EVIDENCE THAT SPECTRIN BINDS TO MACROMOLECULAR COMPLEXES ON THE INNER SURFACE OF THE RED CELL MEMBRANE

D. LITMAN*, C. J. HSU* AND V. T. MARCHESI
From the Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.

SUMMARY

Spectrin binds to a population of high-affinity sites on the exposed surfaces of inverted vesicles prepared from human red blood cell ghost membranes. Optimal spectrin binding requires the presence of monovalent salts but does not require calcium or magnesium. The band 2 subunit of spectrin, prepared in SDS, can also bind to vesicles, but isolated band 1 is inactive.

Pre-incubation of inverted vesicles with antibodies directed against the cytoplasmic segment of band 3 or against bands 4.1-4.2 inhibits the binding of spectrin to the same vesicles. Antibodies against the cytoplasmic portion of glycophorin A have no effect.

These results suggest that spectrin binds to a protein acceptor on the cytoplasmic surface of the red cell membrane which is close to the cytoplasmic segments of bands 3 and 4.1 and/or 4.2.

INTRODUCTION

One of the major structural elements of the erythrocyte membrane is a water-soluble, high-molecular-weight protein called spectrin (Marchesi, 1979). Previous studies using radiolabelling tracer methods (Marchesi, 1974a) or immunocytochemical techniques (Nicolson, Marchesi & Singer, 1971; Ziparo, Lemay & Marchesi, 1978) have shown that spectrin is attached to the cytoplasmic surface of the erythrocyte membrane, but how and where this protein binds to the membrane is still unclear.

Experiments with chemical cross-linking agents show that membrane-bound spectrins are close to other spectrins, and some are also close to other membrane proteins, particularly Bands 3 and 4.1 (Steck, 1972; Wang & Richards, 1974). Similar results have recently been obtained by analysing the capacity of endogenous red cell transglutaminases to link together neighbouring membrane proteins (Lorand, Weismann, Epel & Bruner-Lorand, 1976; Anderson, Davis & Carraway, 1977; Lorand, Siefring & Lowe-Krentz, 1979). Spectrin also binds to liposomes containing the Band 3 protein (Yu & Branton, 1976), but not to liposomes prepared with phospholipids alone, although others have suggested that spectrin might indeed bind directly to

* Present address: Syva Research Institute, 3221 Porter Drive, Palo Alto, California 94304, U.S.A.

Spectrin binds to inverted vesicles prepared from red blood cell ghosts (Bennett & Branton, 1977), and the properties of this association mimic the binding of spectrin to the intact red cell membrane. The results of this study suggest that spectrin binds to a protein or proteins on the exposed surface of the inside-out vesicles (IOV), since the binding of spectrin to these vesicles is eliminated when the latter are treated with proteases. Such proteolytic treatment cleaves most of the major proteins of the inside-out vesicles, including Band 3. Tryptic digestion of Band 3 releases a 40,000 Dalton peptide from its cytoplasmic segment, but this peptide does not react with spectrin, nor does it influence the binding of spectrin to inverted vesicles. In contrast, another peptide, generated by chymotryptic digestion of red cell ghosts was able to compete for spectrin binding to the inverted vesicles (Bennett, 1978). It has been suggested that this competing peptide is derived from Band 2.1 (Bennett & Stenbuck, 1979; Luna, Kid & Branton, 1979).

The experiments described in this paper represent our attempts to study the binding of spectrin to inside-out vesicles prepared from human ghost membranes. We have studied the characteristics of binding of different spectrin subunits to these membranes and have attempted to identify the binding sites for spectrin by incubating membranes with antibodies directed against the cytoplasmic portions of different membrane proteins. The results of these experiments suggest that spectrin associates with the inner surface of the red cell membrane by non-covalent associations with a multi-protein complex containing Bands 3 and 4.1–4.2.

MATERIALS AND METHODS

Membrane and vesicle preparations

Unless otherwise stated all procedures were performed at 0–4 °C. Haemoglobin-free erythrocyte membranes were prepared from freshly drawn human blood essentially according to the procedure of Fairbanks, Steck & Wallach (1971). Hypotonic lysis was carried out by adding 1 vol. of washed red cells to 40 vol. of 5 mM sodium phosphate, pH 8–0 containing 0.03 mM phenylmethane-sulphonyl fluoride (PMSF). Resealed ghosts were prepared from freshly prepared unsealed ghosts by incubation at 37 °C, in 40 vol. of PBS (5 mM sodium phosphate, pH 7.4, 150 mM NaCl), for 45 min. Resealed ghosts were recovered by centrifugation at 14,000 rev/min in a Beckman JA 14 rotor and washed twice in PBS prior to being stored in same at 0 °C.

Inside-out vesicles (IOV) were prepared by the procedure of Kant & Steck (1973) with the following minor modifications. Washed erythrocyte ghosts (40 ml) were suspended in 40 vol. of vesiculation buffer (0.5 mM sodium phosphate, pH 8–9, 0.5 mM β-mercaptoethanol (βMe), 0.03 mM PMSF). Membranes were stirred at 4 °C for 4–6 h until spontaneous endovesiculation was evident by phase-contrast microscopy. Vesicles were then pelleted by centrifugation at 14,000 rev/min for 25 min with a JA 14 rotor. The pellets obtained were resuspended in 1 vol. vesiculation buffer and passed through a 27-gauge needle until a homogeneous population of small IOV was seen by phase-contrast microscopy (3–5 passages). These largely unsealed IOV were stored in vesiculation buffer at 0 °C for up to 10 days.

Sealed IOV were obtained by density gradient sedimentation of IOV on 37-ml linear dextran T70 gradients in vesiculation buffer, \( \rho = 1.05 \) to \( 1.05 \) g/cm³, for 10 h at 27,000 rev/min in the SW 27 Beckman rotor. The low density band at \( \rho = 1.01 \) g/cm³ was aspirated, washed twice in 10 vol. of vesiculation buffer and stored at 0 °C in same.
Spectrin binding to red cell membrane

Spectrin extraction

Spectrin was extracted from washed erythrocyte membranes by incubating ghost membranes for 30 min at 37 °C in 5 vol. of 0.1 mM ethylene diamine tetra-acetic acid (EDTA), 0.5 mM βMe, 0.03 mM PMSF, titrated to pH 9.5. Centrifugation at 50,000 rev/min for 1 h in a 60Ti rotor for 60 min yields a supernatant containing spectrin (Bands 1 and 2) and Band 5. This crude extract was concentrated by isoelectric precipitation at pH 5.1, by the addition of 1 M HCl. The mixture was allowed to stand for 60 min on ice, after which the precipitated protein was collected by centrifugation for 5 min at 5000 rev/min in the JA 14 rotor at 14 °C. The pellet was recovered as described below.

SDS purification of spectrin

The isoelectric precipitate of crude spectrin extract (containing spectrin and Band 5) was redissolved in 1 % sodium dodecylsulphate (SDS), 5 mM Tris, pH 9.0, 0.5 mM βMe, 0.5 mM EDTA, 0.03 mM PMSF, 0.02 % NaN3 and applied to a 5 x 90 cm column of Sepharose 4B equilibrated in the same buffer but containing 0.1 % SDS. SDS gel electrophoresis was used to monitor the column. Those fractions containing pure spectrin (Bands 1 or 2) were pooled, dialysed exhaustively against 2 mM sodium phosphate, pH 9.0, 0.5 mM βMe, 0.5 mM EDTA, 0.03 mM PMSF, 0.02 % NaN3, at 4 °C for 4 days, and then concentrated by pressure dialysis in an Amicon cell with an XM100 membrane to ~ 1 mg/ml. SDS-purified spectrin was stored at 0 °C in dialysis buffer and was found to be stable for up to 6 weeks.

Isotonic purification of spectrin

Spectrin can be purified in the absence of SDS by column chromatography of crude spectrin extract (prior to isoelectric precipitation) on Sepharose 4B (2.5 x 90 cm) equilibrated with 25 mM Tris.HCl pH 9.5, 100 mM NaCl, 0.5 mM βMe, 0.5 mM EDTA, 0.02 % NaN3, 0.03 mM PMSF at 22 °C. The column is developed at 13.5 ml/h; 45-ml fractions are collected and monitored for protein composition as described above. Fractions containing pure spectrin are pooled, dialysed against 5 mM sodium phosphate, pH 9.0, 0.03 mM PMSF, and stored in same at 0 °C for up to 4 weeks.

Iodination procedures

Lactoperoxidase (LPO)-catalysed enzymic iodination was performed according to published procedures (Morrison, Mueller & Huber, 1974). Spectrin dialysed against 20 mM Tris.HCl pH 7.4 (1 mg/ml) was iodinated at 4 °C in a reaction mixture containing (per ml): 5 μg lactoperoxidase (LPO), 0.2-1 mCi 125I (carrier-free), 20 μl 10-6 M NaI, 50 μl 0.8 mM H2O2 added in 5 10-μl aliquots 2 min apart. The reaction was terminated by the addition of βMe to 10 mM and NaN3 to 0.02 %. Iodide was separated from spectrin by dialysis (1000 vol., 5 changes) against 2 mM sodium phosphate pH 9.0, 1 mM βMe, 0.02 % NaN3, 0.03 mM PMSF. The first dialysis medium contained 1 mM NaI.

Chemical iodination via the Bolton–Hunter reagent (monooiodo 3-(4 hydroxyphenyl) propionic acid N-hydroxy succinimide ester) was performed as follows: 1 mg spectrin in 50 mM sodium borate pH 8.5 was added to 0.2 mCi of dry Bolton–Hunter reagent at 0 °C. The reaction was allowed to proceed at 0 °C for 60 min. Spectrin was separated from unreacted reagent on 1.5 x 12-cm columns of Sephadex G50 in 50 mM sodium phosphate pH 7.0 and dialysed against 2 mM sodium phosphate pH 9.0, 0.5 mM βMe, 0.03 mM PMSF.

Spectrin-binding assay

Iodinated spectrin (0-20 μg) was incubated with 20-60 μg of inside-out vesicles, or resealed ghosts in a total volume of 0.2 ml of 10 mM sodium phosphate, pH 7.0, 80 mM NaCl, 1 mg/ml bovine serum albumin (BSA) for 20 min at 37 °C in a 400-μl microfuge tube (Brinkman). Following incubation the vesicles and bound spectrin were separated from free spectrin by centrifugation at 18,000 rev/min in the JA 20 Beckman rotor at 15 °C. The supernatant,
containing unbound spectrin, was aspirated with a drawn Pasteur pipette and the bound spectrin in the pellet quantitated by gamma counting in a Beckman Biogamma I counter.

**Proteolytic digestion of inside-out vesicles**

Inside-out vesicles at a final concentration of 1 mg protein/ml were digested by mercapto-papain (Sigma) in 1.5 ml of 20 mM sodium phosphate pH 7.0, 2 mM EDTA, 5 mM cysteine at 0 °C for 60 min with concentrations of papain ranging from 0.08 μg/ml. Three volumes of ice-cold 20 mM sodium phosphate pH 7.0, 5 mM NEM (Eastman) were added to terminate the reaction and the vesicles were recovered by centrifugation for 15 min at 15,000 rev/min in a JA 20 rotor, washed twice more in phosphate buffer without NEM, and resuspended in 1 ml of 20 mM sodium phosphate, pH 7.0. Aliquots of resuspended vesicles were tested for protein composition (SDS gels), protein content (Lowry protein determination), and spectrin binding competence. Protein released by papain treatment was estimated by Lowry determinations of supernatants.

Inside-out vesicles at 1 mg protein/ml were digested with trypsin (Worthington) for 30 min at 37 °C, in 1 ml of 10 mM sodium phosphate pH 7.0. The reactions (containing 0-0.4 μg trypsin/ml) were terminated by the addition of 10 μl of 0.15 M PMSF, followed by incubation at room temperature for 15 min. Vesicles were washed as above in 3 volumes of 10 mM sodium phosphate, pH 7.0, repeated 3 times, and suspended in 1 volume of same. PMSF-treated trypsin was ineffective in digesting inside-out vesicles (by the criteria of SDS-PAGE) or in inhibiting spectrin binding to inside-out vesicles.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Electrophoresis was performed on gels containing 5.6% acrylamide (including 0.19% N,N'-methylenebis-(acrylamide) and 0.2% SDS according to the procedure of Fairbanks et al. (1971) and modified by Steck & Yu (1973). Gels were stained with Coomassie Blue R (Sigma) and major membrane polypeptides were enumerated according to their characteristic mobilities according to published staining patterns (Steck, 1974).

**Preparation of antisera**

Antiserum directed against the C terminal cytoplasmic extension of glycophorin A were prepared as described previously (Cotmore, Furthmayr & Marchesi, 1977). Antiserum directed against the cytoplasmic extension of Band 3 has been extensively characterized (Fukuda, Eshdat, Tarone & Marchesi, 1978). Antisera against purified Bands 4.1 and 4.2 were raised in white New Zealand rabbits, the characterization and production of which is described elsewhere (Hsu, Litman & Marchesi, in preparation). IgG-enriched fractions were obtained from 33% saturation ammonium sulphate precipitates.

**Binding of IgG fractions of antisera to vesicles and ghosts**

None to 800 μg of 125I-labelled IgG fractions of antisera directed against glycophorin A Band 3, and a mixture of Bands 4.1 and 4.2 were incubated with 20-40 μg of inside-out vesicles or resealed ghosts for 30 min at 37 °C in 400-μl microfuge tubes. Bound IgG was separated from free IgG by centrifugation of the reaction mixtures for 15 min at 18,000 rev/min in the JA 20 rotor. The cpm in the vesicle-containing pellet were determined after aspiration of the supernatants. Radiolabelled nonimmune IgG was always used as a control for specificity.

**Circular dichroism measurements**

The far-u.v. circular dichroism spectra were recorded with a Cary Model 61 recording circular dichrometer at ambient temperature. The instrument was standardized using twice-recrystallized d-10 camphorsulphonic acid. Cylindrical quartz cuvettes of path length 1 mm were used, recording at time constant 3 and at a full scale range of 0.1° or 0.05°. The sample protein concentrations (0.1-0.2 mg/ml) were determined by amino acid analysis with a Durrum D-500 autoanalyser. The measured ellipticity, θ, in degrees, was normalized to molar residue ellipticity, (θ), in deg. cm² dmol⁻¹ using a mean residue weight of 113.4. The CD (circular dichroism) spectra were analysed using the method of Hsu, Litman & Marchesi (in preparation).
Spectrin binding to red cell membrane

in the wavelength region 245 to 210 nm at 1-nm intervals to obtain the secondary structural parameters. The computation is a non-linear regression analysis by means of stepwise Gauss–Newton iteration performed on an IBM 370/67 computer with the BMDP-3R statistical package.

Other procedures

Protein was determined by the method of Lowry, using crystalline BSA as standard. Acetylcholine esterase and NADH cytochrome c oxidoreductase were assayed according to published procedures (Kant & Steck, 1973). The isolation and purification of Bands 4.1 and 4.2 is described elsewhere (Hsu, Litman & Marchesi, in preparation).

Materials

Mercuripapain (E.C. 3.4.10) was obtained from Sigma Chemical Co. Trypsin (TLCK-treated) was obtained from Worthington. Reagents for polyacrylamide gel electrophoresis were obtained from Eastman. All reagents and solvents were reagent grade or better from Fisher, Eastman, Baker or Mallinckrodt.

RESULTS AND DISCUSSION

Spectrin preparation

The spectrin polypeptides can be isolated at more than 95% purity from EDTA extracts of red cell membranes by gel exclusion chromatography at alkaline pH. Two chromatographic procedures were developed to prepare preparative amounts of purified spectrin. The first procedure, which utilizes a buffer containing 0.1% SDS, fractionates spectrin chains on the basis of their monomer size on Sepharose 4B. Band 1 and Band 2 peaks overlap and elute in one peak, peak B, within the included volume of the column as shown in Fig. 1 A. Fractions containing equimolar amounts of Band 1 and Band 2 in peak B are pooled and dialysed to remove essentially all the SDS associated with the spectrin chains.

Chromatography of crude spectrin extract (Methods) on Sepharose 4B in isotonic salt at alkaline pH results in sharp peak of spectrin which elutes within the included volume of the column (Fig. 1 B). SDS-PAGE of successive column fractions (data not shown) reveals that the spectrin subunits are present in equimolar amounts across the peak. Both the isotonic chromatography and the SDS gel filtration procedures yield spectrin preparations that are more than 95% pure, and are stable both chemically and functionally (as defined by the ability to associate selectively with inside-out vesicles) for up to 6 weeks at 0°C.

Spectrin purified in the presence of SDS seems to regain its secondary structure after the SDS is removed by dialysis, since its circular dichroic spectrum is essentially indistinguishable from that of spectrin which was not exposed to SDS (Fig. 2). Spectrin is clearly sensitive to denaturing agents (Schechter, Sharp, Reynolds & Tanford, 1976) and does undergo dramatic conformational changes in the presence of SDS (Fig. 2). We do not know whether SDS-treated spectrin has recovered all of its native conformation, but SDS-treated spectrin does recover its capacity to bind to the red cell membrane (as described below).

Purified spectrin was radiolabelled either by lactoperoxidase-catalysed iodination which results principally in the iodination of tyrosine residues, or by reaction with
Fig. 1. A, gel exclusion chromatography of crude spectrin extract on Sepharose 4B in the presence of 0.1% SDS. 30 ml of spectrin extract (100 mg protein) were applied to a 4 x 90-cm column equilibrated with 5 mM Tris.HCl pH 9.0, 0.5 mM EDTA, 0.5 mM βMe, 0.03 mM PMSF, 0.1% SDS. 13.5-ml fractions were collected at the rate of 54 ml/h. Phenol red was used as a marker for included volume. Fractions 57 to 65 in peak B were pooled, dialysed, and concentrated to $A_{280}$ = 1.0 to give purified SDS-spectrin.

B, gel exclusion chromatography on a 2.5 x 90-cm sepharose 4B column in the presence of 25 mM Tris.HCl pH 9.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM βMe, 0.03 mM PMSF, 0.02% NaN3 eluting at 17 ml/h. 4.5-ml fractions were collected. Fractions 57 to 65 in peak B were pooled and concentrated to ~1 mg/ml. In both columns peak A represents aggregated spectrin and Band 5, peak B represents spectrin (Bands 1 and 2) and peak C represents Band 5 and other minor membrane components.
iodinated 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester which principally acylates lysine residues.

**Inside-out vesicles**

The procedure of Kant & Steck (1973) with minor modifications has been used to prepare inside-out vesicles by low-ionic-strength vesiculation of erythrocyte ghosts. In the course of the low-ionic-strength treatment ghosts can be seen by phase-contrast microscopy to undergo endovesiculation. Concomittant with membrane inversion, spectrin (Bands 1 and 2) as well as Band 5 are nearly quantitatively removed (Fig. 3). No other differences in protein composition are detectable by Coomassie Blue or PAS staining of SDS gels of inside-out vesicles (IOV).

Freeze-fracture electron microscopy of vesicle preparations confirms their inverted nature. Thin-section studies of similar preparations show a mixed population of vesicles, half of which appear to contain other vesicles sealed within the outer membrane.

A subpopulation of IOV that are sealed to small molecules can be isolated by dextran density gradient centrifugation. This population is identical to the total IOV preparation with respect to protein composition and morphology and has been used to confirm the inside-out orientation of the membrane. Eighty-five percent of IOV isolated from dextran gradients are both sealed and inside-out as determined by

![CD spectra of spectrin](image-url)
Fig. 3. SDS gels of normal human erythrocyte ghosts (A) and membrane fragments (B) obtained after extraction in vesiculation buffer.
acetylcholine esterase activity, an enzymic activity associated with the external surface. NADH-cytochrome c oxidoreductase, an enzyme associated with the cytoplasmic surface, is 100% accessible to substrate in sealed IOV as compared with 14% accessibility in resealed ghosts (data not shown). Both sealed and unsealed IOV display the same affinity and selectivity for spectrin binding as shown below.

**Spectrin association with inside-out vesicles**

Purified 125I-spectrin binds selectively to the cytoplasmic surface of membrane vesicles as a function of salt concentration as shown in Fig. 4. Binding to IOV is highly sensitive to monovalent salt concentration but only minimally affected by the presence of 1 mM Mg²⁺ at optimal salt concentrations. Binding of spectrin to resealed ghosts is less than 15% that of IOV, demonstrating physiological specificity of binding.

![Graph showing the effect of monovalent salt concentration on spectrin binding to IOV and resealed ghosts](image-url)

Fig. 4. The effect of monovalent salt concentration on spectrin binding to IOV (▲, ●) or resealed ghosts (△, ○) in the presence (▲, △) or absence (○, ●) of 1 mM MgCl₂. 10 μg of 125I-spectrin were incubated with 35 μg of vesicles or resealed ghosts for 20 min at 37 °C in a total volume of 200 μl of 5 mM sodium phosphate pH 7.0.

The binding of spectrin to IOV is dependent on spectrin concentration and is saturable at approximately 150–200 μg spectrin/mg of vesicle protein (Fig. 5A). Binding of spectrin to resealed ghosts is less than 15% that of IOV, and it increases linearly with spectrin concentration and shows no observable saturation. This binding to resealed ghosts is not significantly different from the amount of spectrin pelleted in the absence of vesicles and represents the background inherent in the binding assay. Specific spectrin binding is defined therefore as the amount bound to IOV minus the amount sedimenting with resealed ghosts.
Fig. 5. A, the binding of purified radiolabelled spectrin to IOV (●—●) or resealed ghosts (○—○) as a function of spectrin concentration. The indicated amount of spectrin was incubated with 35 μg of vesicles or ghosts in 200 μl of 10 mM sodium phosphate, pH 7.0, 80 mM NaCl and assayed as described in Methods.

B, the binding of spectrin (9.8 μg) to increasing concentrations of IOV (●—●), or resealed ghosts (○—○) as indicated. Reactions were as in Fig. 5A, incubation was for 20 min at 37°C.
Fig. 5B shows that the binding of spectrin is dependent on the concentration of IOV and that nearly 80% of the 125I-spectrin is able to bind to IOV.

To show that spectrin is actually bound to IOV as opposed to being adventitiously precipitated in the presence of vesicles, reaction mixtures containing iodinated spectrin and vesicles were subjected to density sedimentation on discontinuous dextran gradients. As can be seen from the gradient profile in Fig. 6, sealed vesicles and spectrin bound to vesicles band at the ρ = 1.01:1.035 density interface. Unbound spectrin sediments to the bottom of the gradient (ρ = 1.04) along with unsealed vesicles.

Fig. 6. Density gradient isolation of reconstituted vesicles. Two-milligrammes of radiolabelled spectrin were incubated with (■—■) or without (▲—▲) 2 mg of IOV in 2 ml of 80 mM Tris.HCl, pH 7.0. After a 20-min incubation at 37 °C the reaction mixtures were diluted with 1.6 ml of dextran T70, ρ = 1.1, in 80 mM Tris.HCl, pH 7.0; 0.8 ml of the resulting solutions, ρ = 1.044, were sandwiched between the ρ = 1.05 and ρ = 1.035 density layers of a discontinuous dextran density gradient consisting of 0.25 ml ρ = 1.05, 2.5 ml ρ = 1.035, and 0.5 ml ρ = 1.01 in the same buffer. The gradients prepared in 45-ml cellulose nitrate tubes were centrifuged for 3 h at 50,000 rev/min in the SW56 Beckman swinging bucket rotor. The sedimentation was terminated without mechanical braking, the gradients were removed by piercing the bottom of the tube with a needle and collecting 200-μl fractions (3 drops/fraction). After dilution with 0.8 ml of 20 mM Tris.HCl pH 7.0 the radioactivity and turbidity (absorbance at 600 nm) were determined for each fraction. ■—■, ▲—▲, 125I cpm; ○—○, absorbance at 600 nm. In this experiment the reconstituted vesicles, isolated at the ρ = 1.035:ρ = 1.01 interface, were composed of 180 μg spectrin/mg of vesicle protein.
This method of density flotation afforded clean separation of bound from free spectrin and thus independently confirms that spectrin is associated with the IOV surface.

SDS gels of reconstituted vesicles isolated from dextran gradients show that the spectrin bands are present in equimolar amounts accounting for up to 20% of the total vesicle protein (data not shown). Furthermore the specific activity of the bound spectrin is 94% that of the initial spectrin preparation. These observations suggest that both chains of spectrin are associating in physiological amounts and that the behaviour observed is a property of the bulk spectrin preparation and not due to a class of atypically labelled molecules.

<table>
<thead>
<tr>
<th>Table 1. Characterization of binding assay requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Activity</td>
</tr>
<tr>
<td>I Buffer</td>
</tr>
<tr>
<td>80 mM Phosphate pH 7.0, Na+</td>
</tr>
<tr>
<td>80 mM Phosphate pH 7.0, K+</td>
</tr>
<tr>
<td>80 mM Tris, pH 7.0</td>
</tr>
<tr>
<td>10 mM Phosphate pH 7.0, NaCl</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+ 5 mM EDTA</td>
</tr>
<tr>
<td>+ 5 mM EGTA</td>
</tr>
<tr>
<td>+ 1.0 mM MgCl₂</td>
</tr>
<tr>
<td>+ 0.1 mM MgCl₂</td>
</tr>
<tr>
<td>+ 1.0 mM CaCl₂</td>
</tr>
<tr>
<td>+ 0.1 mM CaCl₂</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+ 1 mM ATP</td>
</tr>
<tr>
<td>+ 1 mM ATP, 1 mM Mg²⁺</td>
</tr>
<tr>
<td>+ 5 mM ADP</td>
</tr>
</tbody>
</table>

These data provide convincing evidence that spectrin binding to IOV is specific for the cytoplasmic surface of the erythrocyte membrane and has many of the features one would expect for physiological binding, such as salt dependence and saturability. The binding is not influenced by choice of buffers or monovalent salt, as shown in Table 1. Divalent cations, metal chelators, and adenine nucleotides also have little effect on spectrin binding.

**Thermal denaturation of spectrin**

The ability of spectrin to bind selectively to inside-out vesicles is rapidly lost as a result of heating of spectrin in the presence of 80 mM NaCl, as shown in Fig. 7. In agreement with the results of Bennett & Branton (1977) and earlier calorimetric studies (Brandts et al. 1977) we find that spectrin heated in the presence of salt shows a cooperative thermal inactivation centred around 50 °C. This is in marked contrast to the behaviour of spectrin heated in the presence of low salt (0.5 mM phosphate, pH 9.0, 0.5 mM β-Me) which retains 30% activity after 10 min at 98 °C. Circular
dichroism (CD) spectra of heated and unheated spectrin in PBS show a substantial loss of secondary structure resulting from heat treatment, as shown in Fig. 8.

The thermal denaturation observed and the data obtained from CD show that spectrin must be in a native folded conformation to bind to IOV. The data are consistent with the hypothesis that spectrin at low ionic strength is in a partially unfolded state which is converted rapidly to the native state as a result of restoration of tonicity. The ability of the low-ionic-strength state to withstand thermal denaturation yet rapidly recover its capacity to bind to membranes in response to salt makes it an ideal storage form of active, purified spectrin.

**The release of spectrin from reconstituted vesicles**

Spectrin is extracted from washed erythrocyte ghosts by incubating the membranes in low-ionic-strength buffers and divalent metal chelators at low ionic strength. This process is enhanced by alkaline pH and elevated temperatures (Marchesi, 1974). Table 2 demonstrates that such procedures are also effective in releasing spectrin from reconstituted IOV. Spectrin release appears to be an inverse function of ionic strength.
D. Litman, C. J. Hsu and V. T. Marchesi

(Experiment I), the process being stimulated by elevated temperatures and inhibited by 1 mM MgCl₂ (Experiments II and III). These observations parallel those on ghosts mentioned above and strongly suggest that the mode of attachment of spectrin to IOV is physiologically significant.

![Graph](image)

Fig. 8. CD spectra of thermal-denatured spectrin. (—) is spectrin (0.204 mg/ml in PBS) purified by the SDS gel filtration method (Fig. 1). (---) is the same protein solution heated at 65 °C for 20 min. The signal to noise ratios at 222 nm were 25:1, at 208 nm were 20:1 for both spectra, path length = 1 mm.

Table 2. Spectrin release from reconstituted vesicles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 5P7-o, 80 mM NaCl, 37 °C</td>
<td>13</td>
</tr>
<tr>
<td>5P7-o, 20 mM NaCl, 37 °C</td>
<td>25</td>
</tr>
<tr>
<td>5P7-o</td>
<td>45</td>
</tr>
<tr>
<td>o·5P7-o</td>
<td>51</td>
</tr>
<tr>
<td>II o·3P9-o, 0 °C</td>
<td>44</td>
</tr>
<tr>
<td>o·5P9-o, 37 °C</td>
<td>75</td>
</tr>
<tr>
<td>o·3P9-o, 37 °C, 1 mM Mg²⁺</td>
<td>47</td>
</tr>
<tr>
<td>III o·1 mM EDTA, o·5 mM β-Me, 0 °C</td>
<td>23</td>
</tr>
<tr>
<td>o·1 mM EDTA, o·5 mM β-Me, 37 °C</td>
<td>80</td>
</tr>
<tr>
<td>o·1 mM EDTA, o·5 mM β-Me, 1 mM MgCl₂, 37 °C</td>
<td>20</td>
</tr>
</tbody>
</table>

Equilibrium analysis of spectrin binding

Scatchard analysis (Scatchard, 1949) of spectrin-binding isotherms reveals a population of high-affinity binding sites on the cytoplasmic surface of inside-out vesicles as seen in Fig. 9. Up to 200 μg of spectrin can bind per mg of vesicle protein at saturation with an association constant, $K_a$, greater than $10^7$. The IOV therefore
are able to reconstitute approximately 75% of the spectrin typically associated with washed erythrocyte ghosts. The decrease in binding capacity of the IOV may reflect some loss of acceptor sites or the fact that some of the cytoplasmic surface is inaccessible to spectrin molecules by virtue of being within other vesicles. Plotting the binding data according to the Hill equation, shown in Fig. 9B, shows a straight line with a slope of 1.01. The slope, or Hill coefficient C, is an empirical measure of the cooperativity of a binding process. A slope of 1.0 is characteristic of a non-cooperative or stochastic process in which the binding of one spectrin dimer does not affect the affinity of subsequent spectrin binding.

Scatchard analysis can be used as an analytical tool to describe and compare the binding characteristics of different types of spectrin or vesicle preparations. In Experiment I of Table 3 we show that sealed and unsealed IOV bind spectrin with the same affinity, but that there are more exposed acceptor sites in populations of unsealed vesicles. Experiment II clearly shows that SDS-spectrin and isotonic spectrin have nearly identical association constants and saturate vesicle acceptor sites to the same extent. This observation suggests strongly that the binding site or sites
of spectrin renature after SDS removal and assume an active conformation, in agree-
ment with circular dichroism data presented above. By this same approach we have
shown that spectrin binding to IOV is not affected by radiolabelling procedures.
Bolton–Hunter modifications of lysines and LPO-catalysed iodination of tyrosines
yield spectrin populations that have equivalent binding properties (data not shown).
The inclusion of 1 mM MgCl₂ in the binding reactions has no appreciable effect on
the association constant or the extent of spectrin binding (data not shown).

Table 3. Affinity of sealed and unsealed IOV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K_a$</th>
<th>$\mu g/ml$ vesicle protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I SDS–Spectrin, LPO labelled, binding to</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Sealed inside-out vesicles</td>
<td>$1.6 \pm 0.8 \times 10^7$</td>
<td>$94 \pm 18$ $n = 5$</td>
</tr>
<tr>
<td>(b) Unsealed inside-out vesicles</td>
<td>$1.5 \pm 0.7 \times 10^7$</td>
<td>$165 \pm 50$ $n = 4$</td>
</tr>
<tr>
<td>II (a) SDS–Spectrin, IOV</td>
<td>$1.2 \times 10^7$</td>
<td>$210 \mu g c = 0.98$</td>
</tr>
<tr>
<td>(b) Isotonic–Spectrin, IOV</td>
<td>$1.1 \times 10^7$</td>
<td>$205 \mu g c = 1.01$</td>
</tr>
</tbody>
</table>

The results presented above show that spectrin associates selectively and with high
affinity to the cytoplasmic surface of the red cell membrane. SDS and isotonic
preparations are equally competent as ligands, binding is not affected by which
amino acid is labelled with iodine, and magnesium at 1 mM does not appreciably
affect spectrin binding.

Binding of specific spectrin subunits

There is evidence that spectrin exists both in situ and in solution as an $\alpha$, $\beta$ dimer
of Bands 1 and 2 (Kirkpatrick, 1976). There is also evidence that these polypeptides
are chemically and immunologically distinct (Hsu, Lemay, Eshdat & Marchesi,
1979; Kirkpatrick, Rose & LaCelle, 1978). Isolating the individual spectrin chains
by preparative SDS techniques enables us to address the question as to which of the
subunits is responsible for membrane association. Preparations of iodinated subunits,
greater than 90% pure as judged by SDS-PAGE, were assayed for their capacity to
bind selectively to IOV in the standard spectrin binding assay. As shown in Fig. 10A,
B, the 2 subunits behave quite differently when analysed by Scatchard plots. Band 2
binds selectively to IOV with high affinity, $K_a = 4.3 \times 10^6$, and exhibits saturation at
$109 \mu g$ Band 2/mg vesicle protein. The Hill coefficient of Band 2 binding is 1.06,
indicative of a non-cooperative process. Band 1, unlike Band 2, shows no saturable
binding to IOV. Band 1 also shows a greater tendency to aggregate and interact non-
specifically with right-side-out vesicles (data not shown).

On the basis of these results we suggest that the Band 2 moiety of spectrin is chiefly
responsible for spectrin association with red cell membranes. This thesis is supported
by the fact that Band 2 binds selectively with high affinity to the same number of
sites as are occupied by spectrin dimer in analogous reconstitution assays. Further-
more, Band 2 is capable of competing with labelled spectrin for vesicle-binding sites.
Spectrin binding to red cell membrane

The affinity constant for Band 2 binding is less than half that of spectrin dimer but this property may be modified as a result of a Band 1:Band 2 association, or this may be a reflexion of some loss of activity in the course of Band 2 preparation.

![Graph](image)

Fig. 10. The selective binding of spectrin Band 2 to 49 µg of IOV plotted according to the Scatchard equation as described above. In this particular experiment $n$ was determined to be 120 µg/mg vesicles protein, and $K_a$ was found to be $4.0 \times 10^3$. The data plotted according to the Hill equation are shown in the inset. $C$ was determined to be 1.6. A Scatchard analysis of the binding of purified spectrin Band 1 to 49 µg of IOV is shown in Fig. B.

Proteolytic digestion of inside-out vesicles

In order to identify the site or sites on the cytoplasmic surface of the membrane which bind spectrin, we have subjected IOV to controlled proteolytic digestion and compared the kinetics of protein digestion with those of vesicle inactivation. The suspicion that protein components are involved in spectrin binding is strengthened by the observations that sulphhydryl reagents inhibit spectrin association (Hsu, Litman & Marchesi, in preparation), and that protein perturbants which release peripheral proteins from inside-out vesicles, for example, $p$-chloromercuribenzenesulphonate, dimethyl maleic anhydride, NaOH, and lithium diiodosalicylate, all inactivate IOV with respect to spectrin binding (Litman, Hsu & Marchesi, unpublished observations).

When submicrogramme amounts of trypsin or papain are incubated with IOV, they become incapable of binding spectrin. Concomitant with loss of spectrin binding,
the membrane proteins are degraded at different rates. Membrane polypeptides can be roughly segregated into 3 kinetic classes as follows. Class 1: those polypeptides which are degraded much faster than the loss of binding activity (these include Band 3 and Band 2.1); class 2: those proteins which degrade with approximately the same kinetics as vesicle inactivation, principally band 4.1, and class 3: those polypeptides which are degraded much slower than vesicle inactivation (these include Bands 1, 2, 4.2, 6, 7 and the glycophorins (PAS 1, 2, 3)). Gels stained for carbohydrate show that there is no detectable proteolysis of glycophorin A by any of the concentrations of papain or trypsin used.

These results suggest that one or more protein components are critically involved in spectrin binding. Class 3 proteins, which remain undigested after the inactivation of vesicle binding, are not likely to be directly involved in spectrin interaction. Class 1 and class 2 proteins, which include Bands 3, 4.1, and 2.1, are the best candidates for the spectrin anchor by virtue of their digestion kinetics. Although Bands 3 and 2.1 are digested more rapidly than loss of binding activity it is conceivable that hydrophobic segments of these proteins and their cytoplasmic extensions remain with the lipid bilayer, and contain the spectrin-binding region of the molecule. On the basis of these digestion experiments and other evidence that will be presented below it seems reasonable to suppose that Band 4.1 is at or near the spectrin-binding site. Digestion by trypsin, although qualitatively different in some respects, yields data entirely consistent with the results of the papain digestion.

Blocking of spectrin acceptor sites by immune IgG

The ability of specific antisera to block spectrin binding to IOV was tested with the IgG fractions of rabbit and sheep antisera directed against Bands 3, 4.1 and 4.2, and glycophorin A. Anti-Band 3, sera raised in sheep, was characterized as being directed exclusively against the cytoplasmic extension of the Band 3 molecule (Fukuda et al. 1978). Antiglycophorin sera are specific for the C-terminal cytoplasmic extension of glycophorin A and has been characterized previously (Cotmore et al. 1977). Anti 4.1 and 4.2 sera were raised in rabbits in response to immunization with a mixture of purified Bands 4.1 and 4.2.

Iodinated IgG fractions of immune sera against Bands 3, 4.1 and 4.2, and glycophorin A all bind to IOV in a concentration-dependent manner and show specificity for the cytoplasmic membrane surface. Fig. 11A shows the binding of anti-Band 3 IgG to IOV. The specific binding, defined as the binding of immune IgG minus that of nonimmune IgG exceeds 500 µg IgG bound/mg of vesicle protein at saturation. Increasing amounts of anti-B3 IgG, but not nonimmune IgG, inhibit spectrin binding to IOV to the extent of nearly 80% as shown in Fig. 11B. Similar results are obtained with anti 4.1 and 4.2 IgG. Anti-glycophorin A IgG, although capable of binding to IOV, shows no inhibition of spectrin binding above nonimmune controls (data not shown).

The specific inhibition of spectrin binding, defined as the inhibition by immune IgG minus the inhibition by nonimmune IgG, plotted as a function of IgG bound per mg of vesicle protein is shown in Fig. 12. It is clear from this figure that both anti-
Spectrin binding to red cell membrane

Band 3 and anti 4.1 and 4.2 inhibit spectrin binding as a monotonic function of the amount of IgG bound. Anti-glycophorin A, in contrast, is ineffective in competing for or blocking spectrin-binding sites. The fact that the specific inhibition increases linearly as a function of IgG bound without an appreciable lag suggests that individual IgG-binding events are responsible for blocking the spectrin-binding sites. The lack of cooperativity of the inhibition as well as the negative glycophorin control make it unlikely that inhibition is due to an artifact arising from accumulated bulk of IgG bound to the vesicle surface. This is also suggested by the different slopes of the inhibition curves, which indicate that the nature of the determinant to which the IgG is bound is critical in effecting the inhibition.

The specificity and linearity of the IgG-mediated inhibition, especially at sub-saturating amounts of bound IgG, suggest that both Bands 3 and 4.1, 4.2 are

Fig. 11. A, the binding of 125I-labelled sheep anti-Band 3 IgG to 47 μg of IOV is shown as a function of IgG concentration. Indicated amounts of immune (■—■) or nonimmune (○—○) IgG were incubated with IOV for 30 min at 37 °C in 250 μl of PBS. Vesicles were pelleted at 18000 rev/min in the JA 14 rotor for 15 min. Supernatants were removed and the IgG was quantified in the pellet by counting. Δ—Δ denotes specific binding (i.e. μg immune IgG bound—μg nonimmune IgG bound).

B, anti-Band 3 IgG-mediated inhibition of spectrin binding to IOV. Unlabelled immune anti-Band 3 IgG or nonimmune IgG were incubated with vesicles as in A. After incubation 9.5 μg of 125I-spectrin were added to each sample and incubation was continued for 20 min, at which time the vesicles were pelleted to determine the amount of bound spectrin. The amount of bound spectrin in the absence of immune or non-immune IgG was taken as 100 % binding or 0 % inhibition. All points are averages of duplicate determinations.
either part of or near the spectrin-binding locus. Although this experiment does not exclude the participation of other spectrin-binding proteins, it suggests that the binding site must either include or be proximal to Bands 3 and 4.1, 4.2.

In conclusion, these experiments confirm the general impression gained from earlier labelling studies and from more recent studies on binding to IOV (Bennett & Branton, 1978), that spectrin binds to a population of high-affinity sites along the inner surface of the red cell membrane. The binding of spectrin to the inner surfaces of ghost membranes is dependent upon a minimum salt concentration for maximum binding, but the binding is not dependent on the presence of either calcium or magnesium. Of the 2 spectrin subunits (Bands 1 and 2) only the smaller subunit (Band 2) can bind to the inside-out ghost membrane when isolated separately, and it does so with almost the same avidity as both chains together. Purified Band 1 cannot bind alone nor can it compete with the dimeric form. The binding of native spectrin (Bands 1 and 2) can be completely inhibited if spectrin is denatured by heat treatment.

Preincubation of red cell ghost membranes with proteolytic enzymes blocks the capacity of these membranes to bind spectrin molecules. If this proteolytic digestion is carried out in a stepwise fashion it can be seen that some membrane proteins are more likely to be involved in the spectrin-binding site than others. This impression was confirmed by more direct experiments using specific antibodies directed against the cytoplasmic segments of certain membrane proteins. On the basis of antibody-competition studies it appears that Bands 3 and 4.1 and/or 4.2 must be at or near the

Fig. 12. The effect of binding increasing amounts of anti-Band 3 IgG (●—●), anti 4.1, 4.2 IgG (○—○), and anti-glycophorin A (▲—▲) on the ability of IOV to bind spectrin. The specific inhibition is defined as the inhibition of spectrin binding in the presence of immune IgG minus the inhibition in the presence of nonimmune IgG. IgG-binding reactions and spectrin binding assays were performed as described in Fig. 11 A, B and in Methods.
Spectrin binding to red cell membrane

Spectrin-binding site. Since recent studies implicate Band 2.1 as one of the membrane proteins involved with spectrin binding (Luna et al. 1979; Bennett & Stenbuck, 1979), the present findings suggest that spectrin attaches to an exposed segment on the Band 2.1 molecule which is located immediately adjacent to or perhaps in some kind of noncovalent complex with Bands 3 and 4.1-4.2.

Taken together these findings demonstrate that spectrin associates with specific proteins of the red cell membrane when it is recombined with inverted membrane vesicles. It is likely that these associations mimic those in the intact cell, but it is still far from clear how spectrin stabilizes the lipid bilayer or how it may be involved in maintaining the shape of red blood cells. It is also worth pointing out that these reconstitution studies do not provide any hints as to how or even whether actin plays any role in spectrin associations with the membrane (Sheetz & Singer, 1977). These experiments also do not rule out direct associations between spectrin and specific membrane lipids.

This work was supported by Membrane Center Grant GM-21714.

REFERENCES


(Received 9 July 1979)