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ISOLATION OF AN ANKYRIN-BAND 3 OLIGOMER FROM HUMAN ERYTHROCYTE MEMBRANES *

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A cytoskeleton-associated population of band 3 has been isolated in milligram quantities from human erythrocyte membranes as a stable complex with ankyrin. The major population of band 3 (free band 3) was solubilized from ghosts with 0.1 M KCl/Triton X-100. A detergent-insoluble assembly of proteins (cytoskeletons) contained 10–15% of the band 3, as well as ankyrin, spectrin, band 4.1, actin and other minor polypeptides. The remaining band 3 and ankyrin were extracted in a 1:1 molar ratio from the cytoskeletons with 1 M KCl/Triton X-100, and were copurified with the same 1:1 stoichiometry during DEAE-chromatography, and gel filtration. Free band 3 was isolated by the same procedures, and was clearly resolved from ankyrin-associated band 3 on DEAE-chromatography and gel filtration. Direct evidence that ankyrin and band 3 were associated in a complex was provided by immunoprecipitation with anti-ankyrin IgG of band 3 from the native complex, but not of free band 3 or after denaturation of the complex. Ankyrin-associated band 3 contained a reactive site for H_2DIDS (the dihydroanalog of 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid) and thus has an anion transport site. Comparison of ^{125}I-labeled α-chymotryptic peptide fragments of free band 3 and ankyrin-associated band 3 revealed extensive homology with 28 out of 30 identical fragments. The ankyrin-band 3 oligomer is arranged as an αβ dimer with one polypeptide chain of each component, based on the molecular weight calculated from hydrodynamic parameters in dilute solution. Free band 3 behaved under the same conditions as a homodimer. Ankyrin-associated band 3 was capable of band 3 dimerization at concentrations of 1–3 μM, since chemical cross-linking of the oligomer with Cu^{2+}/o-phenanthroline produced a 190000 M_r band 3 dimer on SDS gels.

Introduction

Human erythrocyte ankyrin (band 2.1) is a 215000 M_r polypeptide localized on the cytoplasmic surface of the membrane, and contains the high affinity membrane attachment site for spectrin [2–5]. Ankyrin also is associated with band 3 in Triton X-100 extracts of spectrin-depleted vesicles [6], and ankyrin binds with high affinity to a purified 43000 M_r proteolytic fragment derived from the cytoplasmic domain of band 3 [7]. Ankyrin reassociates with inside-out vesicles at a site which involves band 3, since binding is blocked by the 43000 M_r fragment of band 3, by antibody against the 43000 M_r fragment, and by selective cleavage of band 3 with α-chymotrypsin [7,8]. The linkage of band 3, a membrane-spanning protein, to ankyrin which in turn is associated with spectrin provides a mechanism for attaching the membrane bilayer to the underlying assembly of cyto-

* The nomenclature for erythrocyte membrane proteins is according to Steck [1].

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate, PAS, periodic acid-Schiff; H_2DIDS, the dihydro analog of 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid.

skeletal proteins composed of spectrin, actin and band 4.1. The ankyrin-band 3 linkage is important for maintaining normal cell structure, since erythrocytes with altered membrane binding sites for ankyrin have bizarre shapes and are abnormally fragile [9].

Ankyrin is associated with band 3 in detergent extracts in a 1 : 1 molar ratio, and thus only 10–15% of band 3, which is present in a 8–10-fold excess over ankyrin, is involved directly in the complex with ankyrin. Ankyrin-linked band 3 and the major population of band 3 are cleaved by cyanogen bromide to similar fragments, and are both reduced to a 60000 M_r fragment by external digestion of erythrocytes with α-chymotrypsin [6]. Ankyrin-linked band 3 probably contains a 43000 M_r cytoplasmic domain, since antibody directed gainst the 43000 M_r fragment immunoprecipitated an ankyrin-band 3 complex from detergent extracts [7]. It is still possible that the ankyrin-associated band 3 differs from the major band 3 population in amino acid sequence, while retaining similar domains. The present report describes a procedure for preparative isolation of ankyrin-associated band 3 as an ankyrin-band 3 oligomer which should be useful in further detailed studies. Initial characterization of the ankyrin-associated band 3 demonstrated a reactive site for H_2DIDS indicating the presence of an anion transport site [10,11], and showed that ^{125}I-labelled α-chymotryptic peptides of ankyrin-associated band 3 are very similar to those of free band 3.

Experimental procedure

Materials. Na^{125}I (carrier-free) was from Amersham, and ^{125}I-labeled Bolton Hunter reagent [12] was from New England Nuclear. α-Chymotrypsin (56 units/mg), trypsin (205 units/mg), and pancreatic trypsin inhibitor were from Worthington Biochemicals. PMSF, dithiothreitol, pepstatin A, diisopropylfluorophosphate, and *Escherichia coli* β-galactosidase were from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine, SDS, Coomassie brilliant blue, ^2H_2O (99.8 mol%), Bromophenol blue, and hydroxyapatite were from Bio Rad Laboratories. Ultrogel AcA22 was from LKB, rabbit muscle aldolase, horse spleen ferritin, bovine thyroid thyroglobulin, Dextran 500, and Blue dextran 2000 were from Pharmacia. Triton X-100 was from Packard, and DEAE-cellulose (DE52) was from Whatman. Heat-denatured formaldehyde-fixed Protein A-bearing staphylococci were from CalBiochemicals; bovine liver catalase, yeast alcohol dehydrogenase, and horse heart cytochrome *c* were from Boehringer Mannheim, and bovine serum albumin (Pentex) was from Miles. Plastic thin-layer sheets coated with 0.1 mm cellulose were from E. Merck ^{3}H-labeled H_2DIDS ([^{3}H]H_2DIDS) and unlabeled H_2DIDS were kindly provided by Dr. Rothstein, The Hospital for Sick Children, Toronto, Canada. Affinity-purified anti-ankyrin IgG was prepared from serum of rabbits immunized with ankyrin purified by SDS electrophoresis as described [13].

Methods. Erythrocytes were isolated from freshly drawn blood anticoagulated with acid citrate-dextrose by sedimentation twice at $1 \times g$ in 4 vol. of 150 mM NaCl, 5 mM sodium phosphate, pH 7.5, 0.75% (w/v) Dextran 500 [3]. The resulting cells were essentially free of contamination with other cell types, and were washed twice in 150 mM NaCl and then incubated overnight at 2°C with 10 vol. of 150 mM NaCl, 5 mM sodium phosphate, 100 μg/ml PMSF (dissolved at 200 mg/ml in dimethyl sulfoxide), pH 7.5 in order to minimize proteolysis. Erythrocyte ghosts were prepared by hypotonic lysis in 10 vol. of 7.5 mM sodium phosphate, 1 mM sodium EDTA, 20 μg/ml PMSF, 4 μg/ml pepstatin A, pH 7.5, and were pelleted 20 min at $20000 \times g$ (max). The ghosts were washed once in lysis buffer, and three more times in phosphate buffer alone.

The Stokes radius of the ankyrin-band 3 oligomer and free band 3 was determined by gel filtration on an Ultrogel AcA22 column (0.8×60 cm) equilibrated with 0.1 M KCl, 10 mM sodium phosphate, 1 mM sodium EDTA, 0.2 mM dithiothreitol, 1 mM sodium azide, 0.2% (v/v) Triton X-100, pH 7.5 with 0.6 ml fractions at a flow rate of 4 ml/h. Standard proteins were bovine thyroid thyroglobulin (R_s 85 Å) horse spleen ferritin cross-linked by reaction with Cu^{2+}/*o*-phenanthroline (0.5 mM, 10 min at 2°C) to prevent dissociation in the presence of Triton X-100 (R_s 61 Å), *E. coli* β-galactosidase (R_s 68 Å), bovine

liver catalase (R_s 52 Å), and rabbit muscle aldolase (R_s 48 Å). The plot of $(-\log K_{av})^{1/2}$ vs. R_s for these proteins was linear. The sedimentation coefficient was estimated by rate zonal sedimentation on 5–20% linear sucrose gradients in a SW50 rotor [14], with the modification of Reynolds and Tanford [15] of substituting ^2H_2O (density 1.10) for H_2O to compensate for association of Triton X-100 ($\bar{v} = 0.908$) [16] with protein. The gradients contained 0.1 M KCl, 5 mM sodium phosphate, 1 mM EDTA, 0.2 mM dithiothreitol, 0.2% Triton X-100, p^{2}H 7.8 in ^2H_2O (98 mol%), and standard proteins were bovine liver catalase ($s_{20,w}$ 11.3), yeast alcohol dehydrogenase (7.6 S), bovine serum albumin (4.6 S) and horse heart cytochrome *c* (1.75 S).

Samples of dilute protein in Triton X-100 solution were prepared for electrophoresis and other procedures requiring removal of detergent by adsorption onto hydroxyapatite columns (0.8 × 3 cm) followed by washing with 10 mM sodium phosphate, 1 mM dithiothreitol, 1 mM sodium azide, pH 7.5 and 0.2% (w/v) SDS until the A_{280} was less than 0.01. The proteins were then eluted with 0.5 M sodium phosphate, 1 mM dithiothreitol, 0.2% SDS, pH 7.5.

Amino acid analysis was performed on a Durrum D-500 instrument by Michael Herbstreith in the laboratory of Dr. Allen Roses, Duke University School of Medicine. Samples of ankyrin-band 3 oligomer (50–75 μg) were hydrolysed for 22 h at 100°C in constant boiling HCl with 2 mg/ml phenol and 0.2 mg/ml dithiothreitol to minimize damage to phenylalanine, tyrosine and methionine. Membrane protein was estimated by the method of Lowry et al. [17] and protein content of samples in Triton X-100 was measured by the method of Flores [18], using bovine albumin as a standard in both cases. SDS electrophoresis was performed essentially by the method of Fairbanks et al. [19].

Results

Ankyrin-associated band 3 has been previously isolated by immunoprecipitation of Triton X-100 extracts of spectrin-depleted vesicles with anti-ankyrin IgG [6]. This method is adequate for analytical procedures, but is limited by the requirement for antibody and the harsh conditions required to dissociate the antibody-ankyrin-band 3 complex. An alternative strategy for isolation of ankyrin-linked band 3 was suggested by the observation that a fraction of band 3 remained tightly associated along with ankyrin in Triton X-100-extracted erythrocyte ghosts [6] and whole erythrocytes [20]. Further extraction of the detergent-insoluble material (cytoskeletons) with 1 M KCl in the presence of Triton X-100 released ankyrin and band 3 as well as other, minor polypeptides [6,20] (Fig. 1). It is thus possible by selective extraction to separate band 3 into a fraction which is readily solubilized from ghosts and presumably interacts weakly or not at all with cytoskeletal proteins (free band 3) and another population which is firmly associated with the cytoskeleton. It has been suggested that the band 3 and ankyrin which are co-extracted from cytoskeletons with 1 M KCl/Triton X-100 are associated with each other [6,20]. Direct evidence is not available demonstrating such a linkage, and the cytoskeleton-associated band 3 has not been examined in detail to establish how this polypeptide is related to the major population of band 3. In order to address these questions, it was important to isolate and characterize the cytoskeleton-associated population of band 3.

Preparative scale isolation of ankyrin-band 3 oligomer

Extraction of erythrocyte ghosts with Triton X-100 in 0.1 M KCl solubilized about 80% of band 3 and band 4.2, but did not remove the cytoskeletal proteins spectrin (bands 1, 2), ankyrin (bands 2.1, 2.2, 2.3), bands 4.1, and 4.9 or actin (band 5) (Fig. 1). The sialoglycoproteins (PAS-1,2,3) were almost completely solubilized by Triton X-100 (not shown), as has been observed previously [21]. The band 3 which remained after extraction was not simply partitioning between a free and cytoskeletal-associated form since subsequent extraction removed only an additional 5–8% of the band 3, and the remainder persisted after three washes in detergent in a constant ratio to the other cytoskeletal proteins [6] (not shown). Extraction of cytoskeletons with 1 M KCl in the presence of Triton X-100 solublized the remaining band 3 and ankyrin in a 0.97:1 molar ratio, respectively, as well as band 4.2 and band 7, but did not

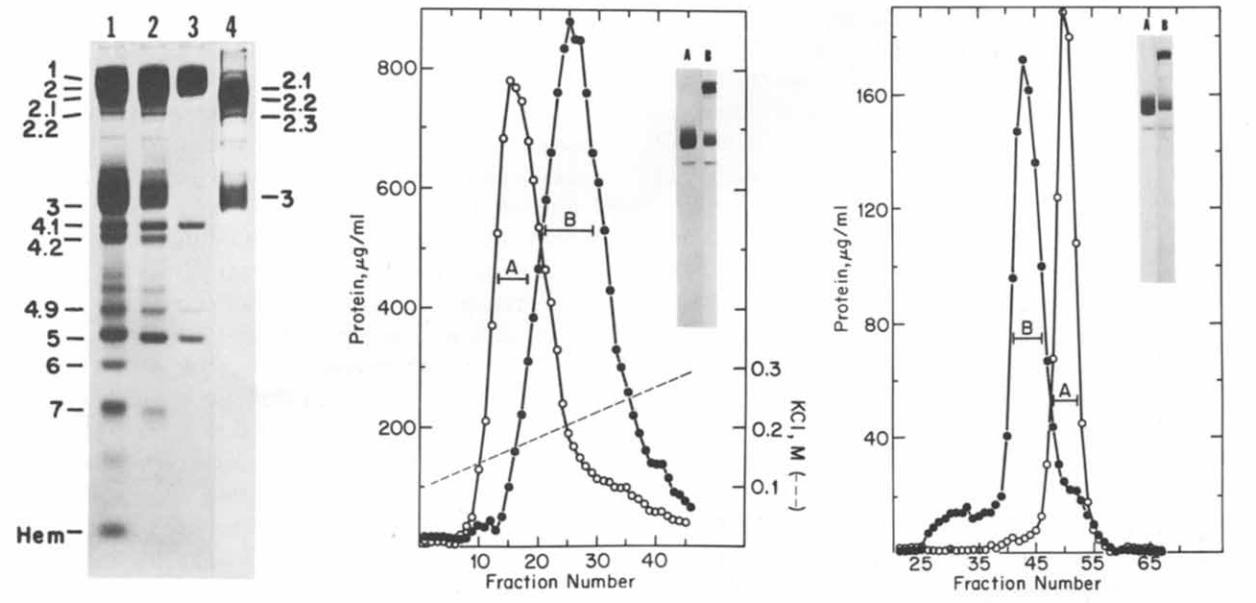


Fig. 1. Isolation of ankyrin-band 3 oligomer by selective extraction (left panel), DEAE-chromatography (middle panel), and gel filtration (right panel). Free band 3 was extracted from erythrocyte ghosts (108 ml) by incubation for 10 min at 2°C in 12 vol. of 0.1 M KCl, 7.5 mM sodium phopshate, 1 mM sodium EDTA, 0.5 mM dithiothreitol, 20 μg/ml PMSF, 0.5% (v/v) Triton X-100, pH 7.5 (buffer A), followed by centrifugation (20 min, 20000× *g*). The pellets (lane 2, left panel) were washed twice more in the same solution, and then ankyrin-band 3 oligomer was extracted by incubation for 30 min at 2°C in 100 ml of buffer A plus 1 M KCl and 2 μg/ml pepstatin A, followed by centrifugation (30 min, 45000× *g*). The supernatant (lane 4, left panel) was dialysed against buffer A, and adsorbed batchwise with 10 ml of DE52-cellulose equilibrated with the same buffer, which was then packed into a 1×12 cm column, washed, and eluted with a linear gradient of 50 ml buffer A versus 50 ml 0.3 M KCl in buffer A. The peak (22 ml) (peak B, middle panel) was applied to a 5×60 cm column of AcA22 Ultrogel equilibrated with buffer A minus PMSF, and 15.2-ml fractions collected (45 ml/h) (right panel). The supernatant from the first detergent extraction (free band 3) was subjected to the same procedures for comparison with ankyrin-band 3 oligomer, and is designated by A in the middle and right panels. Samples were analysed by SDS electrophoresis on a 7% polyacrylamide 3 mm thick slab gel which was stained with Coomassie blue (left panel): lane 1, ghosts (10 μl); lane 2, Triton X-100 extracted ghosts in same volume as ghosts (10 μl); lane 3, 1 M KCl/Triton-extracted ghosts (10 μl); lane 4, supernatant from 1 M KCl/Triton X-100 extraction (35 μl). The final recovery of oligomer was 12 mg from 405 mg of ghost membrane protein. This represents a yield of about 44% based on the estimate that band 2.1 represents 4.7% of membrane protein [3], the M_r of band 3 and band 2.1 are 95000 and 215000, respectively, and that each band 2.1 was associated with one band 3 molecule.

disassemble the spectrin-actin-band 4.1 complex (Figs. 1 and 2, Table I).

The solubilized proteins were dialysed, and chromatographed on DEAE-cellulose with a linear gradient of 0.1–0.3 M KCl (Fig. 1). Ankyrin and band 3 eluted as the major peak, and were present in a ratio of 1 mol ankyrin per 0.94 mol band 3. Bands 2.2 and 2.3 which are degradation products of ankyrin [2–5], were enriched in fractions at lower salt concentrations, and these fractions were not pooled even though band 3 was present. A small amount of material comigrating with band 4.2 also was observed, but this polypeptide was present only at 12% of the molar amounts of band 3 and ankyrin. The band 3 extracted from ghosts with 0.1 M KCl/Triton X-100 was also chromatographed under identical conditions, and eluted at a lower concentration of salt than the cytoskeleton-associated band 3 (Fig. 1). Cytoskeleton-associated band 3 from DEAE-cellulose fractions was applied to an AcA22 Ultrogel gel filtration column, and eluted as a single major symmetrical peak containing band 3 and ankyrin in a 0.93:1 molar ratio, respectively, and band 4.2 in 7% of the molar amount of ankyrin (Fig. 1, 2, Table I). Free band 3 from DEAE-cellulose chromatography was fractionated on the same column, and eluted later than the cytoskeleton-associated band

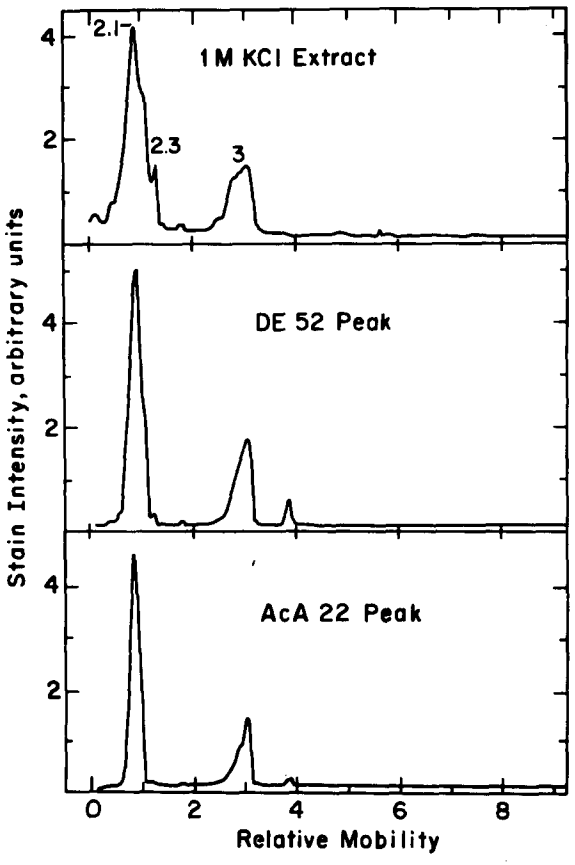


Fig. 2. Densitometer scans of Coomassie blue-stained SDS gels of ankyrin-band 3 oligomer following selective extraction, DEAE-chromatography, and gel filtration. SDS gels of ankyrin-band 3 oligomer were prepared at various stages of purification (Fig. 1), and scanned.

TABLE I

RELATIVE MOLAR RATIOS OF OLIGOMER POLYPEPTIDES

Estimated from densitometry of Coomassie-blue-stained SDS gels (Fig. 2), assuming molecular weights of 215000 for band 2.1, 95000 for band 3, and 72000 for band 4.2, and assuming that these proteins are stained equally by Coomassie blue.

Fraction	Band		
	2.1 [a]	3	4.2
1 M KCl/Triton X-100 extract	1	0.94	–
DE52 peak	1	0.97	0.12
AcA22 peak	1	0.93	0.07

[a] Bands 2.2 and 2.3 also were present in the 1 M KCl/Triton X-100 extract and are included with 2.1, assuming molecular weights of 190000 for band 2.2 and 170000 for band 2.3.

3 (1.9 V_0 versus 1.6 V_0), indicating a smaller effective Stokes radius.

The cytoskeleton-associated band 3 was resolved from free band 3 and retained an equimolar amount of ankyrin during selective extraction and purification. It is therefore likely that ankyrin and band 3 were associated together in a stable complex. The association of ankyrin with band 3 was tested further by immunoprecipitating native and denatured ^{125}I-labeled-cytoskeleton-band 3 with anti-ankyrin IgG (Fig. 3). Band 3 and ankyrin were both immunoprecipitated from the native preparation. The antibody reaction was specific for ankyrin since no complex was formed in the presence of unlabeled ankyrin. The co-precipitation of band 3 with ankyrin required linkage between band 3 and ankyrin since free band 3 was not immunoprecipitated (not shown) and since immunoprecipitation of the denatured cytoskeleton-associated band 3 yielded an unaltered amount of ankyrin but almost no band 3 (Fig. 3). Cytoskeleton-associated band 3 is thus tightly bound to ankyrin by a linkage requiring a native protein conformation, and will be referred to as ankyrin-associated band 3.

The final recovery of ankyrin-associated band 3 was 12 mg from 100 ml erythrocyte ghosts, which is approx. a 40% yield (Fig. 1). The procedure could be readily adapted to a larger scale, and is limited primarily by the amount of ghosts which can be prepared.

A polypeptide comigrating with band 4.2 persisted during the purification of free band 3 and ankyrin-associated band 3 and was present in variable amounts in different preparations. 10–20% of the 72000 M_r band in ankyrin-associated band 3 was a proteolytic fragment of ankyrin, since some of this band was immunoprecipitated by anti-ankyrin IgG, and the reaction did not occur with preimmune IgG or in the presence of ankyrin (not shown). The remainder of the 72000 M_r band most likely was band 4.2, since two-dimensional peptide maps of band 4.2 from ghosts, free band 3 and ankyrin-associated band 3 were nearly identical (not shown). It is known that band 4.2 is associated with band 3 in detergent solution [23] and it is likely that such an interaction was occurring during the isolation steps.

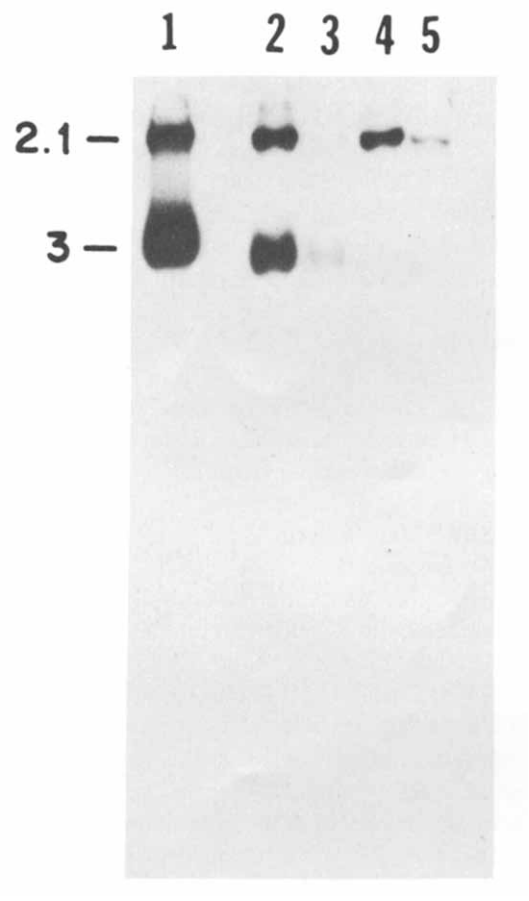


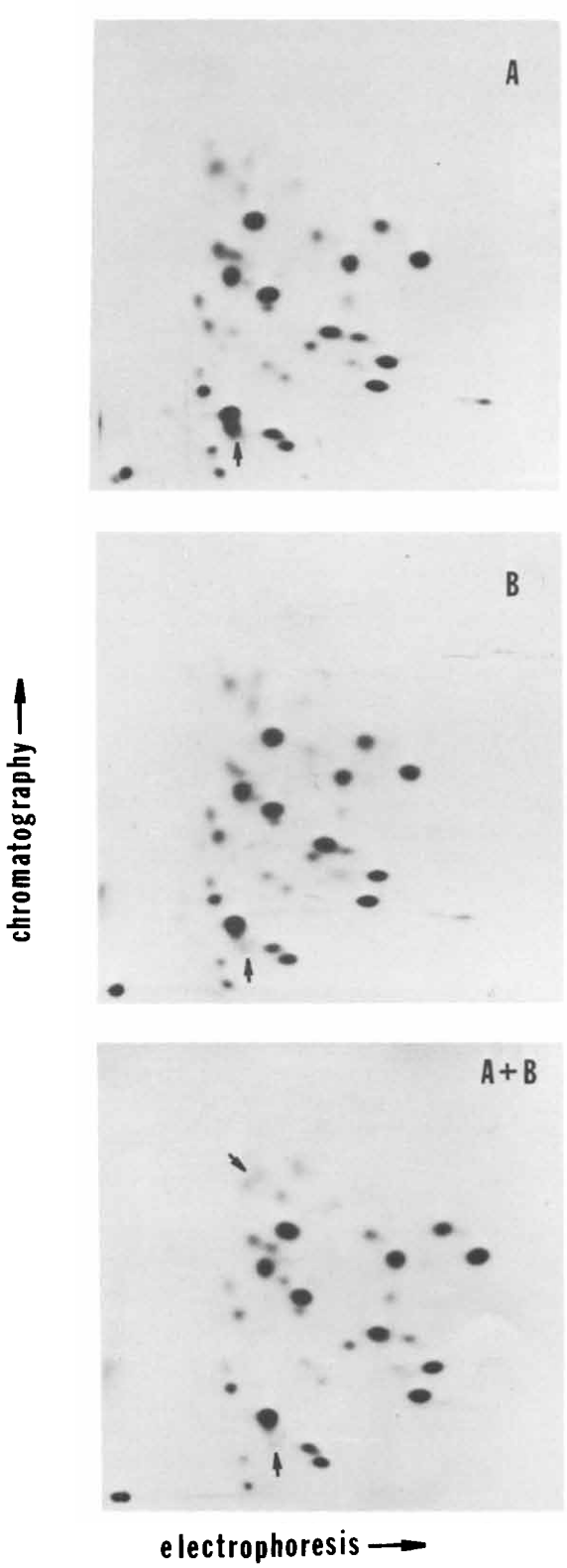
Fig. 3. Immunoprecipitation of native and denatured ankyrin-band 3 oligomer with monospecific anti-ankyrin IgG. Ankyrin-band 3 oligomer (Fig. 1) radiolabeled with ^{125}I-Bolton Hunter reagent [3,7] (67000 cpm/μg) was either untreated or heated 10 min at 70°C with 0.5% (w/v) SDS. Samples (2.6 μg) were incubated 4 h at 2°C with monospecific anti-ankyrin IgG (10 μg/ml) in the presence and absence of 50 μg/ml unlabeled ankyrin in a 0.2 ml volume containing 250 μg/ml bovine serum albumin, 2 μg/ml pancreatic trypsin inhibitor, 150 mM NaCl, 10 mM sodium phosphate, 1 mM sodium EDTA, 0.2% (v/v) Triton X-100, and 0.2 μl Protein A-bearing staphylococci [22]. The final concentration of SDS in the denatured sampels was 0.05%, which does not interfere with immunoprecipitation as long as Triton X-100 and albumin are present. The staphylococci with adsorbed IgG were washed twice in incubation buffer minus albumin and trypsin inhibitor, and the immune complexes solubilized for electrophoresis by addition of 30 μl of 1% SDS, 20 mM dithiothreitol, 20 mM Tris-HCl, pH 8 followed by heating 20 min at 60°C. The samples were analysed on a 7% polyacrylamide 1.5 mm thick slab gel, which was dried and developed by autoradiography. Lane 1, starting oligomer; lane 2, immunoprecipitate of native oligomer; lane 3, immunoprecipitate of native oligomer and unlabeled ankyrin; lane 4, immunoprecipitate of denatured oligomer; lane 5, immunoprecipitate of denatured oligomer and unlabeled ankyrin.

Comparison of free band 3 and ankyrin-associated band 3

Ankyrin-associated band 3 and free band 3 have a similar electrophoretic mobility, but could otherwise be unrelated. Peptide maps of ^{125}I-labeled free and ankyrin-associated band 3 purified by SDS electrophoresis demonstrate that these polypeptides have a high degree of homology. Thirty ^{125}I-labeled peptide fragments produced by digestion with α-chymotrypsin were resolved by electrophoresis and chromatography, and 28 of these fragments comigrate exactly in mixtures of digest from free band 3 and ankyrin-associated band 3 (Fig. 4). The only reproducible difference between the two proteins was that free band 3 contained a fragment not present in ankyrin-band 3, and that a minor fragment exhibited a different mobility in the two preparations. Tryptic digests also were analysed, and exhibited no detectable differences between free and ankyrin-associated band 3 (not shown).

Peptide maps with ^{125}I-labeled proteins suffer from several limitations which should be kept in mind in interpreting this data. Peptide fragments which are not iodinated (i.e., those not containing tyrosine or histidine) will not be visualized. Furthermore, peptides differing only in the sequence of amino acids will be poorly resolved. Finally, differences in peptide mobility can result from post-translational modifications and reflect a different environment rather than a different primary amino acid sequence. The minor differences in peptides between band 3 fractions could result from the different purification procedures or from the fact that ankyrin-associated band 3 is bound to ankyrin in erythrocytes and protected from glucosylation, deamidation, or proteolysis which occur in these cells. On the other hand, these proteins may have a major difference in sequence in an unlabeled peptide. A completely satisfying resolution of the extent of homology will require determination of the amino acid sequence of free and ankyrin-associated band 3. Nevertheless, the present peptide mapping demonstrates that these proteins are highly homologous, if not identical.

A major function of band 3 is transport of anions, and the anion transport site can be covalently labeled with [^{3}H]H_2DIDS [10]. The presence of such a site in ankyrin-associated band 3



was examined by reacting erythrocytes with [^{3}H]H_2DIDS, and measuring incorporation of label into the two populations of band 3 (Table II). Controls for nonspecific reaction of H_2DIDS, which is capable of combining with any primary amine, were erythrocytes which had been reacted with unlabeled H_2DIDS to block specific sites. About 0.9 mol of [^{3}H]H_2DIDS was incorporated per mol of free band 3, and 0.8 mol were coupled per mol of ankyrin-associated band 3. The ankyrin-band 3 thus contains an anion transport site, although the function of this site in transport remains to be evaluated.

Physical properties of free band 3 and ankyrin-band 3 oligomer

A molecular weight of 258000 was estimated for the ankyrin-band 3 complex based on a sedimentation coefficient determined in linear sucrose gradients in Triton X-100 with ^2H_2O to compensate for detergent binding to protein [15], a Stokes radius measured by gel filtration, and a partial specific volume calculated from the amino

Fig. 4. Two-dimensional peptide maps of ^{125}I-labeled free band 3 (A), ankyrin-associated band 3 (B), and a 1 : 1 mixture of free band 3 and ankyrin-associated band 3 (A + B). Free band 3 and ankyrin-band 3 oligomer were purified by selective extraction and DEAE-chromatography (Fig. 1) from erythrocytes incubated 12 h at 2°C with diisopropylfluorophosphate (0.05% v/v) and PMSF (50 μg/ml) to minimize proteolysis. The proteins were separated from Triton X-100 by hydroxyapatite chromatography (Methods) and denatured (10 min, 70°C) in 0.1% SDS, 1 mM dithiothreitol, 10 mM sodium phosphate, pH 7.5. The denatured proteins (6 μg) were reacted with 1 mCi carrier-free Na^{125}I (90% incorporation of ^{125}I) using Chloramine-T as an oxidant [24], mixed with 20 μg unlabeled protein, and electrophoresed on an 8% polyacrylamide 1.5 mm thick slab gel. Band 3 was identified by staining with Coomassie blue, and this area of the gel was excised, equilibrated with 50 mM ammonium acetate, 2 mM sodium azide, and incubated in 0.5 ml of this buffer for 18 h at 37°C with two additions of α-chymotrypsin (25 μg). The supernatants containing 80% of the radioactivity were lyophilized, and dissolved in acetic acid/formic acid/water (15 : 5 : 80, v/v) (electrophoresis buffer) at $6 \cdot 10^5$ cpm/μl. 1-μl aliquots were applied to 14.5 × 19 cm cellulose-coated thin-layer plates, then electrophoresed at 900 V for 55 min, and after drying, chromatographed in *n*-butanol/pyridine/acetic acid/water (32.5 : 25 : 5 : 20, v/v) [25]. The plates were dried and developed by autoradiography. One sample (A + B) contained an equal volume of digest from free band 3 and ankyrin-associated band 3. The maps are nearly identical except for spots marked by the arrows.

TABLE II

[^{3}H]H_2DIDS LABELING OF FREE BAND 3 AND ANKYRIN-ASSOCIATED BAND 3

[^{3}H]H_2DIDS (0.2 mCi/mmol) was added to a final concentration of 4 μM to washed erythrocytes (12 ml, 25% hematocrit) in 150 mM NaCl, 5 mM sodium phosphate, pH 7.5 which were either untreated or had been preincubated (30 min, 37°C) with 100 μM unlabeled H_2DIDS to block specific reactive sites. After 30 min at 37°C, the cells were washed, and ghosts and membrane extracts prepared as described (Fig. 1). Samples were analysed on SDS gels, which were stained with Coomassie blue, and the relative amounts of band 3 estimated by densitometry. [^{3}H]H_2DIDS incorporation was determined by excising the area of the gels corresponding to band 3, dissolving these in 15% H_2O_2 (5 h at 60°C), and measuring ^{3}H in the dried samples using Bray's solution as a liquid scintillant.

Band 3 source	[^{3}H]H_2DIDS incorporated [a]
Ghosts	0.6 ± 0.05
0.1 M KCl/Triton X-100 extract	0.9 ± 0.10
1 M KCl/Triton X-100 extract	0.8 ± 0.09

[a] Expressed as mol [^{3}H]H_2DIDS incorporated per mol band 3, and corrected for nonspecific incorporation by subtraction of the values obtained with cells pretreated with unlabeled H_2DIDS; mean of triplicate determinates + half range.

acid composition of the oligomer (Table III). This value is quite close to the sum of the molecular weights of 169000 calculated for ankyrin from hydrodynamic properties [3] and 95000 estimated for band 3 from SDS electrophoresis. The reason that the molecular weights of ankyrin and ankyrin-band 3 oligomer are apparently lower than expected from SDS electrophoresis may be due to anomalous behavior during gel filtration, which has been observed previously with other large, asymmetric proteins [29]. It should be emphasized that the estimates of Stokes radius and sedimentation coefficient for the oligomer suffer from several limitations. The apparent Stokes radius estimated by gel filtration is the effective size of protein and associated detergent and thus is larger than the value for protein alone. The sedimentation coefficient determined by sedimentation on sucrose gradients is relatively inaccurate. Furthermore, both measurements are based on the assumption that the oligomer is not undergoing association-dissociation equilibria. A more precise determination of the Stokes radius and M_r would be by

TABLE III

SUMMARY OF PHYSICAL PROPERTIES OF BAND 3-ANKYRIN OLIGOMER AND FREE BAND 3

Property	Value	
	Oligomer	Band 3
Stokes radius R_s [a] (Å)	69	61
Sedimentation coefficient, $s^0_{20,w}$ [b] (S)	8.9	7.0
Partial specific volume, $\bar{v}$ [c] (cm^3/g)	0.73	0.75
M_r, calculated	258000	194000
Frictional ratio, f/f_0 [d]	1.51	1.46

[a] From gel filtration on AcA22 Ultrogel (Methods).

[b] From sedimentation on linear sucrose gradients in ^2H_2O (Methods).

[c] Calculated from amino acid composition [26] determined for the oligomer (Methods) and from published data for band 3 [27].

[d] Calculated from equations:

$$M_r = \frac{6\pi N R_s s_{20,w}}{(1 - \bar{v}\rho_{20,w})}$$

and

$$f/f_0 = R_s\left(\frac{4\pi N}{3M_r(v + \delta/\rho_{20,w})}\right)^{1/3}$$

where δ was assumed to be 0.2 g of solvent per g of protein [28].

combined sedimentation equilibrium and sedimentation velocity measurements or direct measurement of the radius of gyration by quasi-elastic light scattering. The instruments for such measurements were not available, however.

The estimate of an M_r of 258000 and a 1:1 molar ratio of ankyrin to band 3 in the oligomer indicate that the predominant form of the oligomer is an $\alpha\beta$ dimer with a single polypeptide chain of band 3 and of ankyrin. A $(\alpha\beta)_2$ tetramer would have an apparent M_r of 520000 and would be readily distinguished from a dimer. Similarly, a (band 3)$_2$-ankyrin or (band 3)-(ankyrin)$_2$ arrangement would have different stoichiometries and can be excluded. Band 3 is known to be a stable dimer in solution as well as in ghost membranes [23,30,31]. Furthermore, free band 3, isolated un-

der identical conditions, has a molecular weight of 194000 calculated from parameters determined in teh same way as for the ankyrin-band 3 oligomer, and this M_r is very close to the value of 190000 expected for a homodimer (Table III).The possibility that the band 3 component of the oligomer was unable to self-associate was examined by chemically cross-linking relatively concentrated solutions of oligomer (1–3 μM) with *o*-phenanthroline/Cu^{2+} [30]. Band 3 in the oligomer was cross-linked to a 190000 M_r dimer and this was reversed by reduction with dithiothreitol (not shown). The ankyrin-associated band 3 is thus capable of band 3 dimerization, but these dimers apparently dissociate at the low protein concentrations of 0.1–0.3 μM employed in measurement of hydrodynamic values. Free band 3 is more stable as a dimer, since this complex did not dissociate even at the lower concentrations.

Discussion

This report describes isolation of milligram quantities of the cytoskeleton-associated population of band 3 as a stable complex with ankyrin. Band 3 and ankyrin were extracted from erythrocyte cytoskeletons in a 1 : 1 molar ratio, and maintained this stoichiometry during DEAE-chromatography and gel filtration (Figs. 1 and 2, Table I). These procedures clearly resolved free band 3 from the oligomer form since free band 3 was extracted from erythrocytes while oligomeric band 3 remained associated, and free band 3 was eluted at lower salt concentrations in DEAE-chromatography and exhibited a smaller Stokes radius in gel filtration. Direct evidence that ankyrin and band 3 were associated was provided by immunoprecipitation with anti-ankyrin IgG of band 3 in the native complex, but not after denaturation or as free band 3 (Fig. 3). Ankyrin-associated band 3 contained 28/30 identical α-chymotryptic ^{125}I-labeled peptide fragments with free band 3 (Fig. 4), reacted with [^{3}H]H_2DIDS (Table II), and was capable of band 3-dimerization (not shown).

Measurements of reassociation of ankyrin with inside-out vesicles have demonstrated that the membrane capacity for ankyrin is at most 25% of that expected if each band 3 polypeptide could bind to one ankyrin molecule [7–9]. A possible explanation for the lower capacity could be that ankyrin binds selectively to a sub-population of band 3 which differs in some substantial way from other band 3 polypeptides. If such a difference does exist between band 3 populations, it does not reside in the ankyrin binding site since free band 3 incorporated into liposomes was capable of binding to ankyrin [8]. The present study limits further the possible difference between free and ankyrin-associated band 3. Peptide maps of the two populations are nearly identical, but cannot rule out a subtle difference in amino acid sequence such as occurs in the different forms of actin [32].

A possible difference between the two forms of band 3 may be in the site for band 3 dimerization, which is localized in the membrane-bound domain [33]. Ankyrin-band 3 is capable of band 3 dimerization, as determined by chemical cross-linking at 1–3 μM of oligomer, but exists in a monomeric form at lower concentrations, even though free band 3 still is arranged as a dimer under the same conditions (Table III). The lack of band 3 dimerization of the ankyrin-associated band 3 could result either from a perturbation due to binding of ankyrin or to an alteration in a specific domain of band 3. If the latter mechanism were true, then ankyrin-preferred band 3 would be in a somewhat different environment in the membrane than the more completely dimerized band 3 population, and could selectively bind to ankyrin.

Heterogeneity in band 3 has been suggested previously [34], but this is the first example of isolation of a functionally defined sub-population of band 3. Future work which will be possible with this preparation includes study of transport activity and ultrastructure of free band 3 and ankyrin-associated band 3 following incorporation of these proteins into liposomes, examination of the two populations for post-translational modifications such as phosphorylation and carboxymethylation [35] and detailed structural analysis with monoclonal antibodies and determination of amino acid sequence. It will also be important to study the biosynthesis of the two populations during maturation of erythrocytes and to understand how the appearance of band 3 is coordinated with that of spectrin and ankyrin.

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