Reassociation of Ankyrin with Band 3 in Erythrocyte Membranes and in Lipid Vesicles*

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The binding of human erythrocyte ankyrin (band 2.1) to the erythrocyte membrane has been characterized by reassociating purified ankyrin with ankyrin-depleted inside-out vesicles. Ankyrin reassociates at high affinity with a limited number of protease-sensitive sites located only on the cytoplasmic side of the erythrocyte membrane. Deparating the vesicles of band 4.2 does not affect their binding capacity. A 45,000-dalton polypeptide derived from the cytoplasmic portion of band 3 competitively inhibits the binding of ankyrin to inside-out vesicles. Although the bulk of band 3 molecules appear to have the potential for binding ankyrin, only a fraction of the band 3 molecules in native membranes or in reconstituted liposomes actually provides accessible high affinity ankyrin binding sites.

Studies of the interactions between intrinsic and extrinsic proteins of the human erythrocyte membrane have shown that ankyrin (band 2.1) (1) and perhaps a family of related polypeptides (bands 2.2 to 2.6) (2, 3) provide high affinity binding sites that link spectrin (4) to the cytoplasmic surface of the erythrocyte membrane (5–7). Using purified components, the binding between ankyrin and spectrin has been confirmed by studies of proteins in solution and by direct, electron microscopic visualization of the spectrin-ankyrin complex (8, 9). These studies also showed that attachment of ankyrin to ankyrin-depleted vesicles enhanced the vesicles' spectrin binding properties, but the association between ankyrin and the cytoplasmic face of the membrane remained to be elucidated.

Both Sheetz (10) and Bennett and Stenbuck (7) have shown that ankyrin is associated with bands 3 and 4.2 when erythrocytes or inside-out vesicles are treated with the nonionic detergent, Triton X-100, at various salt concentrations. Here we show that ankyrin reassociates at high affinity with a limited number of protease-sensitive sites located only on the cytoplasmic side of the erythrocyte membrane. This reassociation does not require band 4.2 and occurs at physiological ionic strength and pH. Competition experiments provide evidence that band 3 is the polypeptide that anchors ankyrin to the membrane.

EXPERIMENTAL PROCEDURES

Materials—Fresh human blood was obtained from the Northeast Regional Red Cross Blood Program and was used within 2 days. Diisopropyl fluorophosphate (DFP),2 dithiothreitol, N-(Tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid (Tes), Sepharose 4B, ammonium sulfate, type III, and Triton X-100 were obtained from Sigma, ultrapure urea from Schwarz/Mann, and DE52 from Whatman. DE52 was cyclized at least two times in acid and base and equilibrated with 20 mM KCl buffer. Bovine pancreatic trypsin (recrystallized three times from bovine pancreas) and trypsin (crystallized two times) were obtained from Sigma and dissolved in buffer just before use.3 Bolton-Hunter reagent was purchased from Amersharm or New England Nuclear. Phosphatidylcholine from egg yolk was obtained from Sigma and was used as received. SM 2 Bio-Beads were purchased from Bio-Rad, washed in methanol, and stored in the same buffer in which they were to be used.

Ghost (11) were prepared from fresh human blood after washing the erythrocytes three times in phosphate-buffered saline (150 mM NaCl, 5 mM NaPO4, 1 mM Na2EDTA, and 3 mM NaN3, pH 7.5). The subsequent lysing and washing solution was 5 mM NaPO4, pH 7.6, containing 1 mM Na2EDTA. Ghosts were incubated overnight in phosphate-buffered saline with 0.4 mM (0.0675%) v/v isopropyfluorophosphate at 0°C to remove band 6 and to inactivate endogenous proteases. After washing in excess phosphate-buffered saline, membranes were stored at 0°C in a 20 mM KCl buffer (20 mM KCl, 1 mM sodium phosphate, 1 mM Na2EDTA, and 3 mM sodium azide, pH 7.6). Resealed ghosts were prepared by warming the band 6-depleted ghost membranes for 15 min at 37°C. Permeability to macromolecules was ascertained by mixing ghosts with 3% (w/v) dextran (84,000 daltons, Sigma) dissolved in 20 mM KCl buffer. After this treatment, ghosts appeared bright by phase contrast microscopy, indicating their impermeability to the added dextran (12).

Preparation of Membrane Vesicles—Right-side-out vesicles were prepared by forcing erythrocyte ghosts in 20 mM KCl buffer through a 26-gauge hypodermic needle. The resultant vesicles were washed with 20 mM KCl buffer and resuspended in this same buffer containing 0.4 mM DFP. To prepare inside-out vesicles (13), band 6-depleted ghosts were washed twice at 4°C with 15 volumes of a low salt buffer containing 1 mM Tes and 0.1 mM Na2EDTA, pH 8.5. They were then incubated at 37°C for 30 min in 15 volumes of this low salt buffer containing 0.4 mM DFP. The resultant vesicles were centrifuged at 4°C (30 min at 40,000 x g) and the pellets were washed once more in approximately 20 volumes of low salt buffer (without DFP), then in 20 mM KCl buffer, and finally resuspended in 20 mM KCl buffer to the original volume of the membrane pellet prior to removal of band 6 (final protein concentration approximately 2 mg/ml). The inside-out orientation of membrane faces was ascertained by freeze-fracture electron microscopy (13).

KCI-stripped Vesicles—To remove band 4.1 and 2.1 simultaneously, inside-out vesicles were extracted in 1 M KCl at pH 7.6 and

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1 Nomenclature for human erythrocyte polypeptides according to Steck (22).

2 The abbreviations used are: DFP, diisopropyl fluorophosphate, Tes, N-(Tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid.

3 Bolton-Hunter reagent was purchased from Amersharm or New England Nuclear.

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previously described (8) and then resuspended in 20 mM KCl buffer to their original volume.

Urea-Stripped Vesicles—To remove bands 2.1, 4.1, and 4.2, inside-out vesicles were incubated for 30 min at 37°C in 20 volumes of 1 M KCl buffer containing 2.5 M urea and 0.1 M glycine, pH 7.6, with 0.4 mM DFP. The suspension was centrifuged at 40,000 × g for 30 min, the supernatant discarded, and the pellet resuspended in a small volume of 20 mM KCl buffer containing 1 mM diithiothreitol. After dilution to 20 volumes with 20 mM KCl buffer, the vesicles were again centrifuged, the supernatant discarded, and the pellets resuspended to their original volume in the same buffer. The membrane suspension was then dialyzed for 3 days at 4°C against 20 mM KCl buffer containing 0.2 mM diithiothreitol.

Membrane Reassociation Assays for Ankyrin and Spectrin—The reassociation of ankyrin with membranes was measured by incubating purified protein labeled with 125I-Bolton-Hunter reagent (8) with vesicles in an incubation buffer (total volume 200 µl) consisting of 130 mM KCl, 10 mM NaCl, 5 mM NaPO4, 1 mM NaEDTA, 3 mM NaN3, and 1 mM diithiothreitol, pH 7.5, at 0°C. Bovine serum albumin (0.1 mg/ml) was included to prevent adsorption of labeled proteins to tubes during incubations.

Complete incubation mixtures were swirled gently at 4°C for 30 min. One hundred fifty microliters from each mixture was then transferred to a 400-µl microfuge tube that contained 10% (w/v) sucrose dissolved in incubation buffer. An air space separated the 150-µl sample at the top of the tube from the 250-µl sucrose solution at the bottom of the tube. After a total incubation time of 90 min, centrifugation was initiated (18,000 rpm, Sorvall SS-34 rotor) to separate the free 2.1 from the bound 2.1 which sedimented with the vesicles. Calculation of bound and free ligand included a correction for the fraction of the incubation mixture which was not sampled.

RESULTS

Preparation and Selective Extraction of Inside-out Vesicles—Treatment of ghosts with Tes-EDTA-DFP at 37°C released 80 to 90% of the spectrin and actin (Fig. 1, a and b). While the use of EDTA-DFP to elute spectrin and actin resulted in slightly more residual spectrin than if Tes or 0.3 mM phosphate alone were used (not shown), the inclusion of EDTA and DFP during the preparation and storage of inside-out vesicles and of purified ankyrin prevented proteolytic degradation. The residual spectrin was probably trapped

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29) of membrane polypeptides (a to d) and ankyrin purification steps (e to h). a, Erythrocyte ghosts (16.4 µg of protein) after removal of band 6 (see "Experimental Procedures"); b, inside-out vesicles, 11.8 µg of protein; c, KCl-stripped vesicles, 9.2 µg of protein; d, KCl-urea-stripped vesicles, 8.9 µg of protein; e, supernatant obtained by KCl stripping of inside-out vesicles; f, supernatant re-purified protein inside-out vesicles by pre-extracted band 4.1 with 1 M KCl and 0.4 M urea, pH 8.5 (see "Experimental Procedures"); g, supernatant obtained by KCl stripping of pre-extracted inside-out vesicles; h, ankyrin after purification on DEAE-cellulose.
within sealed vesicles or unfragmented ghosts because it was not affected by high salt extraction (Fig. 1, a and d) or by proteolysis of the inside-out vesicles with chymotrypsin (see Fig. 6b) (5). Analysis of freeze-fractured replicas (Fig. 2) indicated that more than 85% of the vesicles prepared with Triton X-EDTA-DFP were inside-out.

Inside-out vesicles incubated with 1 M KCl at 37°C (henceforth referred to as KCl-stripped vesicles) released 65 to 80% of band 4.1 and ankyrin and a variable amount (typically 20 to 30%) of band 4.2; inside-out vesicles incubated in 1 M KCl and 2.5 M urea released 70 to 80% of bands 4.1 and ankyrin, as well as 70 to 80% of band 4.2 (Fig. 1, c and d). Band 4.1 was selectively extracted using 1 M KCl and 0.4 M urea at 0°C so that the ankyrin subsequently extracted at 37°C with KCl could be purified by a single passage over DEAE (Fig. 1, e to h).

Ankyrin Binding to Inside-out Vesicles—We expected that ankyrin would reassociate with inside-out vesicles which had been stripped of this polypeptide without denaturing its binding sites. Preliminary experiments indicated that KCl-stripped vesicles bound ankyrin more effectively than NaOH-stripped membranes (prepared as described in Ref. 15). KCl-stripped vesicles were, therefore, used to characterize the binding of purified ankyrin.

To compare the binding capacity of variably-stripped vesicles, the membrane concentrations were always adjusted to compare the same number of vesicles (as estimated from band 3 content) and scaled per milligram equivalent. The milligram equivalent was defined as the quantity of inside-out vesicles that contained 0.33 mg of band 3, as estimated from densitometric scans of SDS-polyacrylamide gels. One-milligram equivalent of inside-out vesicles contained 1 mg of total protein, while 1 mg equivalent of KCl-stripped vesicles contained approximately 0.8 mg of total protein.

To determine what percentage of our purified ankyrin was capable of reassociating with the KCl-stripped vesicles, the labeled ligand was incubated at a single concentration with increasing amounts of stripped vesicles (Fig. 3a). At least 70% of the labeled ankyrin bound to the KCl-stripped vesicles; less than 20% bound to the heat-denatured vesicles although no aggregation of membranes or change in the electrophoretic profile of the membrane polypeptides was apparent after heat denaturation (not shown). The relationship between the concentration of native vesicles and the percentage of ankyrin which was bound was approximately linear below 40-μg equivalents of membrane protein (200-μg equivalents/ml in the reassociation assay). For this reason, our reassociation assays were consistently carried out with between 10 and 30-μg equivalents/sample.

KCl-stripped vesicles bound 125I-labeled ankyrin with high affinity ($K_d$ = approximately 5 to $8 \times 10^{-8}$ M, Fig. 3, b and c). Analysis according to Scatchard (16) showed that the high affinity binding to stripped vesicles saturated at approximately 95 μg of ankyrin/mg equivalent of membrane protein. This amounted to approximately 1 mol of ankyrin/8 mol of band 3. In some experiments, of which Fig. 3 is an example, the Scatchard plots at high ankyrin concentrations hinted at the presence of additional lower affinity ankyrin binding sites. Heat-denatured vesicles bound little 125I-ankyrin and resealed ghosts bound even less. The binding to heat-denatured vesicles and to resealed ghosts exhibited low affinity and did not appear to be saturable. Inside-out vesicles that had not been stripped of their native ankyrin bound less labeled ankyrin with lower apparent affinity than did the KCl-stripped vesicles. This result would be expected if added labeled and native unlabeled ankyrin competed for the same binding sites.

To confirm that the labeling process had not modified the reassociation characteristics of ankyrin, competition experiments were performed with unlabeled ankyrin (Fig. 4). Unlabeled ankyrin competed with labeled ankyrin ($K_i$ = 11 μg/ml or $5 \times 10^{-8}$ M) and up to 90% of the 125I-ankyrin binding was eliminated by a 65-fold excess of unlabeled ankyrin. This competitive activity was totally lost if the unlabeled ankyrin was heated at 65°C for as little as 2 min after the addition of excess crystalline dithiothreitol to preclude oxidative cross-linking (data not shown). Since the apparent $K_i$ derived from these experiments is similar to the $K_d$ calculated from the binding of labeled ligand (Fig. 3c), it is apparent that iodination of the molecule has not significantly changed the reassociation characteristics of ankyrin.

Although most reassociation assays were carried out at 0-4°C, we compared the rate of binding and the saturation levels achieved at 0-4°C versus 20°C and 37°C. The protease inhibitor DFP was included in these assays and quantification of the labeled polypeptides on sodium dodecyl sulfate-polyacrylamide gels following the reassociation assays indicated that proteolytic degradation of the sensitive ankyrin polypeptide had not occurred. As expected, the 20°C and 37°C samples reached equilibrium faster than the cold samples, but exhibited a slight decrease (about 10%) in the maximum number of binding sites. The binding affinity was not affected by temperature. These experiments also indicated that at 0-4°C, equilibrium binding was reached within 45 to 60 min.

The effect of ionic strength on the reassociation of ankyrin was also tested (Fig. 5). In contrast to the binding of spectrin to spectrin-depleted inside-out vesicles, where binding affinity is increased at least 3-fold in phosphate-buffered saline compared to 20 mM buffer, the affinity of ankyrin for stripped membranes was the same at these two salt concentrations. As expected, binding was substantially reduced in 0.5 M KCl and almost totally abolished in 1 M KCl.

3 W. R. Hargreaves, unpublished data.
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Fig. 3. Reassociation of purified ankyrin with erythrocyte membranes. a, Effect of increasing quantities of vesicles on the binding of \(^{125}\)I-ankyrin to KCl-stripped vesicles (■) or to heat denatured (100°C, 2 min) KCl-stripped vesicles (○). \(^{125}\)I-ankyrin (1.42 pg) was incubated with the vesicles as described under "Experimental Procedures"; the microgram equivalent is defined in the text. b, Influence of increasing concentrations of \(^{125}\)I-ankyrin on the binding of \(^{125}\)I-ankyrin to 30-µg equivalent of KCl-stripped vesicles (○), inside-out vesicles (●), heat-denatured inside-out vesicles (▲), and resealed ghosts (□). c, Data from b plotted according to Scatchard (16). Binding to heat-denatured vesicles has been subtracted to correct for nonspecific binding.

Fig. 4. Competitive displacement of the \(^{125}\)I-ankyrin binding to KCl-stripped vesicles by unlabeled ankyrin. a, Ninety percent inhibition of the binding of \(^{125}\)I-ankyrin is achieved with a 65-fold molar excess (196 µg/ml) of unlabeled ankyrin. 100% = 7.7 µg of \(^{125}\)I-ankyrin bound/mg equivalent. b, Dixon plot (30) shows competitive inhibition of \(^{125}\)I-ankyrin binding when vesicles (6.8-µg equivalent) were incubated with increasing amounts of unlabeled ankyrin prior to the addition of 3.4 µg/ml (●), 6.9 µg/ml (△), or 10.2 µg/ml (□).

To assess the nature of the high affinity binding site for ankyrin, KCl-stripped vesicles were treated briefly with α-chymotrypsin (Fig. 6, a and b). This enzyme readily cleaves the intrinsic erythrocyte membrane polypeptide, band 3 (14), which comprises the major polypeptide component of KCl-stripped membranes. Band 4.2, which is also prominent in these membranes, is not noticeably affected by brief treatment with α-chymotrypsin.

Treating the vesicles with α-chymotrypsin substantially reduced their ability to bind ankyrin. KCl-stripped vesicles were treated briefly with α-chymotrypsin (Fig. 6, a and b). This enzyme readily cleaves the intrinsic erythrocyte membrane polypeptide, band 3 (14), which comprises the major polypeptide component of KCl-stripped membranes. Band 4.2, which is also prominent in these membranes, is not noticeably affected by brief treatment with α-chymotrypsin.

Fig. 5. Effect of ionic strength on the reassociation of ankyrin. Twenty-eight-microgram equivalents of KCl-stripped vesicles were incubated with labeled ankyrin in buffer containing 5 mM phosphate, 1 mM NaEDTA, 3 mM NaN₃, and 1 mM dithiothreitol, pH 7.5, with 20 mM KCl (○), 140 mM KCl (●), 0.5 M KCl (▲), or 1 M KCl (□).

To the sum of ankyrin bound by the two kinds of vesicles alone (Fig. 7). Thus, residual chymotrypsin activity could not account for the reduction in ankyrin binding to chymotrypsin-treated vesicles. A second experiment to control for proteolysis used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the amount of radioactivity in the relevant stained bands (not shown). No differences were found between samples which contained protease-treated vesicles and control samples which contained only unproteolyzed vesicles.

While it appeared that the presence of intact band 3 was correlated with the competence of stripped membranes to bind ankyrin, we investigated the possible role of band 4.2 in this reassociation phenomenon. Inside-out vesicles were selectively extracted to remove either little or most of the band...
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4.2, along with ankyrin and band 4.1 (see Fig. 1, c and d). Binding of ankyrin was unaffected by these treatments (Fig. 8). We concluded that band 4.2 is not required for the high affinity binding of ankyrin to KCl-stripped vesicles.

Competition experiments were used to confirm that band 3 was the membrane binding site for ankyrin. The 45,000-dalton water-soluble polypeptide that is known to be a fragment derived from the cytoplasmic portion of band 3 (2, 14) competed effectively with KCl-stripped vesicles for the binding of ankyrin (Fig. 9), whereas neither band 6 nor heat-treated 45,000-dalton fragment (2 min at 100°C) showed any effect. However, the apparent inhibition constant \( K_i \approx 5 \times 10^{-7} \) m, Fig. 9) suggests that the band 3 fragment binds to ankyrin approximately 10 times less avidly than does ankyrin to the intact membrane site.

Because band 3 is present in a 4- to 8-fold molar excess over ankyrin monomers in the intact erythrocyte membrane (17), and because the reassociation of ankyrin with the KCl-stripped vesicles was limited to a similar stoichiometry, it became apparent that some mechanism, perhaps denaturation, must be limiting the interaction of ankyrin with band 3 in the membrane. The most likely step during which a large subset of band 3 molecules might be denatured, leaving a minor subset completely protected, occurs during the elution of spectrin and actin with low salt at 37°C. This step occurs before ankyrin is stripped from the vesicles. We reasoned that if this low ionic strength treatment was able to denature unoccupied ankyrin binding sites, those binding sites that were occupied by ankyrin might remain undenatured. However, low ionic strength treatment of stripped inside-out vesicles that lacked ankyrin had absolutely no effect on the subsequent reassociation of ankyrin (data not shown).

To determine whether discrete subpopulations of band 3 accounted for the limited binding of ankyrin, we attempted to separate the putative binding-incompetent population of band 3 molecules from the binding-competent molecules. Ghosts were extracted with detergent (0.5% Triton X-100) in 150 mM KCl, an ionic strength which is known to maximize both spectrin-ankyrin and ankyrin-membrane interactions. The
limited population of band 3 molecules that was solubilized from ghosts under this condition was assumed to contain band 3 molecules which were unassociated with the membrane cytoskeleton (10). These band 3 molecules were likely to be the binding-incompetent molecules, if such molecules existed. The competence of this limited population of band 3 molecules

![Fig. 9. Competitive inhibition of ankyrin binding to KCl-stripped vesicles by the water-soluble, cytoplasmic domain of band 3.](image)

![Fig. 10. Binding inhibition by the water-soluble domain of band 3 previously extracted in Triton X-100.](image)

![Fig. 11. Association of ankyrin with band 3 reconstituted into phosphatidylcholine vesicles. A detergent extract containing band 3 was prepared by incubating ghosts in 1% Triton X-100 in phosphate-buffered saline. The solubilized membrane polypeptides were reconstituted into phosphatidylcholine vesicles (see "Experimental Procedures") and then either these vesicles (○) or standard KCl-stripped vesicles (□) were incubated with increasing concentrations of [125I]-ankyrin. The binding of ankyrin is shown in the form of a Scatchard plot, corrected for nonspecific binding to heat-denatured membranes.

To ascertain directly whether the limited population of band 3, solubilized from ghosts with detergent at physiological ionic strength, was capable of the same high affinity interaction with ankyrin that we had observed with KCl-stripped vesicles, we incubated with increasing amounts of the water-soluble fragment prior to the addition of 2 pg/ml (○), 4 pg/ml (△), or 8 pg/ml (□) of [125I]-ankyrin. Values have been corrected for nonspecific binding to heat-denatured vesicles.

**DISCUSSION**

Our results show that ankyrin binds with high affinity to a limited number of heat-sensitive and protease-sensitive sites
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which are located on the cytoplasmic surface of the erythrocyte membrane. Competition experiments using the 45,000-dalton cytoplasmic fragment of band 3 indicate that these sites are part of the cytoplasmic portion of the transmembrane protein, band 3 (18). Thus, our studies using inside-out vesicles, purified ankyrin, and the water-soluble fragment of band 3 accord with and clarify studies of Bennett and Stenbuck (7) which show that ankyrin is bound to a complex of band 3 and band 4.2 in detergent extracts of erythrocyte membranes.

The band 6 and band 4.2 polypeptides also have binding sites on band 3 (19) and their attachment could also modulate the interaction between band 3 and ankyrin. However, band 6 does not bind to inside-out vesicles at physiological ionic strength and pH (20) and does not affect the binding of ankyrin to membranes under such conditions (Fig. 9). Pretreatment of our vesicles with urea to extract band 4.2 had no effect on the binding of ankyrin (Fig. 8). Thus, under the conditions employed in this study, band 4.2 also appears to play no role in binding ankyrin to the membrane or to band 3.

Extrapolation of our binding data indicates that at saturating levels of ankyrin, the stoichiometry of ankyrin that binds with high affinity to band 3 in the vesicles is approximately 1 mol of ankyrin/8 mol of band 3. Although this is nearly the same stoichiometry found in freshly prepared erythrocyte ghosts (17), the basis of this stoichiometry is puzzling if, as observed in Triton solutions, 1 band 3 molecule can bind 1 ankyrin molecule (7). Since the polypeptide moieties of the band 3 molecules are thought to be identical (7, 14, 21), one would expect all of them to provide potential binding sites for ankyrin. Limited synthesis of ankyrin could account for the stoichiometry in the native cell, but other reasons must be sought to explain why less than 15% of the band 3 molecules in our inside-out vesicles appear to provide binding sites for ankyrin.

In spite of the biochemical evidence suggesting that most band 3 molecules share a common polypeptide sequence, we considered the possibility that not all band 3 molecules contain an ankyrin binding site. We extracted ghosts with detergent under conditions where only those band 3 molecules that were not linked to ankyrin would have been solubilized. After being reconstituted into lipid vesicles, these "noncytoskeletal" band 3 molecules bound substantial amounts of ankyrin with an affinity similar to that exhibited by the total band 3 population in stripped inside-out vesicles, although negative cooperativity or the presence of additional lower affinity ankyrin binding sites was suggested by the curved Scatchard plot (Fig. 11b). Furthermore, a 45,000-dalton, water-soluble fragment of the "noncytoskeletal" band 3 inhibited the binding of labeled ankyrin to inside-out vesicles as effectively as the fragment derived directly from intact, stripped membranes or from a Triton extract of the latter. Thus, those band 3 molecules which are not bound to ankyrin in the membrane do have the capacity to bind ankyrin with high affinity.

One explanation why less than 15% of the band 3 molecules in our inside-out vesicles appear to provide binding sites for ankyrin is that this estimate assumes all of the band 3 in our KCl-stripped vesicles is accessible. When one takes into account the presence of right-side-out vesicles (10 to 15%) and the presence of residual ankyrin (20 to 30% of the native ankyrin content), accessible band 3 may be only 50% of the total band 3 content. But, even when this correction is made, not all of the accessible band 3 molecules in our inside-out vesicles provide binding sites for ankyrin. At most, only 1 out of 4 band 3 molecules in our inside-out vesicles or reconstituted vesicles binds labeled ankyrin with high affinity.

One plausible explanation for the limited number of ankyrin binding sites in stripped vesicles is that steric factors further limit accessibility to the potential binding sites. There is general agreement that band 3 forms a stable dimer in the erythrocyte membrane (22) in Triton X-100 extracts (20) and after reconstitution into lipid vesicles (23). Cross-linking studies show that higher order oligomers may also exist (24, 25), and recent reanalyses of fluorescence depolarization data (26) and of the number of intramembrane particles in the erythrocyte membrane (27) strongly suggest that band 3 may exist as a tetramer. If band 3 exists in membranes as a tetramer, the self-association of band 3 dimers to form tetramers could effectively shield all but one binding site for ankyrin or create a new, shared binding complex. The latter hypothesis is consistent with the lack of obvious negative cooperativity when ankyrin associates with stripped membranes and the relatively poor inhibition of ankyrin binding by the monomeric cytoplasmic fragment of band 3. The apparent negative cooperativity seen in ankyrin reassociation with reconstituted band 3 is consistent with incomplete reformation of band 3 oligomers after detergent treatment.

In spite of the fact that ankyrin binds with high affinity and specificity to band 3 sites on the cytoplasmic surface of ankyrin-depleted inside-out vesicles, evidence is required to show that this reassociation fully reconstitutes the native association of ankyrin with the membrane. Reconstitution of function is one of the most useful criteria for establishing the biological relevance of ligand binding. The only known function of ankyrin is related to its spectrin binding capacity (8, 28). Thus far, our investigations have shown that reassociating ankyrin with KCl-stripped vesicles which do not bind spectrin does in fact reconstitute some of the binding capacity (8). But further studies in which [32P]spectrin was reassociated with membrane-bound 125I-ankyrin suggest that as much as 50% of the membrane-associated ankyrin may be unavailable for spectrin binding. Thus, the factors that limit the association of ankyrin to its attachment sites on the membrane and that restrict the spectrin binding capacity of membrane-associated ankyrin remain subjects for future research.

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Note Added in Proof—A report describing the association of ankyrin with the cytoplasmic domain of band 3 appeared after this manuscript was submitted (Bennett, V., and Stenbuck, P. J. (1980) J. Biol. Chem. 254, 6424–6432).

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