Relationships of the Spectrin Complex of Human Erythrocyte Membranes to the Actomyosins of Muscle Cells†

Michael P. Sheetz,‡ Richard G. Painter,§ and S. J. Singer*

ABSTRACT: Important similarities are reported between human smooth muscle actomyosin and the human erythrocyte spectrin complex, primarily components 1, 2, and 5 (Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606). The actin-like protein, component 5, is identical with human uterine actin in its ability to form 50–70-Å filaments, to stimulate myosin ATPase activity, and to bind rabbit heavy meromyosin specifically. Antibodies to human smooth muscle myosin(uterine) were prepared which were monospecific. A weak but specific cross-reaction of these antisera with components 1 and/or 2 (spectrin) was characterized and at least 25% of the antimyosin antibodies showed a low affinity reaction with spectrin. Antibodies generated against a soluble complex of spectrin components 1 and 2 reacted only with component 1 and did not cross-react with myosin. In addition to these structural similarities between smooth muscle actomyosin and the spectrin complex, we have found that spectrin is involved in ATP-dependent erythrocyte shape changes (Sheetz, M. P., Painter, R. G., and Singer, S. J. (1976b), *Cold Spring Harbor Symp. Cell Motility* (in press) and, therefore, the spectrin complex is also a mechanochemical protein system.

Eukaryotic cells in general contain proteins that are similar to the contractile proteins of muscle cells (see Pollard and Weihing, 1974, for review). These proteins are thought to be involved in the contractile and mechanochemical activities of nonmuscle cells, such as are involved in cell motility and cytokinesis. The human erythrocyte is a relatively simple eukaryotic cell with limited cellular activities, but it does exhibit some mechanochemical features: its unique biconcave disk shape and the changes in this shape that are ATP dependent (Weed et al., 1969; Sheetz et al., 1976a,b) are expressions of such features. It has been postulated for some time (Onishi, 1962) that erythrocytes possess an actomyosin-like protein system, and the protein complex known as spectrin (Marchesi and Steers, 1968) or tektin (Mazia and Ruby, 1968) has been proposed to be such a system (Guidotti, 1972). The spectrin complex consists of several protein components which are peripherally associated with the cytoplasmic face of the erythrocyte membrane (Nicolson et al., 1971), the major components of which include bands 1, 2, and 5 in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis designations of Fairbanks et al. (1971). The similarity in molecular weights of components 1 and 2 (together called spectrin) to muscle myosin and of component 5 to muscle actin, together with the filamentous nature of the spectrin complex in electron micrographs of erythrocyte ghosts (Marchesi and Palade, 1967; Nicolson et al., 1971), have been the bases for proposing that the spectrin complex is related to muscle actomyosins. On the other hand, this evidence is quite indirect, and, in fact, on closer examination of their respective ATPase activities and other properties, components 1 and 2 show substantial differences from muscle myosins. In this paper, we provide direct evidence, however, that the spectrin complex and muscle actomyosins are indeed structurally related. We show by several criteria that component 5 is closely related to muscle actin, and that antibodies to smooth muscle myosin from the human uterus cross-react weakly but specifically with components 1 or 2 of the spectrin complex.

The conclusions from these studies have been reported briefly (Singer, 1974; Sheetz et al., 1976b). Since our work was completed, component 5 has independently been identified as actin by Tilney and Detmers (1975).

Materials and Methods

Purification of Erythrocyte Actin (Component 5). Outdated human erythrocytes were washed three times with isotonic Tris[1]-NaCl (20.0 mM Tris-HCl and 146 mM NaCl, pH 7.4) to remove plasma and the buffy coat. Cells were lysed by 1:10 dilution into 10 mM Tris, pH 7.4, and washed twice by centrifugation in this buffer at 15 000 rpm for 10 min in an SS-34 rotor. Then ghosts were washed once with 10 volumes of 0.5 M KCl–10 mM Tris, pH 7.4, and subsequently two distilled water washes were performed to remove the salt. The acetone powder of these membranes was prepared by mixing the final ghosts with 2 volumes of cold dry acetone (0 °C), centrifuging at 15 000 rpm for 10 min, washing twice with dry acetone, and then drying the pellet in a vacuum desiccator. The acetone powder was extracted twice with 20 ml of buffer A (0.2 mM Tris-HCl, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol, pH 8.0) per g of powder at 0 °C for 30 min. The extracts were pooled and concentrated by vacuum dialysis to 1–2 mg/ml protein. To polymerize the actin, 2 mM $MgCl_2$ and 50 mM KCl were added and the sample was incubated at room temperature for 2 h and subsequently at 4 °C for 16 h. The filamentous protein was pelleted by centrifugation at 80 000*g* for 3 h. The pellet was resuspended in buffer A, dialyzed against buffer A for 2 days, and centrifuged at 80 000 *g* for 3 h. This

† From the Department of Biology, University of California at San Diego, La Jolla, California 92093. *Received April 8, 1976.* This research was supported by National Institutes of Health Grants GM-15971 and AI-06659.

‡ Dernham Junior Fellow of the American Cancer Society, 1972–1974. Present address: Department of Physiology, University of Connecticut School of Medicine, Farmington, Connecticut 06032.

§ United States Public Health Service Postdoctoral Fellow, 1972–1974. Present address: Syntex Research, Department of Cell Biology, Palo Alto, California 94304.

[1] Abbreviations used: HMM, heavy meromyosin; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

cycle of polymerization and depolymerization resulted in the loss of some protein material. Two cycles were adequate to produce a preparation that was >95% pure.

Rabbit Actin and Heavy Meromyosin (HMM). Rabbit skeletal actin was prepared as described elsewhere (Spudich and Watt, 1971) and was judged free of troponin, tropomyosin, and myosin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Rabbit skeletal muscle myosin was prepared by a published procedure (Tonomura et al., 1966) and its HMM fragment prepared by limited tryptic digestion (Lowey and Cohen, 1962).

Human Uterine Actomyosin, Myosin, and Actin. Human smooth muscle actomyosin was prepared from a normal human uterus (Knierem et al., 1967). Uterine myosin and actin were prepared from actomyosin basically following the procedures of Adelstein et al. (1971). Actomyosin was dissolved in 0.6 M KCl, 5 mM $MgCl_2$, and 15 mM Tris-HCl (pH 7.5) (buffer B) and immediately ultracentrifuged at 2 °C (100 000*g*; 2 h) after the addition of 5 mM ATP. The clear supernatant was further purified by ammonium sulfate fractionation taking the 30–55% saturated ammonium sulfate fraction which was subsequently dialyzed overnight against buffer B. After the addition of 5 mM ATP, the protein was immediately applied to a column of Sepharose 4B equilibrated with buffer B containing 0.5 mM ATP–0.5 mM $MgCl_2$. The elution pattern was similar to that of Adelstein et al. (1971) with the first peak consisting of pure myosin and the second peak consisting of actin. Both proteins were assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and each was found to be free of the other.

ATPase Assays. Measurement of the ATPase activities of myosin and HMM in the presence, or absence, of actin and erythrocyte component 5 was carried out in 1.0 ml of 0.06 M KCl, 10 mM Tris-HCl (pH 7), 2 mM $MgCl_2$, 0.2 mM EGTA, and 2 mM ATP as described by Spudich and Watt (1971). Inorganic orthophosphate was assayed by the method of Fiske and Subbarow (1925). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate was performed using 5.6% acrylamide gels essentially following the methods of Fairbanks et al. (1971). All samples were reduced with 1% β-mercaptoethanol and incubated on a boiling water bath for 2 min immediately after the addition of 2% sodium dodecyl sulfate. Gels were stained for protein with Coomassie blue.

Electron Microscopy. Actin and actin–HMM complexes were examined with a Phillips EM 300 electron microscope after negative staining with 1% uranyl acetate (pH 4.5) as described by Huxley (1963).

Antihuman Uterine Myosin Antiserum. Rabbits were immunized with an initial injection in the popliteal lymph node of 25 μg of purified myosin. Boosting injections of 100 μg of myosin in isotonic saline were made intradermally 1 month after the initial immunization. The antisera were characterized by Ouchterlony plate reaction showing identity of the antigen precipitated in a crude uterine extract with purified myosin. Likewise antisera after precipitation with an equivalent amount of purified myosin showed no further reaction with the crude uterine extract.

Preparation of Spectrin Components 1 and 2. Spectrin was prepared from white erythrocyte ghosts by dialysis of ghosts at 1 mg/ml protein against 5×10^{-4} M NaN_3, pH 7.4, at 0 °C for 16 h. The membranes were centrifuged and the supernatant fraction which was normally 0.3 mg/ml in protein was taken as the crude spectrin extract. From scans of gels stained with the quantitative dye, fast green, spectrin was estimated to be 60% of the protein present. Separation of the two bands of the spectrin was accomplished by slicing and eluting unfixed sodium dodecyl sulfate polyacrylamide gels of the crude material. The purity of the isolated spectrin bands was determined by polyacrylamide gel electrophoresis.

Characterization of Antihuman Spectrin Antibody. This antigen affinity column purified γ globulin was analyzed as described earlier (Nicolson and Painter, 1973).

Microcomplement Fixation Assay. The procedure of Levine and van Vunakis (1967) was used without modification.

Quantitative Precipitin Assay. Samples of spectrin extract which had been made isotonic (0.15 M KCl) were added to a constant volume of antiserum and the total volume was made up to a constant value with an isotonic KCl solution. Solutions were mixed thoroughly and incubated at 37 °C for 2 h and 4 °C for 24 h before pelleting in a table-top centrifuge at 4 °C. Pellets were washed twice by resuspension and centrifugation before solubilization with 1% sodium dodecyl sulfate to a final volume of 0.4 ml. The A_{280} of the solubilized pellets was measured and converted to protein concentration by the factor of 1.25 A units mg^{-1} ml^{-1}. Subsequently sodium dodecyl sulfate solubilized samples were reduced and applied to sodium dodecyl sulfate–polyacrylamide gels (3.25% acrylamide).

Chemicals and Reagents. ATP (sodium salt), sodium dodecyl sulfate, and dithiothreitol were obtained from Sigma Chemical Co. Uranyl acetate was obtained from Polysciences, Inc. Acetone was analytical grade. $(NH_4)_2SO_4$ was enzyme grade (Mann Research Laboratories). All other chemicals were reagent grade or better.

Results

A. Actin

Isolation and Purity of Erythrocyte Actin (Component 5). At each step in the purification of actin from erythrocyte ghosts sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out and the patterns were compared with those of the original membranes (Figure 1). The gel of the acetone powder of the high-salt-treated membranes (Figure 1A) is the same as that of the whole membrane, except for the absence of component 6 in the former case. This component, which is known to be a subunit of glyceraldehyde-3-phosphate dehydrogenase (Shin and Carraway, 1973), was removed by the high-salt extraction and this treatment aided in subsequent purification of component 5. Extraction of the acetone powder with buffer A removed primarily component 5 along with traces of components 1 and 2 (Figure 1D). These impurities were largely removed by the subsequent KCl–$MgCl_2$ polymerization steps (Figures 1E and 1F) and the final actin was judged 95% homogeneous by the electrophoretic criterion (Figure 1F). The purified protein coelectrophoresed with rabbit actin, human uterine actin, and component 5 of the whole erythrocyte ghost.

The overall yield of actin was 6–8 mg/g of the acetone powder protein compared with a theoretical yield of about 50 mg/g, corresponding to a 15% recovery of the total membrane component 5. The majority of the loss occurred in the initial extraction step of the acetone powder where 75–80% of the component 5 remained with the membranes (Figure 1C). The low recovery is most probably due to entrapment of the component 5 in denatured protein in the acetone powder, rather than to any molecular heterogeneity of that component. Actin

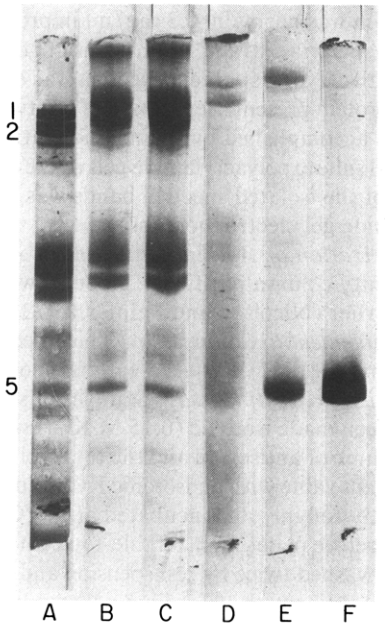

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gels (5.6% acrylamide) of the steps in the purification of erythrocyte actin. (A) The red cell ghost; (B) acetone powder of the red cell ghost; (C) extracted acetone powder (pellet); (D) extract of the acetone powder (supernatant); (E) pellet of first polymerization with 50 mM KCl and 5 mM $MgCl_2$; (F) purified actin.

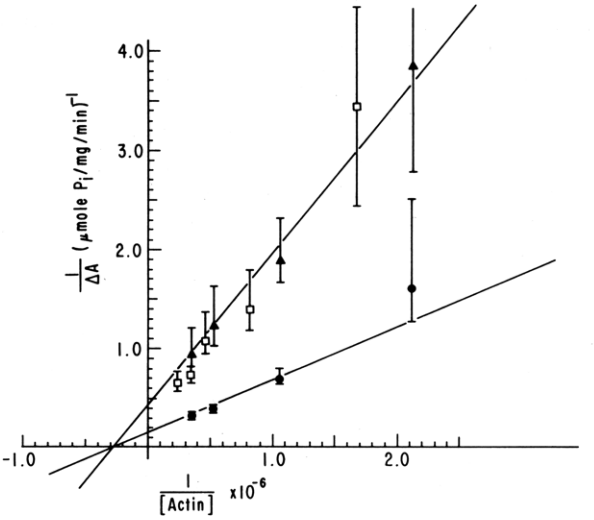

FIGURE 2: Double-reciprocal plot of rabbit skeletal HMM ATPase activation by actin from (▲) human erythrocytes, (□) human uterus, and (●) rabbit skeletal muscle. $\Delta A = A_a - A_0$, where A_a = ATPase activity with actin and A_0 = control ATPase activity.

recovery in other tissues is also low (Pollard and Weihing, 1974).

ATPase Activation. One unique functional property of actins from a wide variety of sources is their stimulatory effect on the intrinsic ATPase activity of myosin or the proteolytic fragment of myosin, HMM. As shown in Figure 2, purified component 5 does stimulate the ATPase activity of rabbit HMM. Although the stimulation is not as great as with rabbit actin, this appears to reflect a species difference since human uterine actin has the same quantitative effect as the erythrocyte actin on the rabbit HMM ATPase.

Similar experiments (not shown) were performed with human uterine myosin instead of rabbit HMM and they showed a greater degree of stimulation with the human uterine and erythrocyte actins than with rabbit actin.

Electron Microscopy. Another unique property of actin is its ability to form extensive filaments, 50–70 Å in diameter, which will bind HMM to form so-called arrowhead complexes (Huxley, 1963). This complex formation is prevented by pyrophosphate or ATP. Filaments of component 5 formed by polymerization in KCl–$MgCl_2$ become decorated with HMM (Figure 3B) but not in the presence of pyrophosphate (Figure 3A). These bare filaments and HMM-decorated filaments are indistinguishable from similar filaments prepared with rabbit actin instead of component 5. When erythrocyte components 1 and 2, which had been separated from coextracted component 5 by partition gel chromatography, were added to polymerized component 5 in the absence of pyrophosphate, no discernible interaction occurred with the filaments (Figure 3D). Spectrin components 1 and 2 alone under the same ionic conditions formed an irregular set of short filamentous structures (Figure 3C).

B. Spectrin (Components 1 and 2)

Antimyosin Antibody Cross-Reaction with Spectrin. Of four rabbits immunized with human uterine myosin, three of them made a significant antibody response as shown in the Ouchterlony diffusion experiments in the right half of Figure 4. In the same figure it is demonstrated that rabbit antispectrin antibodies did not react with the myosin, while they of course reacted intensely with homologous spectrin as shown on the left side of Figure 4. Diffuse precipitin bands can be observed between the three positive antimyosin antibody preparations and spectrin on the left side of the figure. That particular rabbit serum (113) which did not react with myosin did not react with spectrin either. The experiments in Figure 4 were all carried out in 0.5 M KCl to solubilize the myosin. If the double diffusions with spectrin were performed in 0.15 M KCl, the diffuse precipitin band formed between the antimyosin antibodies and spectrin was sharpened up (Figure 5) and was confluent with the stronger band formed between antispectrin antibodies and spectrin. Furthermore, if the antimyosin antibody preparation was first absorbed with myosin at the equivalence point, it no longer gave any double-diffusion precipitin band with either myosin or spectrin in an experiment similar to that shown in Figure 5.

In order to measure the extent of this cross-reaction, quantitative precipitation experiments were carried out between the antimyosin antibodies and spectrin or myosin. The cross-reaction exhibited a typical precipitin curve (Figure 6). Under these conditions at equivalence, 50 μg of spectrin was precipitated out of the 550 μg present in the original solution. Similarly, 100 μg of the antimyosin antibodies was precipitated by spectrin at this equivalence point, of the 400 μg of antibody which could be precipitated by the homologous antigen, myosin. The extent of cross-reaction was therefore of the order of 25% under these conditions. The cross-reaction was also observed in microcomplement fixation experiments at an antimyosin antibody concentration 16-fold greater than that required for 80% complement fixation with myosin; but at only an 8-fold greater concentration of antimyosin antibody, no complement fixation with spectrin was observed.

The most direct demonstration that the cross-reaction was specific was obtained from experiments in which antigen–antibody precipitates formed at the respective equivalence points in the reactions were dissolved in sodium dodecyl sulfate and β-mercaptoethanol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Under these conditions, the protein bands found on the gel should correspond to

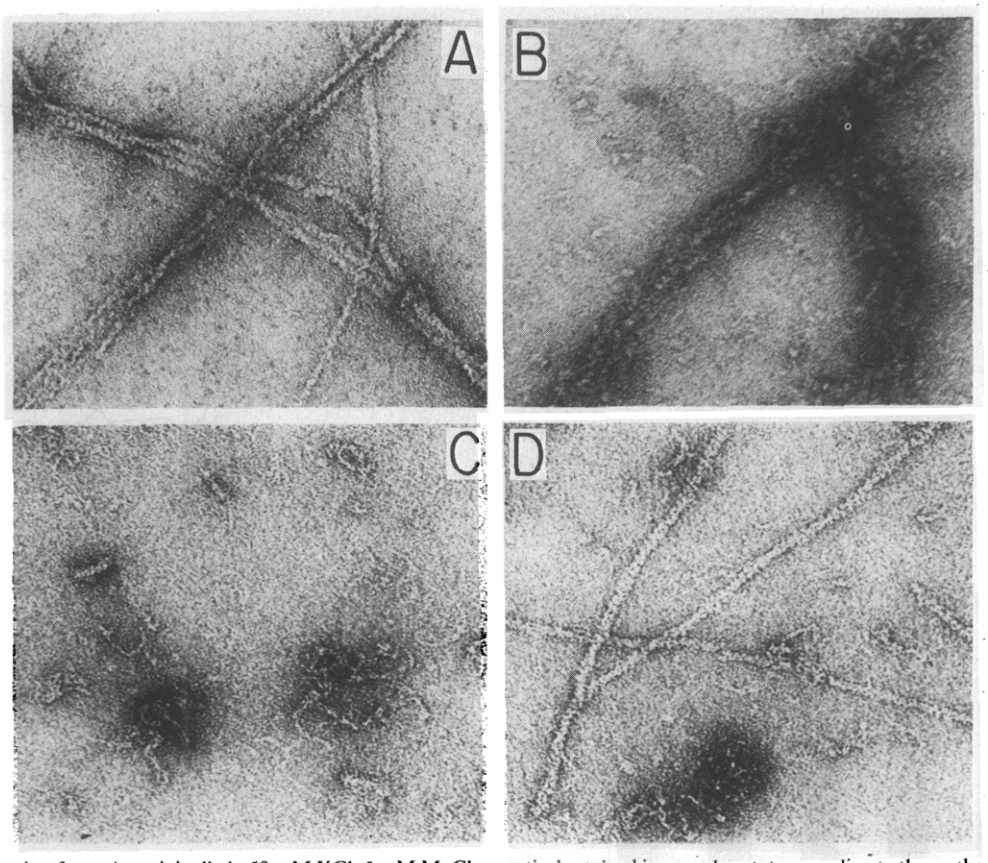

FIGURE 3: Micrographs of proteins originally in 50 mM KCl–5 mM $MgCl_2$ negatively stained in uranyl acetate according to the method of Huxley (1963) (A) Human erythrocyte actin plus rabbit striated muscle HMM in the presence of 20 mM pyrophosphate; (B) human erythrocyte actin plus rabbit striated muscle HMM in the absence of pyrophosphate; (C) purified human erythrocyte spectrin; (D) human erythrocyte actin and purified spectrin in the absence of pyrophosphate.

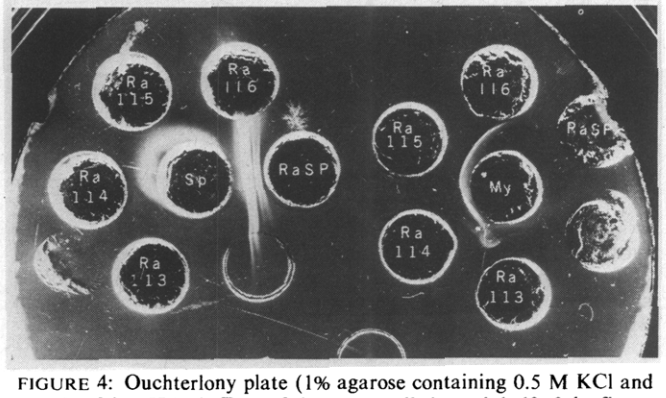

FIGURE 4: Ouchterlony plate (1% agarose containing 0.5 M KCl and 10 mM Tris, pH 7.4). Four of the outer wells in each half of the figure contain the isolated γ globulins (at four times their serum concentrations) of four rabbits (113–116) immunized with human uterine myosin. The fifth outer well contains affinity column purified antibodies to human erythrocyte spectrin (RaSp). These antisera were placed around wells containing 0.5 mg/ml myosin (My) or 2 mg/ml spectrin extract (Sp) in the same buffer.

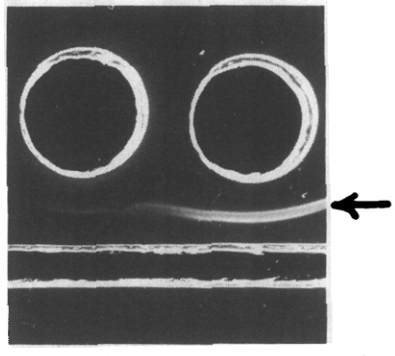

FIGURE 5: This Ouchterlony plate (1% agarose containing 0.15 M KCl and 10 mM Tris, pH 7.4) shows the precipitin line of identity (arrow) between antibodies directed to human uterine myosin (rabbit 114) in the left well and human erythrocyte spectrin in the right well when they are both diffused against the spectrin extract of hemoglobin-free ghosts in the trough below the wells.

the specific antigen in the precipitate, together with the dissociated heavy (H) and light (L) chains of the antibody. In Figure 7, these gels are shown. Gels A and E correspond to the smooth muscle myosin and erythrocyte spectrin preparations, respectively, used in the antigen–antibody precipitations; the antigen preparations used here were not as pure as were those used for immunization. It may be observed in gel C that the cross-reactive precipitate formed between antimyosin antibodies and spectrin contained only spectrin components 1 and 2, and H and L chains of the antibody. (The mobility of spectrin on such gels is slower than that of myosin, and the two proteins are therefore easily distinguished). Furthermore the weight ratio of spectrin to antimyosin antibody chains (Figure 7C) was clearly about the same (2:1) as the ratio of antigen to antibody in the homologous precipitates (Figures 7B and 7D). These results show conclusively that the antimyosin antibodies cross-react specifically with spectrin.

Specificity of Antispectrin Antibody. The antibody to spectrin has been characterized previously to show its specificity for spectrin alone of the erythrocyte membrane components. There remained, however, the question whether the antibodies were directed against component 1 or 2 or both, and

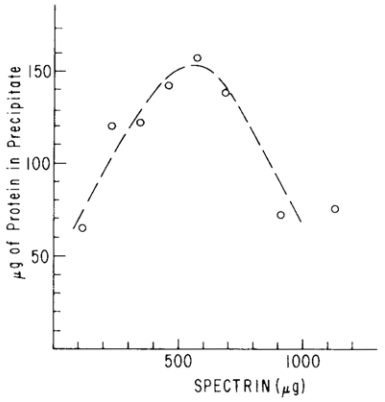

FIGURE 6: Quantitative precipitin curve obtained by using a constant amount of antimyosin antibody (~400 μg when precipitated with uterine myosin) and a variable amount of spectrin in a constant volume and ionic strength. The crude spectrin extract was used in these experiments and the concentration of spectrin in the extract was used in the plot.

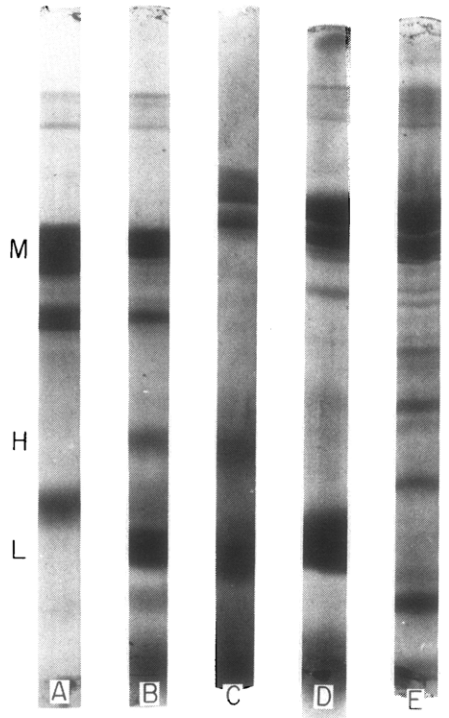

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gels of samples employed in the quantitative cross-reaction experiments. (A) Purified myosin (major band M plus some degradation products); (B) precipitate at equivalence in 0.25 M KCl of purified myosin and the rabbit antimyosin serum; (C) precipitate at equivalence of the spectrin extract and the rabbit antimyosin serum; (D) precipitate of spectrin extract with rabbit antispectrin gamma globulin; (E) spectrin extract. H and L are the heavy and light chains, respectively, of the dissociated antibodies.

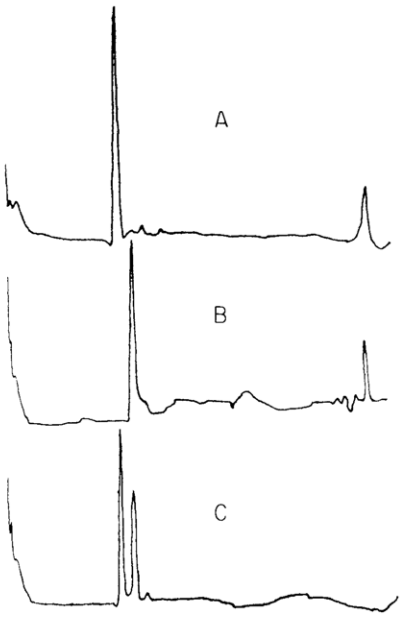

FIGURE 8: Densitometer scans at 550 nm of Coomassie blue stained sodium dodecyl sulfate–polyacrylamide gels (3.25% acrylamide) of (A) purified component 1 of spectrin, (B) purified component 2 of spectrin, (C) mixture of purified components 1 and 2.

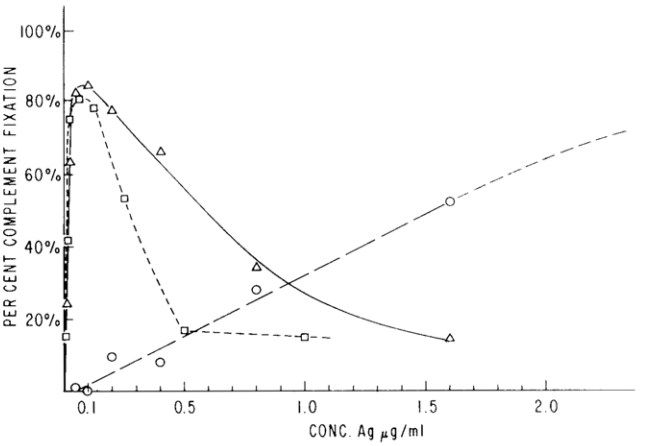

FIGURE 9: Complement fixation curve showing the percentage fixation of the antispectrin antibody vs. the concentration of component 1 in a crude spectrin extract (△), of component 1 in pure form (□), and of component 2 in pure form (○).

to what extent components 1 and 2 were cross-reactive. The antiserum used was unusual in that its reaction with spectrin was not affected by sodium dodecyl sulfate denaturation of spectrin after the sodium dodecyl sulfate was dialysed away. Because complete antigenic activity was present in the sodium dodecyl sulfate denatured spectrin, it was possible to separate components 1 and 2 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and assay their individual reactions with the antibody by microcomplement fixation. Components 1 and 2 were eluted from unstained gel slices and their purity was verified by rerunning a portion of the samples on sodium dodecyl sulfate–polyacrylamide gels (Figure 8). As shown in Figure 9 there was no detectable reaction of the antispectrin antibody with component 2 of spectrin at low concentrations and the complement fixation at higher concentrations probably represented trace contamination by component 1. There was, however, reaction of the antibody with component 1 to the same extent as that with native spectrin (not treated with sodium dodecyl sulfate) in the crude spectrin extract. This shows that there is no significant cross-reaction of antibodies to component 1 with component 2.

There were insufficient amounts of these isolated components to test against the antimyosin antibodies to determine whether the cross-reaction was specific for either component 1 or 2, or both.

Discussion

It has been suggested previously that there are actomyosin-like proteins in the erythrocyte (Onishi, 1962; Guidotti, 1972), but these reports have been rendered ambiguous by the

lack of sufficiently definitive correlations between erythrocyte and muscle proteins. In this paper, we have provided such definitive correlations.

Component 5 of human erythrocyte membranes is an actin. That the molecular weights of component 5 and muscle actin are essentially the same, about 43 000, has been noted previously. But in addition, we have shown that upon isolation of pure component 5 it is as effective as human uterine actin in stimulating the ATPase activity of rabbit HMM. Furthermore, by the well-established and specific criterion of Huxley (1963), when component 5 is treated in the manner that converts globular actin to fibrous actin, it forms filaments that are identical in appearance with those of muscle actin. These are "decorated" in arrowhead complexes upon addition of HMM (Figure 3). Similar results were independently reported by Tilney and Detmers (1975).

Human erythrocyte spectrin (components 1 and 2) must contain polypeptide structures that are similar to structures on human smooth muscle myosin. This is concluded from our demonstration that antimyosin antibodies cross-react weakly but specifically with spectrin. We do not know, however, whether this cross-reaction is specific to component 1 or 2, or both. The two components of spectrin appear to be significantly different. It is known that component 2 is specifically photoaffinity labeled with a photoreactive analogue of ATP (Haley and Hoffman, 1974), which strongly suggests that it has a binding site for ATP, and is phosphorylated both in intact cells and in ghosts (Williams, 1972; Avruch and Fairbanks, 1974; Sheetz and Singer, to be published), whereas component 1 is not modified in such experiments. Furthermore, in this paper we have demonstrated (Figures 8 and 9) that rabbit antispectrin antibodies prepared against a soluble complex of components 1 and 2 react only with component 1. The inability of the antispectrin antibodies to cross-react with smooth muscle myosin, whereas the antimyosin antibodies cross-react with spectrin, allows for the possibility that an antigenic similarity between smooth muscle myosin and spectrin component 2 exists, but it would require the preparation of separate and specific antibodies to components 1 and 2 to establish this point. Despite the clear differences between components 1 and 2, however, it is well-established that the two molecules are present in situ on the membrane, as well as in solution, as a 1:1 species (see Steck, 1974, for review). In particular, although we have shown that the antispectrin antibodies are directed exclusively to component 1, in aqueous solutions *both* components 1 and 2 are stoichiometrically precipitated by the antibodies (Figure 7D, as well as Figure 7C). Having established that a definite structural relationship exists between smooth muscle myosin and spectrin, we must, however, emphasize the many significant differences between these proteins. Spectrin does not appear to be associated with light chains that are a characteristic feature of myosins (Pollard and Weihing, 1974). Myosins have Mg^{2+}-ATPase activities which are stimulated by actin, but spectrin has only marginal, if any, such activity, and in our hands shows no stimulated activity in the presence of erythrocyte or muscle actin. Finally, myosins generally can be aggregated into elongated filaments, but we have been unable to form similar filaments from spectrin. On the other hand, freshly prepared spectrin has been reported to stimulate actin polymerization in vitro (Pinder et al., 1975).

Despite these marked differences, we nevertheless suggest that contractile proteins in nonmuscle cells and the spectrin complex in erythrocytes may exercise related mechanochemical functions on their respective plasma membranes. We have previously shown that spectrin components 1 and 2 are peripherally associated with the cytoplasmic surface of the human erythrocyte membrane (Nicolson et al., 1971) and that a smooth muscle myosin-like antigenic component is present in a similar fashion on the cytoplasmic surface of the plasma membrane of human fibroblasts (Painter et al., 1975). In addition, the actomyosin proteins in fibroblasts very likely function in contractile activity of the cell membrane, and there is good evidence (Sheetz and Singer, to be published) that the spectrin complex on erythrocyte membranes is a major controlling element in determining the shape and other properties of the erythrocyte. More recently, a new protein component has been found in smooth muscle and in nonmuscle cells (Wang et al., 1975) present on intracellular filaments in these cells. This protein, named filamin, is distinct from both myosin and spectrin, but has properties more closely resembling those of spectrin than of myosin. The detailed functional relationships among these contractile proteins remain to be elucidated.

References

Adelstein, R. S., Pollard, T. D., and Kuel, W. M. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2703.

Avruch, J., and Fairbanks, G. (1974), *Biochemistry 13,* 5507.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.

Guidotti, G. (1972), *Annu. Rev. Biochem. 41,* 731.

Haley, B. E., and Hoffman, J. F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3367.

Hamoir, G. (1973), *Phil. Trans. R. Soc. London, B Ser. 265,* 169.

Huxley, H. E. (1963), *J. Mol. Biol. 7,* 281.

Knierem, H. J., Kao, V. C., and Wissler, R. W. (1967), *Arch. Pathol. 84,* 118.

Levine, L., and van Vunakis, H. (1967), *Methods Enzymol. 11,* 928–936.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4,* 293.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Marchesi, V. T., and Palade, G. E. (1967), *J. Cell Biol. 35,* 385.

Marchesi, V. T., and Steers, E., Jr. (1968), *Science 159,* 203.

Mazia, D., and Ruby, A. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 1005.

Nicolson, G. L., Marchesi, V. T., and Singer, S. J. (1971), *J. Cell Biol. 51,* 265.

Nicolson, G. L., and Painter, R. G. (1973), *J. Cell Biol. 59,* 395.

Onishi, T. (1962), *J. Biochem. (Tokyo) 52,* 307.

Painter, R. G., Sheetz, M., and Singer, S. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1359.

Pinder, J. C., Bray, D., and Gratzer, W. B. (1975), *Nature (London) 258,* 765.

Pollard, T. D., and Weihing, R. R. (1974), *CRC Crit. Rev. Biochem. 2,* 1.

Sheetz, M. P., Painter, R. G., and Singer, S. J. (1976a), *J. Cell Biol. 70,* 193.

Sheetz, M. P., Painter, R. G., and Singer, S. J. (1976b), *Cold Spring Harbor Symp. Cell Motility* (in press).

Shin, B. C., and Carraway, K. L. (1973), *J. Biol. Chem. 248,* 1436.

Singer, S. J. (1974), *Annu. Rev. Biochem. 43,* 806.

Spudich, J. A., and Watt, S. J. (1971), *J. Biol. Chem. 246,* 4866.
Steck, T. L. (1974), *J. Cell Biol. 62,* 1.
Tilney, L. G., and Detmers, P. (1975), *J. Cell Biol. 66,* 508.
Tonomura, Y., Appel, P., and Morales, M. (1966), *Biochemistry 5,* 515.
Wang, K., Ash, J. F., and Singer, S. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4483.
Weed, R. I., La Celle, P. L., and Merrill, E. W. (1969), *J. Clin. Invest. 48,* 795.
Williams, R. O. (1972), *Biochem. Biophys. Res. Commun. 47,* 671.

Sulfhydryl Group Modification of Sarcoplasmic Reticulum Membranes†

Alexander J. Murphy‡

ABSTRACT: Modification of calcium-translocating sarcoplasmic reticulum membranes (SR) with 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) reveals four classes (kinetic sets) of sulfhydryl groups. Of the 25 mol/1.5×10^5 g of SR protein (i.e., containing 1 mol of ATPase protein) estimated in the presence of sodium dodecyl sulfate, 8 mol are unreactive, while 7, 8, and 2 mol display pseudo-first-order rate constants (k_1) of 0.16, 0.68, and 8.3 min^{-1}, respectively (25 °C, pH 7.8, 4 mM Nbs_2). Under these conditions, the Ca-ATPase activity is lost with $k_1 = 0.73$ min^{-1}, whereas the Ca-independent ATPase activity is essentially unchanged. These results are little changed by the presence of Mg^{2+} or Ba^{2+} in the modification mixture, while Ca^{2+} or Sr^{2+} causes all 16–17 reactable sulfhydryls to be modified with $k_1 = 0.50$ and 0.53 min^{-1}, respectively. The corresponding values for the loss of Ca-ATPase activity are 0.53 and 0.67 min^{-1}; this suggests that blocking of only one of the 16–17 SH groups inactivates the enzyme, i.e., that there is a single "essential" SH group. The midpoint of the transition between the Ca^{2+}-free and Ca^{2+}-modification patterns occurs at a free Ca^{2+} concentration of about 0.9 μM, implying that it is Ca^{2+} binding at the active sites ($K_D = 0.1$–1.0 μM), rather than at the low-affinity nonspecific sites, that effects a conformation change in the ATPase protein (which contains >90% of the cysteines). A calcium-induced conformation change is also suggested by increased ultraviolet absorbance spectrum of the purified ATPase protein upon calcium binding. If protein–lipid interaction is disrupted with deoxycholate or Triton X-100 (which does not destroy the Ca-ATPase activity and hence presumably leaves the tertiary structure of the ATPase protein largely intact), 95% of the sulfhydryls react with Nbs_2 considerably faster; thus, at 2 mg/ml of deoxycholate, 14 groups react with $k_1 > 20$, 5 with $k_1 = 2.3$, and 5 with $k_1 = 0.4$ min^{-1}. These results suggest that the inaccessibility of SH groups in the absence of detergents is due to extensive interaction of the bilayer phospholipids with the ATPase protein.

Sarcoplasmic reticulum membranes, isolated in vitro as vesicles, are of interest both because of their biological role in the release and uptake of calcium in muscle, and, more generally, because their relatively few protein components and limited range of activities make them well suited for study as a model for active transport and protein–bilayer interactions (for a recent review, see Mac Lennan and Holland, 1975). As the functional unit which effects calcium translocation across SR[1] membranes, the ATPase protein appears to be intimately associated with the phospholipid bilayer, as evidenced by investigations using electron spin resonance (Inesi et al., 1973), detergent solubilization (Mac Lennan, 1970), electron microscopy (Deamer and Baskin, 1969; Inesi and Scales, 1974), and tryptic digestion (Migala et al., 1973; Thorley-Lawson and Green, 1973; Stewart and Mac Lennan, 1974; Inesi and Scales, 1974; Louis et al., 1974). The latter two techniques further suggest that the ATPase is more strongly associated with outer, cytoplasmic phospholipids of the bilayer and projects from this surface into the aqueous phase. This asymmetric, cytoplasmic localization has also been inferred from x-ray diffraction experiments on centrifugally packed SR membranes (Dupont et al., 1973); similar studies (Worthington and Liu, 1973; Liu and Worthington, 1974), however, have been interpreted to indicate predominant association with the inner half of the bilayer.

The reactivity of protein functional groups, being dependent on their environment, has been widely used to measure their degree of accessibility, and to clarify their roles in maintaining the structural and functional integrity of globular proteins. For membrane-bound proteins there is the additional potential for estimating the nature and extent of interaction with the phospholipids. Furthermore, to the degree that structural changes result from the presence of, e.g., cations, substrates, or detergents, these alterations may be detected from the extent and rate of chemical modification. In this paper is reported a kinetic study of the reaction of SR with the sulfhydryl group specific reagent Nbs_2 (some of the features of these SH groups were reported on earlier by Hasselbach and Seraydarian,

† From the Laboratory of Physiology and Biophysics and the Department of Biochemistry, School of Dentistry, University of the Pacific, San Francisco, California 94115. *Received July 11, 1975.* This work was supported by grants from the National Institutes of Health (AM 16,445 and HL 16,607).

‡ Career Development Awardee of the National Institute of Arthritis, Metabolism and Digestive Diseases (AM 28709).

[1] Abbreviations used are: SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Doc, sodium deoxycholate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ADP, ATP, adenosine di- and triphosphates; ATPase, adenosine triphosphatase.