ISOLATION AND CHARACTERIZATION OF A WATER-SOLUBLE PROTEIN FROM BOVINE ERYTHROCYTE MEMBRANES*

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SUMMARY. A protein from the water-soluble fraction of bovine erythrocyte ghosts has been purified and characterized. This protein contains two high molecular weight polypeptide chains (MW ∿ 220,000 and 240,000); its physical properties suggest that it is a rod-like molecule. It makes up at least one-fifth of the total ghost protein.

When erythrocyte ghosts are extracted with aqueous solutions at low ionic strengths, a major portion of the membrane associated protein can be solubilized (1-5) Mazia and Ruby (1) designated these water-soluble proteins as tektins. One such protein (spectrin) has been purified and studied (2), and other components have been partially characterized (3,5). This report describes the isolation and characterizati of the major protein of the water-soluble fraction from bovine erythrocyte ghosts; it was given the name tektin A.

METHODS. Ghosts were prepared from fresh bovine blood as described by Mazia and Ruby (1). Water soluble proteins were freed from the ghosts by an overnight dialysis at 4°C against deionized water which had been adjusted to pH 7.5-8.0 by addition of dilute NH_4OH. The preparation was then centrifuged at 60,000 rpm for 90' in the A-321 rotor of an International B-60 preparative ultracentrifuge. The supernatant fluid (WSP fraction) was collected and was concentrated by pressure dialysis. Tektin A was separated from other proteins in the WSP fraction by gel filtration on a Bio-Gel P-300 column (200 cm × 1 cm). Fractions containing tektin A were pooled and were concentrated by pressure dialysis. Protein concentrations were determined by the method of Lowry (6) using bovine plasma albumin as a standard. The protein concentration of purified tektin A was determined by measuring the absorbance at 280 nm and using an extinction coefficient ($E^{280}_{1\%}$= 10.1) determined by the method of Babul and Stellwagen (7). Carbohydrate was measured by the phenol-sulfuric acid assay of Dubois et al. (8) after a mild acid hydrolysis of the protein. Lipid was extracted by the method of Bligh and Dyer (9). Phosphorus was measured as described by Bartlett (10).

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Electrophoresis in sodium dodecyl sulfate (SDS) buffer was carried out according to the procedure of Shapiro et al. (11); electrophoresis in non-denaturing buffer was performed as described by Davis (12), using small-pore gels containing 3.25% acrylamide. Viscosity measurements were made at 20.55° in a constant temperature water bath, using a capillary viscometer with a 1 ml. capacity and a water outflow time of 100 seconds. Sedimentation velocity studies were performed in a Spinco Model E analytical ultracentrifuge using schlieren optics. The apparent specific volume, $\bar{v}$, of tektin A was calculated from the amino acid composition; a value of 0.732 cc/g was found (13).

RESULTS. Purification of tektin A. Chromatography of the WSP fraction from bovine erythrocyte membranes on a Bio-Gel P-300 column leads to two major peaks of protein (Fig. 1). The first contains high molecular weight proteins, the second primarily

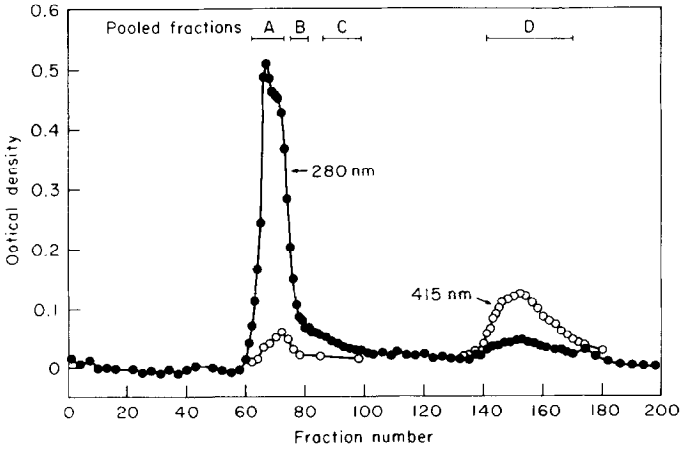

Fig. 1. Elution profile of water-soluble proteins from a P-300 column. The buffer was 1 mM imidazole glycyl glycine, pH 7.3. One ml fractions were collected. The main peak falls in the void volume and contains primarily tektin A. The second peak contains free hemoglobin.

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hemoglobin, as indicated by its absorbance at 415 nm. Analysis of the pooled fractions from the gel chromatography by SDS-polyacrylamide gel electrophoresis (Fig. 2) reveals that the major protein component of the WSP fraction, which is obtained in the void volume peak, contains two polypeptide chains of very high molecular weight. I shall refer to this protein component as tektin A, and to the two large polypeptide components as α' and α in order of decreasing size. A small amount of hemoglobin was invariably found at chromatograph with the tektin A peak (cf absorbance at 415 nm in Fig. 1). Other protein components of the WSP fraction were obtained in later column fractions; their relative elution positions corresponded to the size of their polypeptide chains as measured by SDS gel electrophoresis.

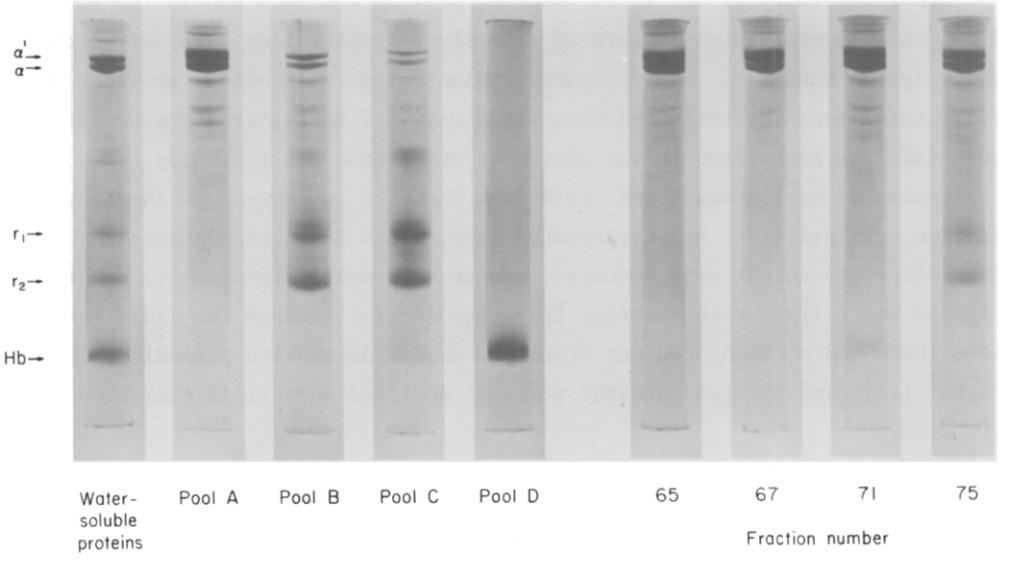

Fig. 2. SDS gel showing the fractionation of water-soluble proteins on a P-300 column. The gels contain 5% acrylamide; they show the composition of the pooled fractions seen in Fig. 1, and of individual fractions from the main peak. The sample applied to the column is shown as "water-soluble proteins."

Breakdown of the α and α' chains during isolation may be seen by comparing the starting material with individual peak fractions and with the pooled and concentrated peak fractions (Pool A). The bands labeled r_1 and r_2 are the most rapidly solubilized proteins of the WSP fraction; other column chromatography experiments have shown that r_1 and r_2 are components of two different protein molecules.

The fractions containing tektin A also contain traces of protein components of slightly higher mobility than α and α'. These appear to be due to limited proteolysis of α and α' during isolation. Tektin A fractions which initially contain essentially no polypeptide components other than α and α' rapidly accumulate smaller components on incubation at 25°C. This type of breakdown is accelerated by the presence of denaturing solvents such as 1% SDS or 6 M guanidine hydrochloride; it is largely prevented by the addition of 1 mM EDTA.

The α and α' components were always present in equal amounts in tektin A fractions, as judged from the intensity of staining on SDS gels. A variety of non-denaturing fractionation procedures failed to separate the α and α' chains or change their proportions; these included precipitation of tektin A by salt or at low pH, and polyacrylamide gel electrophoresis of the molecule under non-denaturing conditions. The native molecule moved as a single band on the polyacrylamide gels; when the protein present in this band was eluted in SDS and electrophoresed on SDS gels, only the α and α' components were found.

About one-third of the total membrane protein was obtained in the WSP fraction; 85-90% of this protein was recovered from the gel filtration step, of which 70-80% was pooled as tektin A. These figures indicate that tektin A is at least one-fifth of the total ghost protein; the true value may well be higher since purity rather than yield was emphasized in the choice of conditions for isolation.

Traces of carbohydrate and lipid were found to be present in the tektin A fraction obtained after P-300 chromatography; about 2% neutral sugars and 4% phospholipid by weight were detected. These components are probably due to contamination by small lipid-rich membrane fragments carried through the isolation procedure, since the α and α' bands did not stain for carbohydrate with periodic acid Schiff reagent (14), and the gel patterns were not modified when lipid-extracted samples were used.

Properties of tektin A. The solubility of purified tektin A is quite sensitive to variations in the solvent. The protein aggregates at KCl concentrations as low as 8 mM, as shown by formation of multiple boundaries in sedimentation velocity experiments in the analytical ultracentrifuge. In 0.1 M KCl, 80% of the protein is sedimented by centrifugation for 1 hour at 100,000 × g. The effect of divalent cations is even more striking; in 5 mM $CaCl_2$ 80% of the protein is sedimented by centrifugation for 10 minutes at 5000 × g. Tektin A is also precipitated if the pH of the solution is lowered to 4.5. The protein is quite soluble in 80% ethanol. These data suggest that tektin A has a highly hydrophobic character, and aggregates whenever a small fraction of its charged groups are blocked. The strong response to even rather low salt concentrations suggests that the salt ions are bound tightly to sites on tektin A.

Studies of the α and α' chains of tektin A. SDS gels indicate that the α and α' chains are prominent components of fresh erythrocytes dissolved in SDS and of ghosts isolated by other techniques (13). As polypeptide chains, α and α' are unusually large; other reports have suggested they might be aggregates of smaller components (4,15). This possibility was examined by subjecting tektin A to a variety of stringent denaturing conditions and following the effects by SDS gel electrophoresis. Disulfide bonds were cleaved and sulfhydryl groups blocked either by reduction and alkylation (16) or sulfonation (26). Denaturing treatments included exposure to 6 M guanidine-HCl or 8 M urea, or boiling in 3% SDS plus 3% mercaptoethanol. Neither the apparent size nor the amount of α and α' was changed by any of these treatments; thus α and α' behave as single polypeptide chains.

The approximate molecular weights of α and α' were determined by comparison of their relative mobilities on SDS gels with those of other high molecular weight polypeptide chains (11); chains used as standards were myosin, MW $\sim$ 200,000 (26), E. coli RNA polymerase subunits, MW 165,000, 155,000, and 95,000 (27), and β-galactosidase, MW 130,000 (28). The accuracy of the extrapolation is limited

chiefly by the uncertainty of the molecular weights of the standards. The molecular weight values found were 220,000 for α and 240,000 for α'.

What is the size and structure of the tektin A molecule? The unusual solubility properties of this protein make it difficult to determine the molecular weight of the native molecule. Both sedimentation equilibrium and velocity measurements must be carried out at elevated ionic strength to avoid errors due to the primary charge effect (17). However, tektin A aggregates at salt concentrations well below those routinely employed. At low ionic strength ($\mu \sim .01$) tektin A has a sedimentation coefficient, extrapolated to zero protein concentration, of approximately $s_{20,w} = 8$ S. The significance of this figure is difficult to evaluate since the extent of the possible primary charge effect is not known. However, extensive studies of the sedimentation properties of the WSP fraction at elevated ionic strengths (29) suggest an upper limit of $s_{20,w} = 8$-10 S for the tektin A component.

Viscosity measurements indicate that the molecule is highly asymmetric. The intrinsic viscosity at low ionic strength (1 mM Tris, pH 7.7, 1 mM EDTA) is 139 cc/g, a value characteristic of elongated molecules. From this result and the assumption of a rod-like particle, an axial ratio of 45 may be calculated (18). In 0.1 M NaCl the reduced viscosity values become variable, even for successive measurements of the outflow time of a single sample; a slight decrease (down to 100-120 cc/g) was usually found. The formation of heterogeneous aggregates under these conditions could account for the variability observed.

Although the molecular weight may be calculated from the sedimentation coefficient and intrinsic viscosity (19), for tektin A such a calculation cannot currently yield an exact value because of uncertainty with respect to the shape factor, β, and the true value of the sedimentation coefficient. The approximate molecular weight indicated for a sedimentation coefficient of 8 S, assuming a value of 2.9 for β, is 6×10^5. Subunit structures for tektin A consisting of from 2 to 4 equivalents of the α and α' subunits would be within the range of this figure. Although a 3-chain structure ($\alpha\alpha'_2$ or $\alpha_2\alpha'$) would provide the best fit, this type of structure is unlikely because α and α' always appear to be present in equal amounts.

The subunit structure of tektin A was examined by a new method recently described by Davies and Stark (20). A diimidoester, dimethylsuberimidate (DMS), is incubated with the protein molecule and forms amidine cross-links between polypeptide chains. At low protein concentrations, only intermolecular cross-links are formed. The number of polypeptide chains present in the native molecule is revealed by separating the cross-linked chains by SDS gel electrophoresis and comparing the molecular weight of the cross-linked bands with those of the original polypeptide components. This technique was first applied to myosin, which is known to be a rod-like molecule containing two high molecular weight (∿ 200,000) polypeptide chains. The relative mobility of the cross-linked myosin chains (MW ∿ 400,000)

provided a standard with which to compare the results of tektin A.

Cross-linking of tektin A at various DMS and protein concentrations always resulted in an equal shift of the α and α' bands to the higher molecular weight forms. This observation, and the fact that α and α' were readily cross-linked at low protein concentrations (0.2 mg/ml), both support the assumption that α and α' are polypeptide chains of a single protein molecule and are present in equal amounts in that molecule.

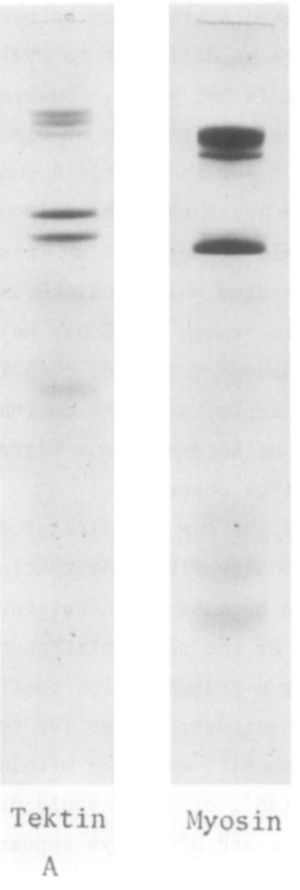

Fig. 3. DMS cross-linking of tektin A and myosin. Cross-linking was performed at tektin A and myosin concentrations of 0.5 mg/ml; DMS was 1.0 mg/ml. The gels contained 3.25% acrylamide. Conditions were chosen to cross-link about half of the molecules; consequently unmodified as well as cross-linked chains are present in both samples. Myosin, which contains two identical high molecular weight polypeptide chains, yields multiple bands with mobilities slightly greater than that appropriate for a dimer when cross-linked. Myosin also contains small, easily-dissociated components (26) seen in the lower portion of the gel. These remain in free form even at DMS concentrations sufficiently high to shift all the large chain components to the cross-linked form; it is therefore unlikely that they contribute to the multiplicity of cross-linked bands observed.

Dimethyl suberimidate was a gift from Dr. George Stark; myosin was obtained from Bruce Patterson.

The mobility of the cross-linked chains of tektin A was consistent with that expected for a dimer, as judged by comparison with the myosin results (Fig. 3). No higher molecular weight forms such as tetramers were observed (except in high salt). However, for both tektin A and myosin, cross-linking yielded multiple bands in the dimer region. One attractive explanation for the appearance of multiple bands is that there are several different species of dimer which differ in conformation due to differences in the position of the DMS cross-links. The constant relationship between the molecular weight of a polypeptide chain and its mobility in SDS gels is critically dependent on the shape of the complex formed between the protein and the SDS (21); chains linked at different points might well differ in the shape they assume in SDS, and consequently in mobility during electrophoresis.

The simplest model for tektin A consistent with these data is a dimer of subunit structure $\alpha\alpha'$; this would represent a native molecule of MW $\sim$ 460,000. However, the possibility of a tetrameric structure ($\alpha_2\ \alpha_2'$) cannot yet be ruled out, since the failure to observe a cross-linked tetramer might reflect steric or chemical constraints in the molecule.

DISCUSSION. Several workers have observed the presence of two polypeptide chains of high MW in erythrocyte membranes (4,15,22,23). These components are present in ghosts prepared from a variety of species (23). These components are not exposed on the outer surface of the erythrocyte membrane as measured by their failure to react with various chemical (15,24), immunological (4), and enzymatic (25) probes. Most of these studies have involved dispersal of whole ghosts in denaturing solvents (15, 22,23); the use of low ionic strength solubilization procedures has made possible examination of the native properties of this molecule and other water-soluble proteins (1-5).

Tektin A may be related to spectrin, which is also released from the membrane at low ionic strength and makes up about 20% of the total ghost protein (2). However the purified spectrin molecule is reported to have a much lower molecular weight (polypeptide chain MW of 140,000), different solubility properties, and a much lower intrinsic viscosity. Although one might postulate that spectrin could be a degradation product of tektin A, this would require a highly specific cleavage of the α and α' chains of tektin A to produce a single 140,000 molecular weight product. Breakdown of the tektin A α and α' chains was observed in the present study, but the breakdown products ranged widely in size; no single major product was seen. Furthermore, SDS gels of whole ghost preparations or the WSP fraction failed to reveal any component corresponding in size to spectrin. Thus the relationship between spectrin and the polypeptide components of erythrocyte ghosts is not clear at the present time.

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