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Biochemical Characterization of Complex Formation by Human Erythrocyte Spectrin, Protein 4.1, and Actin†

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ABSTRACT: Ternary complex formation between the major human erythrocyte membrane skeletal proteins spectrin, protein 4.1, and actin was quantified by measuring cosedimentation of spectrin and band 4.1 with F-actin. Complex formation was dependent upon the concentration of spectrin and band 4.1, each of which promoted the binding of the other to F-actin. Simultaneous measurement of the concentrations of spectrin and band 4.1 in the sedimentable complex showed that a single molecule of band 4.1 was sufficient to promote the binding of a spectrin dimer to F-actin. However, the molar ratio of band 4.1/spectrin in the complex was not fixed, ranging from approximately 0.6 to 2.2 as the relative concentration of added spectrin to band 4.1 was decreased. A mole ratio of 0.6 band 4.1/spectrin suggests that a single molecule of band 4.1 can promote the binding of more than one spectrin dimer to an actin filament. Saturation binding studies showed that in the presence of band 4.1 every actin monomer in a filament could bind at least one molecule of spectrin, yielding ternary complexes with spectrin/actin mole ratios as high as 1.4. Electron microscopy of such complexes showed them to consist of actin filaments heavily decorated with spectrin dimers. Ternary complex formation was not affected by alteration in Mg^{2+} or Ca^{2+} concentration but was markedly inhibited by KCl above 100 mM and nearly abolished by 10 mM 2,3-diphosphoglycerate or 10 mM adenosine 5′-triphosphate. Our data are used to refine the molecular model of the red cell membrane skeleton.

The membrane skeleton of the human red cell consists of an extensively cross-linked, self-associated network of proteins which coats the cytoplasmic surface of the cell membrane. It is commonly held that the membrane skeleton is responsible for the remarkable flexibility and resiliency of the red cell membrane and that it plays a large part in maintaining the shape of the cell and its isolated plasma membrane. The major proteins of the membrane skeleton, spectrin, actin, and band 4.1, as well as other components such as band 4.9, have all been purified and at least partially characterized [reviewed in Cohen (1983)], and studies of the association of these proteins in solution have led to the development of molecular models of the red cell membrane skeleton which share certain common features. Nearly all models show that the membrane skeletal network contains short actin filaments cross-linked by tetramers (or higher oligomers) of spectrin [see, for example, Cohen (1983), Palek & Lux (1983), Goodman & Shiffer (1983), and Gratzer (1983)]. Band 4.1 plays the important role of promoting or strengthening the attachment of spectrin to actin, and the entire complex along with such accessory proteins as band 4.9 is bound to the membrane by the protein ankyrin, which fastens the membrane skeleton to the integral membrane protein band 3.

The development of this model of the membrane skeleton was based largely on studies of binary associations such as spectrin binding to ankyrin, spectrin binding to band 4.1, and spectrin binding to actin in the presence or absence of band 4.1. However, extrapolation from studies of binary interactions may not provide a complete or accurate picture of membrane skeletal organization. The present work provides a preliminary characterization of the ternary spectrin–actin–band 4.1 complex in vitro. This complex is of interest also because defects in its formation have been implicated in the inherited blood disorder hereditary spherocytosis and may also be involved with other red cell diseases as well [reviewed in Palek & Lux

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(1983)]. Recently, the measurement of spectrin–actin–band 4.1 complex formation has been used to characterize several cases of hereditary spherocytosis (Wolfe et al., 1982; Goodman et al., 1982).

In light of the importance of these measurements to the study of red cell membrane skeletal organization and their use as possible diagnostic or screening tools in the laboratory, we have initiated a detailed characterization of spectrin–actin–band 4.1 complex formation. In addition to delineating those variables which are relevant to complex formation, our findings also provide new information on the stoichiometry of spectrin binding to actin and band 4.1.

Materials and Methods

Preparation of Actin. Actin was extracted from rabbit skeletal muscle by the method of Spudich & Watt (1971) with the exception that the F-actin was extracted with 0.8 M KCl rather than 0.6 M KCl prior to sedimentation. ^{3}H-Labeled G-actin was prepared as described (Cohen et al., 1978; Cohen & Foley, 1982). Actin was polymerized at a concentration of 4 mg/mL in 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM adenosine 5′-triphosphate (ATP),[1] 0.1 mM $CaCl_2$, and 1 mM DTT 1 h prior to use and diluted to the appropriate concentration at the start of each experiment.

Preparation of Spectrin Dimers and Band 4.1. Spectrin was prepared by modifications of previously described methods which we found to improve both the actin binding activity and the stability of the protein. All procedures were done at 4 °C unless indicated otherwise. Whole blood was washed 3–4 times in buffered saline containing 145 mM NaCl, 5 mM sodium phosphate, and 0.5 mM EGTA, pH 8.0. Washed cells were lysed in at least 20 volumes of ice-cold 5 mM sodium phosphate and 0.5 mM EGTA, pH 8.0, and the ghosts were washed 3–4 times in this buffer until they were white. After the last wash, the ghost pellets were pooled and incubated for 30 min on ice with DFP (35 μL/100 mL of ghosts). The appropriate volume of DFP was first added to 1 mL of 0.1 M sodium phosphate, pH 8.0, which in turn was added to the ghosts. The ghosts were washed once in 5 mM sodium phosphate and 0.5 mM EGTA, pH 8.0, and once in ice-cold spectrin extraction buffer (0.1 mM EGTA, pH 8.5). The ghost pellets were pooled in a minimum volume, and approximately 0.5 volume of 0.1 mM EGTA, pH 8.5, was added, as well as PMSF (0.25 μL/mL of ghosts of a 40 mg/mL stock solution) and pepstatin A (5 μL/mL of ghosts of a 1 mg/mL stock solution). The mixture was incubated at 37 °C for 30 min with gentle agitation and centrifuged at $150000g_{max}$ for 40 min. The supernatant was carefully removed and adjusted to 5 mM sodium phosphate, 100 mM KCl, 0.06 mM PMSF, and 0.02% NaN_3, pH 7.6 (column buffer), and 25 mL was chromatographed on a 2.5 × 90 cm column of Sepharose 4B. The dimer fractions were pooled, concentrated to ca. 1 mg/mL, and dialyzed overnight against 5 mM sodium phosphate, 0.02% NaN_3, and 1 mM DTT, pH 7.6, prior to use.

For some experiments, spectrin was labeled with *N*-[^{3}H]-ethylmaleimide by a procedure nearly identical with that used for labeling of actin (Cohen et al., 1978). Briefly, spectrin was prepared in the absence of any reducing agents and incubated at a concentration of about 1.0 mg/mL in 5 mM sodium phosphate, pH 7.6, with a 5-fold molar excess of *N*-[^{3}H]ethylmaleimide. The *N*-[^{3}H]ethylmaleimide was first diluted in pentane with unlabeled *N*-ethylmaleimide to achieve a final specific activity of 2 Ci/mmol. The appropriate amount of 2 Ci/mmol of *N*-[^{3}H]ethylmaleimide was then added to a glass test tube and gently evaporated in a fume hood. Spectrin was added to the dried *N*-[^{3}H]ethylmaleimide and incubated with tumbling at room temperature for 30 min. The reaction was terminated by adding a final concentration of 5 mM β-mercaptoethanol, and the protein was dialyzed against 5 mM sodium phosphate, pH 7.6, and 2 mM β-mercaptoethanol for 18 h at 4 °C. The final specific activity of the spectrin was generally in the range of 1000–3000 cpm/μg. Calculations indicated that labeling resulted in the incorporation of approximately 0.7 mol of *N*-ethylmaleimide per mol of spectrin, so that, on the average, each spectrin molecule contained one or fewer modified sulfhydryl groups, out of a total number of approximately 30 (Marchesi et al., 1979; Fuller et al., 1974; Smith & Palek, 1983). Spectrin was clarified by centrifugation at 150000*g* for 30 min prior to being used. To be sure that spectrin molecules used in experiments remained in the dimeric form, spectrin in all studies was prewarmed to 37 °C for 30 min to dissociate any tetramers which might have formed.

^{32}P-Spectrin for use in double-label experiments with ^{125}I-band 4.1 was prepared exactly as described in Bennett & Branton (1977). Crude ^{32}P-spectrin extracts were centrifuged on 5–20% sucrose gradients, and fractions containing pure ^{32}P-spectrin dimers were pooled, dialyzed overnight against 5 mM sodium phosphate, pH 7.6, 0.3 mM NaN_3, and 0.2 mM DTT, and concentrated to ca. 1 mg/mL by ultrafiltration.

Band 4.1 was extracted by a modification of the method of Tyler et al. (1979) which enabled us to improve the yield and to cut the total extraction time to 1.5 days. Whole blood (300 mL) was washed 5 times in 145 mM NaCl, 5 mM sodium phosphate, and 0.5 mM EGTA, pH 8.0 (PBS–EGTA). The packed cells were lysed in 30 volumes of ice-cold 5 mM sodium phosphate and 0.5 mM EGTA, pH 8.0 (5 PB–EGTA), and washed 3 times in this buffer (centrifugation times were 20 min at 12 000 rpm in a Sorvall GSA rotor). The pelleted ghosts were pooled in a minimum volume, and an equal volume of 300 mM NaCl, 10 mM sodium phosphate, and 1 mM EGTA, pH 8.0 (2 × PBS–EGTA) was added. The suspension was further diluted 2-fold with PBS–EGTA, 2 mM DFP was added, and the mixture was incubated on ice for 30 min to extract band 6. The suspension was centrifuged in a GSA rotor as above and washed once in 5 PB–EGTA and once in ice-cold 0.25 mM sodium phosphate and 0.025 mM EGTA, pH 8.0. The pellets were transferred into 40-mL capacity screw-cap centrifuge tubes, diluted with ice-cold 1 mM TES and 0.1 mM EGTA, pH 8.0, and centrifuged in a Sorvall SS 34 rotor at 17 500 rpm for 20 min. After removal of the supernatant, the pellets were diluted with 20 volumes of 1 mM TES and 0.1 mM EGTA (prewarmed to 37 °C) and incubated at 37 °C for 40 min to extract spectrin. The extracted membranes were washed once with 0.25 mM sodium phosphate and 0.025 mM EGTA, pH 8.0, and once with 5 PB–EGTA.

In preparation for the extraction of band 4.1, the membranes from the above wash were pooled and resuspended in a final volume of 40–50 mL with 25 mM sodium phosphate, pH 7.6. A sufficient volume of 1 M borate, pH 8.0, was added (generally 1 mL per 50 mL of ghost suspension) to compensate for the pH drop due to addition of DFP which was then added

[1] Abbreviations: DFP, diisopropyl fluorophosphate; ATP, adenosine 5′-triphosphate; 2,3-DPG, 2,3-diphosphoglycerate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PBS–EGTA, 145 mM NaCl, 5 mM sodium phosphate, pH 8.0, and 0.5 mM EGTA; 5 PB–EGTA, 5 mM sodium phosphate, pH 8.0, and 0.5 mM EGTA; 2× PBS–EGTA, 300 mM NaCl, 10 mM sodium phosphate, pH 8.0, and 1 mM EGTA; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

to a final concentration of 2 mM, followed by addition of dry KCl to a final concentration of 1 M. The mixture was incubated at 37 °C for 35 min and centrifuged for 35 min in screw-cap centrifuge tubes at 160000g_{max}. The supernatant, containing band 4.1, band 2.1, and other minor proteins, was dialyzed overnight against 5 PB–EGTA plus 0.3 mM NaN_3 and 0.2 mM DTT.

After approximately 18 h, the dialysis bag was transferred for 1–2 h into the above dialysis buffer containing in addition 20 mM KCl. The extract was then centrifuged at 160000g for 35 min to remove residual aggregates or membrane fragments. The extract was loaded onto a 5-mL column of Whatman DE-52 preequilibrated with 5 mM sodium phosphate, pH 7.4, 1.0 mM EDTA, 0.2 mM DTT, 0.3 mM NaN_3, and 20 mM KCl (20 mM KCl column buffer), and the column was flushed with this buffer until the OD_{280} of the effluent returned to the base line. The column was then flushed with 60 mM KCl column buffer to elute contaminating proteins, and when the OD_{280} returned to the base line, band 4.1 was eluted with 120 mM KCl column buffer. Band 4.1 was used directly from the column without further treatment. Protein concentration was determined by measuring OD_{280} using an $A^{1\%}_{280nm}$ value of 10. Typical yield from 300 mL of blood ranged from 1.5 to 3.0 mg of pure band 4.1. Analysis of the final product on SDS gels and by immunoblotting using anti-band 4.1 antibodies showed that the protein is identical in appearance [that is, it contains the same family of breakdown products and minor components described in Cohen et al. (1982)] with band 4.1 prepared by the unmodified method of Tyler et al. (1979). Labeling band 4.1 with ^{125}I-labeled Bolton–Hunter reagent was done just after the 160000g centrifugation following the 1 M KCl extraction. Bolton–Hunter reagent (0.25 mCi) was dried under a stream of N_2 and taken up in a volume of 1 M borate, pH 8.5, equal to one-tenth the volume of the extract. This was added to the extract and incubated on ice for 30 min, after which the extract was dialyzed and treated as above.

Measurement of Spectrin–Actin–Band 4.1 Complex Formation. Unless specified otherwise, proteins 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 mM DTT, and 1 mg/mL bovine serum albumin (BSA). All samples of spectrin were preincubated at 37 °C for 1 h prior to the experiment to dissociate any spectrin tetramers which may have formed. All radioactively labeled proteins were centrifuged at 150000g for 20 min prior to use in experiments to remove aggregates which 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 (unless otherwise specified), to sediment F-actin and any associated spectrin or band 4.1. Samples (20 μL) were taken from the meniscus after centrifugation and counted for either ^{125}I, ^{3}H, or ^{32}P; the pellets were discarded. In all binding experiments (except that shown in Figure 4), the final concentration of actin in the reaction mix was 100 μg/mL, of which approximately 80 μg/mL was sedimented by centrifugation both in the presence and in the absence of band 4.1 or spectrin. The amount of actin sedimented was also independent of time up to 2 h of incubation.

In double-label experiments when ^{125}I-band 4.1 and ^{32}P-spectrin were used, samples were counted in a liquid scintillation counter, making the appropriate corrections for ^{32}P crossover into the ^{125}I channel. Protein sedimentation was quantified by subtracting concentrations in the supernate from those in the initial reaction mixtures. The values shown in the figures are the means of duplicate determinations which agreed to within 15%. Variability of binding data from one experiment to another was somewhat greater, but generally, the difference between data from similar experiments was less than 30%.

Electron Microscopy of Proteins. 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 (Cohen et al., 1980; Tyler & Branton, 1980) with 9:1 platinum:carbon from an angle of 5° from the horizontal and the replicas examined in a JEOL 100 S electron microscope.

Results

Figure 1 shows the results of a double-label binding experiment at 4 and 25 °C using metabolically labeled ^{32}P-spectrin and ^{125}I-band 4.1. The basis for the experiment is that F-actin can be sedimented by centrifugation, bringing with it any associated spectrin or band 4.1 which would not otherwise sediment. Figure 1A shows that although there was some binding of spectrin to F-actin without band 4.1 (shown only for 25 °C binding) the addition of 25 μg/mL band 4.1 caused a substantial increase in spectrin binding to actin. (Note that band 4.1 independent spectrin binding has been subtracted; see Figure 1 legend.) In the same experiment, binding of ^{125}I-band 4.1 to spectrin plus F-actin was also measured (Figure 1B). While there was some binding of band 4.1 to F-actin without spectrin (2–3 μg/mL, not shown), binding increased up to 6–8-fold with addition of increasing concentrations of spectrin.

Using the combined binding data for band 4.1 and spectrin from Figure 1A,B, we computed the ratio of band 4.1 to spectrin bound in the ternary spectrin–actin–band 4.1 complex. It should be noted that binary associations, that is, spectrin binding to actin without band 4.1 and band 4.1 binding to actin without spectrin, have been corrected for (see legend to Figure 1) and that the mole ratios in Figure 1C reflect binding to the ternary complex only. Figure 1C shows that at low spectrin concentrations the ratio is slightly above 2.0 mol of band 4.1 per mol of spectrin dimer, while at higher spectrin concentrations the ratio drops to 0.6–0.7 mol of band 4.1 mol of spectrin dimer. The data show that under appropriate conditions a single molecule of band 4.1 is sufficient to induce binding of a spectrin dimer to F-actin. Furthermore, at high concentrations of added spectrin, it appears that there can be as many as two spectrin dimers per band 4.1 in the ternary complex, while at lower concentrations the opposite holds and there may be two band 4.1 molecules per spectrin.

Figure 1 shows that nearly identical binding data and mole ratios were obtained at 4 and 25 °C, the two incubation temperatures tested. This finding makes it highly unlikely that the spectrin dimers used in the 25 °C studies had converted to tetramers during the incubation (which would affect the calculated mole ratios in Figure 1C) because tetramer formation is blocked at 4 °C (Ungewickell & Gratzer, 1978).

An additional factor in the interpretation of binding data such as is shown in Figure 1 is the possibility that the proteins have been in some way modified by the labeling agents used. The following observations rule out any labeling-induced artifacts: (1) While the binding data of Figure 1 were obtained with metabolically labeled ^{32}P-spectrin, identical results were obtained with spectrin labeled with ^{125}I-labeled Bolton–Hunter reagent and *N*-[^{3}H]ethylmaleimide. (2) Unlabeled spectrin

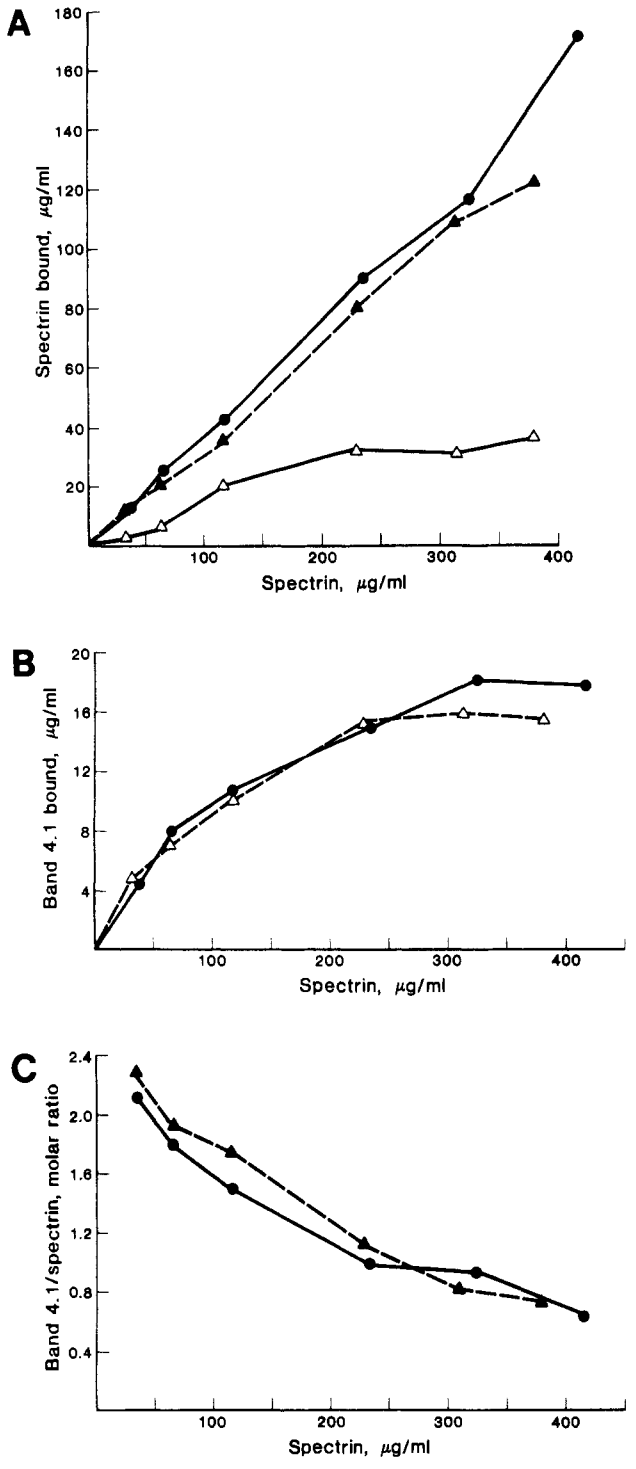

FIGURE 1: Binding of ^{32}P-spectrin and ^{125}I-band 4.1 to F-actin vs. added ^{32}P-spectrin. (A) ^{32}P-Spectrin (1200 cpm/μg) at the indicated concentrations was incubated with ^{125}I-band 4.1 (25 μg/mL) and F-actin (100 μg/mL) in binding buffer as described under Materials and Methods: (●) net (band 4.1 dependent) ^{32}P-spectrin binding to F-actin at 25 °C; (▲) net ^{32}P-spectrin binding to F-actin at 4 °C; (△) ^{32}P-spectrin binding to F-actin at 25 °C in the absence of band 4.1. Net spectrin binding (band 4.1 stimulated) was defined as the binding of spectrin to F-actin in the presence of band 4.1 minus the binding in the absence of band 4.1. (B) Net (spectrin-stimulated) binding of ^{125}I-band 4.1 to F-actin at 4 (△) and 25 °C (●). The data were obtained from the same double-label experiment shown in (A). Net band 4.1 binding was obtained by subtracting ^{125}I-band 4.1 binding to F-actin in the absence of spectrin from each data point. (C) Molar ratio of band 4.1/spectrin in the ternary complex vs. added ^{32}P-spectrin at 4 (▲) and 25 °C (●). Ratios were computed from net binding data of (A) and (B) by using molecular weights of 460000 for spectrin and 80000 for band 4.1.

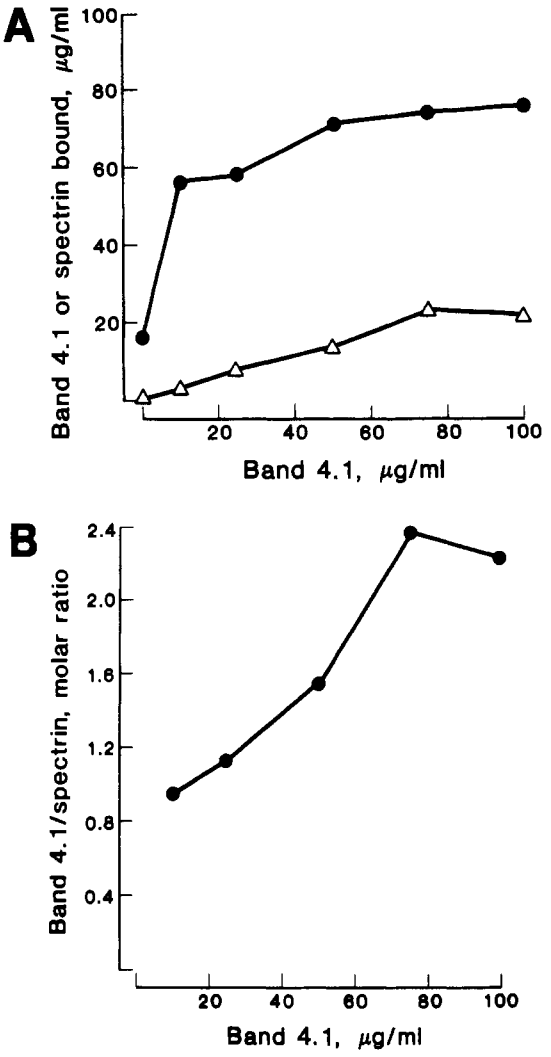

FIGURE 2: Binding of ^{3}H-spectrin and ^{125}I-band 4.1 to F-actin vs. added band 4.1 concentration. (A) ^{3}H-Spectrin (100 μg/mL) was incubated with F-actin (100 μg/mL) and increasing concentrations of band 4.1 for 1 h in binding buffer. Spectrin binding (●) was measured as described under Materials and Methods at 25 °C. The spectrin binding data shown here are total binding data; that is, the amount of spectrin bound to F-actin at 0 μg/mL band 4.1 has not been subtracted to show that there is some spectrin sedimented without band 4.1. Most of this, however, sediments even without F-actin (not shown). In a separate experiment, ^{125}I-band 4.1 was incubated with 100 μg/mL spectrin and 100 μg/mL F-actin at the indicated concentrations for 1 h at 25 °C, and ^{125}I-band 4.1 binding (△) was measured as described under Materials and Methods. The values for band 4.1 represent net, or spectrin-stimulated, band 4.1 binding; that is, the binding of band 4.1 to F-actin in the absence of spectrin has been subtracted at each point. (B) The molar ratio of band 4.1 to spectrin in the complex was computed from the net (band 4.1 stimulated) spectrin binding obtained by subtracting the amount of spectrin sedimenting at 0 μg/mL band 4.1 and net (spectrin-stimulated) band 4.1 binding.

dimers (or band 4.1) competed for ^{125}I-spectrin binding (or ^{125}I-band 4.1 binding) and, upon adjustment of specific activity at each concentration of added cold spectrin (or band 4.1), gave binding data identical with that of labeled spectrin (or band 4.1). (3) Within experimental error, unlabeled band 4.1 promoted the binding of spectrin to actin identically with ^{125}I-band 4.1, and unlabeled spectrin promoted the binding of band 4.1 to actin identically with ^{32}P-spectrin.

Figure 2A shows the dependence on band 4.1 concentration of spectrin binding to F-actin. Consistent with the results of Figure 1, band 4.1 increased the binding of spectrin to F-actin 2–4-fold over a range of concentrations. The apparent plateauing of spectrin binding vs. added band 4.1 probably rep-

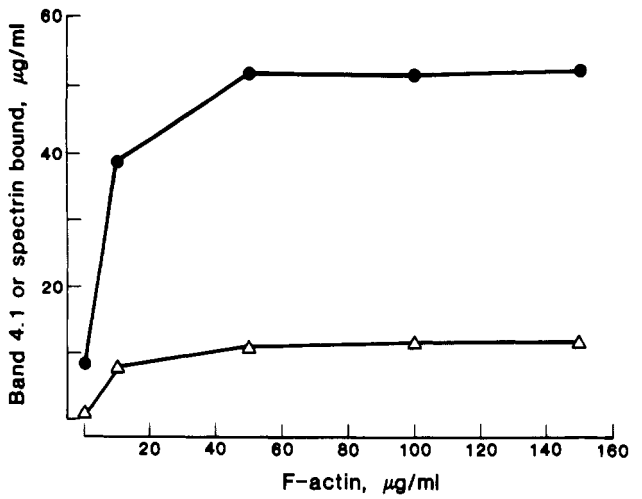

FIGURE 3: F-Actin concentration dependence of spectrin-actin-band 4.1 complex formation. ^{32}P-Spectrin (100 μg/mL) and ^{125}I-band 4.1 (25 μg/mL) were coincubated in this double-label experiment with the indicated concentrations of F-actin in binding buffer. Binding of ^{32}P-spectrin to F-actin in the presence of ^{125}I-band 4.1 was measured as described under Materials and Methods at 25 °C. (●) Net (band 4.1 stimulated) ^{32}P-spectrin binding; (△) net (spectrin-stimulated) ^{125}I-band 4.1 binding.

resents depletion of functional spectrin from the reaction mixture. In a parallel experiment (Figure 2A), also at constant spectrin concentration, band 4.1 binding increased as the concentration of added band 4.1 was increased. Figure 2B shows the molar ratio of band 4.1 to spectrin in the ternary complex vs. increasing concentrations of added band 4.1. At low band 4.1 concentration, the ratio is approximately 0.9 but increases above 2.0 as more band 4.1 is added.

The above findings show that the incorporation of both spectrin and band 4.1 into the ternary spectrin-actin-band 4.1 complex is dependent upon both the spectrin concentration and the band 4.1 concentration. Figure 3 shows the effect of actin concentration on spectrin and band 4.1 incorporation into the complex. Spectrin binding in the presence of band 4.1 increased with added actin concentration up to about 50 μg/mL F-actin, at which concentration binding plateaued. In the absence of band 4.1, spectrin binding to actin was reduced 5-6-fold (not shown) and was only slightly dependent upon F-actin concentration. The figure also shows that band 4.1 binding to F-actin in the presence of a constant amount of spectrin increased with actin concentration. Binding of band 4.1 to F-actin without spectrin showed only a slight dependence on actin concentration (not shown). Calculation shows that the molar ratio of band 4.1 to spectrin in the complex was nearly constant, ranging from 1.4 to 1.5 over the range of actin concentrations studied.

It is interesting to note in Figure 3 that even at an F-actin concentration of 10 μg/mL nearly as much spectrin is sedimented as with 100 μg/mL actin. Subtracting the amount of spectrin sedimenting in the absence of actin, we find a net value of bound spectrin of 31 μg/mL, or 0.062 nmol/mL. To compare this value with the amount of F-actin in the pellet, we measured in a parallel experiment the sedimentation of 10 μg/mL ^{3}H-labeled F-actin in the presence of 100 μg/mL spectrin and 25 μg/mL band 4.1. We found that 6.4 μg/mL or 0.147 nmol/mL sedimented. Thus, there are about two actin monomers for every spectrin molecule in the pellet under these conditions.

This finding suggested that it might be possible for every actin monomer in a filament to bind a spectrin molecule. To test this possibility, we reduced the F-actin concentration to

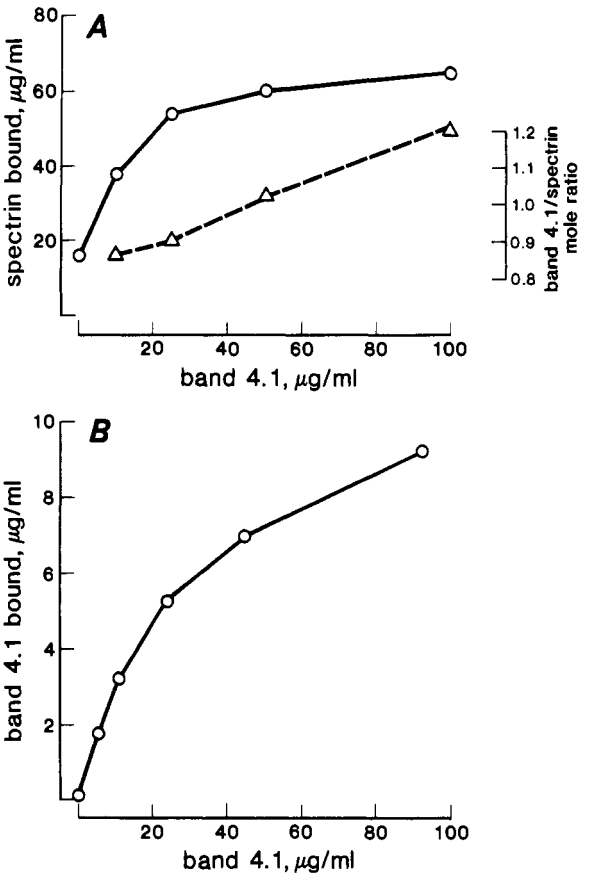

FIGURE 4: Measurement of spectrin binding capacity of F-actin. (A) ^{3}H-Spectrin (150 μg/mL) was incubated with F-actin (5 μg/mL) and the indicated concentrations of band 4.1 in binding buffer for 1 h at 25 °C. Binding was quantified as described under Materials and Methods. (○) ^{3}H-Spectrin binding to F-actin vs. added band 4.1. Molar ratio (△) of band 4.1 [see (B)] to spectrin in the ternary complex computed as described in the legends to Figures 1 and 2. (B) Net (spectrin-stimulated) binding of ^{125}I-band 4.1 to F-actin measured under the same conditions as in (A).

5 μg/mL, increased the spectrin concentration to 150 μg/mL, and measured spectrin binding vs. added band 4.1. Figure 4A shows that spectrin binding saturated at a net value of 50 μg/mL or 0.1 nmol/mL. (The net binding was obtained by subtracting the amount of spectrin sedimented in the absence of band 4.1, that is, the zero point in Figure 4A, from the total spectrin sedimented.) In a parallel experiment using ^{3}H-actin and unlabeled spectrin and band 4.1, we found that 3 μg/mL (or 0.07 nmol/mL) of the added 5 μg/mL actin sedimented with the complex. Thus, the molar ratio of spectrin/actin in the sedimented fraction at saturation is 1.4. Since we do not known whether every actin molecule in the sedimented filaments has spectrin bound to it, this ratio is an average, some actins possibly having more and some fewer bound spectrins. Experiments similar to the above were also conducted at 0 °C to eliminate the possibility of spectrin tetramer formation, and similar experiments were also done at actin concentrations of 10 and 25 μg/mL. In all cases, spectrin binding saturated at spectrin/actin mole ratios of 1.1-1.4. The results suggest that the actin monomers in a filament are capable of binding at least one spectrin molecule each.

It is interesting to note that 5 μg/mL is almost certainly below the critical concentration of actin under our experimental conditions and that in the absence of spectrin and band 4.1 little or no actin sediments. Addition of spectrin and band 4.1 results in the sedimentation of 3 μg/mL actin, suggesting that these proteins stabilize actin to depolymerization (since actin is added to the reaction from a concentrated polymerized

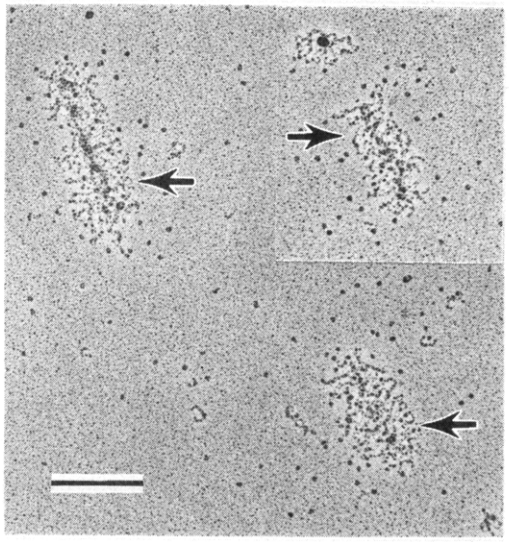

FIGURE 5: Electron micrograph of a low-angle rotary-shadowed replica of short actin filaments (arrows) heavily decorated with spectrin dimers. The specimen was obtained from an incubation containing 5 μg/mL F-actin, 100 μg/mL spectrin, and 50 μg/mL band 4.1. The proteins were incubated for 1 h in binding buffer at 25 °C except that the sodium phosphate buffer was replaced by 2 mM TES, pH 7.6. Specimens were shadowed and replicated as described under Materials and Methods. Bar = 0.2 μm.

stock) or markedly affect its critical concentration.

Measurement of band 4.1 in the complex under conditions identical with those of Figure 4A showed that band 4.1 binding continued to increase even after spectrin binding had plateaued (Figure 4B). This is reflected in a monotonic increase in the ratio of band 4.1 to spectrin incorporated into the complex (Figure 4A). At the highest concentration of band 4.1 tested, there were 1.25 mol of band 4.1 per mol of spectrin. From the above, we conclude that under appropriate conditions spectrin, actin, and band 4.1 can combine to form a complex with approximately 1:1:1 stoichiometry.

Ultrastructure of Spectrin–Actin–Band 4.1 Complexes. Examination of spectrin–actin–band 4.1 mixtures formed under conditions leading to 1/1 or higher molar ratios of spectrin/actin revealed what appeared to be actin filaments heavily decorated by spectrin molecules (Figure 5). In addition, small nodules with attached spectrin molecules were frequently found. These nodules could represent protofilaments of actin with attached molecules of spectrin and band 4.1. As expected, at lower mole ratios of spectrin to actin, actin filaments were only sparsely decorated with spectrin molecules (not shown) as we have described previously (Cohen et al., 1980).

Variables Affecting Complex Formation. Complex formation, or stimulation of spectrin binding to actin by band 4.1, took place rapidly at 25 °C. At the earliest time point that could be measured (about 2 min of incubation), complex formation was already 50% of maximum. After 10 min, it was nearly 90% maximum and showed only minimal change over the next 2 h. Complex formation was relatively insensitive to variations in $MgCl_2$ concentration over the range 0–10 mM in the presence of 120 mM KCl (not shown) but was markedly sensitive to KCl concentration above 100 mM. Figure 6 shows that there was more than a 2-fold reduction in total spectrin bound between 2.5 and 300 mM KCl, or a 10-fold reduction in band 4.1 stimulated (net) spectrin binding. Varying the concentration of Ca^{2+} from 10^{-8} to 10^{-4} M with Ca-EGTA buffers in the presence of 2 mM $MgCl_2$ and 120 mM KCl had no effect upon complex formation (not shown).

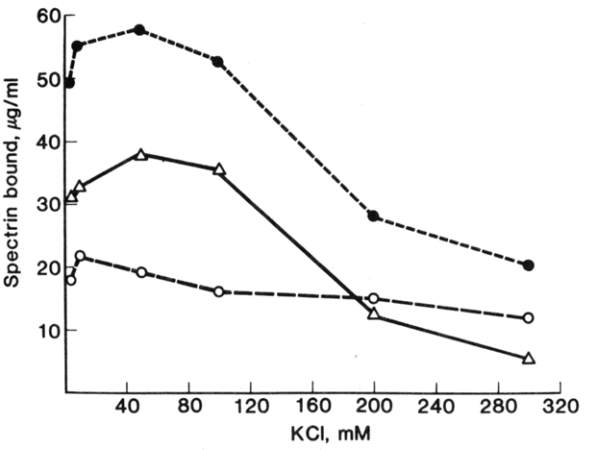

FIGURE 6: KCl concentration dependence of ternary complex formation. ^{125}I-Spectrin (100 μg/mL) was incubated with 100 μg/mL F-actin with (●) or without (○) 7.5 μg/mL band 4.1 at 25 °C for 1 h in binding buffer modified to contain the indicated concentrations of KCl. Net (△) (band 4.1 stimulated) spectrin binding.

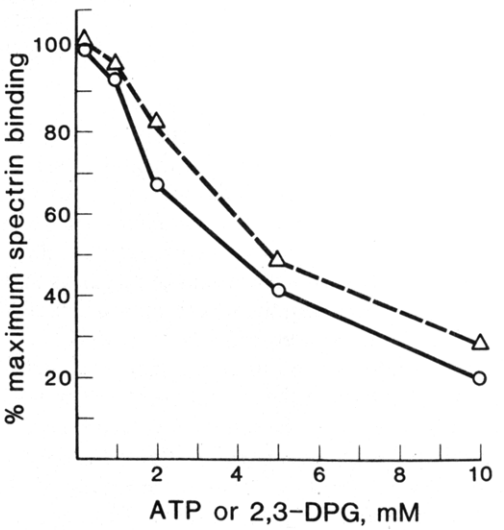

FIGURE 7: ATP and 2,3-DPG concentration dependence of ternary complex formation. ^{125}I-Spectrin (100 μg/mL) was incubated with 7.5 μg/mL band 4.1 and 100 μg/mL F-actin for 1 h at 25 °C in binding buffer modified to contain the indicated final concentrations of ATP (△) or 2,3-DPG (○). Binding was measured as described under Materials and Methods. Values shown represent the percent of net (band 4.1 stimulated) spectrin binding normalized to the values obtained at zero 2,3-DPG or ATP concentration.

One group of agents which did have a prominent effect on net spectrin binding were the poly- and biphosphates ATP and 2,3-DPG. Figure 7 shows that at a concentration of 5 mM these compounds reduced net spectrin binding by 50–60% while at 10 mM they reduced binding by 70–80%. These findings are consistent with the observed effects of these agents on cytoskeletal organization reported by Sheetz and co-workers (Sheetz & Casaly, 1980; Schindler et al., 1980).

Discussion

We have described an experimental system to study complex formation by the major red cell cytoskeletal proteins spectrin, actin, and band 4.1 and have partially defined the dependence of this association on the concentrations of the reacting proteins as well as other variables. We found that complex formation was dependent upon the concentration of spectrin and band 4.1 and that each of these proteins promoted the binding of the other to F-actin. We did observe some binding of both spectrin and band 4.1 to F-actin in the absence of the other

protein, but it is unclear whether such binding has any significance. Brenner & Korn (1979) also measured binding of (sheep) spectrin to F-actin using a sedimentation assay and found binding in the absence of band 4.1; however, their binding studies were done at actin concentration 10-fold higher than those used here. Because our interest was specifically directed toward band 4.1 stimulated spectrin binding, we purposefully did our studies under conditions in which binding without band 4.1 was minimal. We also found a small amount of binding of band 4.1 to F-actin in the absence of spectrin (not shown), but in other studies (Cohen et al., 1984) done at pH 7.0 or below, binding was significantly greater. We are currently investigating further the association of band 4.1 and F-actin.

Simultaneous measurement of the number of moles of spectrin and band 4.1 incorporated into the ternary complex in double-label experiments allowed us to compute the molar ratios of these proteins under a variety of conditions. We found that the molar ratio was not fixed but depended upon the initial relative concentration of the proteins. However, under our conditions, we never found a molar ratio of band 4.1 spectrin to be less than 0.6 or greater than 2.4. Thus, under appropriate circumstances, a single molecule of band 4.1 is sufficient to anchor spectrin to F-actin. Moreover, a molar ratio of 0.6 indicates that a single molecule of band 4.1 can promote the binding of nearly two molecules of spectrin. An alternative explanation for this finding is that within the ternary complex spectrin tetramer or oligomer formation is favored to a greater degree than in solution. Although we have no way to address this question at present, such an occurrence would obviously have profound implications for the organization of spectrin in the membrane skeleton. At the opposite extreme, Figures 1C and 2B show that at high ratios of added band 4.1 to added spectrin there were 2 mol of band 4.1 per spectrin in the ternary complex. Tyler et al. (1980) also showed that spectrin dimers could bind 2 mol of band 4.1 under conditions of band 4.1 excess, as have other authors (Wolfe et al., 1982; Goodman et al., 1982). While binding of band 4.1 dimers to spectrin could also lead to a 2:1 stoichiometry, analysis of band 4.1 by ultracentrifugation on sucrose gradients shows it to be exclusively monomeric (our unpublished results; Tyler et al., 1979).

We have recently shown (Cohen et al., 1984; Cohen & Langley, 1984) that each of spectrin's polypeptide chains separately is capable of binding a molecule of band 4.1, providing a possible explanation for the observed 2:1 band 4.1:spectrin stoichiometry. Since only one molecule of band 4.1 is needed to promote spectrin binding to actin and since the mole ratio of band 4.1 to spectrin in vivo is 1 to 1, it may be that spectrin can attach to actin by only one of its chains and that the α and β chains are interchangeable in this regard. However, recent work from our laboratory has also shown that α and β chains separately, even in the presence of band 4.1, fail to bind to actin, while recombinant dimers made from the separated chains do bind (Cohen & Langley, 1984).

Our results also show that every, or nearly every, actin monomer in a filament can bind at least one spectrin molecule when band 4.1 is present. However, it should be noted that in vivo other erythrocyte actin binding proteins may modulate the stoichiometry of spectrin–actin binding. Band 4.9 (Siegel & Branton, 1982) and erythrocyte tropomyosin (Fowler & Bennett, 1983) may both have important effects on these associations. The ability of spectrin to bind to each actin monomer in a filament distinguishes it, and perhaps nonerythroid spectrins as well, from other types of actin binding proteins. Certain actin binding proteins, such as capping or severing proteins, are thought to bind to filament ends and have extremely low protein/filament ratios while other proteins such as tropomyosin bind to as many as seven actin monomers in a filament at the same time. Perhaps another distinguishing feature is the requirement that band 4.1 be present to promote spectrin binding to actin. Although nonerythroid cells do contain band 4.1 (Spiegel et al., 1982; Cohen et al., 1982), the nonerythroid band 4.1 seems, at least in fibroblasts, to have a distribution distinct from nonerythroid spectrin (Cohen, 1983; Cohen et al., 1982).

Spectrin–actin–band 4.1 complexes formed at mole ratios of spectrin to actin close to 1 to 1 have a highly heterogeneous appearance when viewed by electron microscopy. Some appear as spiderlike structures as previously described (Cohen et al., 1980) while others appear to consist of short actin filaments heavily decorated with spectrin dimers. Although the length of actin filaments which may exist in the red cell membrane skeleton is uncertain, it is likely they are short, perhaps containing fewer than 20 monomers each (Cohen, 1983; Pinder & Gratzer, 1982). Further, since the stoichiometry of spectrin:actin in the skeleton is approximately 3:5, it is likely that such filaments which do exist would be heavily decorated with spectrin molecules. Thus, structures similar to those shown in Figure 5 may be relevant to the make-up of the membrane skeleton. As we have shown previously (Cohen et al., 1980), substituting spectrin tetramers for dimers in such mixtures leads to the formation of highly aggregated two- or three-dimensional structures which resemble the structure of the skeleton as seen by electron microscopy [see, for example, Cohen (1983)].

Our results showed that while $MgCl_2$ had little effect on complex formation, KCl above 100 mM significantly reduced specific binding of spectrin to F-actin. Modulation of cytoskeletal stability by fluctuations in KCl concentration may be important in circumstances of slightly increased or slightly reduced intracellular ion content [see, for example, Wiley et al. (1975)]. Also of interest was the striking effect of ATP and 2,3-DPG on complex formation. Those agents at concentrations of 5 mM reduced band 4.1 stimulated spectrin binding to F-actin by 50%, and at higher concentrations by 70–80%. Wolfe et al. (1981) have also reported an effect of ATP and 2,3-DPG on ternary complex formation but found that 2,3-DPG was significantly more potent than ATP. The effects of 2,3-DPG are consistent with the findings of Sheetz & Casaly (1980), who showed that 4 mM 2,3-DPG caused extensive dissociation of spectrin from isolated Triton shells. Schindler et al. (1980) also showed an effect of 2,3-DPG, although at higher concentration, on the lateral mobility of red cell membrane proteins. This latter phenomenon is presumably an indirect effect of cytoskeletal disruption by 2,3-DPG. As noted by Sheetz & Casaly (1980), the concentration of 2,3-DPG, but not of ATP, which produces significant perturbations is within the physiological intracellular range, making it possible that this molecule may modulate cytoskeletal associations in vivo. Moreover, only a slight perturbation of the spectrin–actin–band 4.1 complex may be necessary to produce a major effect upon membrane mechanical stability or deformability. Thus, even a 10% or 20% increase or decrease in membrane skeletal interconnections induced by a shift in 2,3-DPG concentration or even intracellular KCl concentration could have important consequences for cellular function.

One agent which had no demonstrable effect upon ternary complex formation was Ca^{2+}. This was surprising in light of

previous findings that mixtures of spectrin, F-actin, and band 4.1 exhibited Ca^{2+}-dependent changes in low-shear viscosity (Cohen & Korsgren, 1980; Fowler & Taylor, 1980) and that the association of band 4.1 and F-actin is, under some conditions, Ca^{2+} sensitive (Cohen et al., 1984). It may be that low-shear viscosity is sensitive to a type of interaction which is not measured by our sedimentation assay but is of physiological importance. The distinction, if any, between associations measured by these two methods is a matter for future investigation.

In conclusion, our results are consistent with a model of the red cell membrane skeleton in which an individual molecule of band 4.1 promotes the binding of a spectrin molecule to a monomer of actin within a short filament. It seems likely that both the α and the β chains of spectrin participate in the binding, perhaps by associating with the same shared molecule of band 4.1 or alternatively by sharing a band 4.1 molecule with an adjacent dimer. The choice between these alternatives awaits further work on the association of spectrin with band 4.1.

Registry No. ATP, 56-65-5; 2,3-DPG, 138-81-8; potassium, 7440-09-7.

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