Selective Association of Spectrin with the Cytoplasmic Surface of Human Erythrocyte Plasma Membranes

Quantitative Determination with Purified $^{32}$P-Spectrin*

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A specific association between spectrin and the inner surface of the human erythrocyte membrane has been examined by measuring the binding of purified $^{32}$P-spectrin to inside out, spectrin-depleted vesicles and to right side out ghost vesicles. Spectrin was labeled by incubating erythrocytes with $^{32}$P, and eluted from the ghost membranes by extraction in 0.3 mM NaPO$_4$, pH 7.6. $^{32}$P-Spectrin was separated from actin and other proteins and isolated in a nonaggregated state as a $S_{10,w} = 7$ S (in 0.3 mM NaPO$_4$) or $S_{10,w} = 8$ S (in 20 mM KCl, 0.3 mM NaPO$_4$) protein after sedimentation on linear sucrose gradients.

Binding of $^{32}$P-spectrin to inverted vesicles devoid of spectrin and actin was at least 10-fold greater than to right side out membranes, and exhibited different properties. Association with inside out vesicles was slow, was decreased to the value for right side out vesicles at high pH, or after heating spectrin above 50° prior to assay, and was saturable with increasing levels of spectrin. Binding to inverted vesicles was rapid, unaffected by pH or by heating spectrin, and rose linearly with the concentration of spectrin. Scatchard plots of binding to inverted vesicles were linear at pH 7.6, with a $K_D$ of 45 $\mu$g/ml, while at pH 6.6, plots were curvilinear and consistent with two types of interactions with a $K_D$ of 1 and 19 $\mu$g/ml, respectively. The maximal binding capacity at both pH values was about 200 $\mu$g of spectrin/mg of membrane protein. Unlabeled spectrin competed for binding with 50% displacement at 27 $\mu$g/ml.

$^{32}$P-Spectrin dissociated and associated with inverted vesicles with an identical dependence on ionic strength as observed for elution of native spectrin from ghosts. MgCl$_2$, CaCl$_2$ (1 to 4 mM) and EDTA (0.5 to 1 mM) had little effect on binding in the presence of 20 mM KCl, while at low ionic strength, MgCl$_2$ (1 mM) increased binding and inhibited dissociation to the same extent as 10 to 20 mM KC1.

Binding was abolished by pretreatment of vesicles with 0.1 M acetic acid, or with 0.1 $\mu$g/ml of trypsin. The periodic aci-Schiff-staining bands were unaffected by trypsin digestion which destroyed binding; mild digestion, which decreased binding only 50%, converted Band 3 almost completely to a membrane-bound 50,000-dalton fragment resistant to further proteolysis.

These experiments suggest that attachment of spectrin to the cytoplasmic surface of the membrane results from a selective protein-protein interaction which is independent of erythrocyte actin. A direct role of the major sialoglycoprotein or Band 3 as a membrane binding site appears unlikely.

Biological membranes are currently viewed, to a first approximation, as dynamic structures whose components are capable of rapid lateral diffusion within the two dimensional plane of a phospholipid bilayer (1-3). Membrane proteins, however, may exhibit a restricted distribution such as the static arrays observed in neuromuscular (4) and gap (5) junctions. Moreover, the lateral diffusion of membrane molecules can be nonrandom, as in formation of "caps" of labeled surface components of lymphocytes over one pole of the cell (6). These examples and others (reviewed in Ref. 7 and 8) indicate possibilities for long range interactions and regulation in the membrane which are not predicted on the basis of a highly fluid and disordered environment.

The human erythrocyte may provide a model system for studying the mechanisms that can influence long range interactions in cell membranes. Evidence is accumulating that spectrin (Bands 1 and 2) (9-11), and possibly erythrocyte actin (12), which are peripheral membrane proteins located on the cytoplasmic surface of erythrocyte ghosts (13), may be involved in control of the lateral distribution of the major membrane glycoproteins. Antispectrin immunoglobulin trapped within erythrocyte ghosts induces clustering of colloidal iron hydroxide binding sites (14, 15), which are thought to represent the sialoglycoproteins. Previous reports from this laboratory (16-19) have focused on the topography of the intramembrane particles revealed by freeze-fracture electron microscopy (20-21). These structures represent proteins intercalated into the membrane bilayer (19, 22-24) and are associated with receptors for anti-A blood group antibody (25), influenza virus (26), phytohemagglutinin (26, 27) colloidal iron hydroxide (28), and concanavalin A (29). The particles are normally dispersed in the plane of the membrane but after pretreatment of ghosts under conditions that dissociate spectrin and actin, rearrange-

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Nomenclature for human erythrocyte proteins according to Stock (30).
The attachment of particles to form clusters may be induced by manipulation of pH or ionic strength (16). A direct association between spectrin (and possibly actin) and the intramembrane particles has been proposed (17) because it was found that conditions which promote precipitation of extracted spectrin and actin (low pH, Ca**+, Mg**+, basic proteins) also induce aggregation of particles in membranes partially depleted of spectrin. This hypothesis is supported by the recent demonstration that aggregation of particles reconstituted in liposomes proceeds only at low pH and after binding of spectrin and erythrocyte actin (18, 19).

An alternative explanation for restraint of intramembrane particles is that the cytoplasmic portions of these structures are simply trapped within a meshwork of spectrin and actin without the formation of specific protein-protein associations (16, 30, 31). Little is known of the molecular nature of the spectrin-membrane association, the role, if any, that erythrocyte actin plays in this interaction, or the details of the organization of spectrin and actin on the inner membrane surface. Thus, on the basis of available data, neither entrapment nor binding of spectrin to the intramembrane particles can be excluded. Some cross-linking studies (32) indicate a close proximity between spectrin and multiple membrane proteins, although the affinity and specificity of these interactions cannot be determined.

The present experiments have been directed towards elucidating in biochemical terms the nature of the attachment of spectrin to the erythrocyte membrane. The approach adopted here has been to measure the interaction of purified spectrin with inside-out membrane vesicles depleted completely of spectrin and actin. A specific association between spectrin and the cytoplasmic surface of the membrane is characterized, and features are described strongly supporting a selective, proteinn-protein interaction. Analogous studies have been reported previously for binding of aldolase (33) and glyceraldehyde-3-phosphate dehydrogenase (34-36) to erythrocyte ghosts.

**EXPERIMENTAL PROCEDURES**

**Materials**

Carrier-free [**P**]orthophosphoric acid in 0.02 N HCl and Na**3** in 0.1 N NaOH were obtained from New England Nuclear. Trypsin (recrystallized three times), bovine liver catalase, and phosphatase B were from Worthington Biochemicals; 5'-ATP, adenosine, phenylmethylsulfonyl fluoride, dithiothreitol, bovine serum albumin, human fibrinogen, human IgG, ovomucoid, and wheat germ lectin were purchased from Sigma. Dextran T100, dextran blue, and 4% agarose were purchased from Pharmacia, and benzylpenicillin, sodium salt (2525 units/ml) was from Nutritional Biochemicals. Wheat germ agglutinin as a membrane marker (42, 43). The microsomal fraction of human liver was prepared according to Marchesi (37) and radioiodinated as described by Cuatrecasas (38).

**Methods**

**Preparation of Erythrocyte Ghosts—** Freshly drawn blood, anticoagulated in acid citrate/dextrose, is centrifuged (2 min at 900 x g) and the red cells resuspended in 310 mosM NaCl. Following three washes in this solution, the erythrocytes are lysed in 20 to 30 volumes of 20 mosM NaPO₄, pH 7.6, at 0°C (39), and immediately centrifuged (10 min at 2°, 18,000 rpm) in a Sorvall SS34 rotor. After carefully aspirating the viscous bulb of granulocyte debris, which may contain protease activity (40), the membrane pellet is resuspended in the same buffer and centrifuged once more. The ghosts are washed three to four times in a similar manner until white.

**Purification of Spectrin—** Erythrocyte ghosts from 3 ml of packed cells are prepared and given a final wash in 0.3 mosM NaPO₄, pH 7.6, at 0°C. The membranes are resuspended in a final volume of 3 ml in 0.3 mosM NaPO₄, pH 7.6, incubated for 25 min at 37°C, and the suspension centrifuged (15 min, 225,000 x g). The resulting supernatant, (A₂₈₀ = 0.4 to 0.6) is referred to in this report as the low ionic strength extract, contains 60 to 80% of the erythrocyte spectrin and almost all of Band 5 or erythrocyte actin. KCl is added to this solution to a final concentration of 20 mm, and 0.8 to 1 ml portions are layered onto 12 ml linear sucrose gradients (5 to 20%, w/v) in SW 40 centrifuge tubes with a 0.5 ml cushion of 20% sucrose and containing 30 mosM NaCl, pH 7.6. Following centrifugation at 20,000 x g for 18 h at 40,000 rpm in a SW 40 rotor, fractions (0.8 ml) are collected, and protein estimated by absorbance at 280 nm. The major peak usually has 0.14 to 0.25 mg/ml of protein, and contains only Bands 1 and 2 when analyzed by SDS-polyacrylamide electrophoresis. Such preparations may be stored at least 3 weeks at 4°C without detectable degradation or appearance of new bands.

**Preparation of **[**P**]Spectrin—** Three milliliters of packed erythrocytes from freshly drawn blood are washed twice in 310 mosM NaCl and resuspended in 40 ml of a solution containing NaCl (130 mm), KCl (3 mm), NaHCO₃ (20 mm), MgCl₂ (2 mm), CaCl₂ (1 mm), and benzylpenicillin (200 units/ml) at pH 7.5. The cells are partially depleted of ATP and other phosphorylated metabolites (41) during a 3- to 5-h incubation at 37°C, and then pelleted and added to 8 ml of a solution containing NaCl (130 mm), NaHCO₃ (25 mm), KCl (3.7 mm), MgCl₂ (2.5 mm), CaCl₂ (1.2 mm), glucose (10 mm), glucose (12 mm), benzylpenicillin (400 units/ml), and Na**3** (0.62 mm, 2.5 Ci/mmol) at a pH of 7.5. Phosphorylation of membrane components with **[**P**]** proceeds during incubation for 12 to 15 h at 37°C, after which time the cells are washed twice in cold 310 mosM NaCl, and ghosts prepared as described above.

**[**P**]Spectrin is purified as described above with the modification that in addition the sucrose gradients contain ATP (50 μM), dithiothreitol (1 mM), and MgCl₂ (0.1 mM). These components increase the stability of spectrin as determined by binding to inverted vesicles (see below), but do not alter sedimentation behavior at 2°C or the purity of the protein. The specific activity varies from 0.0 to 3600 cpm/μg of protein, or 0.2 to 0.4 Ci/mmol of spectrin, assuming a molecular weight of 460,000. The binding to inverted vesicles of **[**P**]**spectrin stored at 0°C may decrease by up to 60% after 3 days, although no proteolysis is detected by electrophoresis, and **[**P**]** labeling is still intact. The experiments reported here were routinely completed within 48 h of preparation.

**Assay of **[**P**]**Spectrin Binding to Membrane Vesicles—** **[**P**]**spectrin (5 to 20 μg, 700 to 1600 cpm/μg) is incubated at 0°C in plastic test tubes (12 x 75 mm) in a 0.25-m1 volume containing 20 to 50 μg of membrane protein, and various concentrations of KCl, NaPO₄, dithiothreitol, MgCl₂, and sucrose as described in the figure legends. The free and membrane-bound **[**P**]**spectrin are separated by layering 0.2 ml of the samples over 0.2 ml of 20% sucrose, 20 mM KCl, 0.7 mM NaPO₄, pH 7.6, in polyethylene microfuge tubes (0.5-ml capacity), followed by centrifugation at 4°C for 45 min in a Sorvall SS34 rotor. Under these conditions, more than 90% of the membrane vesicles are pelleted as determined with **[**3**]**-labeled wheat germ agglutinin as a membrane marker (42, 43). The microfuge tubes are frozen in liquid nitrogen and the tips containing the membrane pellets cut off and placed in glass vials containing 5 ml of Aquasol, and the radioactivity determined in a Beckman liquid scintillation counter. Blank samples containing no membranes exhibit no Cpm counts above background levels. Recoverability of the values obtained by this method is usually within 5%. Unless indicated otherwise, data points were determined in duplicate.

**Preparation of Spectrin-depleted Inverted Vesicles—** Ghosts prepared from 5 ml of packed erythrocytes are resuspended in 30 volumes of 0.3 mosM NaPO₄, pH 7.6, and incubated for 30 min at 37°C. This incubation dissociates spectrin and actin from the membranes and reduces the ghosts to small vesicles (<1 μm). Following centrifugation (40 min at 19,000 rpm, SS34 rotor), the membrane pellet is resuspended in 10 to 15 ml of 0.3 mosM NaPO₄, pH 7.6, and laid over 15 ml of T110 dextran (10% w/v) in 0.3 mosM NaPO₄, pH 7.6, 0.5 mosM NaCl. The vesicles are then centrifuged for either 30 min at 50,000 rpm in a 60 Ti rotor, or overnight at 75,000 rpm in a SW 27 rotor. Two major membrane bands are detectable, one at the dextran-buffer interface containing about 15% of the membranes and retaining 1 to 2% of Bands 1 and 2, and the other (about 80%) in a loosely packed pellet which is nearly completely lacking spectrin. Occasionally the membranes are resolved into two bands which have not been characterized. The vesicles which pelleted through the dextran were washed once in 90 mosM KCl, 0.7 mosM NaPO₄, 0.5 mosM NaCl, 3 mosM KCl, and 5 mosM NaCl, pH 7.6, following which the sediments were collected, diluted with 0.1 mosM NaCl, and centrifuged as before.

The abbreviations used are: SDS, sodium dodecyl sulfate; Aₐ, absorbance.
Ai% of 10.1 (ll), or by intrinsic fluorescence (excite 295 nm, emit which case the Lowry method was used. SDS-polyacrylamide elec-
ermame the protein content of spectrin and membranes directly, in
cytoghosts at low ionic strength in the absence of divalent
metal ions has been noted previously (g-12,16,40,47-49). The

denudated inverted vesicles. Freeze-fracture electron microscopy (Fig.
ts from fresh blood were suspended in a 0.2-ml volume containing
NaPO4, 0.3 mm, pH 7.6, 0.2 mg/ml of membrane protein, and
various concentrations of KCl. After incubation for 30 min at 37°,
the samples were sonicated three times for 1 s at 30 watts, layered onto
0.2 ml of 20% sucrose in microfuge tubes (0.5-ml capacity), and
centrifuged (30 min at 20,000 × g). The resulting membrane pellets
were analyzed by electrophoresis in the presence of 0.2% SDS on 5%
polyacrylamide gels. Following staining with Coomassie blue, the
gels were scanned at 550 nm, and the area under peaks correspond-
ting to spectrin (Bands 1 and 2) was estimated relative to that of Band
3. The values are expressed as the percentage (area Bands 1 + 2/area
Band 3) of a control sample maintained at 0° in 20 mM KCl, and were
determined in duplicate with an average half-range of ±8%. Con-
trols, in which the extracted spectrin was centrifuged without mem-
brane, indicated that less than 4% of spectrin was pelleted through
the 20% sucrose when centrifuged in the absence of membrane.

Membrane protein was estimated by the method of Lowry et al.
(46) with bovine serum albumin as a standard, while spectrin
concentrations were determined by absorbance at 280 nm assuming an
A 280 of 10.1 (11), or by intrinsic fluorescence (excite 295 nm, emit at
338 nm, 10 nm band widths). Occasionally it was necessary to com-
pare the protein content of spectrin and membranes directly, in
which case the Lowry method was used. SDS-polyacrylamide elec-
trophoresis was performed essentially as described by Fairbanks et
al. (40). Freeze-fracture electron microscopy was conducted as previ-
ously described (17-19).

RESULTS

The elution of spectrin and erythrocyte actin from erythro-
cyte ghosts at low ionic strength in the absence of divalent
metal ions has been noted previously (9-12, 16, 40, 47-49). The
quantitative dependence of spectrin dissociation on salt concen-
tration at pH 7.6 (Fig. 2), indicates an abrupt transition in
stability of the membrane-spectrin complex below about 10
mm KCl; erythrocyte actin is eluted in a similar manner (not
shown). These measurements were performed after a 25-min
incubation at 37°, at which time elution is essentially complete
(40). A similar dependence on ionic strength is evident at
lower temperatures, although the time required for equilib-
rium is increased. Qualitatively similar results have been
obtained with other salts, including NaCl, NaPO4, and even
the ionic detergent, sodium cholate. Although fresh blood has
been used in this study, nearly identical measurements have
been made with blood aged for 7 weeks at 4°. This procedure
for eluting spectrin and erythrocyte actin avoids use of EDTA,
which, in the absence of Ca2+ or Mg2+, promotes irreversible
loss of function of muscle actin (50).

Rate Zonal Sedimentation of Spectrin

Spectrin and actin, eluted from erythrocyte ghosts at low
ionic strength, sediment as two separate proteins on linear
sucrose gradients. More than 50% of the spectrin migrates at a
S15, w of about 8 in 20 mM KCl and 7 at low salt (Fig. 3A),
assuming a partial specific volume of 0.73 (47). Some rapidly
sedimenting protein which may represent aggregated mate-
rial is present under both conditions. Analysis of gradient
fractions by SDS-polyacrylamide electrophoresis demonstrates
that the peaks of spectrin in 20 mM KCl (Fig. 4E) and in low
salt (not shown) contain no detectable actin or significant
amounts of protein other than Bands 1 and 2. Erythrocyte
actin is distributed in Fractions 11 and 12 in both gradients.
Sedimentation on sucrose gradients provides a simple means
of purifying spectrin to electrophoretic homogeneity in a non-
aggregated form; such preparations are used exclusively in
this report.

V. Bennett and D. Branton, unpublished observation.
The basis of the ionic strength-dependent change in sedimentation velocity for spectrin is not known. The change is reversible (Fig. 3B), and other proteins examined, including bovine serum albumin, beef liver catalase, human IgG, and erythrocyte actin exhibited no change in sedimentation behavior under these conditions. Other experiments with spectrin in low and high ionic strength media\(^4\) also indicate sedimentation changes in the analytical ultracentrifuge as well as shape changes of the molecules visualized by electron microscopy. The important finding, in terms of this study, is that spectrin which has been isolated and purified undergoes a change in sedimentation characteristics dependent on ionic strength of the medium. These findings indicate spectrin is unlikely that the presence of in vivo bound spectrin will interfere with possible binding to the external surface of these vesicles since this spectrin is known to be localized on the cytoplasmic face of the membrane (13).

Preliminary results demonstrated that binding of spectrin to inverted vesicles could be detected by SDS-polyacrylamide electrophoresis. Bands 1 and 2 recombined in approximately equal ratios, and the binding was abolished completely by prior digestion of vesicles with trypsin. A reassociation assay was subsequently developed on the basis of radioactivity measurements. Many reports have demonstrated a cyclic AMP-independent phosphorylation of a protein co-migrating with Band 2 of spectrin following incubation of erythrocyte ghosts with \(\gamma-^{32}\)P]ATP (53-58). Furthermore, approximately 2 atoms of bound phosphate/polypeptide chain have been reported in preparations of spectrin purified in the presence of sodium deoxycholate (59). These findings indicate spectrin may be phosphorylated in vivo, and suggest that radiolabeled, native spectrin may be obtained by incubating erythrocytes with \(^{32}\)P, followed by the usual purification procedure. Spectrin prepared in this way exhibits a major peak of radioactivity co-migrating with Band 2, and a smaller amount (about 20%)

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\(^4\) D. M. Shotton and D. Branton, manuscript in preparation.
with Band 1 (Fig. 5, Table I). This $^{32}$P-labeled protein is eluted from erythrocyte membranes under the same conditions as spectrin (Fig. 12) and co-migrates with spectrin during sedimentation on sucrose gradients, and on gel filtration on 4% polyacrylamide gels. Three-milliliter packed erythrocytes from freshly drawn blood were partially ATP-depleted and then incubated for 14 h at 37° with [32P] (see "Methods"). Ghosts were prepared and [32P] spectrin isolated and purified as described under "Methods." Duplicate samples (50 μl) of ghosts (65 μg of protein, 980,000 cpm), low ionic strength extract (25 μg, 49,000 cpm) and puriﬁed spectrin (8 μg, 5,900 cpm) were analyzed by electrophoresis on 5% polyacrylamide gels in the presence of 0.2% SDS. One set of gels was sliced into 1-mm sections without staining and the other was stained with Coomassie blue. The arrows and numerals represent the position of protein bands on the gels stained with Coomassie blue. TD, tracking dye. Counts (cpm) (above) were determined from the total radioactivity recovered from the gels.

**Distribution of $^{32}$P label on SDS-polyacrylamide gels following incubation of erythrocytes with $^{32}$P.** A, erythrocyte ghosts; B, low ionic strength extract of erythrocyte ghosts; C, spectrin after sucrose gradient sedimentation. Three-milliliter packed erythrocytes from freshly drawn blood were partially ATP-depleted and then incubated for 14 h at 37° with [32P] (see "Methods"). Ghosts were prepared and [32P] spectrin isolated and purified as described under "Methods." Duplicate samples (50 μl) of ghosts (65 μg of protein, 980,000 cpm), low ionic strength extract (25 μg, 49,000 cpm) and puriﬁed spectrin (8 μg, 5,900 cpm) were analyzed by electrophoresis on 5% polyacrylamide gels in the presence of 0.2% SDS. One set of gels was sliced into 1-mm sections without staining and the other was stained with Coomassie blue. The arrows and numerals represent the position of protein bands on the gels stained with Coomassie blue. TD, tracking dye. Counts (cpm) (above) were determined from the total radioactivity recovered from the gels.

**Fig. 5. Distribution of $^{32}$P label on SDS-polyacrylamide gels following incubation of erythrocytes with $^{32}$P.** A, erythrocyte ghosts; B, low ionic strength extract of erythrocyte ghosts; C, spectrin after sucrose gradient sedimentation. Three-milliliter packed erythrocytes from freshly drawn blood were partially ATP-depleted and then incubated for 14 h at 37° with [32P] (see "Methods"). Ghosts were prepared and [32P] spectrin isolated and purified as described under "Methods." Duplicate samples (50 μl) of ghosts (65 μg of protein, 980,000 cpm), low ionic strength extract (25 μg, 49,000 cpm) and puriﬁed spectrin (8 μg, 5,900 cpm) were analyzed by electrophoresis on 5% polyacrylamide gels in the presence of 0.2% SDS. One set of gels was sliced into 1-mm sections without staining and the other was stained with Coomassie blue. The arrows and numerals represent the position of protein bands on the gels stained with Coomassie blue. TD, tracking dye. Counts (cpm) (above) were determined from the total radioactivity recovered from the gels.

**Table I**

| Fraction                  | [32P]Spectrin specific activity | [% in spectrin] | Subunit distribution of [32P] |
|---------------------------|---------------------------------|-----------------|-----------------------------|
| [32P]Spectrin alone       | 4.9 ± 0.8                       | 82%            | 19%                         |
| [32P]Spectrin and vesicles| 4.7 ± 0.7                       | 78%            | 15%                         |
| Membrane pellet           | 4.5 ± 0.2                       | 93%            | 20%                         |

* Gels were stained with Coomassie-blue and scanned with a Joyce Loebl densitometer, the bands were cut out, and the radioactivity was determined. Data is expressed as counts per minute per mm² of area of Bands 1 and 2 under densitometer scan ± 1 S.D. of triplicate determinations. The average recovered from Bands 1 and 2 was 1830 cpm for spectrin alone, 887 cpm for the supernatant, and 3517 cpm for the pellet.

* Expressed as percentage of total gel radioactivity obtained in Bands 1 and 2; average of three gels.

* Expressed as percentage of radioactivity in Bands 1 and 2; average of three gels.

**Specificity of Binding** — An important feature of a meaningful reassociation of spectrin is that binding should be restricted to the inner surface of the membrane. Binding of [32P]spectrin, measured under standard conditions, is at least 10-fold greater for inverted spectrin-depleted vesicles than right side out spectrin-containing membranes (Fig. 6). Furthermore, the association with inverted vesicles exhibits significant qualitative differences from that with everted membranes (see below), and over 80% of the binding capacity of inverted vesicles is lost following pretreatment of membranes with 0.1 M acetic acid (Fig. 6), or after mild digestion with trypsin (Table II). The loss of binding with acid could be due to denaturation of the "receptor" site(s) for spectrin or to elution of the peripheral membrane proteins which occurs with this treatment (60).³

**General Features** — The binding of [32P]spectrin to inverted vesicles progresses slowly at 0°, and is maximal at about 90 min, whereas the interaction with right side out vesicles is nearly complete at the earliest time examined (12 min) (Fig. 7). Electrophoresis of membrane-bound and free [32P]spectrin following a 90-min incubation demonstrates no significant dephosphorylation, or redistribution of label (Table II). Also, no proteolysis of spectrin or membrane proteins can be detected. The binding of spectrin to inverted vesicles increases linearly with membrane protein within the range of about 5 to 40 μg (Fig. 6).

The binding of spectrin to inverted vesicles, but not right side out membranes, is affected by pH (Fig. 8). Association is greatest at pH 6.6, decreases by about 50% at pH 7.6, and

![Image](http://www.jbc.org/)}
**[32P]Spectrin Binding to Inverted Human Erythrocyte Membranes**

Fig. 6 (left). Influence of increasing amounts of membrane protein on binding of [32P]spectrin to spectrin-depleted inverted vesicles before (A) and after (●) exposure to 0.1 M acetic acid, and to right side out vesicles (○). [32P]Spectrin (8.2 µg, 750 cpm/µg) was incubated for 90 min at 0°C in a 0.2-ml volume containing KCl (20 mM), NaPO₄ (0.7 mM, pH 7.6), MgCl₂ (1 mM), dithiothreitol (1 mM), sucrose (2.5%), and various amounts of membrane protein. Some of the inverted vesicles (●) were preincubated for 30 min at 0°C with 0.1 M acetic acid and washed once with 0.1 M NaPO₄, pH 7.6, and once with the incubation buffer. Membrane-bound radioactivity was determined as described under "Methods."

FIG. 7 (center). Time course of [32P]spectrin binding to inverted vesicles (●) and to right side out vesicles (○). [32P]Spectrin (6.6 µg, 800 cpm/µg) was added at various times to samples at 0°C (final volume 0.25 ml) containing KCl (20 mM), MgCl₂ (1 mM), dithiothreitol (1 mM), sucrose (3%), and either inverted vesicles (●) (27 µg of protein) or right side out vesicles (○) (52 µg of protein). As described under "Methods," 0.2-ml portions of the suspensions were analyzed for membrane-bound radioactivity. The incubation was assumed to extend until the centrifuge rotor attained maximal velocity.

FIG. 8 (right). Influence of pH on binding of [32P]spectrin to inverted vesicles (●) and to right side out vesicles (○). [32P]Spectrin (5.2 µg, 1,250 cpm/µg) was incubated for 90 min at 0°C in suspensions (0.25 ml) containing KCl (20 mM), MgCl₂ (1 mM), dithiothreitol (1 mM), sucrose (3%), either inverted vesicles (40 µg of membrane protein) (●) or right side out vesicles (51 µg of membrane protein) (○), and NaPO₄ (10 mM) at various pH values. Membrane-bound radioactivity of 0.2-ml aliquots was determined as described under "Methods."

**Dependence on Spectrin Concentration** — [32P]Spectrin binds to inverted vesicles in a saturable manner, while the extent of association with right side out membranes increases linearly with spectrin concentration (Fig. 10A). Scatchard plots of data corrected for the binding to right side out membranes (Fig. 10B) indicate at least two types of interactions at pH 6.6, with an approximate Kₛ for each of 4 to 19 µg/ml respectively. At pH 7.6, membrane protein are estimated by the Lowry method (46) with the concentration of spectrin and membranes assayed in the same determination.

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[5] Kₛ values are expressed in terms of protein concentration determined by A₅₅₀ values for maximal spectrin binding per mg of membrane protein are estimated by the Lowry method (46) with the concentration of spectrin and membranes assayed in the same determination.
the plot is linear and the $K_D$ is about 45 µg/ml (Fig. 10B). The inverted vesicles have an extrapolated capacity of about 200 µg of spectrin/mg of membrane protein at both pH values. The basis for the complexity of the binding isotherm at pH 6.6 is not clear. Negative cooperativity appears unlikely since a Hill plot is linear with a slope of nearly unity (Fig. 10B, inset), although more data are required to establish this point.

Competitive displacement of $[^{32}P]$spectrin binding by purified, unlabeled spectrin (Fig. 11) demonstrates that binding is saturable, and provides another means of estimating the affinity of the interaction. Fifty per cent of the binding is displaced by about 27 µg/ml of spectrin at pH 7.6, which is thus a minimal value for the $K_D$ under these conditions.

**Effect of Ionic Strength and Divalent Metal Ions on Association and Dissociation of $[^{32}P]$Spectrin**

Dissociation of reconstituted $[^{32}P]$spectrin from inverted vesicles depends on ionic strength (Fig. 12) just as does elution of spectrin from erythrocyte ghosts (Fig. 2). Association of $[^{32}P]$spectrin with inverted vesicles also depends critically on the salt concentration. Virtually no binding occurs with 0.3 mM NaPO$_4$, pH 7.6, while binding is maximal at 10 mM KCl, MgCl$_2$ (1 mM), dithiothreitol (1 mM), sucrose (5%), and either inverted vesicles (14 µg of protein) (A, B) or right side out vesicles (17 µg of protein) (C, D), membrane-bound radioactivity was determined as described under "Methods." The data are expressed as the average of duplicate determinations ± the half-range.

**TABLE II**

| Membrane treatment | $[^{32}P]$Spectrin binding | Membrane protein released |
|---------------------|-----------------------------|---------------------------|
| No addition         | 27 ± 1                      | 7                         |
| Trypsin, 0.1 µg/ml, 30 min, 0º | 13 ± 0.4                    | 11                        |
| Trypsin, 0.1 µg/ml, 10 min, 24º | 6 ± 0.2                     | 19                        |
| Trypsin, 0.1 µg/ml, 20 min, 24º | 4 ± 0.1                     | 21                        |
| Trypsin, 0.1 µg/ml, 30 min, 24º | 3 ± 0.1                     | 26                        |

Measured $[^{32}P]$Spectrin (7 µg, 1,100 cpm/µg) was incubated for 90 min at 0º in a suspension (0.25 ml) containing KCl (20 mM), MgCl$_2$ (1 mM), dithiothreitol (0.5 mM), NaPO$_4$ (2 mM pH 7.6), and 38 to 51 µg of membrane protein, and membrane-bound radioactivity determined (see "Methods"). Data is expressed as the average of duplicate determinations ± the half-range.

**Fig. 11.** Competitive displacement of $[^{32}P]$spectrin binding to inverted vesicles with purified unlabeled spectrin. $[^{32}P]$Spectrin (5.3 µg, 870 cpm/µg) was incubated for 90 min at 0º in 0.2-ml suspensions containing KCl (20 mM), NaPO$_4$ (0.7 mM, pH 7.6), MgCl$_2$ (1 mM), dithiothreitol (1 mM), sucrose (5%), either inverted vesicles (21 µg of protein) or right side out vesicles (41 µg of protein), and various concentrations of unlabeled spectrin purified by sucrose density gradient centrifugation. Membrane-bound radioactivity was determined (see "Methods"), and the specific binding to inverted vesicles calculated by subtracting the values for binding to right side out membranes, which did not exceed 5 µg/mg of membrane protein.

**Effect of Digestion with Trypsin**

Digestion of inverted vesicles with 0.1 µg/ml of trypsin for 30 min at 24º, followed by addition of phenylmethyalsulfonyl fluoride and thorough wash-
ing, abolishes over 90% of the binding by[^32P]spectrin (Table II) from inverted vesicles. Association (A) was determined by addition of[^32P]spectrin (10 μg, 1,065 cpm/μg) to samples (0.25 ml) containing finally NaPO₄ (0.3 mM, pH 7.6), dithiothreitol (0.4 mM), sucrose (4%), either inverted vesicles (30 μg of protein) or right-side out vesicles (32 μg of protein), and various concentrations of KCl. After 120 min at 0°, membrane-bound radioactivity was measured (see “Methods”) and the specific binding of[^32P]spectrin calculated by subtracting values for binding to right-side out vesicles from those of inverted vesicles. Dissociation of reconstituted[^32P]spectrin (B) was measured by diluting aliquots (0.1 ml) of a suspension containing[^32P]spectrin (98 μg/ml, 1,052 cpm/μg), KCl (20 mM), NaPO₄ (0.7 mM, pH 7.6), MgCl₂ (0.8 mM), dithiothreitol (0.8 mM), sucrose (5%), and inverted vesicles (0.39 mg/ml) to 8 ml after a 90-min incubation at 0°. The diluted samples, which contained NaPO₄ (0.7 mM, pH 7.6) and various concentrations of KCl, were incubated for 25 min at 37° and centrifuged for 60 min at 40,000 × g. The supernatants were aspirated and the membrane-bound radioactivity determined. The data are expressed as the percentage of[^32P]spectrin bound relative to the value determined for control vesicles; b, vesicles digested for 30 min at 0°; c, vesicles after 20 min at 24°.

**DISCUSSION**

This report demonstrates the feasibility of directly examining the interaction between spectrin and the inner membrane surface by measuring the binding of purified[^32P]spectrin to inverted vesicles. This approach is analogous, in principle, to previous studies with ligands, such as polypeptide hormones, which associate with the cell surface (66). Spectrin, however, does not exist normally in solution, and it is important to

**TABLE III**

| Addition | [^32P]spectrin binding (μg/mg membrane protein) |
|----------|-----------------------------------------------|
| **Experiment 1** | |
| 20 mM KCl | 17 ± 0.5 |
| + MgCl₂, 1 mM | 22 ± 1.4 |
| + MgCl₂, 2 mM | 96 ± 1.5 |
| + MgCl₂, 4 mM | 27 ± 0.6 |
| + CaCl₂, 1 mM | 22 ± 1.3 |
| + CaCl₂, 2 mM | 23 ± 1.2 |
| + CaCl₂, 4 mM | 27 ± 4 |
| + EDTA, 0.5 mM | 15 ± 2 |
| + EDTA, 1 mM | 13 ± 0.6 |
| **Experiment 2** | |
| 0.3 mM NaPO₄, pH 7.6 | 17 ± 0.1 |
| + MgCl₂, 1 mM | 20 ± 0.9 |
| + KCl, 2.5 mM | < 0.5 |
| + MgCl₂, 1 mM | 20 ± 1.4 |
| + KCl, 5 mM | 15 ± 0.7 |
| + MgCl₂, 1 mM | 20 ± 0.8 |
| + KCl, 10 mM | 21 ± 0.4 |
| + MgCl₂, 1 mM | 25 ± 0.5 |
| + KCl, 20 mM | 21 ± 0.4 |
| + MgCl₂, 1 mM | 25 ± 0.5 |

**Fig. 12.** Influence of increasing concentrations of KCl on the binding of[^32P]spectrin (A) and the dissociation of reconstituted[^32P]spectrin (B) from inverted vesicles. Association (A) was determined by addition of[^32P]spectrin (10 μg, 1,065 cpm/μg) to samples (0.25 ml) containing finally NaPO₄ (0.3 mM, pH 7.6), dithiothreitol (0.4 mM), sucrose (4%), either inverted vesicles (30 μg of protein) or right-side out vesicles (32 μg of protein), and various concentrations of KCl. After 120 min at 0°, membrane-bound radioactivity was measured (see “Methods”) and the specific binding of[^32P]spectrin calculated by subtracting values for binding to right-side out vesicles from those of inverted vesicles. Dissociation of reconstituted[^32P]spectrin (B) was measured by diluting aliquots (0.1 ml) of a suspension containing[^32P]spectrin (98 μg/ml, 1,052 cpm/μg), KCl (20 mM), NaPO₄ (0.7 mM, pH 7.6), MgCl₂ (0.8 mM), dithiothreitol (0.8 mM), sucrose (5%), and inverted vesicles (0.39 mg/ml) to 8 ml after a 90-min incubation at 0°. The diluted samples, which contained NaPO₄ (0.7 mM, pH 7.6) and various concentrations of KCl, were incubated for 25 min at 37° and centrifuged for 60 min at 40,000 × g. The supernatants were aspirated and the membrane-bound radioactivity determined. The data are expressed as the percentage of[^32P]spectrin bound relative to the value determined for control vesicles; b, vesicles digested for 30 min at 0°; c, vesicles after 20 min at 24°.

**Fig. 13.** SDS-polyacrylamide electrophoresis of inverted vesicles following limited proteolysis with trypsin. Membrane samples were digested with 0.1 μg/ml of trypsin as described in Table II, and analyzed for[^32P]spectrin binding (Table II) and by electrophoresis of 50-μl samples containing 40 to 50 μg of protein, (see "Methods", Fig 2). Panel A, densitometer scans of Coomassie blue-stained gels of a, control vesicles; b, vesicles digested for 30 min at 0°; c, vesicles after 20 min at 24°. Panel B, densitometer scans of samples as in Panel A stained by the periodic acid-Schiff (PAS) reaction. TD, tracking dye.
Spectrin has generally been obtained from a low ionic strength extract of erythrocyte ghosts which has been noted to form filaments or fibrils with addition of 50 to 100 mM salt and/ or 1 to 3 mM Ca²⁺ or Mg²⁺ (10, 12, 48, 67, 68). Such crude extracts contain Band 5 (M₉ = 43,000) in levels as high as 10%, which is about equimolar to the amount of spectrin, i.e. Bands 1 and 2, in the extract. Band 5 resembles muscle actin in that it forms filaments with the addition of salt, and is decorated with heavy meromyosin (12). Although erythrocyte actin may be separated from spectrin by gel filtration under some conditions (11, 47, 50), others have reported co-migration of these proteins (48, 69) as well as evidence for functionally significant interactions between spectrin and actin (12, 69). Band 5 and spectrin have very similar isoelectric points, and are both precipitated over a similar range of pH values (17). Thus, separation of spectrin from actin can be difficult but is essential if the ambiguities of filament formation and association with other peripheral proteins are to be distinguished from the intrinsic behavior of spectrin.

The physical state of spectrin in solution is presently uncertain. Cross-linking studies (11, 31) indicate association between Bands 1 and 2 rather than between 1 and 1 or 2 and 2, but the possibility of a higher polymer of (1–2), or (1–2), cannot be excluded. The stability and relative proportions of (1–2), and free 1 and 2 is not clear (49, 59, 70). A further difficulty is that isolated spectrin readily self-associates to form large aggregates (11, 47), although this may be prevented by the presence of detergents (59).

Spectrin utilized in this study was routinely purified by sedimentation on linear sucrose gradients in which Bands 1 and 2 migrate together as a single peak well separated from Band 5 and aggregated material. The availability of pure, nonaggregated spectrin which can be labeled with [³²P]phosphate, and a simple and rapid means of separating free and membrane-bound spectrin make binding studies technically possible. Evaluating such data is more difficult, however. No measurable membrane function is known to be lost or modified by elution of spectrin, and it is likely that in the intact cell spectrin performs a structural role which cannot be reproduced once vesicles are formed. Furthermore, spectrin is presumably attached to the membrane in vivo, and the binding process examined here has no demonstrated relevance to any known physiological event. In view of the lack of functional correlates of spectrin binding, it is essential to establish criteria which will distinguish specific and possibly biologically relevant, interactions from nonspecific adsorption. These features would be expected for a selective association resembling the attachment of spectrin in erythrocytes. (a) The ionic dependence of dissociation and association of [³²P]spectrin should be the same as in native membranes. (b) Binding should be limited to the inner surface of the membrane. (c) Binding should be abolished by treatments of spectrin, and possibly vesicles, which denature proteins. (d) Binding should be saturable at a level similar to the amount of spectrin present in vivo, and exhibit an appropriate affinity.

The binding of [³²P]spectrin observed in this study demonstrates properties consistent with these expectations. [³²P]Spectrin dissociates and associates with a nearly identical dependence on salt concentrations as observed for elution of spectrin from ghosts (Fig. 2, 12). MgCl₂ at 1 mM has the same effect on binding and dissociation of [³²P]spectrin as 10 to 20 mM KCl, which is in accord with reports that divalent metal ions prevent elution of spectrin from ghosts (40, 49).

The equilibrium constant for the [³²P]spectrin-membrane association is about 45 μg/ml at pH 7.6, while at pH 6.5, the high affinity component of binding occurs with a K₅₀ of 4 μg/ml (see Fig. 10). These concentrations correspond to roughly 10⁻⁷ M and 10⁻⁸ M, respectively, if a molecular weight of 460,000 is assumed, and are in the same range as obtained for association of glyceraldehyde-3-phosphate-dehydrogenase with erythrocyte membranes (34, 36). Inside out vesicles bind maximally about 200 μg of spectrin/mg of membrane protein in either pH, which is close to the amount of spectrin present in erythrocyte ghosts (30). Unlabeled spectrin competes effectively for [³²P]spectrin binding (Fig. 11).

These features of spectrin binding are necessary aspects of a specific association, but each one is insufficient in itself to provide a clear demonstration that actual reconstitution is occurring. However, considered together, the experiments strongly support the hypothesis that spectrin is reattaching to the original membrane site to which it is bound in the erythrocyte ghost. If such an interpretation is accepted, the loss of binding following digestion of vesicles with trypsin and pre-treatment with dilute acetic acid indicates that the attachment of spectrin to the membrane is due primarily to a protein-protein association. Functionally important interactions between spectrin and phospholipids may also occur in addition. Although spectrin does not associate with liposomes composed of phosphatidylcholine (18, 19), binding to extracted erythrocyte lipids has been detected. This interaction is not abolished by heat-denaturing spectrin, and its possible biological significance is not clear.

The affinity for binding of spectrin (10⁻⁷ to 10⁻⁸ M) suggests a relatively rapid rate of dissociation from its membrane site(s); a half-life of about 15 min at 0°, for example, can be calculated from the time course (Fig. 7) for a spectrin-membrane complex at pH 7.6 with a K₅₀ of 10⁻⁷ M. The apparent stability of the attachment of spectrin in unsealed ghosts indicates that additional interactions of spectrin with itself or other proteins (or both) may be operative in vivo but are not reconstituted under our experimental conditions. Thus, because the actual configuration of spectrin in erythrocytes may be intermediate between a loosely bound, self-sufficient network and a form irreversibly attached to the membrane, any controls which spectrin exerts on the topography of transmembrane elements (7, 14–19) should be considered in dynamic terms where the residence time of spectrin with its membrane site is finite and may be subject to regulation. It is pertinent that a significant lateral motion has been observed for intrinsic erythrocyte proteins labeled with D. M. Shotton and D. Branton, manuscript submitted for publication.

6 D. M. Shotton and D. Branton, manuscript submitted for publication.

7 V. Fowler and D. Branton, manuscript submitted for publication.
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has recently been reported (71).

In view of these considerations, it is of considerable interest to determine which membrane protein(s) are involved in the interaction with spectrin. The possible candidates for a binding site are limited presumably to the proteins or glycoproteins present in at least 2 x 10^6 copies/erythrocyte, which is the approximate number of spectrin molecules (i.e., Bands 1 and 2) (30, 40). Nearly total loss of binding following proteolysis of inverted vesicles is obtained with no significant change in the periodic acid-Schiff-staining profile. After partial digestion only half the binding capacity is lost, but Band 3 is almost completely reduced to a membrane-bound, 50,000-dalton peptide, and high molecular weight proteins in the region of band 2.1 disappear. Further digestion does not affect the 50,000-dalton fragment of Band 3, but does abolish binding. These results suggest that neither the major sialoglycoproteins nor an intermediate protein to which spectrin is bound directly.

A polypeptide has been partially purified from the tryptic digest of inverted vesicles which blocks 50% of spectrin binding at a concentration of 35 μg/ml; essentially no inhibition is obtained with the purified 45,000-dalton portion of Band 3, released in soluble form by trypsin (64), even at levels as high as 300 μg/ml. Competition for spectrin binding thus provides an assay to monitor isolation procedures, and it should be possible with this approach to identify and purify the protein(s) involved in the association of spectrin with the membrane.

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