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Functional Characterization of Human Erythrocyte Spectrin α and β Chains: Association with Actin and Erythrocyte Protein 4.1†

Carl M. Cohen* and Robert C. Langley, Jr.

ABSTRACT: Human erythrocyte spectrin α and β chains were purified by preparative sodium dodecyl sulfate gel electrophoresis and also by DEAE-cellulose chromatography in the presence of urea. The purified chains behaved as individual monomers on sucrose gradients and did not form homodimers. Recombination of the chains led to the formation of α–β heterodimers with sedimentation characteristics identical with native α–β dimers. The binding of ^{125}I-labeled band 4.1 to α and β chains was measured by sucrose gradient rate zonal sedimentation and by quantitative immunoassay. It was found that both α and β chains associated with ^{125}I-labeled band 4.1 in a nearly identical manner over the range of band 4.1 concentration studied. The association was abolished by heat denaturation of the spectrin chains or by denaturation of band 4.1 with a 40-fold molar excess of *N*-ethylmaleimide. As expected, purified β chains but not α chains bound to ^{125}I-labeled ankyrin as measured by a quantitative radioimmunoassay. The binding of purified α chains, β chains, and recombinant α–β heterodimers to F-actin was measured in the presence of band 4.1. We found that α or β chains separately exhibited no band 4.1 dependent association with F-actin but that α–β heterodimers formed by recombination of the chains did. We conclude that spectrin binding to F-actin in the presence of band 4.1 requires the participation of both of spectrin's polypeptide chains.

The spectrin molecule is the major structural protein of the red cell membrane and constitutes approximately 75% by weight of red cell membrane skeletons obtained by Triton extraction of ghosts (Sheetz & Sawyer, 1978; Lux et al, 1976; Branton et al., 1981). Although the importance of spectrin to the maintenance of red cell shape and membrane integrity has been appreciated for some time, the biochemistry of this protein has only recently been explored in any detail [see Knowles et al. (1983) for a review]. Recent work has shown that spectrin is a heterodimeric protein containing a 240 000-dalton α chain and a 220 000-dalton β chain that associate with one another at multiple sites (Morrow et al., 1980) along their length. As visualized by low-angle rotary shadowing (Shotton et al., 1979; Tyler & Branton, 1980), the dimer has a contour length of 1000 Å, is 50 Å wide, and, because of its heterogeneous appearance as seen by low-angle rotary shadowing, is thought to be highly flexible. Under appropriate conditions, spectrin dimers readily self-associate in a head to head fashion to form tetramers (Shotton et al., 1979; Ungewickell & Gratzer, 1978) that are 2000 Å long, as well as various higher oligomeric structures (Morrow et al., 1981). Tetrmers and higher oligomers are thought to be the predominant form of spectrin in the membrane skeleton in situ.

Spectrin has two well-defined functions within the membrane skeleton. First, it fastens itself and the entire membrane skeleton to the membrane by its association with ankyrin, which in turn binds to the integral membrane protein band 3, thus securing the membrane skeleton to the lipid bilayer (Bennett, 1982). Second, spectrin tetramers bind to and cross-link short actin filaments and in this way produce the two-dimensional network that constitutes the membrane skeleton [see Cohen (1983) for a review]. The binding of spectrin to actin is promoted by the protein band 4.1, which is essential for stabilizing the otherwise weak association between these two proteins [reviewed in Cohen (1983)].

The partial localization of the functional sites on spectrin for these and other associations has been achieved through a combination of biochemical and electron microscopic techniques. It is known that spectrin's binding site for ankyrin is located on the β chain, about 200 Å from the head of the chain (that end which participates in the dimer–dimer association), and that spectrin's binding sites for actin and band 4.1 are located at the tail of the molecule (Branton et al., 1981; Cohen, 1983). Beyond this however, little is known. The focus of this work is to define which of the polypeptide chains of spectrin are responsible for binding to band 4.1 and actin. Our results show that while spectrin α and β chains may be different in many other respects, their association with band 4.1 and possibly actin seems to be remarkably alike.

Materials and Methods

Protein Preparations. Human erythrocyte spectrin and band 4.1 were prepared from freshly drawn human blood as described previously (Cohen & Foley, 1982; Tyler et al., 1972). Actin was extracted from rabbit muscle by the method of Spudich & Watt (1971) and handled as described by Cohen & Foley (1982). Ankyrin was prepared as a byproduct of band 4.1 purification as described by Tyler et al. (1979).

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Purification of Spectrin α and β Chains by Preparative Sodium Dodecyl Sulfate Gel Electrophoresis. Sepharose 4B column purified spectrin was concentrated to 1 mg/mL and dialyzed overnight against 5 mM sodium phosphate, pH 8.0, 1 mM DTT.[1] Prior to solubilization of the protein sample (4 mg) for electrophoresis, 10% of the sample was fluorescently labeled with dansyl chloride. This was done by adjusting an aliquot of the protein to 0.1 M Tris-HCl, pH 8.2–5% SDS and adding, with vigorous vortexing, 20 μL of 10% w/v dansyl chloride (in acetone)/mL of protein. The sample was adjusted to 1% β-mercaptoethanol and boiled for 1 min, cooled to 20 °C, and combined with the remaining 90% of the protein to be solubilized. The 4-mg protein sample was then brought to 50 mM Tris-HCl, pH 7.0, 2 mM EDTA, 1% SDS, 0.25 M sucrose, and 40 mM DTT and heated for 20 min at 37 °C in order to solubilize the protein.

The solubilized protein sample was electrophoresed at 70 V for approximately 18 h in a 5%, 17 × 14 × 0.3 cm SDS–polyacrylamide gel with the buffer system described by Laemmli (1970). The two bands corresponding to the α and β spectrin chains were visualized with a UV light and cut out, and the proteins were electrophoretically eluted with an ISCO electrophoretic protein concentration apparatus. Briefly, the polyacrylamide gel strips containing the proteins were homogenized by forcing them through a 20-mL plastic syringe directly into the sample chamber (cathode) of the elution apparatus, which was then filled with 10 mM Tris–acetate, pH 8.8. The proteins were electrophoretically eluted from the gel into the collection chamber (anode) with 25–35 mA at 100–150 V in 10 mM Tris–acetate, pH 8.8, as the electrode buffer. The sample and collection wells were separated by a small piece of glass wool to prevent the gel pieces from migrating into the collection chamber. The eluted proteins were harvested under UV illumination from the collection chamber at 20-min intervals for 2 h or until no fluorescence remained in the gel.

The eluted spectrin chains were dialyzed overnight against 5 mM sodium phosphate, pH 8.0–1 mM DTT, concentrated by pressure filtration to 0.5–1.0 mg/mL, and dialyzed 24–64 h against the same buffer. Residual SDS was removed by passing the protein solutions through 2 mL of Exracti-Gel D (Pierce) in a 1.2 × 3.0 cm column, which was preequilibrated and eluted with 5 mM sodium phosphate, pH 8.0–1 mM DTT. The spectrin chains eluted in the void volume and were collected as one fraction, which was then concentrated to 1 mg/mL and dialyzed as before until used.

Purification of Spectrin α and β Chains by Urea–DE-52 Column Chromatography. Spectrin was precipitated with 50% w/v ammonium sulfate, suspended in 20 mM Tris-HCl, pH 8.0–1 mM EDTA to a concentration of 2–3 mg/mL, and dialyzed overnight against two 2-L changes of the same buffer. The dialyzed dimer was diluted with an equal volume of 6.0 M urea, 0.30 M NaCl, 20 mM Tris-HCl, pH 8.0 and 1 mM EDTA and stirred slowly on ice for 2 h to dissociate the dimer into α and β monomers. The mixture of monomers was loaded onto a 2.5 × 25 cm Whatman DE-52 (DEAE-cellulose) column, which was preequilibrated with 3.0 M urea, 0.15 M NaCl, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and the β chain was eluted at 13 mL/h, 4 °C, with the same buffers, as described by Yoshino & Marchesi (1984). The column was flushed overnight (60 5-mL fractions), and the α chain was eluted with a 0.15–0.50 M NaCl gradient in 3.0 M urea, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Aliquots of each fraction were checked for purity on SDS–polyacrylamide gels, and fractions containing only pure α or β chains were pooled and dialyzed overnight against 5 mM sodium phosphate, pH 8.0–1 mM DTT. They were then concentrated to 1 mg/mL and dialyzed in the same buffer until used. For use in binding experiments, spectrin α chains, β chains, or dimers were labeled with ^{125}I by using Bolton–Hunter reagent (New England Nuclear).

SDS Assays. The SDS content of the proteins purified by SDS preparative gel electrophoresis was monitored throughout the procedure by the colorimetric assay described by Waite & Wang (1976). This assay was linear from 1 to 20 μg of SDS and was not afected by the presence of 5 mM sodium phosphate, 1 mM DTT, or up to 10 mg/mL BSA.

Recombination of α and β Chains To Form Renatured Dimers. Equal amounts of α and β chains were mixed and dialyzed at room temperature (22 °C) for 4 h against 6.0 M guanidine hydrochloride, pH 8.0, 0.15 M Tris, 0.1 mM EDTA, and 1 mM DTT followed by an 18-h, 4 °C, dialysis against 20% w/v glycerol, 5 mM sodium phosphate, pH 8.0, and 1 mM DTT. Proteins were then dialyzed against several changes of 5 mM sodium phosphate, pH 7.6–1 mM DTT, 4 °C, until used in experiments.

Binding of Spectrin Chains to Band 4.1 or Ankyrin. Spectrin–band 4.1 association was assessed by two methods: (1) analytical sucrose gradient centrifugation or (2) quantitative immunoassay. For sucrose gradient analysis, proteins were preincubated for 0.5 h at 25 °C in 20 mM KCl, 5 mM sodium phosphate, pH 7.6, and 0.5 mM DTT (unless specified otherwise) at the concentration indicated in the figure legends. Sample aliquots of 250 μL were centrifuged on 5.0-mL linear gradients of 5–25% w/v sucrose in 20 mM KCl, 5 mM sodium phosphate, pH 7.6, and 0.5 mM DTT (unless specified otherwise) at 32000 rpm for 16.5 h in a Beckman SW 50.1 rotor. Gradients were drained from the bottom into 20 fractions, and the protein distribution was monitored by counting the fractions for ^{125}I in a gamma counter.

Binding of spectrin to band 4.1 and ankyrin was also measured by an immunoassay similar to that described by Tyler et al. (1980). Antispectrin antisera for the assays were obtained by immunization of New Zealand rabbits with chromatographically purified spectrin dimers. The antisera obtained were judged specific for spectrin by analysis of electroblots of red cell ghosts. The antisera reacted equivalently with spectrin α chains and spectrin β chains as measured in preliminary immunoassays. The binding assay was performed by incubating 10 μg of spectrin dimers, α chains, or β chains with various concentrations of ^{125}I-labeled band 4.1 or ^{125}I-labeled ankyrin (specified in the figures) in a final volume of 250 μL containing 120 mM KCl, 5 mM sodium phosphate, pH 7.6, 0.1% Triton X-100, 1 mg/mL bovine serum albumin, 0.5 mM DTT, and 1 mM EDTA (binding buffer) for 45 min at 22 °C. At the end of 45 min, 10 μL of antispectrin antiserum was added and the reaction continued for an additional 45 min. At this time, 100 μL of a 10% suspension of Pansorbin (Calbiochem; formalin-fixed Staph A) in binding buffer was added, and the reaction was continued with intermittent agitation for 45 min. The samples were centrifuged at 2400*g* for 10 min and washed 3 times with ice cold binding buffer (1 mL per wash). Pansorbin pellets were counted for ^{125}I in a gamma counter. Correction for nonspecific binding was made by substracting the binding at each concentration of ^{125}I-labeled band 4.1 or ^{125}I-labeled ankyrin of samples that contained everything except spectrin. Such

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

corrections ranged from 20 to 50% of the total observed binding. Binding of ankyrin or band 4.1 was normalized to the amount of spectrin dimer, α chain, or β chain in the Staph A pellet as measured in separate control experiments. All incubations were done in duplicate, and agreement between duplicate samples was within 5% of the average values, which are shown in the text.

Binding of Spectrin to Actin in the Presence of Band 4.1. Proteins at the concentrations indicated in figure legends were coincubated in plastic test tubes at 25 °C for 1 h in binding buffer consisting of 120 mM KCl, 5 mM sodium phosphate, pH 7.6, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mDTT, and 1 mg/mL bovine serum albumin. All samples of spectrin were preincubated at 37 °C for 1 h prior to the experiment to dissociate any spectrin tetramers that may have formed. All radioactively labeled proteins were centrifuged at 150000*g* for 20 min prior to use in experiments to remove aggregates that may have formed. Duplicate 150-μL samples of reaction mixes were spun in a Beckman airfuge at 30 psi for 20 min or in a Beckman 42.2 Ti Rotor for 15 min at 20000 rpm, 4 °C, to sediment F-actin and any associated proteins. Samples (20 μL) were taken from the meniscus after centrifugation and counted for ^{125}I; the pellets were discarded. Protein sedimentation was quantified by subtracting concentrations in the supernate from those in the initial reaction mixtures. Duplicate samples generally agreed to within 10%. The values shown in the figures represent the means of the duplicate determinations.

Electron Microscopy of Proteins. Solutions containing spectrin chains, dimers, or recombinant dimers were diluted to 20 μg/mL in 70% w/v glycerol. Mixtures of spectrin, actin, and band 4.1 were diluted to a final total concentration of 50 μg/mL in 45% v/v glycerol. Samples were sprayed from a N_2-pressurized atomizer onto freshly cleaved mica disks and dried in the vacuum chamber of a Balzers freeze-etch device at 10^{-6} Torr. Samples were rotary shadowed as described previously (Tyler & Branton, 1980; Cohen et al., 1960) with 6:1 platinum–carbon from an angle of 5° from the horizontal and the replicas examined in a JEOL 100 S electron microscope.

Results

Figure 1A shows an SDS–polyacrylamide gel of purified spectrin α chains and β chains obtained by preparative SDS gel electrophoresis. Typically, cross contamination of one chain by the other was less than 5%, although contamination of β chain by α chain was generally undetectable. Similar results were found for spectrin chains purified by DEAE-cellulose chromatography in the presence of urea. The appearance of the chains by low-angle rotary shadow electron microscopy is shown in Figure 1B. The chains generally appeared as extended flexible structures with a 1000-Å countour length, similar to that of spectrin dimers. Spectrin β chains, particularly those obtained from preparative gels, sometimes appeared to be less extended, frequently consisting of nodules with short chains extending from them (not shown). Centrifugation of the urea–DEAE-cellulose purified chains on 5–25% sucrose gradients (Figure 2) showed that the α chains generally migrated in a relatively compact distribution, although the β chains (particularly those from SDS gels) were sometimes more dispersed in distribution, possibly reflecting a more heterogeneous structure.

Comparison with protein standards showed that both chains had a sedimentation coefficient between 6.55 and 7.05 S, the range reflecting some variability from preparation to preparation. For comparison, erythrocyte ankyrin, a 210000-dalton

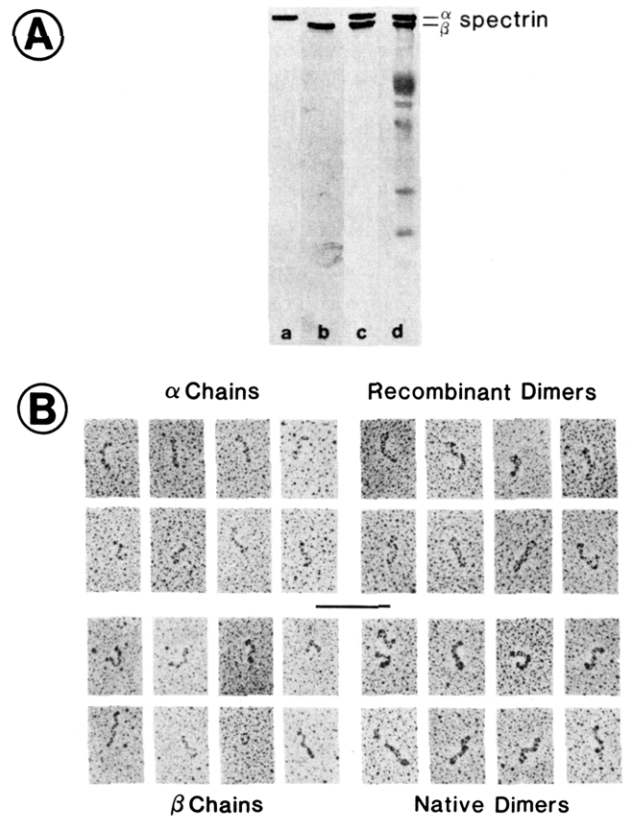

FIGURE 1: (A) SDS–polyacrylamide gels of spectrin α chains (a) and β chains (b) purified by preparative gel electrophoresis, (c) Sepharose 4B purified spectrin dimers, and (d) red cell ghosts. (B) Electron micrograph of low-angle rotary-shadowed α chains, β chains, recombinant α–β heterodimers, and native dimers. Spectrin chains were prepared by the urea–DE-52 cellulose method, and recombinant α–β heterodimers were formed as described under Materials and Methods. Chains and recombinant dimers were further purified by centrifugation on sucrose gradients (see Figure 2), and the peak fractions were diluted to a final protein concentration of 20 μg/mL in 70% w/v glycerol. Samples were sprayed and shadowed for electron microscopy as described under Materials and Methods. Bar = 0.2 μM.

globular protein, has a sedimentation coefficient of 6.9 S (Bennett & Stenbuck, 1980), and native spectrin dimers have a value of 9.7 S (Shotton et al., 1979). Estimates of the sedimentation coefficient of isolated erythrocyte spectrin α and β chains have also been made by Calvert et al. (1980), who reported a value of 5.5 S for the α chain and an unspecified value below 7 S for the β chain. The α chain of brain spectrin has also been reported to have an *s* value of 5.5 S (Davis & Bennett, 1983).

The ability of the α and β chains to recombine into an α–β heterodimer was examined by sucrose gradient centrifugation. Figure 2 shows that urea–DEAE-cellulose purified α and β chains separately migrate to fraction 12 of 5–25% sucrose gradients. Mixture of the α and β chains in equal concentration followed by a brief preincubation resulted in the formation of a species migrating to fraction 7 of the sucrose gradient (Figure 2c), the position of native spectrin dimers (see Figure 3b). A small shoulder in fraction 11 (Figure 2c) probably represents uncombined α and β chains. The percentage of recombination in this experiment was high, approximately 70% of the counts migrating in the position of the recombined dimer. Reassociation of the chains to form a heterodimer was the result of specific intermolecular association of α and β chains since chains purified by the SDS method and not subjected to the renaturation protocol (see Materials and Methods) did not form heterodimers. The ready reassociation of urea-purified α and β chains and the lack of

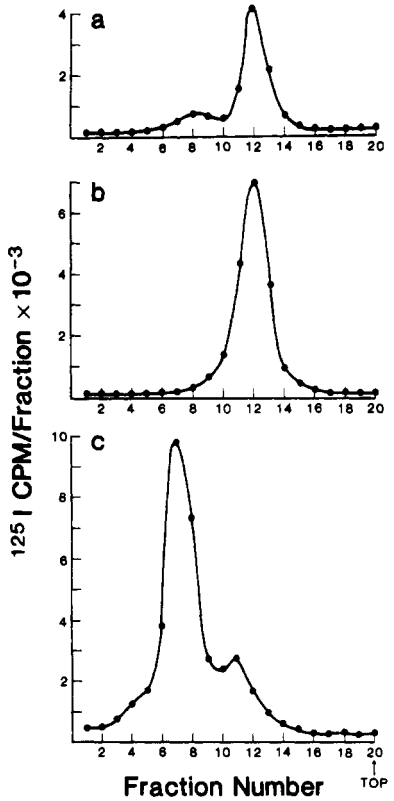

FIGURE 2: Sucrose gradient centrifugation of ^{125}I-labeled α chains (a), ^{125}I-labeled β chains (b), and ^{125}I-labeled α–β heterodimer recombinants (c). Spectrin α and β chains purified by the urea–DE-52 cellulose method were centrifuged on 5–25% sucrose gradients (see Materials and Methods) separately or after a brief preincubation together as described under Materials and Methods. Gradient fractions were counted for ^{125}I to determine protein distribution.

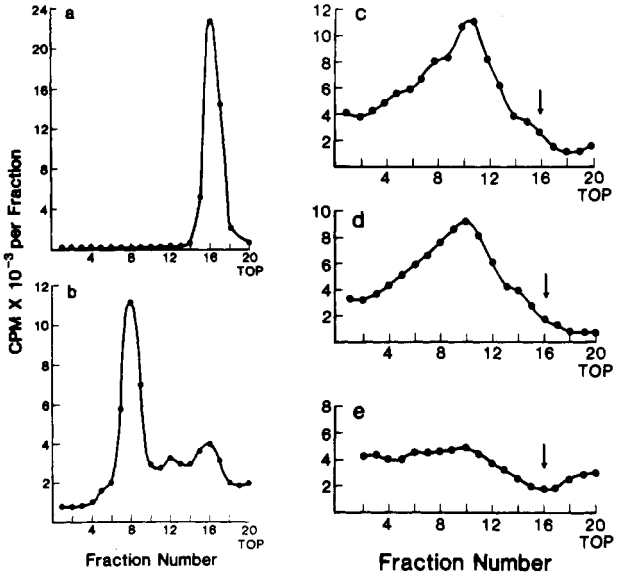

FIGURE 3: Binding of ^{125}I-labeled band 4.1 to spectrin dimers, α chains, and β chains demonstrated by sucrose gradient centrifugation. ^{125}I-Labeled band 4.1 (50 μg/mL) in 20 mM KCl, 5 mM sodium phosphate, pH 7.6, and 0.5 mM DTT was preincubated at 25 °C for 0.5 h alone (a) or in the presence of 50 μg/mL spectrin dimer (b), 50 μg/mL α chains (c), 50 μg/mL β chains (d), or 18 μg/mL α chains plus β chains (e). Proteins were centrifuged on a 5–25% sucrose gradient as described under Materials and Methods. Gradient fractions were counted for ^{125}I to determine protein distribution. Arrows mark the position where band 4.1 would migrate by itself.

significant formation of α_2 or β_2 forms have been noted previously (Calvert et al., 1980). The small secondary peak in the α chain distribution of Figure 2a probably represents an aggregate and was never seen in any other runs.

Table I: SDS Content of Spectrin α and β Chains during Purification

treatment	protein	SDS/protein (mg/mg)[a]	SDS/protein (mol/mol)[a]
solubilized protein	dimer	37.70[b]	65 447.2
electrophoretic elution	band 1	2.90[b]	2415.7
	band 2	3.20[b]	2444.8
overnight dialysis	band 1	0.30	249.9
	band 2	0.31	236.8
concentration (by vacuum filtration)	band 1	0.16	133.3
	band 2	0.12	91.7
64-h dialysis	band 1	0.042	35.0
	band 2	0.025	19.1
Extracti-Gel and concentration	band 1	0.014	11.7
	band 2	0.008	6.1

[a] The SDS content of the solution was measured after the treatment listed. [b] Most of the SDS at this point in the procedure represents unbound SDS since these values greatly exceed the maximal SDS binding capacity (1.4 mg of SDS/mg of protein) for typical proteins (Reynolds & Tanford, 1970).

While most of the experiments shown in the following figures were done with α and β chains purified by preparative gel electrophoresis, all were repeated with the same results when spectrin chains purified by urea–DEAE-cellulose chromatography were used. However, because of the extensive association of SDS with polypeptides, we thought it important to determine whether there was residual SDS associated with the purified chains. Using a sensitive colorimetric assay of SDS (Waite & Wang, 1976), we followed the disappearance of SDS from our α and β chain preparations. Table I shows that the final preparations of spectrin chains contained 8–14 μg of SDS/mg of protein or 0.8–1.4 wt % SDS contamination. On a molar basis, this corresponds to 6–12 mol of SDS/mol of spectrin chain, but since the molecular weight of SDS is 288, this translates into only about 1700–3400 daltons of SDS on a 220 000- or 240 000-dalton polypeptide. The difference in the amount of residual SDS associated with the α and β chains shown in Table I probably is not significant, since in other experiments the values were nearly identical. Our studies comparing SDS- and urea-purified spectrin chains show that this amount of residual SDS has no apparent effect upon the associations we measured, although we cannot rule out subtle perturbations of other functions not detected by our experiments.

Our initial experiments to measure the association of spectrin α and β chains with band 4.1 were done by sucrose gradient centrifugation. Figure 3 shows an experiment demonstrating the binding of purified spectrin dimers to ^{125}I-labeled band 4.1, which by itself migrated as a single peak in fraction 16 of the gradient (Figure 3a). Addition of spectrin dimers (Figure 3b) caused a shift in the position of ^{125}I-labeled band 4.1 to fraction 8, which is the position of spectrin dimers in the gradient; a small peak of unbound ^{125}I-labeled band 4.1 remains in fraction 16. Figure 3c,d shows similar experiments with purified spectrin α and β chains. The data show that both chains cause a shift in the position of ^{125}I-labeled band 4.1 in the gradient from fraction 16 (the position of unbound 4.1) into a broad peak centered on fraction 10. This shows that both spectrin chains bind to ^{125}I-labeled band 4.1. Also shown in Figure 3e is a mixture of α and β chains used to produce a preparation of recombinant spectrin dimers. The mixture also shifts the postition of ^{125}I-labeled band 4.1, but the distribution is much more heterogeneous than that of the α chain–

or β chain–band 4.1 complexes.

Several observations can be made upon the basis of the gradient profiles of Figures 2 and 3. First, the positions of the peaks of the ^{125}I-labeled band 4.1–α chain and –β chain complexes are lower in the gradient then those of the free α and β chains. This is not unexpected since the binding of a single molecule of band 4.1 (80 000 daltons) to each chain (240 000 or 220 000 daltons) would increase the aggregate molecular weight of the complex by 35% over that of the individual chains. Second, the distribution of the ^{125}I-labeled band 4.1–α chain and –β chain complexes is much more disperse than the distribution of the ^{125}I-labeled band 4.1–native spectrin dimer complex. This probably simply reflects the more disperse distribution of the isolated chains realtive to spectrin dimers, although the formation of large aggregates of spectrin chains and band 4.1 cannot be entirely ruled out. The highly dispersed distribution of the ^{125}I-labeled band 4.1–recombinant dimer complex probably relfects a combination of the heterogeneity of the distributions seen in Figure 3c,d as well as low efficiency of dimer formation in this preparation.

Although the isolated α and β chains did associate with band 4.1 under the same conditions as native spectrin dimers, it was nevertheless possible that some or all of the observed binding was due to nonspecific association. To test this possibility, we performed the following experiments. First, α and β chains were denatured by heating to 60 °C for 10 min. This treatment entirely eliminated the binding of ^{125}I-labeled band 4.1 to α and β chains (not shown). Next, we denatured band 4.1, by reacting it with a 40-fold molar excess of *N*-ethylmaleimide. This treatment caused the ^{125}I-labeled band 4.1 to aggregate and migrate to the bottom of a sucrose gradient even in the presence of spectrin dimers, α chains, or β chains. In separate experiments (not shown) using *N*-ethylmaleimide-denatured band 4.1 and ^{125}I-labeled α chains or ^{125}I-labeled β chains, it was found that the migration of spectrin chains in a gradient was not affected by the presence of denatured band 4.1. These experiments show that denaturing either the spectrin chains or band 4.1 eliminates their association with one another.

An additional test of the specificity of the association between ^{125}I-labeled band 4.1 and α and β chains relied upon a paradoxical effect of increased KCl concentration upon the binding of band 4.1 to spectrin in sucrose gradients. The gradients shown thus far were formed in 20 mM KCl, 5 mM sodium phosphate, pH 7.6, and 0.5 mM DTT. However, when the KCl concentration was raised to 120 mM, the binding of ^{125}I-labeled band 4.1 to spectrin dimers was abolished. The reason this is paradoxical is that the binding of ^{125}I-labeled band 4.1 to spectrin can easily be measured in 120 mM KCl by the immunoprecipitation assay used here (see below) and by others (Tyler et al., 1980; Wolfe et al., 1982; Goodman et al., 1982). Whatever the reason for this effect, it was also manifested with spectrin α and β chains. We found that 120 mM KCl completely abolished binding of β chains to band 4.1 and substantially reduced binding of α chains (not shown). This observation suggests that α chains, β chains, and dimers all share certain common features in their binding to band 4.1.

To obtain a more accurate comparison of the binding characteristics of α chains, β chains, and dimers to ^{125}I-labeled band 4.1, we used a quantitative immunoassay similar to that described by Tyler et al. (1980). Figure 4 shows that by this measure both α and β chains bind to ^{125}I-labeled band 4.1 and that both chains bind nearly the same amount of band 4.1 per unit weight as native dimer at each band 4.1 concentration. We are currently obtaining additional data to determine the relative affinities and saturation binding capacity of each chain.

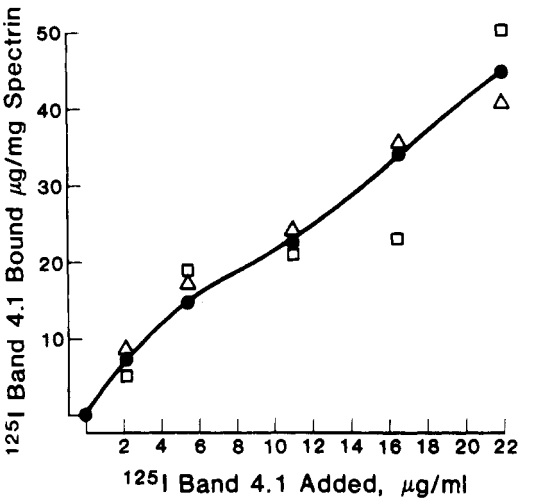

FIGURE 4: Binding of ^{125}I-labeled band 4.1 to spectrin measured by radioimmunoassay. ^{125}I-Labeled band 4.1 at the indicated concentrations was incubated with 10 μg/mL α chains (△), β chains (□), or native dimers (●), and binding was measured as described under Materials and Methods.

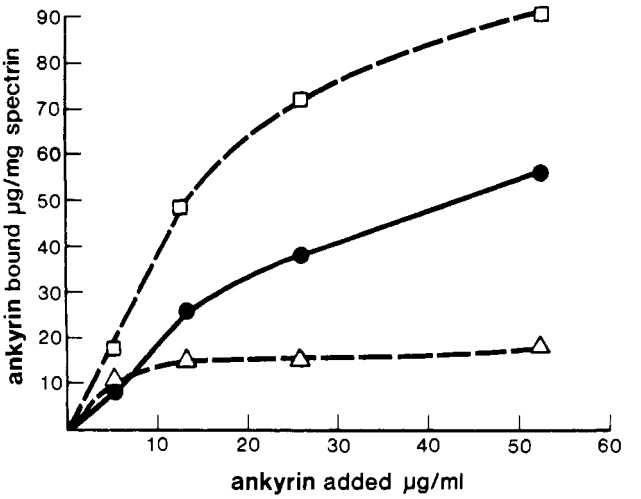

FIGURE 5: Binding of ^{125}I-labeled ankyrin to spectrin measured by radioimmunoassay. ^{125}I-Labeled ankyrin at the indicated concentrations was incubated with 50 μg/mL α chains (△) or β chains (□) or 100 μg/mL native dimers (●), and binding was measured by immunoassay as described under Materials and Methods.

As an additional test of the function of the spectrin chains, we used the immunoassay to determine whether the ankyrin binding site on the isolated β chain was functional. Figure 5 shows that isolated β chains do bind ^{125}I-labeled ankyrin while α chains bind 5–6 times less per unit weight. The data show that β chains bind about twice as much ankyrin per unit weight as α–β dimers, a result that is consistent with the fact that dimers are nearly twice as heavy as β chains but contain the same number of ankyrin binding sites. Thus, we can conclude that the functional region of the β chain responsible for association with ankyrin has been renatured to its native configuration in the β chain.

The final functional characteristic of the isolated spectrin α and β chains that we examined was their ability to associate with F-actin. Using a sedimentation assay, we measured the binding of ^{125}I-labeled α chains, β chains, and recombinant dimers to F-actin in the presence and absence of band 4.1. The binding data shown in Figure 6 represent band 4.1 stimulated binding of spectrin, or spectrin chains, to F-actin. That is, the binding of dimers or chains to F-actin without band 4.1 has

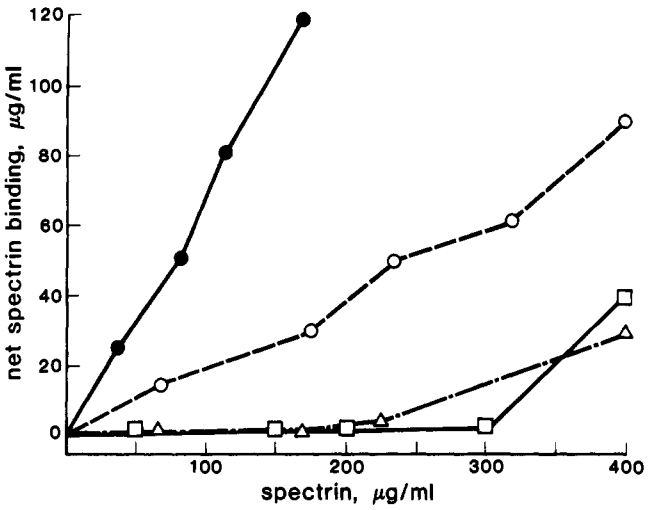

FIGURE 6: Band 4.1 dependent binding of spectrin and spectrin chains to F-actin. ^{125}I-Labeled α chains (△), ^{125}I-labeled β chains (□), native ^{125}I-labeled spectrin dimers (●), or ^{125}I-labeled recombinant dimers (○) were incubated at the indicated concentrations with 25 μg/mL band 4.1 and 100 μg/mL F-actin. Binding of spectrin dimers and chains was measured as described under Materials and Methods. The binding values shown represent net, or band 4.1 stimulated, binding to F-actin. That is, the binding or sedimentation of the proteins in the absence of band 4.1 at each concentration shown has been subtracted.

been subtracted from the total binding to give the net values shown. The data show that α and β chains have little or no band 4.1 stimulated binding to F-actin, except at the highest concentration tested. By contrast, recombinant dimers made from mixing α and β chains exhibited binding to actin at all concentrations tested. However, binding of the recombinant dimers was only 25–50% of that of an equivalent concentration of native dimers. A possible explanation for the difference between recombinant and native dimers might be that the recombinant solutions contain 25–50% of the dimers expected if recombination were 100% effective or that a percentage of the bands or recombinant dimers have denatured band 4.1 or actin binding sites.

However, examination of the recombinant dimers used in this experiment by sucrose gradient centrifugation showed that greater than 70% of the protein migrated with a sedimentation coefficient equivalent to that of spectrin dimers (see Figure 2). Thus, it seems likely that, even though most of the spectrin chains had recombined to dimers, the actin binding site was not renatured in all cases. In summary, these results show that although the α and β chains of spectrin bind band 4.1, they must be reassociated into a dimer in order to bind actin and that the actin binding site is probably a shared property of the combined chains and may be difficult to fully renature.

Discussion

The spectrin molecule is an extended multifunctional protein composed of two polypeptide chains that are folded in such a way as to define four or five structural domains each (Knowles et al., 1983). These domains have been defined on the basis of selective proteolytic attack and, in several cases, have been associated with a particular function or binding site within the molecule (Speicher et al., 1980; Morrow et al., 1980). Our work has focused upon the functional regions of spectrin associated with the binding sites for actin and band 4.1. Although electron microscopy has localized the binding sites for these molecules on or near what has been called the α-5 and β-4 domains (Morrow et al., 1980), it has remained uncertain whether both chains are in fact involved in this association. Our results suggest that although both spectrin α and β chains have binding sites for band 4.1, band 4.1 will not stimulate binding of either chain separately to F-actin. However, when the chains were brought together to form an α–β heterodimer, they did bind to F-actin. A similar conclusion was reached in a preliminary study of erythrocyte spectrin α and β chains by Calvert et al. (1980). Davis & Bennett (1983) also concluded that brain spectrin α and β chains needed to be reassociated in order to bind to F-actin, although their studies were done without band 4.1.

There are several possible interpretations for our finding that only reassociated chains bind F-actin in the presence of band 4.1. For example, it is possible that each of the polypeptide chains has a very weak association with F-actin, even in the presence of band 4.1, and that when the chains recombine these weak associations act in concert to yield a stronger net association. This view is partially supported by the increased binding of F-actin when individual α and β chains are added at the highest concentrations tested in Figure 6. An alternative view might be that neither chain alone binds specifically to F-actin and that the sedimentation of the individual chains at 400 μg/mL in Figure 6 simply reflects an artifact of aggregation. The latter view would imply that spectrin's actin binding site is a shared property of the two chains in concert rather than being a product of the properties of the individual chains. This situation could arise if, for example, combining spectrin's α and β chains in the presence of band 4.1 led to a conformational change at the tail end of the molecule that facilitated its binding to actin. In this case, the functional domain of the spectrin molecule responsible for actin binding might consist of the combined termini of the α and β chains. The choice between these and other possibilities awaits further work.

Our finding that each of spectrin's polypeptide chains binds a molecule of band 4.1 could explain the 2:1 band 4.1 to spectrin stoichiometry found when excess band 4.1 was added to spectrin in solution in the absence (Tyler et al., 1979) or presence (Cohen & Foley, 1983) of F-actin. Although the binding of two band 4.1 molecules per spectrin dimer has been questioned by more recent work (D. Branton, personal communication), we feel that both the relative and absolute concentrations of these proteins play a crucial role in the outcome of such binding studies. In any case, it is doubtful that this 2:1 binding has any physiological relevance because we have found in previous studies (Cohen & Foley, 1983) that only a single band 4.1 molecule is needed to promote the binding of a spectrin dimer to F-actin. Furthermore, in vivo there is only a single band 4.1 molecule per spectrin dimer in the red cell membrane skeleton.

The way in which a single molecule of band 4.1 promotes the association of a spectrin heterodimer with F-actin is unclear. However, one clue to the mechanics of this association may be found in our recent demonstraton that under appropriate conditions a single molecule of band 4.1 can promote the binding of two spectrin dimers to F-actin (Cohen & Foley, 1983). Although there are several possible interpretations for such a finding, one likely explanation is that each band 4.1 molecule is bivalent, capable of binding two or more spectrin dimers. Our current hypothesis holds that these sites represent an α chain site and a β chain site. Although unproven, this hypothesis simply explains several observations. First, it explains how α and β chains individually can bind to band 4.1. Second, it explains how a single band 4.1 molecule can bind two spectrin dimer molecules, possibly binding one by its α chain and one by its β chain. Finally, it suggests that the single

band 4.1 molecule needed to promote the binding of a spectrin heterodimer to F-actin may be shared by both chains, each binding to its own site on the band 4.1 molecule. This α–β–band 4.1 complex may provide the necessary molecular configuration for actin binding that the isolated chains lack. Of course, alternative explanations of these phenomena are possible. One might be that the single band 4.1 needed for actin binding attaches to one or the other of spectrin's chains (but not both) causing the dimer to bind to actin. However, this explanation does not easily deal with the fact that the individual chains do not bind F-actin in the presence of band 4.1 and does not take into account the apparent bivalent nature of band 4.1 (Cohen & Foley, 1983).

There remain several additional features of the association of spectrin α and β chains with F-actin that need further exploration. While the characterization of the association of α and β chains with band 4.1 shown in Figure 4 was representative of several other studies done, we were unable for logistical reasons to measure binding at concentrations of ^{125}I-labeled band 4.1 higher than 20–30 μg/mL. Thus, it is possible that the binding capacity or even the affinity of the two chains for band 4.1 is distinct. Further work is needed to address this question.

Another feature of the spectrin–band 4.1 interaction that requires further work is the apparent inhibitory effect of 120 mM KCl on this association as assayed by sucrose gradient centrifugation. The immunoassay of band 4.1–spectrin binding shown in Figure 4 was measured in 120 mM KCl, as was the band 4.1 stimulated binding of spectrin to actin shown in Figure 6. It is possible that the association constant for spectrin–band 4.1 binding is lower in 120 mM KCl than it is in 20 mM KCl; to our knowledge, these data are not available. If the association constant were lower in 120 mM KCl, it is possible that binding would be reduced more in a sucrose gradient than in a relatively rapid in-solution binding assay.

As indicated under Results, α and β chains purified either by preparative SDS gel electrophoresis or by chromatography on DE-52 cellulose in the presence of urea behaved identically in all of the functional assays we described. The urea method has the advantage that it is rapid and requires far fewer manipulations than the SDS method. Further, the chains purified by this method had a more compact, homogeneous distribution on sucrose gradients, suggesting a more uniform, less aggregated solution of polypeptides than that found by SDS purification. However, the urea method was not easily reproducible in our hands. Most preparations using this method yielded the β chains as expected but gave α chains heavily contaminated by β chains. Manipulation of such variables as pH and urea concentration did not yield consistent improvement. The SDS method, although time consuming, was highly reproducible. We cannot of course be certain that even with removal of all, or nearly all, the SDS that the chains have refolded precisely to their native configuration, an argument that is equally true of the urea-solubilized chains. However, the ability of the chains to form dimers and to bind ankyrin, band 4.1, and actin (when recombined) strongly argues that at least the major functional domains involved in these associations have renatured.

The requirement that both spectrin chains be together in order to bind actin in the presence of band 4.1 has important implications for the biogenesis of the membrane skeleton. It has been reported in one system, that of chick embryo erythroid cells, that spectrin α chains are synthesized at a 3-fold excess over β chains (Blikstad et al., 1983; Moon & Lazarides, 1983). Excess α chains are apparently degraded, being unable to bind to membranes independently of β chains (Moon & Lazarides, 1983). Our results suggest that while the β chains may assemble onto the membrane skeleton independently of α chains, they are probably not functional as structural elements; that is, they cannot bind to actin. Although no direct evidence of the presence of band 4.1 in chick embryo erythroid cells is yet available, a wide variety of non-erythroid cells are known to contain this protein (Cohen et al., 1982; Spiegel et al., 1982; Davies & Cohen, 1983). The synthesis of band 4.1 relative to that of α and β spectrin would also be a crucial determinant in regulating the assembly of the membrane skeleton.

Table II: Functional Associations of Erythrocyte Spectrin α and β Chains

	α chain	β chain	$\alpha\beta$ dimer
	head tail	head tail	head tail
Phosphorylated[b]	no	yes	yes
Ankyrin[c] binding	no	yes	yes
Band 4.1 binding	yes	yes	yes
Actin binding (band 4.1 dependent)	no	no	yes
Calmodulin[d] binding	yes (?)[a]	yes (?)[a]	(?)[a]
Participation in dimer-dimer association[e]	yes	yes	yes

[a] Calmodulin binding to erythrocyte spectrin has been demonstrated only under denaturing conditions (Sears et al., 1982; Glenney et al., 1982) and has been suggested by one group to be near the tail end or β-4 domain of the β chain (Sears et al., 1982). Others (Glenney et al., 1982) have shown that the calmodulin binding site for spectrin and other related proteins is on the α chain. Ultrastructural studies suggest that the calmodulin binding site of brain spectrin is near the head region of the α chain. (★) Approximate location of functional domain on chain (Tsukita et al., 1983). [b] Speicher et al., 1982. [c] Litman et al., 1980; Tyler et al., 1979. [d] Sears et al., 1982; Glenney et al., 1982; Tsukita et al., 1983. [e] Morrow et al., 1980.

Our results also have implications for the molecular model of the red cell membrane skeleton as it is currently understood (Cohen, 1983). Our findings lead us to suggest that the single molecule of band 4.1 needed to promote the binding of a spectrin dimer to F-actin is simultaneously associated with the α and β chains of the dimer and that both chains participate in binding to actin. Whether the chains themselves contact the actin filament or are linked via band 4.1 is still not known. One interesting implication of this suggestion is that if one of the spectrin chains is defective in band 4.1 binding, the dimer may still be able to bind band 4.1. In fact, several cases of hereditary spherocytosis have been reported (Wolfe et al., 1982; Goodman et al., 1982) in which it has been suggested that a fraction of the spectrin molecules in the membrane skeleton fail to bind band 4.1. In this case, our model would suggest that an altered conformation of one chain adversely affects the other so that neither can bind band 4.1 or, less likely, that both chains have molecular defects. Clearly, more work is needed before these or other possibilities can be chosen.

Finally, we note that the spectrin chains are among the largest single polypeptides known. Comparison with other large multifunctional proteins suggests that each spectrin chain could have as many as 8 or 10 separate functional domains, yet Table II shows that only a limited number of such domains have been identified. Moreover, our observations provide the first demonstration of any functional association of spectrin's α chain with any protein besides the β chain. Further study

of spectrin's polypeptide chains is needed to determine whether they participate in other, possibly unexpected, associations.

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