Divalent Cation Binding to Erythrocyte Spectrin†

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ABSTRACT: Erythrocyte spectrin dimers and separated α- and β-spectrin chains bound $^{45}Ca^{2+}$ after electrophoresis on native or sodium dodecyl sulfate–polyacrylamide gels, blotting, and $^{45}Ca^{2+}$ overlay. Flow dialysis and equilibrium dialysis revealed two binding components: high-affinity, Ca^{2+}-specific sites with $k_d = 4 \times 10^{-7}$ M and $n = 100 \pm 20$ per dimer and a low-affinity (millimolar) divalent cation component. Whereas brain spectrin had only four high-affinity sites [Wallis, C. J., Wenegieme, E. F., & Babitch, J. A. (1992) *J. Biol. Chem. 267*, 4333–4337], erythrocyte spectrin had 25-fold more sites per dimer. In addition to possibly modifying spectrin interactions with calcium-dependent protease and actin, as suggested by previous work on the interaction of Ca^{2+} with brain spectrin, the approximately two high-affinity sites per repeating segment of erythrocyte spectrin appear to stabilize a folded conformation of repeat structures indicated by an entropy increase upon binding. These data support the hypothesis that divalent cation binding to erythrocyte spectrin has become specialized to stabilize the membrane skeletal network and the cell, making them flexible but resistant to shear under the stressful conditions of blood circulation.

Models of the membrane skeleton are derived largely from studies of the mammalian erythrocyte (Bennett, 1990) where a two-dimensional network of structural proteins including spectrin, actin, adducin, and proteins 4.1 and 4.9 is assembled just under the membrane. Identified linkers of the erythrocyte membrane to the cytoskeleton include ankyrin, which bridges binding sites on spectrin and band 3 protein, the membrane anion channel (Bennett & Branton, 1977; Luna et al., 1979; Yu & Goodman, 1979; Bennett & Stenbuck, 1980), and protein 4.1, which binds both to spectrin and to glycophorin (Ungewickell et al., 1979; Fowler & Taylor, 1980; Ohanian et al., 1984; Cohen & Langley, 1984; Correas et al., 1986). The erythrocyte membrane skeleton is involved in a variety of cellular processes including the maintenance of membrane receptor distribution and membrane deformability, elasticity, and stability.

Because spectrin interacts with most of the other proteins (as well as self-associating), it undoubtedly plays a central role in membrane skeleton function (Bennett, 1990). Spectrin is an extended, antiparallel heterodimer with both the α and β subunits divisible into three structural domains. Both subunits encode a terminal self-association region whereas most of each subunit consists of approximately 106 amino acid repeats (Speicher & Marchesi, 1984) of unknown function. The carboxyl-terminal portion of α-spectrin contains two putative EF-hand-type (Kretsinger, 1987) calcium-binding sites (Wasenius et al., 1989; Sahr et al., 1990). Recently it was observed that both brain and erythrocyte spectrins bind calcium by $^{45}Ca^{2+}$ overlay of the blotted proteins (Wallis et al., 1992).

The interactions of salts with spectrin have been studied for over 2 decades (Marchesi et al., 1970), but the effects of cations on spectrin still are unclear. Ralston (1976) suggested that the surface charge of spectrin played a role in salt effects. Elgsaeter (1978) demonstrated that spectrin's radius of gyration increased as the ionic strength of the solution was lowered. Spin-labeling studies of spectrin (Cassoly et al., 1980; Lammel & Maier, 1980) showed that Ca^{2+} and Mg^{2+} reduced the surface charge of spectrin and caused the molecule to contract, but the effects on secondary structure were not examined. Also, Gratzer and co-workers (Beaven & Gratzer, 1980; Beaven et al., 1990) demonstrated that spectrin at 1–2 mg/mL is aggregated by millimolar concentrations of several divalent cations through side-to-side associations.

In this study, we observed that erythrocyte spectrin contains at least two types of calcium-binding sites. Higher affinity binding is calcium-specific, and physiological concentrations of Mg^{2+} do not displace calcium at these sites, whereas lower affinity binding occurs with both calcium and magnesium. In both cases, binding produces structural changes in the protein. Considering the physiological variations of erythrocyte internal calcium and magnesium concentration (Bunn et al., 1971; Larsen et al., 1981), these results indicate that fluctuating micromolar calcium and millimolar magnesium binding to spectrin are the basis for the remarkable flexibility of the red blood cell membrane.

EXPERIMENTAL PROCEDURES

Materials. Fresh horse blood was obtained from Beltex Corp., Fort Worth, TX. Human blood was obtained from a healthy volunteer and was used within 2 h.

Purification of Spectrin. Red blood cell spectrin was purified essentially by the method of Gratzer (1982). All steps were performed at 4 °C. Fresh blood was mixed in a 10:1 ratio with 50 mM sodium citrate/3% dextrose (pH 5.0) as anticoagulant. Red blood cells were washed 3 times with 150 mM NaCl/20 mM Tris, pH 7.6, lysed in 5 mM Tris/0.2 mM phenylmethanesulfonyl fluoride (PMSF),[1] pH 7.6, and washed 2 more times in hypotonic buffer. Spectrin was

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; bisANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; MWCO, molecular weight cutoff; EC_{50}, 50% effective concentration; 2,3-DPG, 2,3-diphosphoglycerate.

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extracted from the ghosts by incubation at 4 °C overnight in 0.3 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, and 0.5 mM 2-mercaptoethanol, pH 8.5. The extract was concentrated against Aquacide I and dialyzed against 260 mM KCl, 40 mM NaCl, 20 mM Tris, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.2 mM PMSF, and 1 mM NaN_3, pH 8.0. Spectrin was separated from contaminating proteins by Sepharose CL-4B column chromatography. Protein peaks were determined both by absorbance at 280 nm and by denaturing polyacrylamide gel electrophoresis. The spectrin was pooled, concentrated against Aquacide I, dialyzed against 20 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.3 mM NaN_3, pH 8.0, and stored at 4 °C.

Chelex Treatment of Spectrins. Spectrin solutions were dialyzed for 72 h at 4 °C against 1000 volumes of the final desired buffer, usually 20 mM Hepes, pH 7.14–8.5, containing 8 g/L Chelex-100. All flow-through buffers were chelated by passage through a 2.5 × 12 cm Chelex-100 column.

Polyacrylamide Gel Electrophoresis and Blotting. Routine SDS–PAGE 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 electrophoresis of native spectrin, the slab gel method of Morrow and Haigh (1983) was employed using 3-mm-thick, 2–4% linear gradients in the Hoefer SE 600 apparatus. Native gels were run at 4 °C with 4-W constant power.

Proteins were transferred to Immobilon-P 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, and 10% (v/v) methanol, pH 8.8.

$^{45}Ca^{2+}$ Autoradiography. Calcium binding to proteins on Immobilon-P membranes was performed by the modification of Gültekin and Heermann (1988) of the method of Maruyama et al. (1984) except that the magnesium chloride concentration was varied from 1 to 5 mM. Dried blots were exposed to X-Omat film for 2 days at −70 °C.

Flow Dialysis and Equilibrium Dialysis. These techniques were performed as described previously for brain spectrin (Wallis et al., 1992). Each experiment was performed at least 5 times, and data were pooled for analysis. Protein concentration was maintained ≤1 μM spectrin tetramer to decrease concentration-dependent polymerization or aggregation. The binding parameter "*n*" is reported as the number of binding sites per dimer because that is the lowest molecular weight, unique structural unit.

Fluorescence Measurements. Steady-state fluorescence measurements were made using an SLM 8000C spectrofluorometer (SLM-AMINCO, Urbana, IL) at 22 °C. Excitation and emission band-passes were set at 4 nm. Measurements were made in a "T-cuvet" with the short path length (2 mm) oriented toward the excitation side to minimize inner filter effects. Samples were excited at 365 nm, and emission was measured by scans at 400–650 nm. For titration experiments, emission was measured at 490 nm. The protein was in 25 mM Hepes (pH 8 or 9), and the divalent cation concentration was varied from 0 to 20 mM by titration with $MgCl_2$ or $CaCl_2$.

Circular Dichroic Measurements. Circular dichroic measurements were performed on an Aviv circular dichroism spectrometer, Model 62DS (Lakewood, NJ), equipped with computerized data collection. Samples were diluted to a convenient concentration (0.05–0.15 mg/mL) using 25 mM borate buffer (pH 7.14 or 9.0) and scanned from 260 to 185 nm. $CaCl_2$ or $MgCl_2$ was added and mixed, and the samples were rescanned. The proportions of secondary structure types were determined by use of the program ProSec. For analysis, a molecular weight of 526 000 for the spectrin dimer was used with a mean amino acid weight of 115.

Trypsin Cleavage. Horse red blood cell spectrin was digested with TPCK-treated trypsin at a substrate:enzyme ratio of 100:1 for 30 min. The final protein concentration was 0.71 mg/mL in a reaction buffer containing 50 mM NaCl/10 mM sodium phosphate (pH 7.0). The $MgCl_2$ concentration varied from 0 to 2 mM. The reaction products were separated by denaturing polyacrylamide gel electrophoresis. The percent protein per band was determined by densitometry (Isco Model 1312) in some experiments, or the peptide fragments were blotted and calcium binding was measured as described above.

Other Measurements. The protein concentration for purified spectrin was determined spectrophotometrically using $\epsilon^{1\%}_{280nm} = 10.7$ (Gratzer, 1982) and confirmed using the Lowry protein assay (Lowry et al., 1951). The concentration of bisANS was determined spectrophotometrically at 385 nm using 16 670 M^{-1} cm^{-1} as the extinction coefficient.

RESULTS

For all the experiments reported in this study, red blood cell spectrin was isolated to at least 95% purity as detected by SDS–PAGE, and divalent cations were removed by dialysis against Chelex-100. We have found it necessary to dialyze erythrocyte spectrin at a pH above 8.0 against at least 8 g/L Chelex-100 for 72 h to maximize removal of endogenous cations. Circular dichroic data (below) suggest that this treatment produces much structural reorganization, but we cannot be sure that *all* cations are removed. As confirmed by electrophoresis on native gels (not shown), spectrin was maintained in the tetrameric form for the duration of our experiments by keeping its temperature below 30 °C.

Autoradiography of Electrophoresed and Blotted Erythrocyte Spectrin. Human erythrocyte spectrin transferred from native electrophoresis gels to Immobilon membranes bound calcium in the presence of 1 μM $^{45}Ca^{2+}$ (unpublished experiments). Because $^{45}Ca^{2+}$ binding was detectable in the blotted native protein, calcium binding was not due to SDS adhering to spectrin after denaturing electrophoresis and blotting. We also separated purified erythrocyte spectrin subunits by denaturing gel electrophoresis and examined calcium binding in the presence of increasing Mg^{2+} concentrations after blotting. There was little reduction in $^{45}Ca^{2+}$ binding under these conditions as the [$MgCl_2$] in the blotting buffer was raised from 1 to 5 mM (unpublished experiments). The lack of a Mg^{2+} effect suggests that Ca^{2+} binds to some high-affinity, calcium-specific sites in both subunits.

Flow and Equilibrium Dialysis Measurements of $^{45}Ca^{2+}$ Binding to Erythrocyte Spectrin. By flow dialysis, it was found that spectrin had two $^{45}Ca^{2+}$-binding components (Figure 1). Best-fit analysis showed that models with more than two components had greater residual error and/or negative parameters. The low-affinity (millimolar k_d) binding component of spectrin was not due to binding to the apparatus because it did not occur in experiments without protein or when using identically treated hemoglobin as a nonbinding protein control. Data were analyzed by Simplex and/or Quasi-Newtonian nonlinear regression for the best fit. Figure 1 demonstrates the quality of fit obtained. Low-affinity binding was subtracted from individual experiments before data modeling. By Hill plot (not shown), there did not appear to be cooperativity of binding ($n_H = 1.13 \pm 0.1$).

Flow dialysis measurements for human erythrocyte spectrin when endogenous Ca^{2+} was reduced by treatment with Chelex

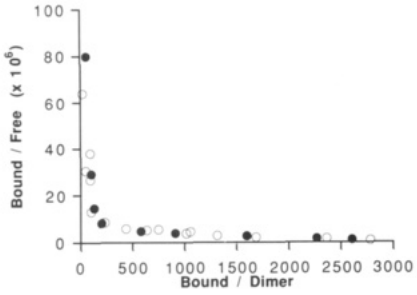

FIGURE 1: Scatchard plot of $^{45}Ca^{2+}$ binding to equine erythrocyte spectrin. Erythrocyte spectrin was prepared by dialysis against 1000 volumes of the desired buffer, usually 20 mM Hepes (pH 8.2) containing 8 g/L Chelex-100, for at least 72 h at 4 °C. All flow-through buffers were chelated by passage through a 2.5 × 12 cm Chelex-100 column. Flow dialysis was performed as described (Wallis et al., 1992). (○) Data; (●) model.

Table I: Binding Constants for the High-Affinity Binding of Ca^{2+} to Human Erythrocyte Spectrin Measured with and without 100 mM KCl[a]

buffer	titration pH	$n \pm$ range[b]	$k_d \pm$ range (M)
25 mM Hepes	8.0	78.9 ± 0.2	$(4 \pm 0.002) \times 10^{-7}$
25 mM Hepes	7.4	98.1 ± 0.1	$(4 \pm 0.004) \times 10^{-7}$
100 mM KCl/ 25 mM Hepes	7.4	116.7 ± 0.2	$(4 \pm 0.008) \times 10^{-7}$

[a] Spectrin was Chelex-treated at pH 8.5 and titrated with calcium at pH 8.0 or 7.4. The flow dialysis method is described under Experimental Procedures. Each value is derived from data from five or more experiments where low-affinity binding was subtracted for each individual experiment. [b] Number of binding sites per dimer.

Table II: Thermodynamic Parameters for the High-Affinity Calcium-Binding Component to Equine Erythrocyte Spectrin Measured at Two Temperatures by Equilibrium Dialysis[a]

parameter	4 °C	25 °C
n[b]	106	90
$k_d \pm$ range (M)	$(9 \pm 0.008) \times 10^{-6}$	$(4 \pm 0.003) \times 10^{-7}$
ΔG_a (kcal mol^{-1})	−6.4	−8.7
ΔS_a[c] (cal mol^{-1} deg^{-1})	111	111
$\Delta H°_a$[c] (kcal mol^{-1})	24.3	

[a] The equilibrium dialysis method is described under Experimental Procedures. [b] n = number of binding sites expressed per dimer. [c] The equation used was $d \ln k_a/dT = \Delta H°/RT^2$.

at pH 8.0 revealed about 100 high-affinity ($k_d = 4 \times 10^{-7}$ M) calcium-binding sites per dimer (Table I). Under the same conditions, the calculated number of sites per dimer and the affinity for the high-affinity sites of equine erythrocyte spectrin were very similar ($n = 89.9$, $k_d = 4 \times 10^{-7}$ M, titrated at pH 8.0). In contrast to Chelex treatment at pH 8.0, when endogenous Ca^{2+} was decreased by Chelex treatment at pH 7.6, there were fewer titratable sites detectable per dimer, but the affinity of the sites remained unchanged. This indicates the tenacious nature of this calcium binding: the pH of the Chelex treatment must be elevated to maximize Ca^{2+} removal from these sites.

The high-affinity binding sites were measured at two temperatures by equilibrium dialysis using calcium-depleted equine erythrocyte spectrin. From these data, the enthalpic and entropic contributions to the binding energy were calculated (Table II). Though it is desirable to make these measurements at several temperatures to obtain very accurate values, nevertheless these data show that high-affinity calcium binding produces an entropy increase for the molecule.

Computer programs and models served to calculate parameters for the low-affinity binding component (Table III).

Table III: Flow Dialysis Data for the Low-Affinity Binding of Ca^{2+} to Erythrocyte Spectrin Chelex-Treated and Titrated with Calcium in Various Solutions[a]

source	solution composition	k_{d_2} (M)
equine	20 mM Hepes, pH 8.0	$(0.7 \pm 0.02) \times 10^{-3}$
equine	20 mM Hepes, pH 7.4	$(1.24 \pm 0.03) \times 10^{-3}$
human	20 mM Hepes, pH 8.0	$(2.0 \pm 0.1) \times 10^{-4}$
human	20 mM Hepes, pH 7.4	$(0.6 \pm 0.2) \times 10^{-4}$
human	25 mM Hepes, pH 7.4	$(0.86 \pm 0.2) \times 10^{-4}$
human	100 mM KCl/20 mM Hepes, pH 7.4	$(1.65 \pm 0.3) \times 10^{-4}$

[a] Flow dialysis is described under Experimental Procedures. Each value is calculated by combining five or more experiments.

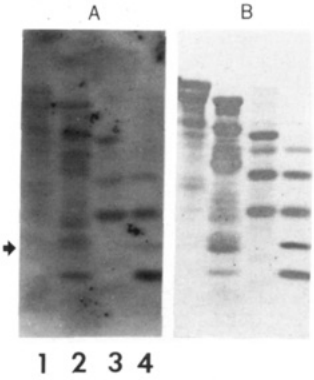

FIGURE 2: Blot and autoradiogram of ^{45}Ca binding to trypsin-digested fragments of equine spectrin. Ten micrograms of spectrin was subjected to digestion at 4 °C by endogenous protease (lane 1) or was digested with 0.1 μg of TPCK-treated trypsin (lane 2) for 30 min at room temperature. One-half volume of 6% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 10 mM Tris, pH 8.0, was added, and samples were run on a 10% polyacrylamide gel and blotted by the method of Maruyama et al. (1984). (A) Autoradiogram. (B) Coomassie blue stained blot. Lanes 3 and 4 are high and low molecular weight markers, respectively. The arrow indicates the position of peptide T28 which binds little ^{45}Ca. Among the molecular weight markers, β-galactosidase, ovalbumin, and soybean trypsin inhibitor bind $^{45}Ca^{2+}$ while bovine serum albumin and carbonic anhydrase do so slightly.

The affinity of these sites appeared to be more variable, depending upon preparation and dialysis conditions, than for the high-affinity sites, and the affinity of calcium for equine spectrin was about 10-fold weaker than for human spectrin. The computed numbers of sites (in the thousands, not shown) obviously are unrealistic because the calculations do not take into account achievement of an absolute end point at a finite number of sites. In other words, computation presumes that low-affinity binding decreases gradually, but, in reality, additional binding is likely to stop abruptly when "all" negatively charged sites on the protein are neutralized. Still, the presence of 100 mM KCl increased the apparent number of millimolar k_d sites 4-fold, indicating that physiological KCl concentrations promote formation of low-affinity divalent cation-binding sites. Most of these sites are likely to be available for binding in situ because proteins that bind to spectrin do so in discrete areas.

Localization of High-Affinity $^{45}Ca^{2+}$-Binding Sites after Proteolysis of Spectrin. $^{45}Ca^{2+}$ binding and autoradiography of Immobilon-bound spectrin fragments produced by digestion with endogenous protease or TPCK-treated trypsin showed that almost all fragments bound $^{45}Ca^{2+}$ with intensities proportional to the amount of protein, even in the presence of 13 mM $MgCl_2$ (Figure 2). The only fragment which bound disproportionately little $^{45}Ca^{2+}$ under these conditions was T28 (arrow), near the carboxyl terminal of the β subunit

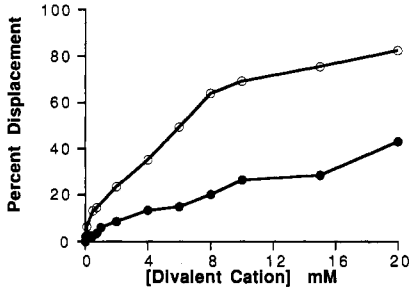

FIGURE 3: Comparison of calcium and magnesium displacement of $^{45}Ca^{2+}$ from equine erythrocyte spectrin. Spectrin was Chelex-100-treated at pH 8.5 and titrated at pH 8.0 with either $CaCl_2$ (○) or $MgCl_2$ (●) to displace bound $^{45}Ca^{2+}$ using the isotope dilution method as described previously (Wallis et al., 1992). The interaction of magnesium-binding sites with calcium was examined by using magnesium as the diluting cation and varying the initial concentration of $^{45}Ca^{2+}$.

Table IV: Circular Dichroic Changes of Equine Erythrocyte Spectrin with Increasing Concentrations of Divalent Cations[a]

Chelex pH	titration pH	cation (concn, mM)	f_R	f_B	f_A	f_T
none	7.14	none	0.250	0	0.625	0.125
7.6	7.14	Ca^{2+} (0.0)	0.300	0	0.681	0.019
7.6	7.14	Ca^{2+} (0.5)	0.251[b]	0	0.697	0.052[b]
8.5	8.0	Ca^{2+} (0.0)	0.137	0	0.863	0
8.5	8.0	Ca^{2+} (0.5)	0.202[b]	0	0.745	0.053[b]
8.5	7.14	Mg^{2+} (0.0)	0.137	0	0.863	0
8.5	7.14	Mg^{2+} (0.5)	0.107	0	0.893	0
8.5	7.14	Mg^{2+} (2.0)	0.156	0	0.844	0
8.5	7.14	Mg^{2+} (4.0)	0.146	0	0.826	0.028[c]

[a] Samples were dialyzed against Chelex-100 to remove endogenous divalent cations where indicated. Circular dichroic measurements were performed as described under Experimental Procedures. f_R, f_B, f_A, and f_T indicate fractions of random coil, β-sheet, α-helix, and turns, respectively. [b] $p < 0.05$ compared to calcium-free. [c] $p < 0.05$ compared to magnesium-free.

(Morrow et al., 1980; Speicher et al., 1982). This indicated that high-affinity calcium binding was widely distributed over the molecule.

Inhibition of $^{45}Ca^{2+}$ Binding to Spectrin by $MgCl_2$. We investigated the effects of Mg^{2+} on calcium binding in more detail. Whereas displacement of bound $^{45}Ca^{2+}$ with unlabeled Ca^{2+} revealed two binding components, titration with $MgCl_2$ had only one component (Figure 3). By Dixon plot (not shown), the K_I of Mg^{2+} for Ca^{2+} binding is 18 mM ($\approx IC_{50}$). Computer analyses revealed that the displacement component had binding parameters comparable to the low-affinity calcium-binding component. This indicates that the low-affinity component consists of "Ca^{2+}/Mg^{2+}" or "divalent cation" sites whereas the high-affinity sites are calcium-specific.

Spectrin Structural Changes Accompanying High-Affinity Ca^{2+} Binding. Circular dichroism of Chelex-treated protein showed that Chelex treatment at pH 8.5 yielded protein very high in helix content (86.3%) with no β-sheet or turn regions measurable, in contrast to untreated spectrin (Table IV). Protein stripped at pH 7.6 and 4 °C yielded protein with spectra closer to untreated spectrin, again indicating the tight binding of endogenous calcium to the high-affinity sites. When 50 μM calcium was added to proteins treated at either pH, both showed an increase in the number of residues involved in turns up to about 240 residues (5.2%) (Table IV); 50 μM $MgCl_2$ does not produce comparable results, indicating the calcium-specific nature of the high-affinity sites, but non-physiological (4 mM) $MgCl_2$ promotes turn formation even

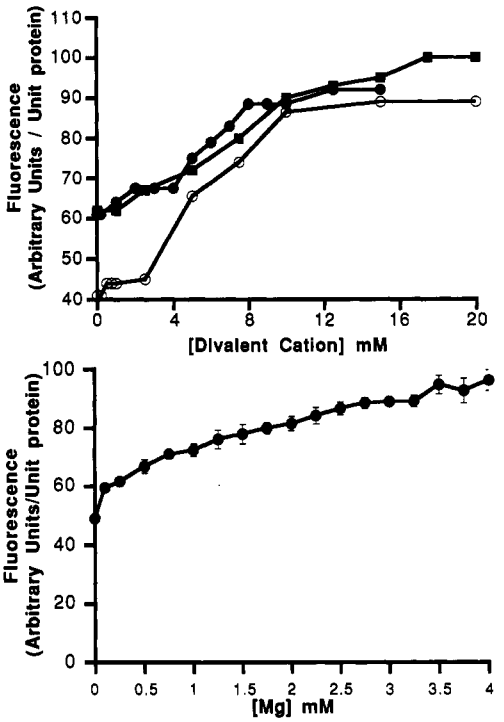

FIGURE 4: Effect of calcium and magnesium concentrations on bisANS fluorescence of equine erythrocyte spectrin. Spectrin was first saturated with bisANS and then titrated with divalent cation until no further increase in fluorescence was detected. (■) Mg^{2+} titration, pH 8.0. (●) Ca^{2+} titration, pH 8.0. (○) Ca^{2+} titration, pH 9.1. Bottom: Detailed titration of equine erythrocyte spectrin with magnesium in the 0–4 mM range.

in the absence of calcium. These results demonstrate a correlation between a change in spectrin secondary structure, particularly a gain of turns, with binding of calcium to the protein.

Investigations of Spectrin Structural Changes with Low-Affinity Divalent Cation Binding. Using bisANS fluorescence to determine the hydrophobicity of the protein, Chelex-treated equine red blood cell spectrin was first titrated with bisANS to demonstrate saturation (not shown). The protein was then titrated with either calcium or magnesium until the fluorescence no longer changed on addition of cation. There was little difference in the influence of Ca^{2+} (EC_{50} = 5 mM) or Mg^{2+} (EC_{50} = 7.5 mM) on fluorescence (Figure 4, upper panel). We have found[2] that reducing the hydrogen ion concentration reduced bisANS fluorescence when it is bound to spectrin. This is seen in Figure 4 as a difference in the initial fluorescence at the two pHs. It is interesting that the maximal hydrophobicity is not changed by increasing the pH. Figure 4 also shows an expanded titration for Mg at 0–4 mM for comparison with the circular dichroic data. Together these data demonstrate that it is both the high- and low-affinity binding abilities of spectrin that are involved in the fluorescence change while high-affinity binding is involved in the secondary structural changes seen by circular dichroism.

By calculating the reduction in θ at 212 and 221 nm as well as a reduction in the θ_{191}–θ_{202} peak, we found a distortion of the helical structure which precedes the appearance of new turn regions.[2] At pH 7, trypsin cleavage in the presence of 0–2 mM $MgCl_2$ demonstrates that increasing [$MgCl_2$] in the physiological concentration range increases the cleavage of spectrin into fragments of approximately the molecular weight of 1–2 repeat units (Figure 5). This suggests that the increased

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

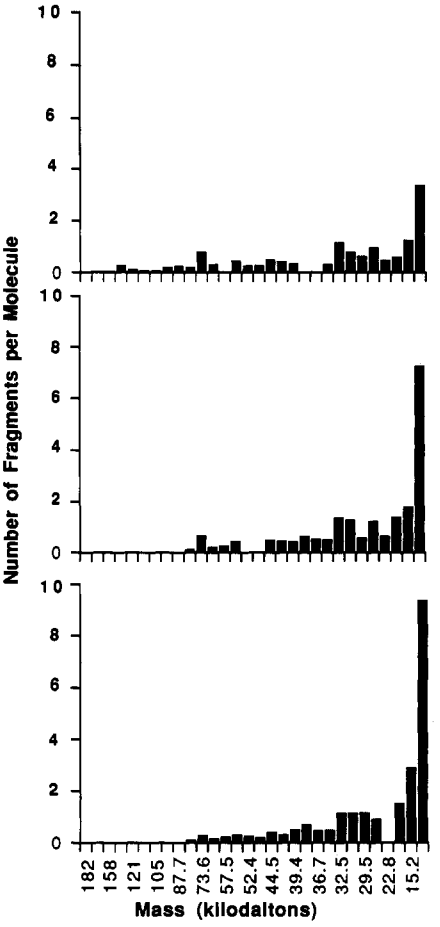

FIGURE 5: Trypsin cleavage of equine erythrocyte spectrin in the presence of three concentrations of $MgCl_2$. The reactions were performed in 50 mM NaCl/10 mM NaH_2PO_4, pH 7.0, for 30 min at an enzyme:spectrin ratio of 1:100 (w/w). Products were separated by SDS–polyacrylamide gel electrophoresis, stained with Coomassie blue, and scanned by UV absorbtion. Data are expressed as the number of fragments produced per intact molecule. Upper panel, no $MgCl_2$; middle panel, 0.5 mM $MgCl_2$; lower panel, 2 mM $MgCl_2$.

helical distortion and/or flexibility indicated by the CD calculations may be in trypsin-sensitive connecting regions between repeat units.

DISCUSSION

Previous Studies of Cation Binding to Spectrin. In 1976, Brauer et al. found two classes of calcium-binding sites in spectrin using a Ca^{2+}-selective electrode. The reliability of their binding estimates was limited by apparent contamination, proteolysis, and the manual techniques used for parameter estimation (Zierler, 1989). However, the low-affinity binding is to spectrin, we observe, because it does not occur in the presence of control protein (hemoglobin) or in the absence of protein. In 1980, Beaven and Gratzer failed to demonstrate binding of calcium to erythrocyte spectrin. There are several reasons for the discrepancy between our work and theirs. First, they isolated their spectrin, in part, by incubation at 37 °C, a treatment that not only stimulates tetramer dissociation but also causes the folding and contraction of spectrin as seen by changes in circular dichroism[2] and suggested from the proposed entropic spring properties of spectrin (Vertessy & Steck, 1989). Second, the treatment they used to remove endogenous cations (1 mM EDTA) was inadequate, and much more extensive treatment with Chelex-100 at higher pH is required. We found that calcium binds very tenaciously to spectrin (Table I). If less than 8 g of Chelex resin/L of dialysis buffer or a dialysis buffer pH below 8.0 was used, the bulk of the tightly bound calcium was not removed. This appears to be due to higher pH extending the molecule,[3] and suggests that a combination of strong chelating ability of the dialysate and electrostatic repulsion between parts of the protein is necessary to remove calcium from turn regions (Table IV). Finally, they used glass-distilled water and stated that "...Ca^{2+} levels in the dialysate were not higher than about 1 μM..." before beginning titrations. At 1 μM, we found $^{45}Ca^{2+}$ bound extensively to blotted spectrins (Wallis et al., 1992). In our experiments, water was purified first by deionization, then by glass distillation, and finally by reverse osmosis, and all solutions were further treated with Chelex-100.

The importance of measuring the binding characteristics of the native protein is emphasized by comparing this work with that of Dubreuil et al. (1991) in which they described binding of calcium to bacterially expressed fragments of *Drosophila* α-spectrin containing EF-hand sequences. While the justification that some properties of spectrin may persist in fragments is understandable, it does not appear to be completely true for calcium binding to spectrin. In this work, we are not proposing that all binding sites are EF-hands. They seem to be related to folding of erythrocyte spectrin (high affinity) and association with acidic residues (low affinity). The much more restricted binding of calcium to brain spectrin (Wallis et al., 1992) than to erythrocyte spectrin suggests differences in the functional roles of calcium binding to these two proteins.

Structural Changes Accompanying Cation Binding. Calcium ions were more potent turn formers than magnesium ions (Table IV), an effect that may relate to the extension and contraction of spectrin described by McGough and Josephs (1990). Using the secondary structure predictions of Davison et al. (1989), it appears[4] that areas between helices 1 and 2, 2 and 3, and 4 and 1 are the most likely sites where calcium ions would promote turn formation. The effects of physiological concentrations of magnesium ions correlate with apparent flexibility changes (Figure 5) at about positions 26–28 in the repeat structure (Davison et al., 1989; Winograd et al., 1991).

Significance of High-Affinity Calcium Binding. The level of free calcium ions in red blood cells is kept very low by the membrane calcium pump (Carafoli, 1991), but in vivo physiological shear stresses considerably increase the leakage of calcium into the cell (Larsen et al., 1981). We propose that an effect of the shear-promoted entry of Ca^{2+} is to bind to spectrin with high affinity which tends to counteract physiological deformation by increasing internal tension of the spectrin "spring" (Vertessy & Steck, 1989; Elgsaeter & Mikkelsen, 1991). Effects of calcium mediated by other proteins are activated at high calcium concentrations to disrupt protein–protein interactions and avoid irreversible damage to the cell (Murakami et al., 1986; Takakuwa & Mohandas, 1988; Tanaka et al., 1991).

Significance of Low-Affinity Magnesium Binding. While internal Ca^{2+} levels are fluctuating in the submicromolar range as the red blood cell moves through the circulatory system, internal magnesium levels fluctuate from 0.25–0.57 mM in oxygenated cells up to 0.67–1.90 mM in deoxygenated cells (Bunn et al., 1971; Gupta et al., 1978; Levy et al., 1988; Ramasamy et al., 1991). This change associates Mg^{2+} with hundreds of sites on erythrocyte spectrin. Determining the

[3] C. J. Wallis and J. A. Babitch, manuscript in preparation.
[4] J. A. Babitch, unpublished analysis.

number of low-affinity sites is obscured by spectrin aggregation which occurs long before the sites are titrated to end point in vitro. Because low-affinity divalent cation sites have been described which contain only a single acidic residue (Nathke et al., 1990), spectrin may contain almost as many low-affinity sites as there are acidic amino acids. The secondary and higher order structures of spectrin are arranged to maximally expose its large number of acidic amino acids (Parry & Cohen, 1991). In addition to being a protein spring (Beaven et al., 1990; McGough & Josephs, 1990; Elgsaeter & Mikkelsen, 1991), spectrin in situ may be viewed as a magnesium buffering sponge which further compacts to resist deformation in the physiological concentration range. Thus, the combination of calcium- and magnesium-binding properties in erythrocyte spectrin has evolved such that each cation, in its own concentration range, increases molecular folding and contraction, raises internal tension, and makes the cell more resistant to deformation and cell damage.

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