Evidence for Multiple Polypeptide Chains in the Membrane Protein Spectrin†

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ABSTRACT: The erythrocyte membrane protein called spectrin was purified and partially characterized. Quantitative and qualitative amino-terminal analysis indicates that the protein is composed of at least three and perhaps six different polypeptide chains. No evidence was found for interchain cross-linking peptides, therefore it is concluded that the subunits of this protein are composed of heterogeneous polypeptides. Amino acid analysis of each subunit of spectrin demonstrates their overall composition to be strikingly similar. Some models are suggested for the polypeptide arrangement of this unusual membrane protein. The membrane protein called tektin A was also purified and compared with spectrin. Immunological analysis, molecular weight determination, and amino acid composition clearly demonstrate that the molecules are the same.

Spectrin is a large molecular weight protein found on the inner surface of the mammalian erythrocyte (Marchesi *et al.*, 1970; Nicolson *et al.*, 1971; Tillack *et al.*, 1970). This protein constitutes about 20–30% of the total red cell ghost protein. It is considered to be a "peripheral membrane protein" (Singer and Nicolson, 1972) because it can be extracted from the membrane by low ionic strength aqueous solvents. In this report, the term "spectrin" is used to define the aqueous extractable molecules from red cell ghosts which migrate as high molecular weight double bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

By using a procedure of erythrocyte washing and extracting that was different from that reported by Marchesi *et al.* (1970), Clarke (1971) isolated and partially characterized an erythrocyte peripheral membrane protein whose physical and chemical properties were somewhat different from those reported for spectrin. Using the nomenclature suggested by Mazia and Ruby (1968), this protein was called tektin A. Interestingly both spectrin and tektin A separate on polyacrylamide gels containing sodium dodecyl sulfate into two subunits of similar molecular weight. Direct comparisons between spectrin and tektin A have not been made, although a number of investigators believe they are probably the same molecular species.

The precise molecular weight of spectrin has been a subject of some controversy. Marchesi *et al.* (1970) estimated the molecular weight of spectrin, by sedimentation equilibrium experiments in guanidine hydrochloride and polyacrylamide gel electrophoresis in sodium dodecyl sulfate, to be *ca.* 140,000. In contrast, the molecular weight of the slowly migrating doublet (spectrin) has been estimated to be nearer 250,000 (Lenard, 1970; Berg, 1969), although these results have been questioned (Trayer *et al.*, 1971) because the sizes were calculated using unreduced proteins as standards. In another study, Clarke (1971) purified the doublet from other membrane proteins prior to determining its molecular weight. Using reduced myosin as a high molecular weight standard, she reported sizes of 240,000 and 220,000, respectively.

Attempts to further dissociate the high molecular weight doublet (spectrin) into smaller components using a variety of conditions have not been successful; therefore, it has been suggested that these are among the largest single polypeptide chains known to exist in eucaryotic organisms (Gwynne and Tanford, 1970). Other investigators (Marchesi *et al.*, 1970) have suggested, however, that some stable covalent cross-linkage (*e.g.*, ϵ-(γ-glutamyl)lysine) may exist in spectrin, such as is seen in fibrin (Pisano *et al.*, 1969, Matacic and Loewy, 1968). This possible explanation received some support when it was shown that an aqueous soluble membrane protein from L cells contained a surprising number of ϵ-(γ-glutamyl)lysine interchain isopeptide cross-linkages (Brickbichler *et al.*, 1973).

In this report we have reevaluated the molecular sizes of the subunits of spectrin. Evidence is presented which shows that the spectrin doublet has multiple amino-terminal amino acids, thereby implying that the subunits cannot be single polypeptide chains. Since the presence of any type of interchain covalent crossbridge (*i.e.*, ϵ-(γ-glutamyl)lysine or ϵ-(β-aspartyl)lysine) was not detected, we suggest that each subunit is probably comprised of several nonidentical large polypeptide chains. Moreover, we have direct evidence demonstrating that spectrin and tektin A are identical molecules. In light of these studies of the subunits, two hypothetical arrangements of the polypeptide chains of spectrin are suggested.

Material and Methods

Preparation of Erythrocyte Membranes. Outdated human blood was used as a source of erythrocytes. The intact cells were washed three times with ice-cold 0.15 M NaCl. After each saline wash the buffy coat was removed so that by the final wash an essentially pure preparation of erythrocytes remained. Following the removal of the last saline wash the packed cells were lysed by pouring them into a rapidly stirring sodium phosphate buffer (8 mM, pH 7.2). The ghosts were washed free of hemoglobin by repeated centrifugation at 20,000*g*. Usually three to four washes were sufficient to remove the majority of hemoglobin, and the resulting membranes were faintly pink.

Extraction and Purification Procedures. SPECTRIN. The membrane extraction procedures were essentially those described by Marchesi *et al.* (1970). The extracted supernatants were pooled and spectrin was concentrated by adding solid am-

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monium sulfate to 50% saturation. The precipitate was collected by centrifugation and dialyzed against TMES buffer (25 mM Tris–5 mM β-mercaptoethanol–1 mM EDTA–50 mM NaCl, pH 8.6). In some experiments the crude spectrin was reprecipitated by slowly adding 0.5 M sodium acetate buffer (pH 5.3). The precipitate was collected by centrifugation at 20,000*g* for 15 min. The protein was redissolved by the addition of 0.1 M Tris (pH 11.0), followed by dialysis against TMES buffer. The crude spectrin was then chromatographed on Sephadex G-200 in an upward flow column (2.5 × 100 cm) using TMES as the eluting buffer. Column fractions containing spectrin were concentrated by low pH precipitation, and resolubilized as described above.

TEKTIN A was extracted and purified from erythrocyte ghosts following the procedures described by Mazia and Ruby (1968). Final purification of tektin A was accomplished by gel filtration on Sephadex G-200 in an upward flow column (2.5 × 50 cm), using imidazole–glycylglycine buffer (1 mM, pH 7.4), as described by Clark (1971).

Polyacrylamide Gel Electrophoresis. Protein samples were routinely analyzed by polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (Weber and Osborn, 1969). In this system spectrin or tektin A migrated into the gel as two closely associated bands (the spectrin doublet). A similar double band has been designated as membrane protein I and membrane protein II by other investigators (Fairbanks *et al.*, 1971). The electrophoresis buffer contained sodium phosphate (0.05 M, pH 7.2) and 0.1% sodium dodecyl sulfate. The gels (0.6 × 10 cm) contained 5% acrylamide (Eastman Chemicals), sodium phosphate (0.05 M, pH 7.2), and 0.1% sodium dodecyl sulfate. The samples (20–25 μl containing 1 μg/ml) were mixed in 5 μl of 20% sodium dodecyl sulfate and 1 μl of 14.3 M β-mercaptoethanol and incubated for 3 min in a boiling-water bath. Electrophoresis was carried out for 8 hr at 6 mA/tube.

The gels were stained by a variation of the procedure of Fairbanks *et al.* (1971). The protein was fixed and some of the sodium dodecyl sulfate eluted by placing the gels overnight in a solution of 25% isopropyl alcohol–10% glacial acetic acid (v/v). They were stained for 8–12 hr in 0.025% Coomassie Brilliant Blue (Sigma Chemical Co.), 10% isopropyl alcohol, and 10% glacial acetic acid, and were then destained in several changes of 10% isopropyl alcohol and 10% glacial acetic acid.

Antibody Preparation and Immunological Analysis. An antiserum against purified spectrin was prepared in a rabbit given a total of 7.5 mg of protein in three injections at 10-day intervals. The initial injection of spectrin mixed with Freunds' complete adjuvant (Difco) was made subcutaneously and in the foot pads. Two subsequent subcutaneous injections were made with spectrin mixed with incomplete to complete (8:1) adjuvant. Double immunodiffusion analyses were carried out in 1% Bacto agar in 0.85% NaCl and 0.1% NaN_3 as described by Ouchterlony (1958).

Amino Acid Analysis. Purified samples of spectrin and tektin A (2.0 mg) were hydrolyzed in 5.7 N HCl at 110° for 18, 48, and 72 hr in sealed, evacuated hydrolysis tubes. Analyses were carried out using a Beckman Model 119 automatic amino acid analyzer equipped with a single column. The total cystine–cysteine content of spectrin was determined using the method of Spencer and Wold (1969), by which proteins are hydrolyzed in the presence of low concentrations of dimethyl sulfoxide.

The amino acid compositions of each of the spectrin subunits were determined according to the method of Houston (1971). The stained protein doublet bands, which were clearly distinguishable from one another, cut out of sodium dodecyl sulfate polyacrylamide gels, and individually hydrolyzed at 110° for 20 hr in 5.7 N HCl.

Molecular Weight Determination. SODIUM DODECYL SULFATE GEL ELECTROPHORESIS. The molecular weights of the subunits of both spectrin and tektin A were determined by polyacrylamide gel electrophoresis in the presence of the detergent sodium dodecyl sulfate (Chrambach and Rodbard, 1972; Weber and Osborn, 1969). The reference proteins used were human haptoglobin 2-2: polymer 3 (285,000) and polymer 2 (228,000) (Fuller *et al.*, 1973), human γ-globulin (150,000), bovine serum albumin (68,000), and ovalbumin (45,000).

SEDIMENTATION EQUILIBRIUM. Molecular weights were determined by the equilibrium method of Yphantis (1964) in a Beckman Model E analytical ultracentrifuge equipped with ultraviolet (uv) optics and scanner. Spectrin (0.2 mg/ml) was dissolved in 25 mM Tris–5 mM β-mercaptoethanol (pH 8.6) and sedimentation equilibrium was performed in a six-channel cell at 20° for 20–24 hr at 10,000 rpm. Molecular weights were calculated from the uv scans using a partial specific volume for spectrin of 0.730 (Marchesi *et al.*, 1970).

Amino-Terminal Analysis. DANSYL CHLORIDE METHOD. The amino-terminal residues of spectrin were determined using a modification of the method of Percy and Buchwald (1972). Three 5 mM aliquots of spectrin were dried under vacuum and taken up in 0.5 ml of 8 M urea–1% sodium dodecyl sulfate. To each sample 0.05 ml of phosphate buffer (0.4 M, pH 8.2) and 0.25 ml of dimethylformamide were added and the tube contents were thoroughly mixed and incubated for 5–8 hr at 37°. Dansyl chloride (Dns-Cl) (5 mg) was dissolved in 0.1 ml of acetonitrile and added to each sample. After 30 min at room temperature, the dansylated proteins were precipitated by adding 10 ml of 10% trichloroacetic acid. The precipitates were collected by centrifugation and washed three times with 5-ml portions of acetone to remove excess Dns-Cl, then dried under nitrogen to remove the acetone, and hydrolyzed for 6 hr in 0.5 ml of 5.7 N hydrochloric acid at 110°. The dansylated amino acids were then chromatographed on 5 × 5 cm polyamide miniplates (Cheng Chin Trading Co., Ltd.). Five different solvent systems were used to aid in the identification of Dns-amino groups.

THIOACETYLTHIOGLYCOLIC ACID METHOD. Quantitative amino-terminal analyses were carried out using the method developed by Mross and Doolittle (1971) and Weinstein and Doolittle (1972). Approximately 20 mg of thioacetylthioglycolic acid was added to 40 nmol of purified spectrin dissolved in 1.0 ml of 6 M guanidine-HCl in 0.1 M Tris (pH 9.5) and 1.0 ml of pyridine. The mixture was incubated at 40° for 1 hr, maintaining the pH at 9.5 with 1.0 M NaOH. The protein was then washed three times using 6 ml of benzene for each wash and three times with 6 ml of 95% aqueous acetone. The resulting precipitate was taken up in 1 ml of water and freeze-dried overnight. The sample was then incubated in 0.2 ml of trifluoroacetic acid at 40° for 20 min and extracted twice with 1.5 ml of 1,2-dichloroethane. The 1,2-dichloroethane extracts were transferred to a hydrolysis tube containing 0.2 ml of 5.7 N HCl and mixed thoroughly. The 1,2-dichloroethane was evaporated under a stream of nitrogen, an additional 0.2 ml of 5.7 N HCl was added, and the sample was hydrolyzed under vacuum at 130° for 4 hr. Amino acid analysis was then carried out on the automatic amino acid analyzer.

Detection of ε-Amino Cross-Linked Lysine. The possible presence of ε-amino cross-linked lysine was investigated using a variation of the chemical method of Pisano *et al.* (1969). This procedure is composed of three steps: (1) cyanoethylation, (2) acid hydrolysis, and (3) quantitation of free lysine.

Spectrin (15 mg) dissolved in 1 ml of 0.1 M NH_4HCO_3 was

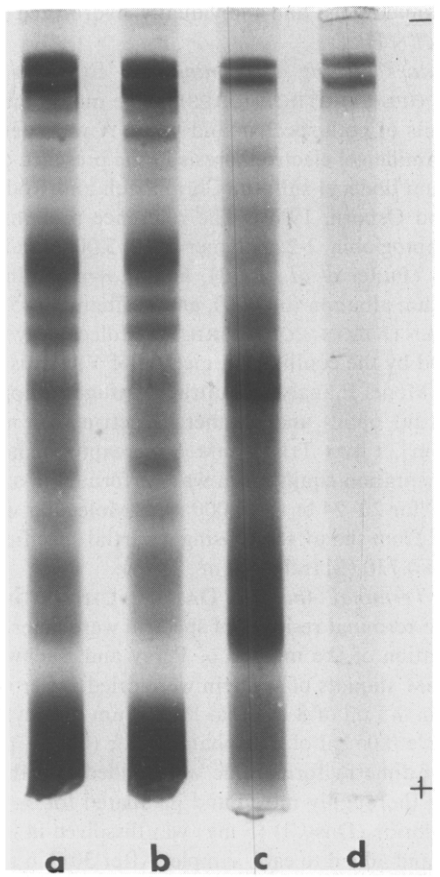

FIGURE 1: Acrylamide gels demonstrating the steps of purification of spectrin: (a) resolubilized red cell ghosts; (b) ghosts after extraction in low ionic strength buffer; (c) concentrated extract; (d) purified spectrin after G-200 chromatography.

incubated with 1 mg of trypsin or chymotrypsin (twice crystallized, Nutritional Biochemicals Corp.) for 24 hr at 37°. The enzymatic digests were then incubated with 200 μl of acrylonitrile and 20 μl of triethylamine in a sealed test tube for 72 hr at 40° with occasional shaking. The tubes were opened and dried under a stream of nitrogen, then hydrolyzed with 1.5 ml of 5.7 N HCl for 20–24 hr. The hydrolyzed samples were applied to the Beckman Model 119 amino acid analyzer in order to determine the quantity of underivatized lysine.

Direct measurement for the presence of ϵ-(γ-glutamyl)lysine was carried out after total enzymatic digestion of spectrin. The

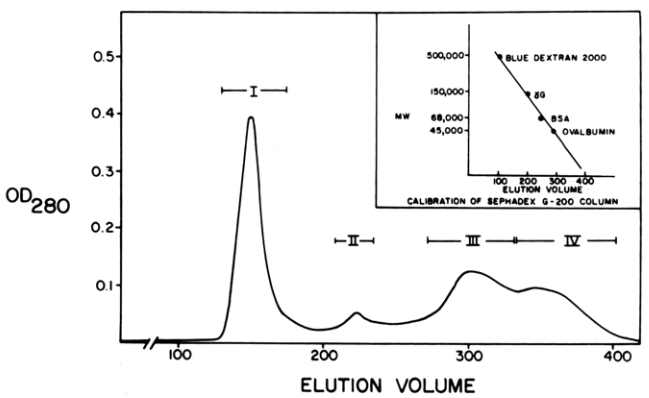

FIGURE 2: Gel chromatography on Sephadex G-200. Peak I represents spectrin and constitutes the majority of the protein. Peak II also shows full immunologic cross-reactivity with anti-spectrin antiserum. Peaks III and IV show no immunological identity with spectrin and constitute protein not investigated in this study. Insert: calibration of Sephadex G-200. Peak II elutes at a position representing a molecular size of *ca.* 110,000.

TABLE I: Molecular Weight of Spectrin Subunits.

	Exptl Mean	No. of Determinations	Deviation	Std Error
SDS[a] gel electrophoresis				
Subunit I	242,901	3	1810	±1045
Subunit II	222,737	3	1864	±1076
Analytical ultracentrifuge	238,942	3	8732	±5041

[a] SDS = sodium dodecyl sulfate.

procedure for the total digestion was that described by Pisano *et al.* (1969) with the exception that the step which called for the use of prolidase was omitted. The enzymes used for digestion included trypsin (Nutritional Biochemicals Corp.), Pronase (Calbiochem), and leucine aminopeptides (Worthington). Following enzymatic hydrolysis, 1.8 nmol of [^{14}C]-ϵ-(γ-glutamyl)lysine was added to the sample as a tracer. The digestion mixture was then subjected to chromatography on a 0.9 × 30 cm Dowex 50-X4 column equilibrated with ammonium acetate buffer (0.1 M, pH 3.8). The sample was loaded on the column and 50 ml of the pH 3.8 buffer was run through the column, then a stepwise shift was made to pH 4.2 ammonium acetate (0.1 M). Fractions of 1 ml were collected throughout, and radioactivity was monitored on 50-μl aliquots. The fractions containing the radioactivity were pooled and freeze-dried. The samples were analyzed directly without further treatment on the Beckman amino acid analyzer, employing pH 3.85 buffer.

Results

Purification of either spectrin or tektin A by the procedures previously outlined resulted in essentially pure protein when analyzed on polyacrylamide gels (Figure 1). Spectrin was eluted near the void volume when Sephadex G-200 column chromatography was performed (Figure 2). Although the major eluting protein is spectrin, there are at least three other protein species fractionated.

When analyzed by double immunodiffusion using antisera directed against spectrin, peaks I and II eluted on Sephadex G-200 show immunological identity. When gel chromatography was carried out on a calibrated Sephadex G-200 column, peak II was shown to have a molecular weight of *ca.* 110,000. This finding was unexpected since all previous data yielded a minimum molecular size of 240,000 and 220,000 for the spectrin doublet. Sodium dodecyl sulfate polyacrylamide gel analysis of peak II revealed only a very small amount of the 110,000 molecular weight species, whereas most of the protein was apparent as the high molecular weight doublet. This information suggests that the protein of peak II spontaneously reassociates into the higher molecular weight subunits.

The molecular weights of the doublet were determined by sodium dodecyl sulfate gel electrophoresis and sedimentation equilibrium. (Table 1). Both techniques yielded results indicating the doublet is in the range of 240,000–220,000. The advantage of the sodium dodecyl sulfate procedure is that it permits molecular weight estimation of each subunit, whereas the sedimentation method yields an average molecular weight for both species. The subunit sizes reported here correspond to those reported by Clarke (1971) and Steck (1972). They are significantly higher than those determined by Marchesi *et al.* (1970).

The amino acid composition of each subunit was determined by first separating the bands on polyacrylamide gels. The sub-

TABLE II: Amino Acid Analyses of Spectrin Subunits.

Amino Acid	Subunit I (240,000)		Subunit II (220,000)		Subunit I & II	
	Mol %[a]	Residues/Mol	Mol %[a]	Residues/Mol	Mol %[a]	Residues/Mol
Lysine	*b*	*b*	*b*	*b*	6.84	287
Histidine	2.85	62	3.04	62	2.86	122
Arginine	5.67	124	5.44	111	5.27	224
Aspartic acid	11.79	257	11.78	240	9.76	414
Threonine	5.20	113	5.84	119	5.09	215
Serine	9.02	197	9.23	188	5.70	241
Glutamic acid	22.12	483	21.96	447	17.61	743
Proline	2.14	47	1.94	39	2.32	97
Glycine	8.09	176	8.31	169	8.32	350
Alanine	9.04	197	8.61	175	8.30	350
Valine	3.93	86	4.44	90	4.39	186
Methionine	0.79	17	0.46	9	1.18	51
Isoleucine	3.66	80	3.54	72	3.67	156
Leucine	12.29	268	11.82	240	12.20	515
Tyrosine	1.63	36	1.14	23	2.31	97
Phenylalanine	3.09	67	2.72	55	3.22	135

[a] Average of three determinations from 20-hr hydrolysis by the method of Houston (1971). [b] Lysine routinely masked by ammonia.

units were visualized by staining and destaining in the usual way. Twelve gels were run simultaneously, each gel having 50 μg of protein on it. The bands were then sliced from the gel and subjected to acid hydrolysis using the precautions suggested by Houston (1971). This procedure permits direct amino acid analysis of isolated protein. Table II demonstrates the amino acid composition and residues per mole of each of the subunits of spectrin.

The purified antiserum directed against spectrin was used to test the immunological similarities between spectrin and tektin A. Double immunodiffusion (Figure 3) demonstrates that complete immunological cross-reactivity exists between spectrin and tektin A and suggests that these proteins are quite likely the same molecular entity. The difference in physical properties reported (Marchesi *et al.*, 1970; Clarke, 1971) probably represents differences in purity and method of preparation.

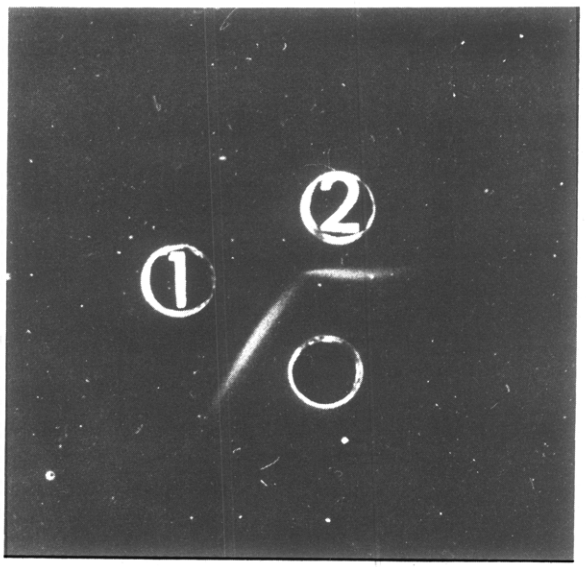

FIGURE 3: Double immunodiffusion precipitin reaction demonstrating full cross-reactivity between (1) tektin A and (2) spectrin using rabbit anti-human spectrin.

When the amino acid composition (Table III) and the electrophoretic mobility on sodium dodecyl sulfate gels (Figure 4) of spectrin and tektin A are compared they are very similar.

The amino terminals of spectrin were determined both qualitatively and quantitatively using two different procedures. Long-term storage of spectrin often results in the appearance of additional electrophoretic bands. Therefore, prior to using any spectrin for amino-terminal analysis, its purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis to ensure that no breakdown products were visible.

TABLE III: Amino Acid Analyses of Spectrin and Tektin A.

Amino Acid	Spectrin[a]		Tektin A[a]	
	Mol %	Residues/Mol	Mol %	Residues/Mol
Lysine	6.6	272	6.2	255
Histidine	3.0	124	2.9	119
Arginine	5.8	239	5.7	234
Aspartic acid	9.4	388	9.3	381
Threonine	4.6	190	5.1	209
Serine	5.0	206	5.4	222
Glutamic acid	15.8	652	15.7	645
Proline	3.3	136	3.4	140
Glycine	5.8	239	6.9	283
Alanine	8.0	330	8.4	345
Valine	5.9	243	5.3	218
Methionine	1.8	74	1.9	78
Isoleucine	3.4	140	3.5	144
Leucine	13.9	573	13.6	558
Tyrosine	2.4	99	2.6	108
Phenylalanine	4.5	186	4.9	201
Cystine–cysteine[b]	0.54	21		

[a] Average of three determinations from 18-, 48-, and 72-hr hydrolysis. [b] Determined by the method of Spencer and Wold (1969).

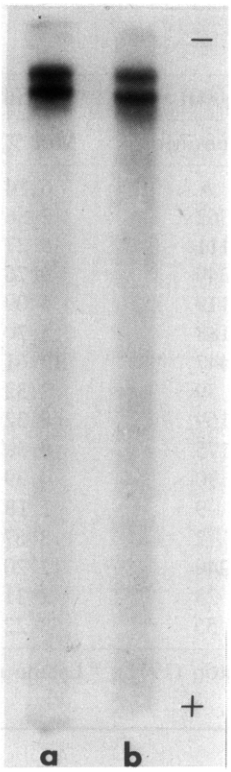

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gels of (a) purified spectrin and (b) tektin A.

Amino-terminal amino acids were determined qualitatively using the dansyl chloride method. Dansyl chloride amino acid standards were chromatographed simultaneously with the spectrin amino terminals. Duplicate samples on three different preparations were analyzed by this method (Table IV). Quantitation of the amino terminals was accomplished using a new coupling reagent (thioacetylthioglycolic acid) following the procedures previously described. Although the quantitative data (Table IV) did not yield the same number of amino terminals as the Dns method, the reproducibility of the results by both the Dns method and thioacetylthioglycolic acid method support the notion that the spectrin doublet has at least three amino-terminal amino acids and perhaps six.

The presence of multiple amino-terminal amino acids of the spectrin subunits suggested that the polypeptide chains may be cross-linked. It has been established that certain "structural" proteins (*e.g.* elastin, collagen, and fibrin) are linked for stability through a peptide bond arising from the ϵ-amino group of lysine and the carboxylamide of glutamine. It has been suggested (Marchesi *et al.*, 1970) that spectrin may have such a cross-linking bridge in it.

Spectrin was examined for the presence of interchain cross-linkages using both enzymatic and chemical procedures. The enzymatic hydrolysis method was used to detect the presence of ϵ-(γ-glutamyl)lysine, since it is known that this dipeptide is not cleaved by the usual proteolytic enzymes. There was no indication of this dipeptide in three different experiments. As a further verification of this finding, the chemical procedure of cyanoethylation was used. This procedure derivatizes all available α- and ϵ-amino groups. To ensure that as many lysine residues as possible were available for cyanoethylation, spectrin was partially degraded with trypsin, prior to adding the cyanoethylating reagents. When this preparation was examined for the presence of underivatized lysines, none were found, verifying the enzymatic hydrolysis data. If there were a lysine or arginine amino terminal to the lysine in an ϵ-(γ-glutamyl)lysine linkage, the α-amino group of that lysine could be available for cyanoethylation following the tryptic digestion. Therefore no free lysine would be detected following acid hydrolysis. To avoid this possible complication, spectrin was partially digested with chymotrypsin prior to cyanoethylation. Neither the enzymatic nor the chemical procedure demonstrated the presence of ϵ-(γ-glutamyl)lysine or ϵ-(β-aspartyl)lysine cross-linkages in spectrin.

TABLE IV: Qualitative and Quantitative Amino-Terminal Analysis.

Protein	Amino-Terminal Residues Identified	Method of Determination	% Recov[a]
Spectrin	Glutamic acid	Dns and TATG	32
	Valine	Dns and TATG	42
	Leucine	Dns and TATG	33
	Methionine	TATG	55
	Phenylalanine	Dns	
	Serine	Dns	
Bovine fibrin control	Glycine	TATG	62
	Tyrosine	TATG	52

[a] Average of 4 determinations using 3 different spectrin preparations (thioacetylthioglycolic acid (TATG) method).

Discussion

The subunits of spectrin or tektin A can be easily separated from all other membrane proteins. Moreover, electrophoresis of the protein in polyacrylamide gels containing sodium dodecyl sulfate at neutral pH demonstrates the presence of two protein bands with molecular sizes of 240,000 and 220,000. It is unlikely that these bands are artifacts of the electrophoretic procedure since they can be demonstrated using either low pH gels containing urea, or high pH gels.

Techniques of isoelectric focusing, preparative gel electrophoresis, and gel chromatography on agarose and ion-exchange resins have not yielded sufficient quantities of each subunit band for detailed comparative investigation. Determination of the amino acid composition of each subunit has been accomplished however, and demonstrates that the subunits are strikingly similar.

The suggestion (Gwynne and Tanford, 1970) that each band of the doublet is a single polypeptide chain is questionable. Our finding the presence of 3–6 α-amino groups/mol wt 450,000 implies, at least, nonidentity of the polypeptides in the molecule. One would expect to see the presence of a stable cross-linking peptide, such as is seen in other structural proteins. The failure to demonstrate the presence of such a stable interchain cross-linkage bond implies that (a) each subunit is comprised of two or more single polypeptide chains of high molecular weight, or (b) each subunit is made up of two or more polypeptide chains of lower molecular weight either (i) linked through a covalent bond which is presently unknown in proteins or (ii) the amino acid sequence of the subunit polypeptide chains is such that it causes an extremely powerful protein–protein interaction to take place which resists harsh dissociating conditions. The presence of lower molecular weight material (*ca.* 110,000) which demonstrates immunological identity with spectrin is difficult to explain. This finding does suggest that there are lower molecular weight forms of spectrin which can be isolated prior to their being incorporated into one or the other of the subunits. It has recently been reported that the high molecular weight doublet can be generated by chromatog-

raphy on Sephadex G-200 of lower molecular weight material obtained from the preparative gel electrophoresis of spectrin (Dunn and Maddy, 1973). Knufermann *et al.* (1973) reported the presence of multiple amino terminals in the aqueous extractable high molecular weight proteins of erythrocytes. With the exception of proline their results and those reported herein are quite similar. The data presented here also appear to favor the existence of polypeptides of smaller size that undergo specific aggregation as a result of strong protein–protein interaction.

At least two possible molecular arrangements of spectrin may be proposed based on the evidence that spectrin contains a minimum of three polypeptide chains, and that spectrin is composed of two subunits which are stable to a variety of harsh treatments known to break quaternary, tertiary, and secondary structures. In the first molecular arrangement spectrin might be composed of two subunits. Each subunit is in turn composed of at least two or three polypeptide chains possessing strong protein–protein interaction, or some as yet to be identified covalent bond. In the second model, it is suggested that both high molecular weight subunits are separate proteins, whose molecular size and amino acid composition differ only slightly, and whose physical properties resemble one another to the extent that they copurify by the techniques thus far employed.

The precise functional role of spectrin is still somewhat obscure. Recent evidence presented by Nicolson and Painter (1973) suggests that spectrin is involved in stabilizing the erythrocyte membrane. The procedures for extraction appear to rely more on a diminution of ionic strength than on the presence of divalent ion chelators. It has been demonstrated in our laboratory (unpublished observations) that spectrin is equally as extractable in low ionic strength buffers as it is in the presence of EDTA or EGTA. Furthermore the *in situ* location of spectrin is on the inner surface of erythrocyte membrane, and perhaps its attachment is due to Ca^{2+} or Mg^{2+} ligand bonding with some integral membrane protein. However, experiments from this laboratory (unpublished observations) demonstrate that even in the presence of 10^{-3} M $MgCl_2$ or $CaCl_2$, spectrin is released from the membrane as long as the ionic strength of the buffer remains low (μ 0.002).

It is clear that spectrin plays an important part in the physiologic health of the erythrocyte, though presently the only known role is supportive; whether spectrin is involved in other functions is still to be determined.

Acknowledgments

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