Temperature and Ionic Effects on the Interaction of Erythroid Spectrin with Phosphatidylserine Membranes†

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ABSTRACT: Specific binding of human erythroid spectrin to large, unilamellar vesicles of bovine brain phosphatidylserine, made by an extrusion technique (LUVETs), has been measured and characterized by a new gel filtration assay. Vesicle-bound spectrin was separated from free spectrin by Sepharose CL-2B chromatography and detected by its intrinsic (tryptophan) or extrinsic (carboxyfluorescein) fluorescence. That the bound spectrin was not an aberrant, adhesive form was shown by the ability of a portion of free spectrin, which had not bound to PS LUVETs during a previous incubation, to bind during a subsequent incubation. Spectrin binding reached a plateau by 30 min of incubation at room temperature and at 37 °C. Binding increased from a low level below 31 °C to about twice as much as 37 °C and to 4–7 times as much between 40 and 43 °C. Similar results were obtained with LUVETs composed of DOPS but not PC. Triton treatment of PS LUVETs and spectrin after incubation of spectrin and vesicles at 40 and 43 °C but prior to chromatography on Sepharose CL-2B eliminated the bound spectrin peak, which thus did not consist of large aggregates of covalently associated spectrin. Binding isotherms fit by nonlinear regression gave an apparent K_d of 0.31 μM and an apparent maximum spectrin binding of 33 nM/mM PS at 25 °C, an apparent K_d of 0.35 μM and an apparent maximum spectrin binding of 40 nM/mM PS at 31 °C, and an apparent K_d of 3.4 μM and an apparent maximum spectrin binding of 113 nM/0.1 mM PS at 37 °C. Resembling the extraction of spectrin from red cell ghosts, dissociation of the spectrin–PS complex was enhanced when the salt content of the buffer was decreased to millimolar concentrations at 25 °C but not at 4 °C.

The original analysis of the viscoelastic behavior of the red cell as bounded by a single-layered wall (Evans & Skalak, 1979) has not yet been affected significantly by the subsequent elucidation of its two-layered structure, i.e., a protein-embedded, lipid membrane supported by a skeletal protein network (Bennett, 1990). Nevertheless, our understanding of the viscoelastic behavior of the red cell should eventually be furthered by knowledge of the molecular structure of its cell wall (Shen, 1989; Steck, 1989; Elgsaeter & Mikkelsen, 1991). Recent progress in the investigation of spectrin, the major component of the skeleton, has been particularly promising in this respect as (1) the complete sequences of the α (Sahr et al., 1990) and β (Winkelmann et al., 1990) subunits of erythroid spectrin are now known, (2) spectrin fragments suitable for structural determination (Winograd et al., 1991) have become available, and (3) successive improvements in model building (Speicher & Marchesi, 1984; Speicher, 1986; Davison et al., 1989; Xu et al., 1990; Parry et al., 1992) have produced a plausible model for the conformation of spectrin.

Less well studied is the interaction of spectrin and other parts of the skeleton with the lipid component of the red cell membrane and, alternatively, with model membranes. Initially interested in the suggestion that some differences in the viscoelastic behavior observed by the micropipet aspiration technique might be explained by the degree to which spectrin interacts with the lipids in the membrane (Markin & Kozlov, 1988), we undertook an investigation of the spectrin–lipid and band 4.1–lipid interactions which might help to determine the merit of this proposal. Findings of the first half of this investigation are presented here, whereas findings of the second half have been published (Takeshita et al., 1993).

After an initial controversy as to whether spectrin can increase the permeability of negatively charged lipid membranes (Sweet & Zull, 1970; Juliano et al., 1971), changes in a variety of membrane parameters have been reported to attend the interaction of spectrin with negatively charged surfaces. Like the induction of vesicle leakage, the increase in the surface pressure of monolayers of negatively charged lipids was interpreted to indicate the penetration of the lipid bilayer by spectrin (Mombers et al., 1980), whereas the identification of sites on spectrin with affinity for fatty acid analogues (Isenberg et al., 1981) suggested structural features responsible for membrane penetration. Unlike the larger increases induced by proteins with greater surface activity, however, the smaller effects of spectrin on surface pressure (Maksymiw et al., 1987; Shiffer et al., 1988) and on vesicle permeability (Subbarao et al., 1991) seem likely now to have arisen from an electrostatic, surface effect with a modest hydrophobic component detectable by specular reflection of neutrons (Johnson et al., 1991). To relate these to other effects on quenching of (anilinonaphthyl)spectrin by spin-labeled lipids and by bovine brain phosphatidylserine (PS)[1] vesicles (Bonnet & Begard, 1984) and on surface pressure measurements, calorimetry, and dilatometry of spectrin-treated lipid (Maksymiw et al., 1987), a quantitative study of the affinity of spectrin for acidic phospholipids appeared warranted,

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; LUVETs, large, unilamellar vesicles made by an extrusion technique; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; TNED, 25 mM Tris, pH 7.6, + 0.1 M NaCl + 0.1 mM EDTA + 0.2 mM DTT; PS, bovine brain phosphatidylserine; DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; CF, carboxyfluorescein; SDS, sodium dodecyl sulfate; Tx, Triton X-100; Tris, tris(hydroxymethyl)aminomethane.

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particularly since the strength of the PS–spectrin interaction has been reported to range from negligible (Morrot et al., 1986; Bitbol et al., 1989) to great (Cohen et al., 1986). For these reasons and to facilitate data gathering, a new gel filtration assay of spectrin–lipid vesicle binding was devised to measure the binding constants and saturation levels of specific binding of spectrin to PS vesicles. In the course of this work, two new features of the interaction of spectrin with PS vesicles were discovered—namely, association increases with temperature, and dissociation is enhanced at low ionic strength at 25 °C as opposed to 4 °C.

MATERIALS AND METHODS

Preparation of Lipid Vesicles. Large, unilamellar vesicles made by an extrusion technique (LUVETs)[1] were prepared as described (MacDonald et al., 1991). Bovine brain phosphatidylserine (PS), dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidylcholine (PC) (Avanti Polar Lipids; Alabaster, AL) in chloroform were dried and left under vacuum for 1 h prior to hydration at a concentration of about 5 mM in 0.2 mL of 25 mM Tris, pH 7.6, + 0.1 M NaCl + 0.1 mM EDTA + 0.2 mM DTT (TNED). After being frozen 10 times in an ethanol/dry ice bath, the vesicles were passed 19 times through a polycarbonate filter with 100-nm pores (Costar, Pleasanton, CA), mounted in a hand-held extrusion device (MacDonald et al., 1991), to generate LUVETs which were stored at 4 °C for no longer than 1 week. Phospholipid concentrations were determined by phosphate assay (Bartlett, 1959).

Preparation and Solubilization of Cytoskeletons. Human red cells were purchased from LifeSource (Glenview, IL) and used within 3 weeks. White cells were removed with a filter (Pall Biomedical Products, Corp., East Hills, NY) prior to washing the red cells by low-speed centrifugation 3 times for 10 min in 0.15 M NaCl + 5 mM sodium phosphate, pH 8, + 1 mM EDTA at 4 °C, as for all subsequent steps except where noted. Chemicals were reagent-grade and usually from Sigma Chemical Co. (St. Louis, MO). Ghosts were prepared by lysing the washed cells in 10 volumes of 5 mM sodium phosphate, pH 8, + 1 mM EDTA + 34 μg/mL PMSF + 2 μg/mL pepstatin prior to pelleting in a Sorvall centrifuge at 27000*g* (Dodge et al., 1963). The ghosts were washed twice in a similar manner. To obtain red cell skeletons (Sheetz, 1979) stripped of band 3, ankyrin, and other accessory proteins (Shen et al., 1984), the ghosts were suspended in 1 volume of 4% (v/v) Triton X-100 + 25 mM Hepes, pH 7, + 2 mM EGTA + 2 mM DTT + 0.3 mg/mL PMSF, layered over 30% (w/v) sucrose (molecular biology grade) + 1.5 M NaCl + 25 mM Hepes, pH 7, + 0.5 mM ATP + 1.25 mM DTT + 0.5 mM EGTA + 0.174 mg/mL PMSF, and centrifuged at 112400*g* for 1 h in an SW28 Beckman rotor.

Isolation and Purification of Spectrin Dimers. To obtain spectrin dimers (Ohanian & Gratzer, 1984), red cell skeletons were dissociated in 1 M Tris (molecular biology grade), pH 7.2, + 0.5 mM EGTA for 10 min at room temperature and centrifuged at 130680*g* for 1 h in an SW50.1 Beckman rotor to pellet undissociated skeletons. The supernatant was chromatographed on a 60 × 2.5 cm column of Sepharose CL-6B (Sigma Chemical Co.) equilibrated with the skeleton dissociation buffer to separate spectrin from band 4.1 and actin. Spectrin was concentrated by addition of solid ammonium sulfate (molecular biology grade) to 50% saturation. The ammonium sulfate precipitate was dissolved in TNED, dialyzed against 1 L of TNED with one change of buffer, and chromatographed on an 80 × 2.6 cm column of Sepharose CL-4B (Sigma Chemical Co.) equilibrated with the same buffer to separate dimers from tetramers and higher order aggregates. Spectrin dimers were concentrated by addition of solid ammonium sulfate to 50% saturation. The ammonium sulfate precipitate was resuspended in TNED at a concentration in the micromolar range, dialyzed against 1 L of TNED with one change of buffer, and centrifuged for 30 min at 27000*g* to remove aggregates. The concentration of spectrin in the supernatant was determined by its specific absorbance of $10.7^{1\%,1cm}$ at 280 nm (Kam et al., 1977). Only the α and β subunits of spectrin were detected on overloaded, 8% polyacrylamide gels by SDS–PAGE (Laemmli, 1970) and Coomassie Blue staining.

Fluorescence Labeling of Spectrin Dimers. For fluorescence labeling, a <1% volume of 5-carboxyfluorescein (and 6-carboxyfluorescein, succinimidyl ester) (Molecular Probes, Inc., Eugene, OR) in DMSO was injected into a vortexed solution of spectrin, previously dialyzed against 1 L of 0.1 M NaCl + 5 mM sodium phosphate, pH 8, with one change. The dye:protein mole ratio was 3, given an extinction coefficient at 491 nm of 66 000 cm^{-1} M^{-1} for carboxyfluorescein (Molecular Probes, Inc.). The mixture was left in the dark on ice for 2 h prior to dialysis against 1 L of TNED for 48 h with one change of buffer. At the end of this time, the dye:protein mole ratio was about 1. After electrophoresis on nondenaturing, 3% polyacrylamide gels (Morrow & Haigh, 1983), CF-labeled spectrin was predominantly in the dimer form, even after 8 months of storage at 4 °C. All carboxyfluorescein bands detected in unfixed gels coincided with Coomassie Blue stained bands of spectrin in fixed gels.

Gel Filtration Assay of Free and Vesicle-Bound Spectrin. Binding of spectrin and CF-spectrin to lipid vesicles was measured by separating the bound and free spectrin on an 11 × 1 cm column of Sepharose CL-2B (Sigma Chemical Co.) equilibrated with TNED. After a column had been poured, an aliquot of vesicles was eluted from it to saturate sites which might nonspecifically absorb lipid. Aliquots of 0.2 mL containing submicromolar spectrin or CF-spectrin or CF-spectrin with or without about 0.1–1 mM lipid vesicles in column buffer were incubated at different temperatures prior to elution from the column at room temperature. The eluant was pumped at a rate of about 1 mL/min through a flow cell, or fractions of eluant were collected for reading in a Farrand fluorometer with the excitation monochromator set at 290 nm for tryptophan and at 485 nm for carboxyfluorescein and the emission monochromator set at 350 nm for tryptophan and at 525 nm for carboxyfluorescein. To monitor tryptophan fluorescence, fractions were treated with 1% Triton prior to fluorescence determination, and corrections for light scattering were made by subtracting readings of identical samples prepared without spectrin. Appropriate interference filters were inserted for carboxyfluorescein measurements. The bound spectrin eluted in about 6 min. The percent recovery of CF-spectrin was determined by recording the fluorescence of a known amount of spectrin pumped through the flow cell without passing through the column. After an adjustment was made for the slightly slower flow rate through the column, recoveries were calculated to range from 70 to 100%, but there was no correlation between the percent recovery and the contents and/or treatment of the sample, so the recoveries of free and bound spectrin were assumed to be proportional to their presence in the samples prior to chromatography. Hence, the amounts of bound and free spectrin were measured by superimposing the chart recorder profile obtained from a sample containing spectrin and vesicles over that obtained from a sample containing spectrin alone in order to trace the free spectrin profile on that of the former sample. Vesicles alone did not contribute any signal between the excitation

and emission wavelengths of carboxyfluorescein, and therefore no correction for their presence was necessary. The areas of free and bound spectrin thus defined were quantified by digitizing them with the SigmaScan program (Jandel Scientific, San Rafael, CA). The amounts of free and bound spectrin were calculated by multiplying the percent free and bound spectrin of the total spectrin, determined from the respective areas of free and bound spectrin, by the amount of spectrin added to the sample.

RESULTS

Gel Filtration Assay of Spectrin or CF-Spectrin Binding to PS Vesicles. To demonstrate that labeling with carboxyfluorescein did not alter the lipid binding properties of spectrin, the elution from Sepharose CL-2B of unlabeled spectrin incubated at room temperature with or without PC or PS LUVETs was monitored as the tryptophan fluorescence of spectrin (Figure 1A). Binding isotherm data collected in this way in one experiment (Figure 5C, open circles) were the same as those obtained in two experiments with CF-spectrin (Figure 5C, closed circles). Although 1% (v/v) Triton X-100 was added to all fractions to reduce light scattering by the vesicles, light scattering by lipid–detergent micelles was significant in the low-wavelength region of tryptophan fluorescence and necessitated subtraction from the spectrin + vesicle containing samples to obtain the profiles shown. It is clear that only the sample containing PS and spectrin (Figure 1A, triangles) but neither the sample containing PC and spectrin (Figure 1A, filled circles) nor spectrin alone (Figure 1A, open circles) gave rise to a peak of spectrin migrating in the position of LUVETs applied alone (fraction 8) and ahead of the free spectrin peak. The identity of the free spectrin peak was affirmed by comparison of its migration on Sepharose CL-2B with that of an aliquot of spectrin dimer freshly eluted from Sepharose CL-4B (not shown). Samples of fractions containing PS-bound and free CF-spectrin were analyzed by SDS–PAGE, and α-spectrin and β-spectrin were seen in lanes of silver-stained (Merril et al., 1983) gels containing either sample but not in the lane containing a sample of the comparable void volume fraction after elution of CF-spectrin incubated without vesicles (not shown).

To expedite the assay of spectrin binding to vesicles, the Sepharose CL-2B eluant of a sample containing CF-spectrin + PS vesicles was pumped through a flow cell in the fluorometer and the elution profile recorded (Figure 1B, filled circles). To show that Triton treatment of the vesicles is not necessary when carboxyfluorescein, rather than tryptophan, fluorescence is used to detect the presence of spectrin, the flow cell profile is compared in Figure 1B with one obtained by reading the carboxyfluorescein fluorescence of 0.34-mL fractions collected during the same Sepharose CL-2B run and subsequently treated with 0.5% (v/v) Triton (Figure 1B, open circles). The LUVETs gave no light-scattering signal in the long-wavelength region of carboxyfluorescein fluorescence, and the profiles are essentially the same, regardless of whether they were monitored in the absence or presence of Triton. Furthermore, the areas of bound spectrin marked "A" and "B" constitute 10.6 and 11.3%, respectively, or virtually the same percentages of the total areas of their respective profiles. The results in Figure 1B affirmed that flow cell monitoring of the Sepharose CL-2B eluant of CF-spectrin incubated with and without vesicles could be relied on for accurate measurement of bound and free spectrin in subsequent experiments.

To show that the bound spectrin is not a PS binding but aberrant form generated during spectrin purification, CF-spectrin was incubated with PS LUVETs, and fractions were

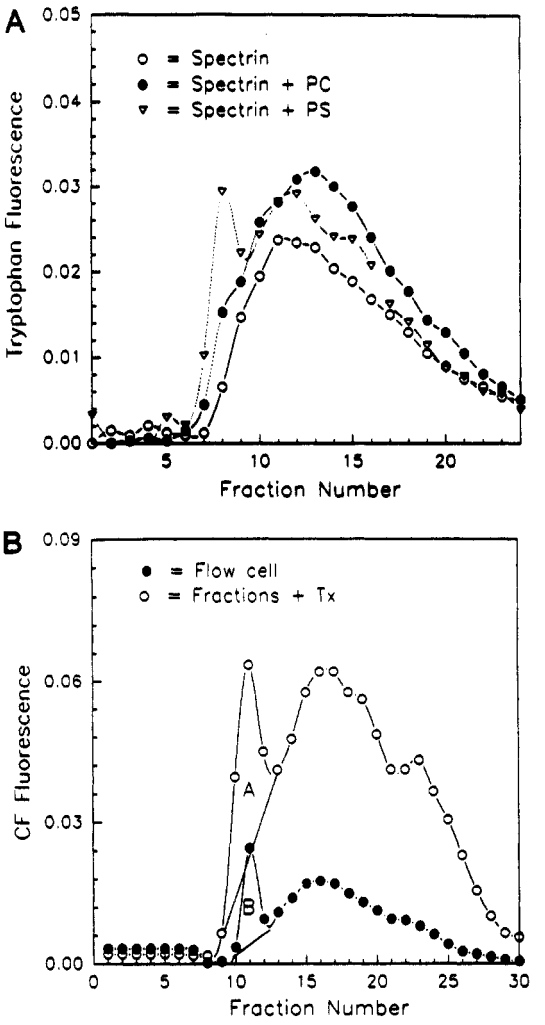

FIGURE 1: (A) Tryptophan fluorescence of fractions eluted from Sepharose CL-2B after application of unlabeled spectrin dimer incubated alone or with PS LUVETs or with PC LUVETs. 0.76 μM spectrin dimer with or without 1 mM PS or PC LUVETs was incubated in 0.2 mL of TNED at room temperature for 10 min prior to application to a Sepharose CL-2B column, collection, collection of 0.45-mL fractions, treatment with 1% Triton, and quantification of bound and free spectrin as described under Materials and Methods. Contributions of light scattering from PS or PC LUVETs alone were subtracted from the appropriate profiles. (B) Comparison of methods of monitoring the Sepharose CL-2B eluant by passage through a flow cell and by reading the carboxyfluorescein fluorescence of fractions treated with 0.5% Triton X-100. 0.43 μM CF-spectrin and 0.6 mM PS LUVETs were incubated in 0.2 mL of TNED at 37 °C for 30 min prior to elution from a Sepharose CL-2B column as described under Materials and Methods. The carboxyfluorescein fluorescence of the eluant was measured either by passage through a flow cell or by manual reading of 0.34-mL fractions treated with 0.5% Triton. "A" and "B" constitute equal fractions of the total areas of their respective scans. Fluorescence is given in arbitrary units.

collected following separation of bound and free spectrin by Sepharose CL-2B chromatography (Figure 2, solid line). Fraction 28 was incubated a second time, either alone (Figure 2, circles) or with a second aliquot of PS LUVETs (Figure 2, squares). A fraction of the previously free CF-spectrin incubated a second time with PS vesicles was detected in the void volume region, indicating the potential of the previously incubated, free spectrin for forming a complex with PS vesicles. No such peak appeared in the void volume region of the profile of CF-spectrin also incubated a second time but without vesicles. The CF-spectrin bound during the second incubation was a much larger fraction of the total than that bound during the first incubation, because the spectrin concentration was much lower during the second incubation than the first

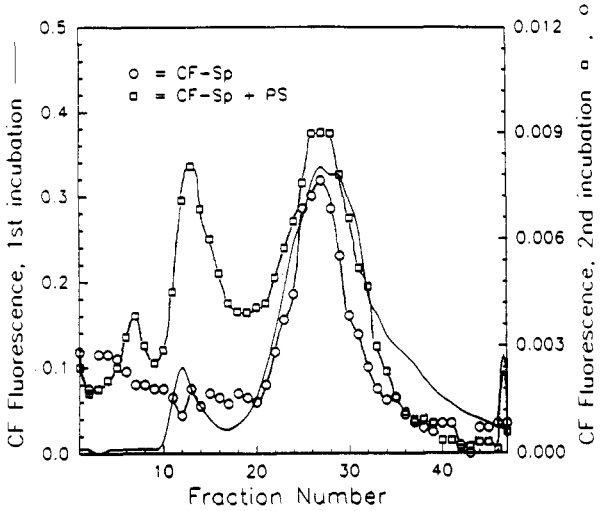

FIGURE 2: CF-spectrin remaining free during a first incubation can bind to PS LUVETs during a second incubation. 1.85 μM CF-spectrin incubated with 0.14 mM PS LUVETs in 0.2 mL of TNED for 30 min at 37 °C was applied to Sepharose CL-2B. 0.34-mL fractions of the eluant were collected (solid line), and their fluorescence was measured and is given in arbitrary units. 0.2 mL of fraction 28 was incubated a second time with (squares) or without (circles) 0.14 mM PS LUVETs for 30 min at 37 °C and applied to Sepharose CL-2B, and the bound spectrin and free spectrin were quantified as described under Materials and Methods. Fluorescence is given in arbitrary units.

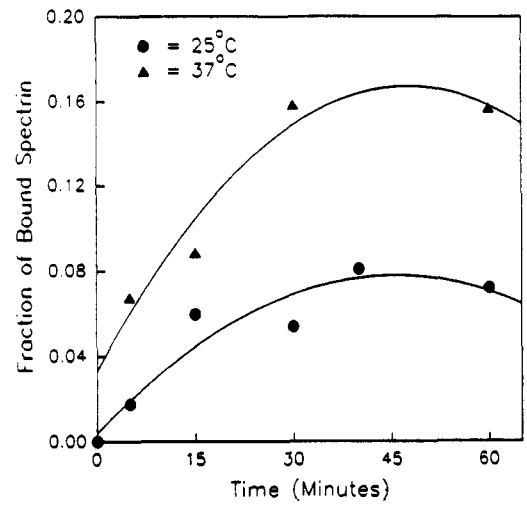

FIGURE 3: Time course of the fraction of CF-spectrin bound to PS LUVETs at 25 and at 37 °C. 0.36 or 0.46 μM CF-spectrin and 1.3 or 1.65 mM PS LUVETs, respectively, were incubated for the indicated lengths of time at 25 and 37 °C prior to separation on Sepharose CL-2B, and bound spectrin and free spectrin were quantified as described under Materials and Methods.

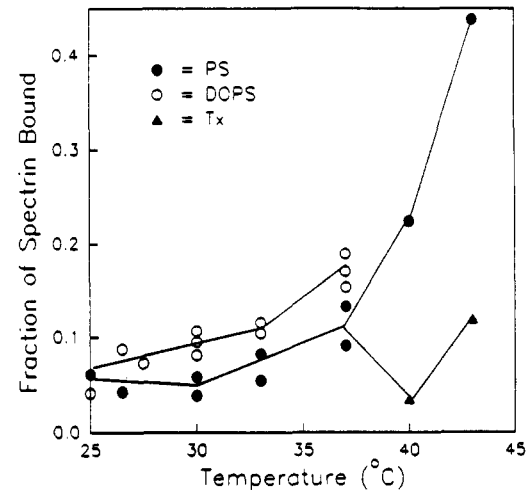

FIGURE 4: Temperature dependence of the fraction of CF-spectrin bound to LUVETs composed of bovine brain PS or DOPS. 0.36 or 0.46 μM CF-spectrin and 1.3 or 1.65 mM LUVETs, respectively, composed of bovine brain PS (filled symbols) or DOPS (open symbols), were incubated for 30 min at the indicated temperatures prior to separation on Sepharose CL-2B, and bound spectrin and free spectrin were quantified as described under Materials and Methods. The triangles represent CF-spectrin + PS samples treated with 0.5% Triton after incubation and prior to Sepharose CL-2B chromatography.

incubation, the spectrin having been diluted during gel filtration of the sample first incubated. CF-spectrin was also incubated at 37 °C in the absence of vesicles, cooled to room temperature, and incubated with PS vesicles at a low concentration at which spectrin–vesicle binding was negligible on incubation at 25 °C but detectable on incubation at 37 °C. No binding occurred (not shown), indicating that 37 °C incubation *per se* of spectrin did not induce an irreversible change in the protein which caused it to bind to PS vesicles.

Time Course of Binding. In preparation for later measurements of the dissociation constant and maximum binding of CF-spectrin to PS vesicles, the time course of binding was determined. Because results of preliminary experiments indicated significantly more binding at 37 °C than at 25 °C, the time course of binding was determined at both temperatures at a CF-spectrin concentration of 0.36 or 0.46 μM and a PS LUVET concentration of 1.3 or 1.65 mM, respectively. The data in Figure 3 which fit second-order regression curves show that the PS vesicles were saturated by 30 min both at 25 °C (circles) and at 37 °C (triangles) and the level of maximum binding of spectrin at 37 °C was about twice that at 25 °C. The relationship between temperature and the binding of CF-spectrin to PS vesicles was studied further.

Temperature Dependence of Binding. Figure 4 shows that a nearly 2-fold increase in binding to PS vesicles (filled circles) was first seen at 37 °C, above which binding increased dramatically. Because spectrin is well-known to undergo an irreversible phase transition at 50 °C (Brandts et al., 1977), CF-spectrin and PS vesicles heated to 40 and 43 °C were treated with 1% (v/v) Triton prior to gel filtration chromatography to determine whether the ostensibly bound material consisted of large, covalently linked aggregates. The peak of PS-bound CF-spectrin in both 40 °C and 43 °C incubated samples was reduced substantially by Triton solubilization of the vesicles (Figure 4, triangles), indicating that these samples did not contain large, covalently linked aggregates of spectrin. Binding could not be measured at 50 °C since incubation of CF-spectrin alone at 50 °C did result in the formation of Triton-insoluble aggregates which eluted in the void volume (not shown). Also measured was the temperature dependence of CF-spectrin binding to LUVETs of the same amount of DOPS, which has a sharp phase transition temperature at −11 °C (Browning & Seelig, 1980), in contrast with bovine brain PS, which has a broad phase transition at 5 °C (Jacobson & Papahadjopoulos, 1975). Although the fraction of CF-spectrin bound per DOPS LUVETs was somewhat higher than that per bovine brain PS LUVETs, the temperature dependence of CF-spectrin bound to DOPS LUVETs (Figure 4, open circles) was nearly the same as to bovine brain PS LUVETs.

Binding Isotherms. Data for binding isotherms were collected under three incubation conditions: (1) 0–1 μM CF-spectrin + 1.25–1.5 mM PS LUVETs was incubated for 30 min at 25 °C; (2) 0–1 μM CF-spectrin + 1.25–1.5 mM PS LUVETs was incubated for 30 min at 31 °C; (3) 0–1.84 μM CF-spectrin + 0.11–0.125 mM PS LUVETs was incubated

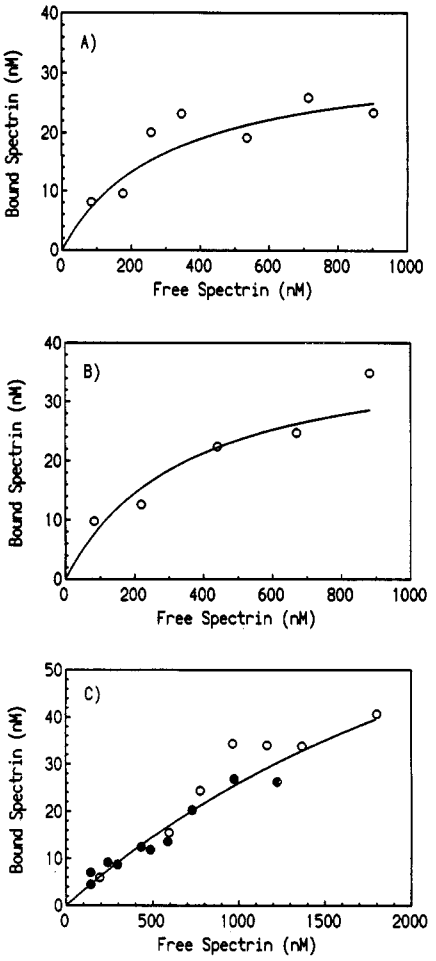

FIGURE 5: Isotherms of CF-spectrin + PS binding. (A) Increasing concentrations of CF-spectrin were incubated with 1.25–1.5 mM PS LUVETs at 25 °C for 30 min prior to separation on Sepharose CL-2B, and free spectrin and bound spectrin were quantified as described under Materials and Methods. (B) Increasing concentrations of CF-spectrin were incubated with 1.25–1.5 mM PS LUVETs at 31 °C for 30 min prior to separation on Sepharose CL-2B, and free spectrin and bound spectrin were quantified as described under Materials and Methods. (C) Increasing concentrations of unlabeled (open circles) or carboxyfluorescein-labeled spectrin (closed circles) were incubated with 0.11–0.125 mM PS LUVETs at 37 °C for 30 min prior to separation on Sepharose CL-2B, and free spectrin and bound spectrin were quantified as described under Materials and Methods. The curves shown in (A), (B), and (C) were obtained by nonlinear regression analysis with the GraphPAD program Inplot, as described under Results.

for 30 min at 37 °C. Figure 5 shows curves fitting the data to the equation describing saturation binding: $Y = AX/(B + X)$, where Y is the bound species, A is the maximum bound, X is the free species, and B is the dissociation constant (K_d). (It was not possible to measure these parameters at the same lipid concentrations at 37 °C as at the two lower temperatures, as binding to 1 mM PS at 37 °C did not begin to approach saturation and binding to 0.1 mM PS at 25 or 31 °C was barely detectable.) Curve-fitting was performed by nonlinear regression (Motulsky & Ransnas, 1987) with the Inplot program (GraphPAD; San Diego, CA). To assess the goodness-of-fit, R^2 values were calculated from the sums of squares of the relative, rather than absolute, deviations of each point from the curve.

The curve for data obtained at 25 °C (Figure 5A) gave an apparent K_d of 3.1×10^{-7} M and an apparent saturation binding concentration of 33 nM/mM PS with an R^2 of 0.92, in close agreement with the curve for data obtained at 31 °C (Figure 5B), which gave an apparent K_d of 3.5×10^{-7} and an apparent saturation binding concentration of 40 nM/mM PS with an R^2 of 0.93. The curve for the data obtained at 37 °C (Figure 5C) gave an apparent K_d of 3.4×10^{-6} M and an apparent saturation binding concentration of 1.13 μM/mM PS with an R^2 of 0.982. As indicated earlier, spectrin monitored by its intrinsic tryptophan fluorescence (Figure 5C, open circles) bound to PS vesicles to the same extent as CF-spectrin monitored by its extrinsic carboxyfluorescein fluorescence (Figure 5C, closed circles). The percent error of all of these values given by the InPlot program ranged from 17 to 52% so that these values must be considered rough estimates. Furthermore, since complete reversibility of complex formation has yet to be demonstrated, these estimates must be termed "apparent". They are nevertheless useful estimates as indicated by their comparing favorably with results of other studies (see Discussion). Limited availability of spectrin precluded gel filtration of free and bound spectrin in the continual presence of an excess of free spectrin, as in the classic Hummel and Dreyer method (1962). Nevertheless, since dissociation of the spectrin–PS vesicle complex was significant only when the ionic strength of the medium was lowered to millimolar concentrations and at 25 °C (see Complex Dissociation below), passage of the complex through a gel filtration column in the presence of progressively lower concentrations of free spectrin did not result in dissociation of the complex and an underestimate of the amount of bound spectrin.

Complex Dissociation. Initial attempts at dissociating complexes of PS and spectrin, which had been isolated either by 1 M Tris dissociation of red cell skeletons (Ohanian and Gratzer, 1984) or by low-salt elution from inside-out ghosts (Bennett, 1983) and concentration by ultrafiltration, were unsuccessful (not shown). Little dissociation occurred when complexes formed by incubating CF-spectrin and PS LUVETs at 37 °C for 20 min were subsequently left at room temperature for up to 7 h. Also, unlabeled spectrin added to samples of previously incubated CF-spectrin and PS LUVETs failed to compete at room temperature with labeled spectrin bound to the vesicles. Since spectrin is often extracted from red cell ghosts by incubation in buffer containing negligible salt and at a faster rate as the temperature is raised (Bennett, 1983), similar treatments were applied to pooled fractions of the spectrin–PS vesicle complex recovered by Sepharose CL-2B chromatography in TNED buffer. After incubation of the complex at different salt concentrations and/or at different temperatures, the complexes were rechromatographed on Sepharose CL-2B in TNED buffer to determine the extent of dissociation. Amounts of bound and free spectrin were estimated from the elution profiles—bound spectrin represented by the area under each curve from fraction 10 to fraction 18 and free spectrin represented by the area under each curve from fraction 18 to fraction 46.

Two conditions stimulated dissociation: (1) *Low ionic strength.* Three aliquots of pooled fractions of the spectrin–PS complex, previously isolated by Sepharose CL-2B chromatography from 1 mM PS LUVETs + 0.75 μM CF-spectrin incubated for 30 min at 37 °C in TNED buffer, were dialyzed at room temperature against two changes of 1 L of either 20 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7, for 4.5 h (Figure 6A, open circles), 3 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7, for 5 h (Figure 6A, filled circles), or 0.3 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7, for 4 h (Figure 6A, open triangles) prior to rechromatography on Sepharose CL-2B with TNED. The free spectrin comprising 31.7% of the recovered complex dialyzed in 20 mM KCl buffer (Figure 6A, open circles) was a contaminant of the pooled fractions containing the complex. If only the peak tube

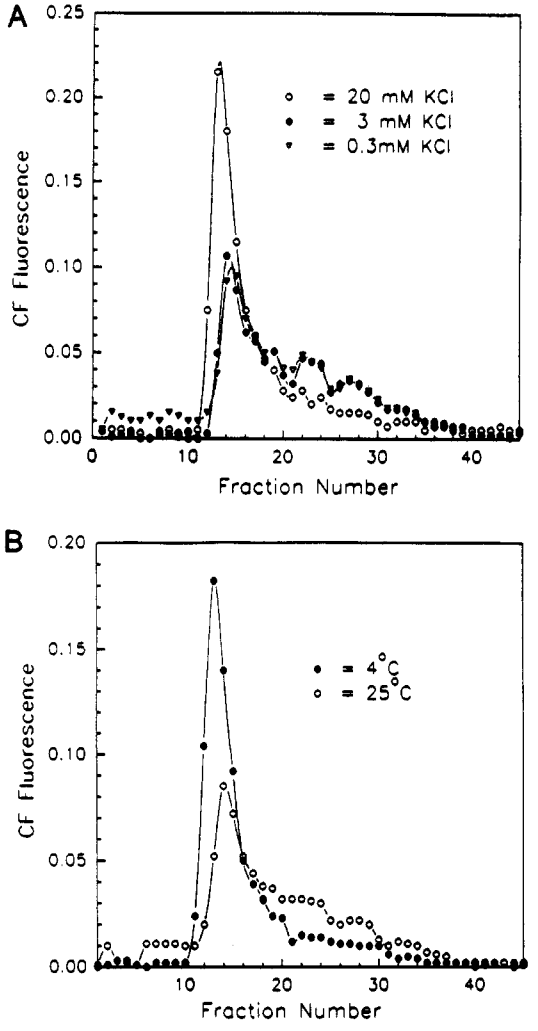

FIGURE 6: (A) Dissociation of CF-spectrin–PS complex at a high vs a low salt concentration. 0.75 μM CF-spectrin and 1 mM PS LUVETs were incubated for 30 min at 37 °C and applied to Sepharose CL-2B. 0.34-mL fractions were collected, and their carboxyfluorescein fluorescence was measured. The peak and preceding fractions containing the CF-spectrin–PS complex were pooled and divided into three aliquots which were dialyzed for 4–5 h at room temperature against two changes of 1 L of (1) 20 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7 (open circles), (2) 3 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7 (closed circles), and (3) 0.3 mM KCl + 0.1 mM EDTA + 0.2 mM DTT, pH 7 (triangles). 0.2 mL of each aliquot was applied to Sepharose CL-2B and eluted with TNED, and the carboxyfluorescein fluorescence of each fraction was measured as described under Materials and Methods. (B) Dissociation of CF-spectrin–PS complex at 0 °C vs. 25 °C. 0.75 μM CF-spectrin and 1 mM PS LUVETs were incubated for 30 min at 37 °C, and the CF-spectrin–PS complex was isolated as described in (A). Two aliquots of the complex were dialyzed for 4.5 h against two changes of 1 L of 0.1 mM EDTA + 0.2 mM DTT, pH 7—one at 4 °C (filled circles) and the other at room temperature (open circles). 0.2 mL of each aliquot was applied to Sepharose CL-2B, and the carboxyfluorescein content of each 0.34-mL fraction was measured as described under Materials and Methods. Fluorescence is given in arbitrary units.

containing the complex is rechromatographed, its elution profile coincides with that of the complex detected by *N*-rhodamine-labeled phosphatidylethanolamine in PS vesicles bound to unlabeled spectrin (not shown) and, thus, includes no free spectrin. In contrast to the pooled complex dialyzed in 20 mM KCl (Figure 6, open circles), identical samples of the pooled complex after dialysis against buffer containing 3 mM KCl (Figure 6A, filled circles) or 0.3 mM KCl (Figure 6A, open triangles) contained 60.2 and 58.1% free spectrin, respectively. Recoveries of free plus bound spectrin in the 3 and 0.3 mM KCl samples were 85 and 87%, respectively, of that in the 20 mM KCl sample. (2) *Higher temperature.* When two aliquots of spectrin–PS vesicle complex were dialyzed for 4.5 h against two changes of 1 L of 0.1 mM EDTA + 0.2 mM DTT, pH 7, more dissociation occurred when dialysis was performed at 25 °C (Figure 6B, open circles) than at 4 °C (Figure 6B, filled circles). Only 23.8% of the complex dialyzed at 4 °C was free spectrin, which was present in the sample before dialysis, whereas 54.4% of the complex dialyzed at 25 °C was free spectrin. Recovery of the free plus bound spectrin in the 25 °C sample was 80% of the free plus bound spectrin in the 4 °C sample.

DISCUSSION

Two properties of specific, spectrin–PS vesicle binding have been demonstrated for the first time by use of a new gel filtration assay: (1) binding of spectrin to PS vesicles at a physiological salt concentration increases with increasing temperature (Figures 3 and 4); (2) dissociation of the spectrin–PS vesicle complex is enhanced by reducing the salt concentration to millimolar levels (Figure 6A) at 25 °C but not at 4 °C (Figure 6B). By nonlinear regression analysis, the apparent K_d of spectrin–PS binding is 3.1×10^{-7} M with apparent saturation of 33 nM/mM PS at 25 °C (Figure 5A), values which are virtually the same at 31 °C—apparent K_d of 3.5×10^{-7} M with apparent saturation of 40 nM/mM PS (Figure 5B). In contrast, at 37 °C the apparent K_d is 3.4×10^{-6} M with apparent saturation of 1.13 μM/mM PS (Figure 5C). The higher apparent K_d but higher apparent saturation at 37 °C than at 25–31 °C indicates that at the higher temperature more spectrin is bound more weakly to PS vesicles in 100 mM NaCl. This difference may be related to the thermocontraction of spectrin detected in previous studies by light scattering (Stokke et al., 1985) and measurements of skeleton size (Johnson et al., 1980; Vertessy & Steck, 1989). The 10–20% increase in spectrin or skeleton size between room temperature and 37 °C is not large enough to account on the basis of size alone for the at least twice as much spectrin bound per amount of PS at 37 °C as at 25–31 °C (Figures 3 and 4). Instead, changes in noncovalent associations accompanying thermocontraction (Vertessy & Steck, 1989) may have facilitated the weaker binding of spectrin to more PS sites as the temperature increased. Spectrin subjected to increasing temperature also displays decreasing steady-state fluorescence anisotropy of its tryptophans (Yoshino & Marchesi, 1984; Clague et al., 1990) and increasing mobilities of nitroxides located at various distances from maleimides conjugated to spectrin (Cassoly et al., 1980), but how increased motion of spectrin would affect binding is uncertain. The rotational correlation time of spectrin bound to red cell membranes is the same as that of free spectrin in solution (Learmonth et al., 1989; Clague et al., 1990).

The binding parameters obtained with the new gel filtration assay are within an order of magnitude of values obtained in three earlier studies in which different means were used to quantify spectrin binding to negatively charged lipid bilayers: (1) Separation of liposome-bound and free spectrin by centrifugation through a dextran cushion gave a K_d of 7×10^{-7} M for PS/PE, 1:2, and a K_d of 2.7×10^{-6} M for PS/PC, 1:2 (Mombers, 1982). (2) By a similar method, we obtained a K_d for spectrin binding to multilamellar PS vesicles of 5.4×10^{-7} M (Takeshita et al., 1993). (3) Quenching of (anilinonaphthyl)spectrin by PS vesicles gave an association constant of 3×10^7 M^{-1} (Bonnet & Begard, 1984). The moderate affinity of spectrin binding to PS vesicles reported in all three studies and the present one indicates that the affinity of spectrin for PS vesicles is 1 order of magnitude or

2 weaker than the affinity of spectrin for proteins associated with the inner surface of ghost membranes [i.e., 10^{-7}–10^{-8} M at 0 °C (Bennett & Branton, 1977)]. Thus, the affinity of spectrin for PS vesicles could approximate that of the residual binding of spectrin to inside-out ghost membranes depleted of ankyrin and 4.1 (Bennett & Branton, 1977).

Very different binding constants have been reported in four other studies: (1) Measured by fluorescence photobleaching, the diffusion coefficient of NBD-PS was the same in intact and skeletal protein-depleted ghost membranes (Morrot et al., 1986). However, the numbers of PS interacting with spectrin may have been too small to be detected in this way. The maximum number of spectrin-bound PS on red cell surfaces could be a tenth of the available PS and, therefore, below the level of detection by photobleaching methods, in spite of most of the spectrin being bound to some PS as indicated in the last paragraph of this Discussion. Video-processed images of negative stain-treated material show the subunits of spectrin to be coiled about each other like two springs (McGough & Josephs, 1990) so that the region of contact between the springs and the bilayer might be limited to only a fraction of each spring. (2) Bitbol et al. (1989) concluded that the same amounts of spectrin were bound to DMPC and to DMPC/DMPS, 1:1, vesicles after the dry lipids had been hydrated in the presence of spectrin containing buffers and centrifuged, since they found the same amounts of spectrin in the supernatants. The 10^{-7} M K_d reported for both types of vesicles is very low for DMPC and must reflect the trapping of free spectrin during vesicle formation and/or pelleting of some free spectrin as well as vesicle-associated spectrin. Line shapes of ^{31}P and ^{2}H NMR spectra of headgroup-labeled DMPC and DMPS in these vesicles—but not quadrupole splittings or spin–lattice relaxation times—were affected by spectrin. Like the inability to detect a difference in the diffusion coefficient of NBD-PS in intact and skeleton-depleted ghosts, the inability to detect effects of spectrin on the conformation and/or dynamics of the PS headgroups in the DMPC/DMPS, 1:1, vesicles might be due to the spectrin-bound PS constituting too small a fraction of the total. (3) At the high end of the spectrum of affinities, nondenaturing PAGE at 4 °C showed that nearly all spectrin dimers bound to sonicated, unilamellar vesicles of PS (Cohen et al., 1986), the concentrations of spectrin and PS vesicles being similar to those in the present study. It may be that the "preferred orientation" (Cantor & Schimmel, 1980) of spectrin and/or PS vesicles induced during electrophoresis greatly enhanced their assocation. (4) Maksymiw et al. (1987) found that spectrin raised the surface pressure of a gel-phase DMPS monolayer by an amount equivalent to an energy of 1.5×10^7 J/mol of spectrin [a typographical error in Maksymiw et al. (1987) gives this value as 10^6 J/mol], which corresponds to an extremely low dissociation constant. Since the difference in the energy of the DMPS monolayer in the presence vs absence of spectrin was measured at a low surface pressure, its magnitude may reflect denaturation of spectrin at the air–water interface, rather than spectrin binding to the DMPS monolayer. In summary, there are reasons for binding affinities measured by photobleaching (Morrot et al., 1986), NMR (Bitbol et al., 1989), nondenaturing PAGE (Cohen et al., 1986), and surface pressure (Maksymiw et al., 1987) methods differing significantly from the moderate affinity measured by centrifugation (Mombers, 1982; Takeshita et al., 1993), fluorescence quenching (Bonnet & Begard, 1984), and gel filtration (Figures 5A–C) methods.

Unlike association of spectrin with PS vesicles, dissociation of a spectrin–PS complex has not been demonstrated previously. The dissociation reported here, however, was not the simple reverse of association as complexes formed in 100 mM NaCl at 37 °C did not dissociate on subsequent incubation at room temperature for up to 7 h and unlabeled spectrin did not compete with labeled spectrin previously bound to PS vesicles under those conditions. Nevertheless, a significant fraction of the complex did dissociate when the salt concentration was reduced to millimolar levels (Figure 6A) at 25 °C but not at 4 °C (Figure 6B). The occurrence of dissociation in millimolar NaCl at 25 °C but not at 4 °C does not conflict with temperature-stimulated association in 100 mM NaCl. On thermodynamic grounds, since dissociation of spectrin from PS vesicles in millimolar NaCl is not the reverse of association of spectrin with PS vesicles in 100 mM NaCl, a temperature increase is not necessarily expected to have opposite effects on dissociation and association. Currently available information on the molecular configuration of spectrin provides support for this conclusion. The conformation of spectrin appears to be different in low and high salt, since spectrin lengthens from 76 to 140 nm (Elgsaeter, 1978) and the quenching of fluorescence of its tryptophans decreases (Subbarao and MacDonald, submitted for publication) when the salt concentration is reduced from 100 to 1 mM. Thus, it is plausible that an increase in temperature could promote the binding to PS of spectrin at 100 mM NaCl but promote the dissociation of a PS–spectrin complex at 1 mM NaCl.

Greater instability of the spectrin–PS complex with decreasing ionic strength may seem unlikely for an interaction which appears to be electrostatic from the preferential binding of spectrin to lipids with a net negative charge but not to lipids with no net charge (Mombers, 1982; Maksymiw et al., 1987; Takeshita et al., 1993; Figure 1A). However, since spectrin bears a net negative charge at neutral pH (Hsu et al., 1979), the stability of the spectrin–PS complex must be affected both by electrostatic repulsion of negatively charged sites on spectrin by the negatively charged bilayer and by electrostatic attraction between positively charged sites on spectrin and the negatively charged bilayer. Therefore, at low ionic strength, electrostatic repulsion plus, possibly, nonelectrostatic forces could exceed electrostatic attraction so as to promote dissociation of the complex formed at high ionic strength, whereas at high ionic strength attraction plus, possibly, nonelectrostatic forces apparently outweigh repulsion.

The resistance to dissociation of the spectrin–PS complex formed in 100 mM NaCl is not surprising, since spectrin is much larger with a more complex structure than small ligands undergoing classical equilibrium binding to a single site. In contrast to the latter, the interaction of spectrin with PS vesicles resembles the surface adsorption of much larger molecules—including proteins [see review by Norde (1986)] and synthetic polymers [e.g., see Johnson and Granick (1992)]. Macromolecules typically desorb much more slowly than they adsorb to surfaces because their attachment by multiple sites can only be reversed on rare, simultaneous dissociation of those sites. Electron microscope images of spectrin bound at different points to PS vesicles (Cohen et al., 1986) show that spectrin can bind to PS vesicles at multiple sites. That these modes of binding may be characteristic of spectrin *in vivo* is indicated by the similarity of the conditions under which spectrin desorbs from PS vesicles (Figure 6A,B) to the conditions under which spectrin can be extracted from inside-out ghost membranes (Bennett, 1983).

The apparent K_ds and saturation levels of spectrin binding to PS indicate that this interaction could occur *in vivo*. Although the affinity of spectrin for PS is an order(s) of magnitude lower than those by which spectrin binds to red

cell membrane proteins, the spectrin–PS interaction may nevertheless be extensive *in vivo* and physiologically important. With a K_d of 3.4×10^{-6} M, the concentration of the spectrin–lipid complex in the red cell is calculated to be 432 μM—i.e., 99% of the spectrin should be bound to phosphatidylserine given that phosphatidylserine is 15.8% of the 3.6 mM phospholipid per red cell (Wintrobe, 1981) and the estimated concentration of spectrin at the inner surface of the red cell membrane is 434 μM (Lux, 1979). The function of this specific interaction between spectrin and phosphatidylserine has yet to be established, but given its apparent extent *in vivo*, this association and that of other skeletal proteins with negatively charged lipids could affect cell shape and, in the case of spectrin, the viscoelasticity of the red cell. The ability to induce substantial spectrin binding to PS vesicles will allow us to study the effects of spectrin on vesicle shape and deformability as a first step toward establishing the function(s) of spectrin–lipid binding in cells.

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