

Identification of proteolytically resistant domains of human erythrocyte spectrin

(peptide maps/chemical domains/peptide alignment/protein structure/membranes)

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ABSTRACT Digestion of purified human erythrocyte spectrin with proteolytic enzymes at 0°C results in the production of intermediate-size peptides that resist further cleavage at 0°C. By two-dimensional peptide analysis of these intermediate peptides it has been determined that five unique peptides are produced by tryptic cleavage of the α subunit of spectrin (band 1); these have apparent molecular weights of 80,000, 46,000, 46,000, 41,000, and 30,000 and account for 97% of the α subunit. Similarly, four unique peptides having apparent molecular weights of 74,000, 65,000, 33,000, and 28,000 account for 90% of the β subunit (band 2). By examining larger peptide fragments, the linear alignment of the unique peptides along each of the spectrin subunits has been established. These results indicate that spectrin is composed of two nonidentical subunits, each containing multiple proteolytically resistant domains. These domains, which may be largely α -helical, seem to be connected by small protease-sensitive segments. The proteolytic resistance of these domains is not influenced by the multimeric state of the spectrin molecule.

Spectrin is the major extrinsic membrane protein of erythrocytes (1). Human erythrocyte spectrin is composed of two high molecular weight polypeptide subunits that have been referred to as band 1 (250,000 daltons) and band 2 (225,000 daltons) on the basis of their mobilities on NaDodSO₄ gels (2); these will be referred to here as the α and β subunits, respectively. The amino acid compositions of the two spectrin subunits are similar (3) but peptide maps of the two subunits indicate significant differences in their primary structure (4-7).

Remarkably little is known about the structure of the two spectrin subunits. Early attempts to analyze spectrin by isoelectric focusing techniques yielded broad, diffuse bands which focused over a relatively wide pH range. On this basis it was suggested that spectrin was composed of multiple polypeptides of roughly equivalent molecular weights (8). More recently it has been found that isoelectric focusing of spectrin yields variable results depending upon the amount of protein being analyzed; sharp bands can be obtained when small amounts of spectrin are focused at approximately pH 5.2 (6). It was also reported that spectrin preparations contained multiple NH₂-terminal amino acids (9-11) although a recent study found only one NH₂-terminal residue per spectrin monomer (12).

In this report we describe the identification of chemical domains within spectrin subunits defined by carefully controlled proteolytic digestion. Each spectrin subunit has been found to contain a set of unique peptides which resist further cleavage at 0°C. The linear alignment of these fragments along the individual subunits has been determined by two-dimensional mapping techniques of overlapping peptides. These results indicate that both subunits of spectrin are composed of unique polypeptides, and each subunit contains relatively large proteolysis-resistant domains.

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We have also identified the domains of spectrin involved in membrane-binding, dimer-tetramer interactions, and noncovalent associations between spectrin monomers (13).

MATERIALS AND METHODS

Extraction and Purification of Spectrin. Spectrin was extracted from hemoglobin-free erythrocyte membranes and precipitated at pH 5.0 as described (14). The precipitate was dissolved in 1% NaDodSO₄/10 mM Tris-HCl/0.5 mM 2-mercaptoethanol/0.5 mM EDTA/0.03 mM phenylmethylsulfonyl fluoride/0.02% NaN₃, pH 9.0 and applied to a 5 × 90 cm column of Sepharose CL-4B (Pharmacia) equilibrated in the same buffer except with NaDodSO₄ at 0.1%. Effluent was monitored at 280 nm, and a flow rate of approximately 60 ml/hr was used. Fractions containing spectrin α and β subunits as determined on polyacrylamide gels were pooled and concentrated on an Amicon cell with an XM-100 membrane. For the preparation of NaDodSO₄-purified spectrin ($\alpha\beta$), fractions were pooled to yield equal amounts of α and β monomers in the final sample. The sample was concentrated to 1-2 mg/ml. NaDodSO₄ was then removed by dialysis against 1 mM Na₃PO₄/0.5 mM 2-mercaptoethanol/0.5 mM EDTA/0.03 mM phenylmethylsulfonyl fluoride/0.02% NaN₃ at 4°C (five changes with a volume ratio \geq 1:100).

Purification of Spectrin Monomers. Fractions from the above column containing α and β subunits were concentrated to 5-10 mg/ml as described above. The sample was chromatographed on two 5 × 90 cm Sepharose CL-4B columns (Pharmacia) fitted with flow adapters and connected in tandem. The buffer was the same as the buffer used for the previous CL-4B column. Sample was recycled at a flow rate of 60 ml/hr, and separation was monitored at 280 nm. When two well-resolved peaks were evident (three or four cycles), the effluent was collected and analyzed on NaDodSO₄/polyacrylamide gels. Fractions containing monomers in sufficient purity (>98% for α and >95% for β) were pooled, concentrated to 1-2 mg/ml, and dialyzed as described above for the preparation of NaDodSO₄-purified spectrin.

Polyacrylamide Gel Electrophoresis. Electrophoresis of fractions from the Sepharose CL-4B column was performed using 6 × 100 mm tube gels containing 5.6% acrylamide and 0.2% NaDodSO₄ according to the procedures of Fairbanks *et al.* (2) as modified by Steck and Yu (15). Gels were stained with Coomassie blue R-250. Proteolytic digests of spectrin monomers and dimers were electrophoresed on 1.5-mm slab gels containing 0.1% NaDodSO₄ by the method of Laemmli (16). Generally, 10% or 12% polyacrylamide gels were used. Two-dimensional polyacrylamide gels were prepared by the method of O'Farrell (17). Lyophilized samples were solubilized and isoelectrically focused by using LKB Ampholines on 4% poly-

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acrylamide gels. Gels containing 60 μg of focused digest were electrophoresed in the second dimension on a 3×110 mm slab gel with 12% polyacrylamide by the method of Laemmli (16). Molecular weights of peptides were determined by their migration on 12% Laemmli gels. Proteins of known molecular weights ranging from 17,000 to 100,000 were used to prepare a standard curve of logarithm of molecular weight versus R_F .

Restricted Proteolytic Digestion of Spectrin (0°C Digests). Spectrin samples were dialyzed against 10 mM sodium phosphate/40 mM NaCl, pH 8.0, and subsequently digested with trypsin (TPCK-trypsin, Worthington) at an enzyme-to-substrate ratio of 1:100 at 0°C . The reaction was terminated by inactivating the trypsin with either 1 mM diisopropyl fluorophosphate or by boiling the sample in gel-solubilizing buffer (3% NaDodSO₄/4 M urea).

Two-Dimensional Peptide Maps. Two-dimensional peptide maps of protein bands from NaDodSO₄/polyacrylamide gels were prepared by the method of Elder *et al.* (18). Na¹²⁵I (carrier-free; 17 Ci/mg; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. Iodinated gel slices were digested with 1 ml of 50 mM ammonium bicarbonate containing either 50 μg of TPCK-trypsin or 50 μg of α -chymotrypsin (Worthington) for 24 hr at 37°C . The supernatant solution typically contained 70–80% of the total incorporated radioactivity. Lyophilized supernatants were dissolved in 20 μl of acetic acid/formic acid/water, 15:5:80, (vol/vol). Aliquots containing 100 cpm/dalton (original peptide molecular mass) were spotted on 20×20 cm cellulose-coated chromatograms (Eastman, 13255 cellulose, without fluorescent indicators). Electrophoresis was carried out with the above buffer for approximately 60 min at 1000 V in a Savant flat-bed electrophoresis apparatus maintained at 10°C . Electrophoresis was terminated when basic fuschin migrated 9.5 cm. Chromatograms were developed in

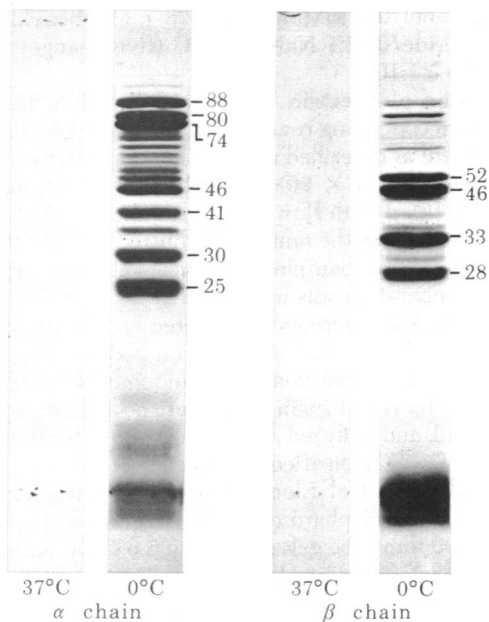


FIG. 1. Electrophoretic analysis of tryptic digests of purified spectrin monomers. Samples were digested in 10 mM sodium phosphate/40 mM NaCl, pH 8.0, with an enzyme/substrate ratio of 1:100, for 20 hr at the temperatures indicated; digestion was terminated by boiling the samples in NaDodSO₄ gel solubilizing buffer for 2 min. The sample was then electrophoresed immediately on 12% NaDodSO₄ slab gels with bromophenol blue as tracking dye. Gels were stained with Coomassie blue. Each lane received 75 μg of protein. Numbers show $M_r \times 10^{-3}$.

the second dimension in butanol/pyridine/acetic acid/water, 32.5:25:5:20, (vol/vol), with 7% 2,5-diphenyloxazole (wt/vol). After drying, a series of exposures (Kodak X-Omat R Film; fluorescent intensifying screens) from 1 to 6 hr were prepared of each map. Exposures with similar grain density of major spots were used for analysis. Three to eight maps of each peptide were made from different gel slices. Background iodination was determined by using polyacrylamide slices that did not contain protein.

RESULTS

Restricted Proteolytic Digestion of Spectrin Subunits (0°C). Purification of individual spectrin subunits by rechromatographing spectrin yielded fractions >98% (α subunit) or >95% (β subunit) pure by densitometric scans of Coomassie

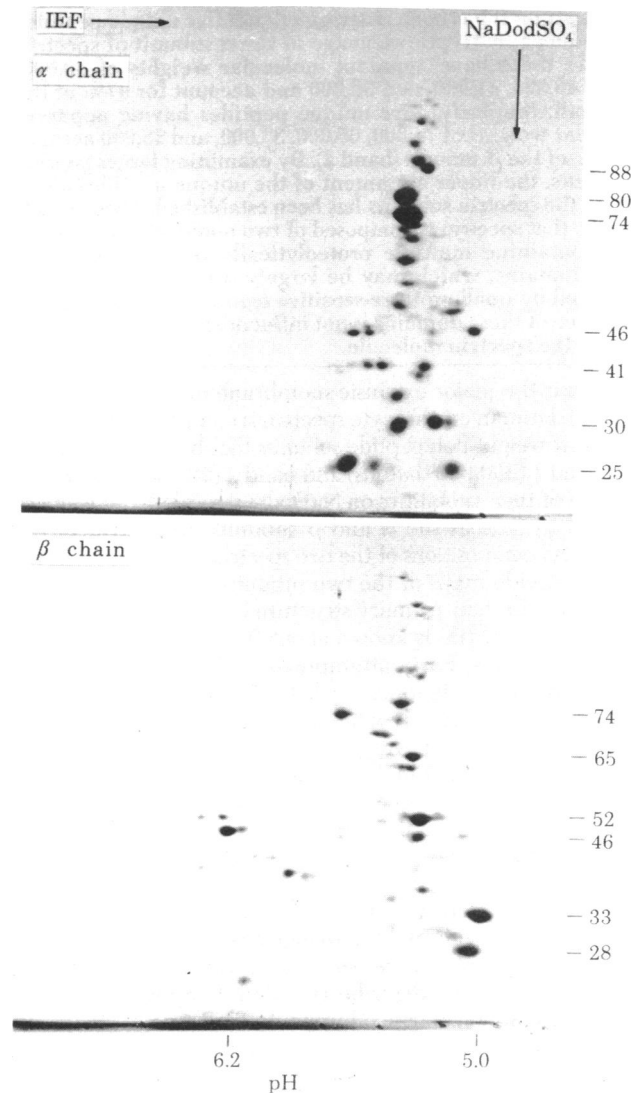


FIG. 2. Two-dimensional separation of tryptic peptides of spectrin monomers. A restricted tryptic digest (0°C) of each spectrin subunit (similar to Fig. 1) was inhibited with 1 mM diisopropyl fluorophosphate at 0°C . A 60- μg sample was focused isoelectrically on 3-mm-diameter tube gels, followed by NaDodSO₄ electrophoresis into an 11% gel. After electrophoresis in the second dimension the gel was stained with Coomassie blue. Peptides of the same molecular weight will be designated by letter suffixes starting at the left (alkaline) side of the gel. For example, the three major peptide spots of the α subunit with apparent molecular weights of 25,000 will subsequently be referred to as 25a, 25b, and 25c, respectively.

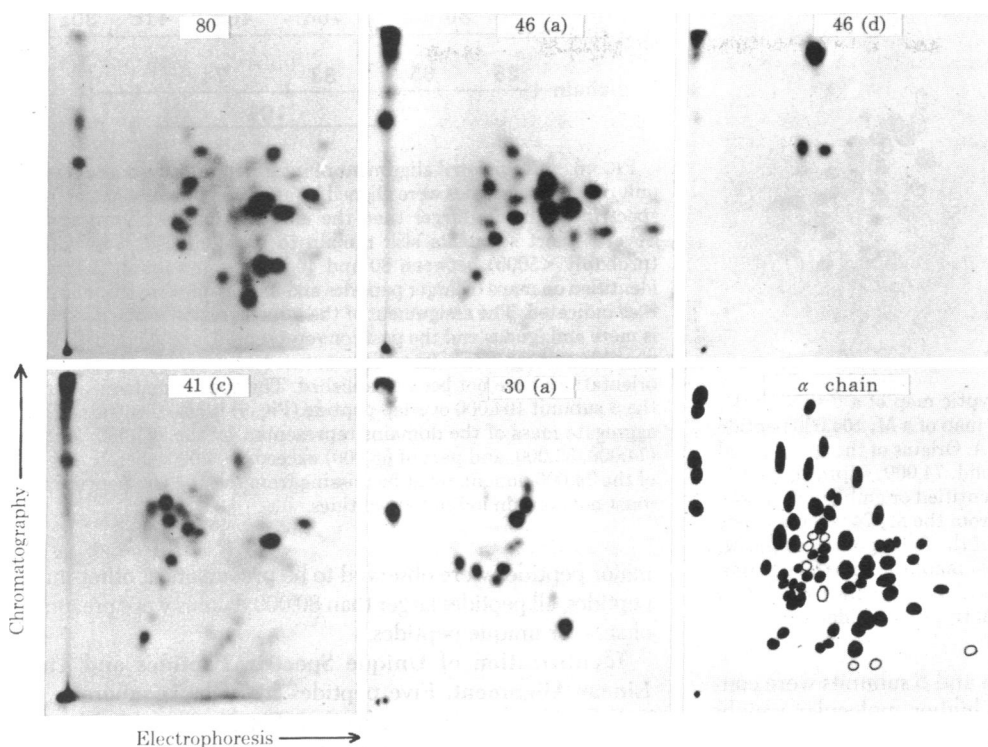


FIG. 3. Two-dimensional chromatryptic maps of unique α subunit tryptic fragments. Intermediate-size tryptic peptides were generated, separated on a two-dimensional polyacrylamide slab gel (Fig. 2), radioiodinated, and then analyzed. The numbers indicate apparent molecular weights ($\times 10^{-3}$) of the intermediate-size peptides. The lower case letters refer to position on two-dimensional polyacrylamide gels (see Fig. 2 legend). (Lower Right) Tracing of a peptide map of the complete α subunit. Solid circles mark spots on the α subunit map found in peptide maps of the five unique intermediate peptides. Variation in migration distances sometimes prevented exact superpositioning of spots when comparing two maps. In most cases this problem was circumvented by identifying the spots on the maps of the entire subunit and using this map as a master template.

blue-stained polyacrylamide gels. The major contaminant in each case was the other subunit.

Several methods of cleaving these purified polypeptides were attempted, but restricted digestion (0°C) with trypsin gave the best results and is reported here. Digestion of isolated spectrin subunits by trypsin at 37°C resulted in extensive degradation; however, when the digestion was carried out at 0°C , intermediate-size peptides were generated, ranging from 88,000 to 25,000 daltons (Fig. 1). These peptides were relatively resistant

to further degradation by trypsin at 0°C . Incubation of the samples for an additional 20 hr at 0°C did not significantly alter the peptides obtained, and addition of another aliquot of trypsin did not cause significant further digestion. When the incubation mixture was warmed to 37°C (after the initial digestion at 0°C), all of the large peptides were further degraded. Modifying the ionic strength of the digestion medium (from 0.01 to 0.15) had only a small effect on the peptide pattern obtained. The results obtained by varying the incubation time and the enzyme/

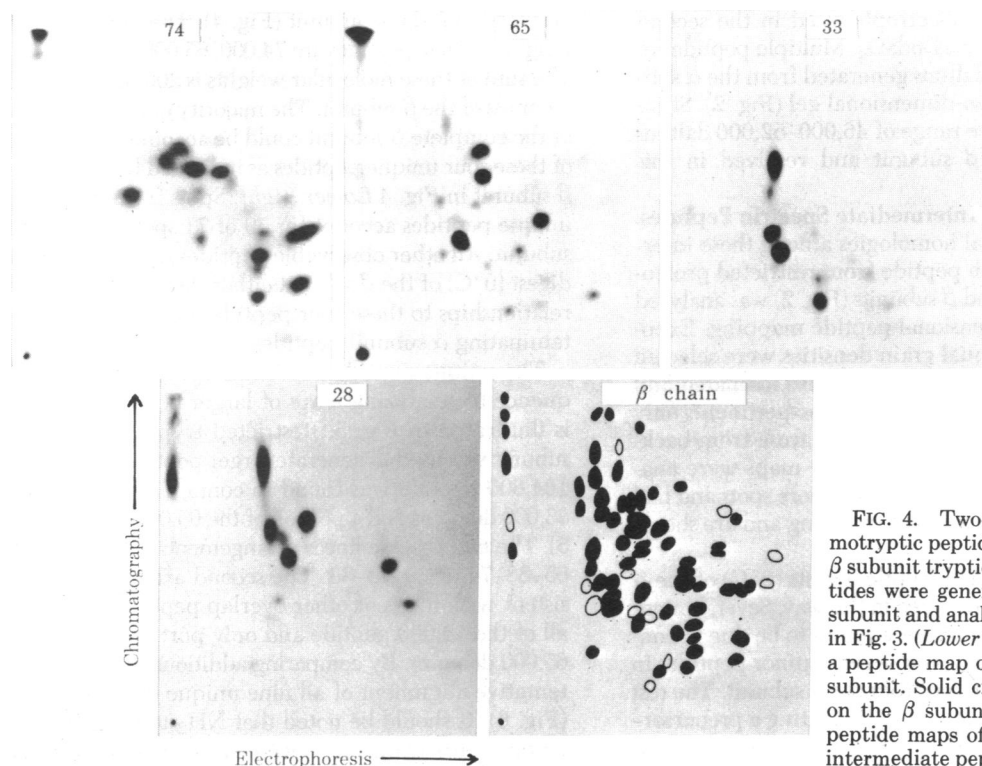


FIG. 4. Two-dimensional chromatryptic peptide maps of unique β subunit tryptic fragments. Peptides were generated from the β subunit and analyzed as described in Fig. 3. (Lower Right) Tracing of a peptide map of the complete β subunit. Solid circles mark spots on the β subunit map found in peptide maps of the four unique intermediate peptides.

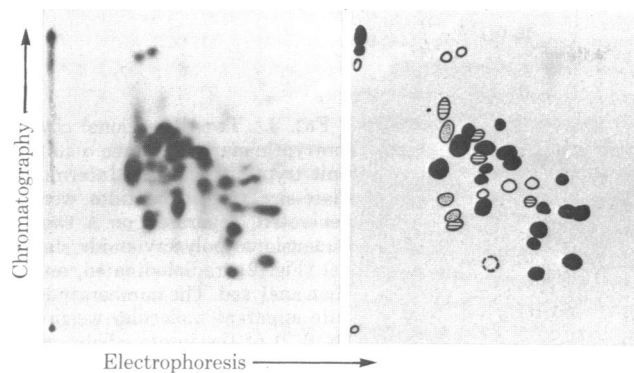


FIG. 5. Two-dimensional chymotryptic map of a β subunit M_r 104,000 overlap peptide. (Left) Peptide map of a M_r 104,000 peptide of β subunit. (Right) Tracing of map in A. Origins of the spots, based upon peptide map comparisons, are: solid, 74,000; stippled, 33,000; hatched, 65,000; open (solid line), unidentified or ambiguous origin; open (dashed line), artifact. All spots from the M_r 74,000 map were present in the M_r 104,000 map. Three of the four spots from the M_r 33,000 map were present in the 104,000 map; the fourth spot (not present on maps of the intact β subunit) is an artifact caused by chymotryptic mapping of the M_r 33,000 tryptic peptide.

substrate ratios suggested that the α and β subunits were converted rapidly through numerous higher molecular weight forms to the intermediate-size peptides shown in Fig. 1; these peptides then underwent a subsequent slower conversion to smaller peptides, many of which were not visible on polyacrylamide gels. Peptides of comparable size were produced by tryptic digestion of NaDodSO₄-purified spectrin dimers and spectrin dimers and tetramers isolated by gel filtration under nondenaturing conditions (data not shown).

Further Resolution of Intermediate-Size Spectrin Peptides. Careful analysis of the peptide patterns obtained from time-course experiments suggested that some of the Coomassie blue-staining bands contained more than one peptide. To resolve these peptides further, a two-dimensional polyacrylamide gel system was used (17). The peptides were first separated by isoelectric focusing and then electrophoresed in the second dimension in the presence of NaDodSO₄. Multiple peptides in the range of 25,000–46,000 daltons generated from the α subunit were resolved on the two-dimensional gel (Fig. 2). Similarly, multiple peptides in the range of 46,000–52,000 daltons were generated from the β subunit and resolved in this system.

Peptide Map Analysis of Intermediate Spectrin Peptides. To explore possible structural homologies among these intermediate-size fragments, each peptide from restricted proteolytic digests (0°C) of the α and β subunits (Fig. 2) was analyzed by high-resolution two-dimensional peptide mapping. Exposures with approximately equal grain densities were selected for comparisons. Acrylamide blanks were also iodinated and analyzed. For exposure levels used in these experiments only a few minor spots on several peptide maps arose from background labeling. Tryptic and chymotryptic maps were analyzed. Chymotryptic maps usually yielded more spots and had less diffuse backgrounds and less spot smearing and are shown here.

The peptides from restricted tryptic digests (0°C) of the α and β subunits (Fig. 2) were analyzed in this way. Several minor peptides in the α subunit digest were found to be due to contaminating β subunit peptides, and several minor peptides in the β subunit digest were derived from the α subunit. The rest of the minor peptides produced maps indicating a precursor-product relationship with major peptide spots. Several of the

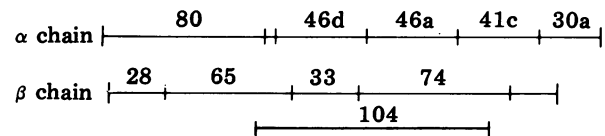


FIG. 6. Provisional alignment of intermediate-size α and β subunit peptides. Peptides were aligned by analysis of peptide maps from spectrin fragments larger than the unique peptides shown above. Several short segments still remain to be identified. The region (probably <5000) between 80 and 46d in the α subunit has been identified on maps of larger peptides and its alignment in the molecule is as indicated. The assignment of the missing region in the β subunit is more ambiguous and the position represented is only one of several possible arrangements (see Discussion). NH₂- and COOH-terminal orientations have not been established. The approximate position of the β subunit 104,000 overlap peptide (Fig. 5) is also illustrated. The aggregate mass of the domains represented by the 104,000 peptide (74,000, 33,000, and part of 65,000) exceeds 104,000; therefore, part of the 74,000 domain must be missing from the 104,000 peptide and must not contain iodinated peptides.

major peptides were observed to be precursors of other major peptides; all peptides larger than 80,000 daltons were precursors of smaller unique peptides.

Identification of Unique Spectrin Peptides and Their Linear Alignment. Five peptides from the α subunit were uniquely different from each other (Fig. 3). These five peptides have apparent molecular weights of 80,000, 46,000, 46,000, 41,000, and 30,000. The sum of molecular weights is 243,000 or approximately 97% of the mass of the α subunit. Most of the spots from a map of the α subunit could be accounted for by the maps of the five unique peptides, as indicated by the tracing of the α subunit map in Fig. 3 Lower Right. Spots from maps of the five unique peptides accounted for 59 of 67 spots in the complete α subunit. All other α subunit peptides smaller than 80,000 daltons were found to be subsets of one of these five peptides, indicating that these unique intermediate-size peptides were slowly digested at 0°C to other fragments.

Similarly, four unique peptides have been identified as being derived from the β subunit (Fig. 4). The apparent molecular weights of these peptides are 74,000, 65,000, 33,000, and 28,000. The sum of these molecular weights is 200,000 or about 90% of the mass of the β subunit. The majority of the spots from a map of the complete β subunit could be accounted for by the maps of these four unique peptides as indicated by the tracing of the β subunit in Fig. 4 Lower Right. Spots from maps of the four unique peptides account for 59 of 71 spots in the complete β subunit. All other observable peptides in the restricted tryptic digest (0°C) of the β subunit either showed precursor-product relationships to these four peptides or were found to be contaminating α subunit peptides.

The unique peptides were tentatively aligned in linear sequence by analyzing maps of larger peptides. This approach is illustrated in Fig. 5. Restricted tryptic digestion of the β subunit was used to generate larger peptides. The map of a M_r 104,000 peptide was found to contain the entire 74,000 and 33,000 domains and a portion of the 65,000 domain (Figs. 4 and 5). The two possible linear arrangements of these domains were 65–33–74 or 65–74–33. The second arrangement was inconsistent with maps of other overlap peptides which contained all of the 33,000 peptide and only portions of the 74,000 and 65,000 domains. By comparing additional overlap peptides, the tentative alignment of all nine unique domains was deduced (Fig. 6). It should be noted that NH₂-to-COOH-terminal orientation has not been established by these experiments.

DISCUSSION

These experiments show that large segments of the polypeptide chains of human erythrocyte spectrin have conformations that resist proteolytic cleavage at 0°C. Five unique intermediate-size peptides have been identified which make up >90% of the α subunit, and four fragments of comparable size make up a similar fraction of the β subunit. These digestion products have been generated from NaDodSO₄-purified α subunit and NaDodSO₄-purified β subunit and from spectrin dimers not subjected to detergents or other denaturing agents.

High-resolution peptide mapping techniques suitable for analyzing gel slices (18) have been used to determine the structural homologies between the large number of different fragments obtained as a result of these digestions. The results summarized here and to be reported elsewhere in more detail have enabled us to identify unique polypeptide segments of both spectrin subunits (Figs. 3 and 4) and to formulate a provisional interpretation of their alignment along each of the spectrin subunits (Fig. 6).

Because both the intermediate-size peptides (Figs. 1 and 2) and their corresponding peptide maps (Figs. 3 and 4) are significantly different from each other, these results confirm that the two subunits of spectrin are significantly different in their primary structure. The β subunit is clearly not a shorter version of the α subunit. These results also provide strong evidence in favor of the idea that each spectrin subunit is a single unique polypeptide rather than a family of structurally similar proteins, as postulated earlier (8). This conclusion is based on the fact that the sum of the molecular weights of the unique intermediate-size peptides closely approximates the mass of each spectrin subunit, and all other peptides result from further cleavage of these peptides. Because these results are based on a peptide mapping technique that relies on the presence of residues that can be iodinated, it is not possible to assess the degree of homology among all peptide segments. Thus, these results must be confirmed by more definitive structural analyses.

It is likely that these intermediate-size peptides reflect an underlying domain structure within the spectrin subunits, even though these peptides are considerably larger than the usual domains of globular proteins. These peptides show a degree of resistance to proteolytic attack that is characteristic of a structural domain. This resistance is a function of the native conformation of the spectrin molecule because it is lost completely when either subunit is exposed to denaturing agents (data not shown). Furthermore when the denatured spectrin subunits are allowed to refold, they do so rapidly and with great fidelity in that their resistance to proteolytic cleavage is restored completely (data not given). Thus, these intermediate-size peptides have both the proteolytic resistance and the refolding properties of domain structures.

Some segments of the spectrin subunits are more resistant to proteolysis at 0°C than are others. An 80,000-dalton fragment which is near an end of the α subunit seems to be one of the most resistant domains. Prolonged proteolysis at 0°C slowly converts the 80,000-dalton peptide to a 74,000-dalton peptide, and this latter peptide resists further degradation under conditions which hydrolyze most of the other spectrin domains to

smaller peptides. This 80,000-dalton fragment is of further interest because it appears to refold rapidly from denaturants, associates with specific β subunit peptides, and is involved in dimer-tetramer interactions (13). It is also noteworthy that portions of the β subunit are not accounted for by this analysis (Fig. 6). We believe that the missing β subunit segment(s) is terminal, based on peptide map overlaps, but it could be located at either or both ends of the β subunit. A 200,000-dalton cleavage product of the β subunit lacks this unidentified segment, consistent with a report (19) that the terminal segments of the β subunit are more sensitive to proteolytic degradation than are internal sites.

The native spectrin dimer is now thought to exist in solution as a 1000-Å flexible rod (20) which contains a substantial amount of α -helical conformation (14). The domain structures described herein are best explained by postulating that the most protease-resistant domains are made up of helical rod-like segments which are connected by small protease-sensitive regions. If the domain structures are distributed segmentally along the polypeptides, they should be ordered as depicted in Fig. 6. Parallel studies show that functional sites can also be assigned to specific domains of the spectrin molecule (13).

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