *J. Cell Biol. 108*, 79–93.
Winkelmann, J. C., Chang, J.-G., Tse, W. T., Scarpa, A. L., Marchesi, V. T., & Forget, B. G. (1990) *J. Biol. Chem. 265*, 11827–11832.
Yin, H. L. (1990) *Adv. Exp. Med. Biol. 255*, 315–324.
Yoshino, H., & Marchesi, V. T. (1984) *J. Biol. Chem. 259*, 4496–4500.