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Spectrin Domains: Proteolytic Susceptibility as a Probe of Protein Structure

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Mild treatment of human erythrocyte spectrin with trypsin produces discrete intermediate-sized peptides. The effects of buffer composition, enzyme-substrate ratio, temperature, and other experimental parameters on the resulting peptide pattern have been examined. Spectrin is capable of regaining its proteolytic resistance after $NaDodSO_4$-induced denaturation, permitting the use of isolated subunits to study spectrin structure and function. Tryptic digestion of isolated subunits also has greatly facilitated the identification of the subunit origin of the intermediate-sized peptides. Isolated subunits could also be recombined to form functional units similar but not identical to the native dimeric form of the molecule. Spectrin apparently is composed of numerous large protease-resistant regions or domains connected by small protease sensitive segments. The structural integrity and accessibility of these sites is minimally affected by oligomeric state or proteolytic digestion conditions. The similarities of sizes, isoelectric points, and amino acid compositions of many intermediate-size peptides from areas of both subunits suggest that at least part of spectrin's structure may have evolved via replication of a single gene. A possible structural repeat of approximately 50,000 daltons is hypothesized.

Key words: spectrin domains, protease-resistant, erythrocyte, membrane, cytoskeleton, structural repeat, domain structure

Spectrin is the predominant component of the erythrocyte cytoskeleton, and as such is thought to play a major role in maintaining the shape and plasticity of the red cell [1–3]. A high affinity membrane binding site [4,5] maintains a tight association of spectrin with the membrane, but interactions with actin and band 4.1 also may be involved [6–9]. Spectrin exists in multiple molecular forms. A dimeric form consists of two polypeptide subunits (α subunit, M_r = 250,000; β subunit, M_r = 220,000) which exist as a flexible rod 1000 Å long [10]. This $\alpha\beta$ dimer can associate to form an $(\alpha\beta)_2$ tetramer and larger forms, depending upon the protein concentration and other factors [11–14].

Until recently little information about the molecular structure of the spectrin subunits has been available. Conflicting results on the identity of the N-terminal amino acids, the isoelectric points, and other structural properties have been reported [15–20] all of which suggested that spectrin was composed of multiple

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polypeptide subunits. On the basis of experiments employing mild trypsin digestion at O°C and two-dimensional cellulose peptide mapping techniques, we recently suggested that spectrin is composed of two nonidentical, homogeneous subunits. Each subunit was represented as a series of domains defined by proteolytic susceptibility [21–23]. Sites of protein–protein interactions and several noncovalent subunit interactions also have been identified [24], and a low-resolution topographical model of the spectrin molecule incorporating these features has been constructed.

The present report describes experiments on isolated spectrin subunits that explore the effects of the oligomeric state and other parameters on the susceptibility of spectrin to mild proteolytic cleavage.

METHODS

Purification of Spectrin

Spectrin was prepared from human erythrocyte ghosts by extraction with 0.1 mM EDTA/0.03 mM phenylmethylsulfonyl fluoride/0.5 mM 2-mercapto ethanol, pH 9.0 as previously described [21,25]. After centrifugation, the supernatant solution, which contained primarily spectrin and actin, was precipitated at pH 5.0–5.2 (adjusted with 1 N acetic acid). The precipitate was dissolved in 1% $NaDodSO_4$/10 mM Tris•HCl/0.5 mM 2-mercaptoethanol/0.5 mM EDTA/0.03 mM phenylmethylsulfonyl fluoride/0.02% NaN_3, pH 9.0, and applied (23°C) to a 5 × 90 cm column of Sepharose CL-4B (Pharmacia) equilibriated in the same buffer except with $NaDodSO_4$ at 0.1% (spectrin column buffer). Effluent was monitored at 280 nm, and a flow rate of 60 ml/hr was used. Fractions were analyzed on 5% polyacrylamide slabs and fractions containing spectrin were pooled and concentrated on an Amicon cell with either an XM-100 or XM-50 membrane. For preparation of $NaDodSO_4$-purified spectrin, fractions were pooled to yield equal amounts of α and β monomers and the sample was concentrated to 1–2 mg/ml. $NaDodSO_4$ was then removed by dialysis against 1 mM Na_3PO_4/0.5 mM 2-mercaptoethanol/0.5 mM EDTA/0.03 mM phenyl-methylsulfonyl fluoride/0.02% NaN_3 (spectrin dialysis buffer) at 4°C (five to six changes with a volume ratio greater than or equal to 1:100 and at least 8 hr between changes).

Spectrin monomers were isolated by concentrating spectrin containing fractions from the above column separation to 10–20 mg/ml as described above. This sample (25–300 mg) was chromatographed on either a single 5 × 90 cm Sepharose CL-4B column or two 5 × 90 cm Sepharose CL-4B columns connected in tandem using the buffer described above. The sample was recycled at 60 ml/min until two partially resolved peaks were obtained (three to five cycles). Samples were processed as described above.

Polyacrylamide Gel Electrophoresis

One-dimensional gel electrophoresis in the presence of $NaDodSO_4$ was performed in 1.5 × 110 mm slab gels following the procedure of Laemmli [26]. Typically, gels with a 5% total acrylamide content were used to analyze intact subunits and 11% polyacrylamide gels were used to analyze peptides.

Two-dimensional polyacrylamide gels were prepared by the method of O'Farrell [27]. Lyophilyzed samples were solubilized and isoelectrically focused using LKB Ampholines on 4% polyacrylamide gels (3 × 100 mm). Gels containing 60–120 μg

of focused peptides were electrophoresed in the second dimension on a 3 × 110 mm slab gel with 11% polyacrylamide by the method of Laemmli. Molecular weights of peptides were determined by their migration on the same gels as proteins of known molecular weights (17,000–100,000). A standard curve of logarithm of molecular weight versus R_f was used to relate the two parameters.

Radiolabeling of Spectrin Subunits

$NaDodSO_4$-purified spectrin (50 mg at 1 mg/ml) was dialyzed in 25 mM sodium phosphate buffer pH 7.6 and incubated with 2.25 ml ENZYMOBEAD Reagent (a solid phase lactoperoxidase-glucose oxidase reagent for radioiodination, Bio-Rad Laboratories), 8 mCi $Na^{125}I$, 44 μl of 1% KI, and 2.6 ml of 0.2 M glucose for 20 min. Reaction was terminated by removing the enzyme coupled beads via filtration (0.45 μm, Millipore Corp.). The labeled protein was desalted on a 2.5 × 40 cm Sephadex G-25 column (Pharmacia) and subsequently dialyzed extensively against spectrin dialysis buffer. $NaDodSO_4$ was added to a final concentration of 1% and the sample concentrated to 5 ml by ultrafiltration using an XM-100 filter. Radioactive isolated subunits were separated by recycling chromatography as described above. After isolation, radioactive subunits were mixed with an equal amount of unlabeled complementary subunit to form a heterodimer ($\alpha\beta$). $NaDodSO_4$ was removed by extensive dialysis against spectrin dialysis buffer.

Restricted Proteolytic Digestion of Spectrin

Spectrin samples were dialyzed into 10 mM sodium phosphate/40 mM NaCl, pH 8.0 and digested with trypsin (TPCK-trypsin, Worthington). Specific conditions of reaction are given in the text. Enzymatic hydrolysis was terminated either by addition of diisopropyl fluorophosphate (1 mM final concentration) or by boiling in $NaDodSO_4$-gel solubilizing buffer (6% $NaDodSO_4$/4 M urea/125 mM Tris HCl/4 mM EDTA/3% 2-mercaptoethanol, pH 6.9).

Two-Dimensional Cellulose Peptide Maps

Two-dimensional cellulose maps were prepared using protein bands excised from polyacrylamide gels that have been fixed and stained with Coomassie blue R-250 dye. Excised protein bands were radiolabeled with $Na^{125}I$ (carrier-free, 17 Ci/mg, New England Nuclear) and peptide mapped as originally described by Elder et al [28] with modifications previously described [21,22].

RESULTS

Relatively large amounts of spectrin (100 mg or more) could be separated from actin and other contaminating proteins by gel filtration in the presence of $NaDodSO_4$ on a Sepharose CL-4B column. After a single passage through a 90-cm long column, gel analysis of the spectrin peak revealed that the lead edge was enriched in the α subunit and the tail edge was enriched in the β subunit, suggesting that pure forms of both could be obtained.

Spectrin monomers were isolated by recycling chromatography in the presence of 0.1% $NaDodSO_4$ (Fig. 1). The resulting pools of isolated α and β subunits were typically approximately 98% and 95% pure, based upon densitometer scans of Coomassie blue stained gels. It was necessary to first prepare $NaDodSO_4$-purified

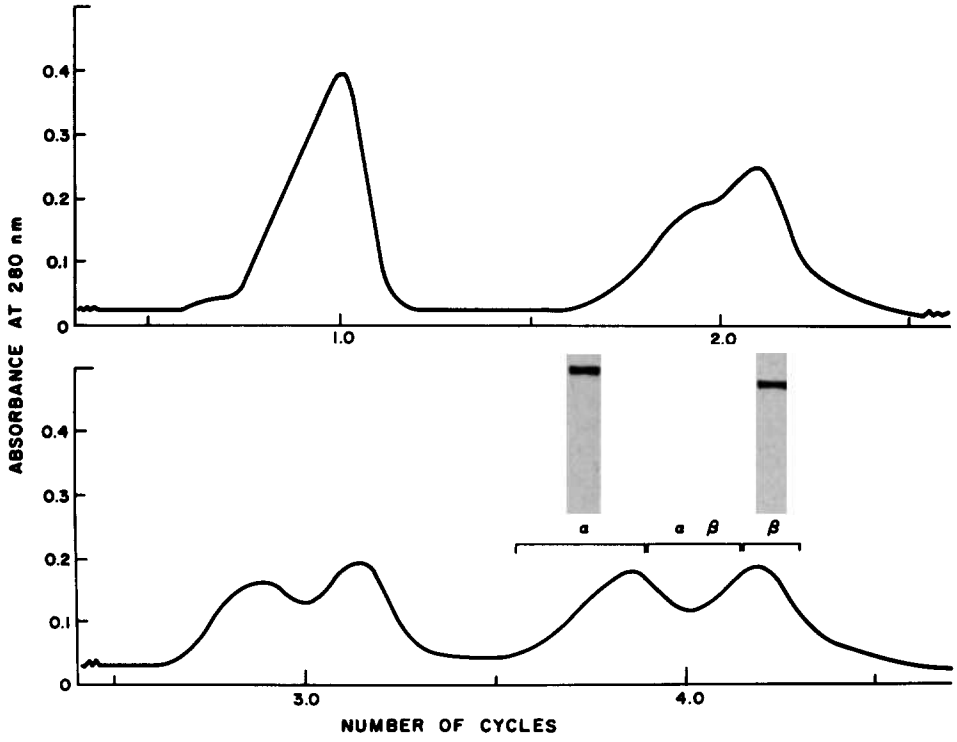

Fig. 1. Separation of spectrin subunits by recycling gel filtration chromatography. Fifty milligrams of $NaDodSO_4$-purified spectrin in approximately 5 ml was applied to a 5 × 90 cm column containing Sepharose CL-4B and recycled at 60 ml/min. Separation was monitored at 280 nm. After three cycles were completed, 11 min fractions were collected and analyzed on 7% polyacrylamide gels. Fractions were combined as indicated and the purity of these pools is indicated by the gel inserts. More than four or five cycles were not practical for a single 90-cm column since band broadening and peak tailing eventually resulted in contamination of the lead edge of the α subunit peak (earliest eluting component) with the tail edge of the β subunit peak from the previous cycle. Chromatographic separation could be improved slightly by connecting two identical columns in tandem. Two column systems were routinely used for separation of larger quantities of spectrin (100–300 mg).

dimer and concentrate that sample before recycling to prevent recontaminating the spectrin with actin and minor peptide components. Recycling chromatography of 100 mg of starting material produced approximately 25 mg of α subunit and 12 mg of β subunit of the purity illustrated in Figure 1. The quantity of β subunit obtained was always approximately one-half the yield of the α subunit and it was less pure as noted above. This was due primarily to tailing of the α subunit into the leading edge of the β subunit peak and contamination of the tailing edge of the peak with minor proteolytic degradation products.

Efficient separation of the subunits required optimum chromatographic conditions, which included minimum dead volume, very homogeneous bed volumes (Pharmacia gel reservoir could not be used to pack column), pulsation-free peristaltic pumping, and a small volume UV monitor flow cell. $NaDodSO_4$ supplied by at least one manufacturer did not permit separation of the α and β subunits by this

method. The reason for this anomaly is not understood and an extensive analysis of $NaDodSO_4$ samples was not undertaken. It was noted, however, that the $NaDodSO_4$ from several other manufacturers produced comparable separations. Electrophoresis grade $NaDodSO_4$ (BioRad Laboratories) was used routinely.

The separation of subunits in $NaDodSO_4$ permitted investigation of the properties of isolated subunits and the properties of recombined subunits after modification of one or both components. Reconstituted dimer could be prepared by combining equimolar amounts (Lowry determination) of isolated subunits in spectrin column buffer and subsequent removal of $NaDodSO_4$ by extensive dialysis using spectrin dialysis buffer as described in Methods. Complete recovery of α-helix and membrane binding capacity using spectrin purified in this manner has been previously reported [20]. Noncovalent associations between subunits apparently do reform after $NaDodSO_4$ removal. Reconstituted dimers were observed by polyacrylamide gel analysis of undenatured samples, gel filtration analysis, and binding of spectrin to inside-out vesicles, although the first two methods might not convincingly distinguish between heterodimers, small aggregates, partially denatured monomers and other possible molecular forms. An analysis of spectrin binding to inside-out vesicles provides the most definitive demonstration of reconstituted functional heterodimer complexes. When reformed dimers were added to vesicles, an equal amount of α and β subunit was associated with the membrane over the full range of receptor saturation levels. Since isolated $NaDodSO_4$-purified β subunit but not isolated α subunit binds to inside-out vesicles [20], α subunit must be associated with the β subunit in reconstituted α, β mixtures. The amount of α subunit associated with the membrane was always approximately equal to the β subunit as estimated by spectrophotometric quantitation of Coomassie blue intensities regardless of the ratio of α to β subunits in the reconstitution mixture. While association of subunits apparently occurs during reconstitution, the level of fidelity for these associations is not known. However, electron micrographs of reconstituted dimers after low angle rotary shadowing produce a more heterogeneous population than native dimers or tetramers (data not given). Some additional properties of reconstituted heterodimers are given below.

Figure 2 illustrates the reconstitution of heterodimers after specifically radiolabeling one or both subunits. Both subunits were associated with the complimentary subunit as demonstrated by binding to inside-out vesicles. These hybrids were subsequently digested with trypsin at 0°C and the resulting patterns were analyzed by Coomassie blue staining and autoradiography of one-dimensional polyacrylamide gels (data not given). Comparison of peptide patterns of these hybrids with peptides from isolated radioactive and nonradioactive monomers showed no difference between reformed dimers and isolated monomers when analyed either by Coomassie blue staining or autoradiography and were similar to results obtained with nonradioactive reconstituted dimers and monomers that were analyzed on more highly resolving two-dimensional gels (see below and Fig. 5).

The effects of varying enzyme-substrate ratios on the peptide patterns produced by trypsin digestion of spectrin for 20 hr at 0°C illustrate the resistance of large segments of the molecule to complete proteolytic cleavage (Fig. 3). The extent of proteolysis increased at higher enzyme concentrations, and some peptides were converted to lower molecular weight forms; but this change was slight over a greater than ten-fold increase in enzyme concentration. An 80,000 dalton peptide derived

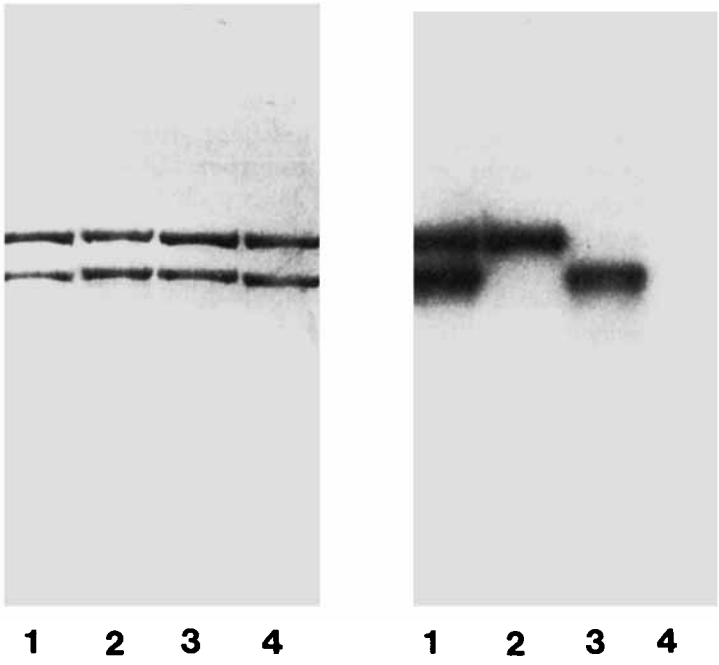

Fig. 2. Specific radiolabeling of a single subunit within a functional dimeric complex. Fifty milligrams of $NaDodSO_4$-purified spectrin was labeled with ^{125}I using lactoperoxidase-catalyzed iodination (BioRad ENZYMOBEAD reagent). Isolated radioactive subunits, purified as described in Methods and Fig. 1, were combined in equal molar ratios with similarly purified unlabeled subunits and $NaDodSO_4$ removed by extensive dialysis. The primary product of reconstitution was a functional heterodimer as detailed in Results. The four lanes on the left side represent Coomassie blue staining of a 5% polyacrylamide gel with the anode at the bottom. Each lane contains 5 μg of: 1, $\alpha^*\beta^*$, 2, $\alpha^*\beta$; 3, $\alpha\beta^*$; 4, $\alpha\beta$ (* indicates ^{125}I-labeled subunit). The four lanes on the right side represent a contact autoradiograph of the same gel. Exposure was for 24 hr at 23°C without intensifying screens.

from the α subunit was almost completely converted to a 74,000 dalton peptide at higher enzyme concentrations (Fig. 3). This conversion from 80,000 to 74,000 daltons could be greatly diminished by using 20 mM Tris•HCl buffer at pH 8.0 instead of phospate buffer. In both cases, the 80,000 dalton and/or 74,000 dalton peptide resisted further proteolysis during longer incubations and at higher enzyme concentrations. Addition of a second aliquot of trypsin after a 20 hr incubation also did not convert this region of the molecule to smaller forms. Other intermediate-size peptides from the α subunit were only slightly less protease resistant than the 80,000 dalton domain as indicated by the persistence of the peptides at 52,000, 46,000, and 41,000 daltons at high concentrations of enzyme (Fig. 3, lane 3). The appearance of 25,000 dalton peptides at higher enzyme concentrations is caused by further proteolysis of several of the domains or regions represented by the 52,000 and 46,000 dalton peptides, indicating that the degree of proteolytic resistance of these regions is somewhat less than that of the 80,000 dalton peptide (for further information on the interrelationship of peptides from both subunits see below and [21,22].

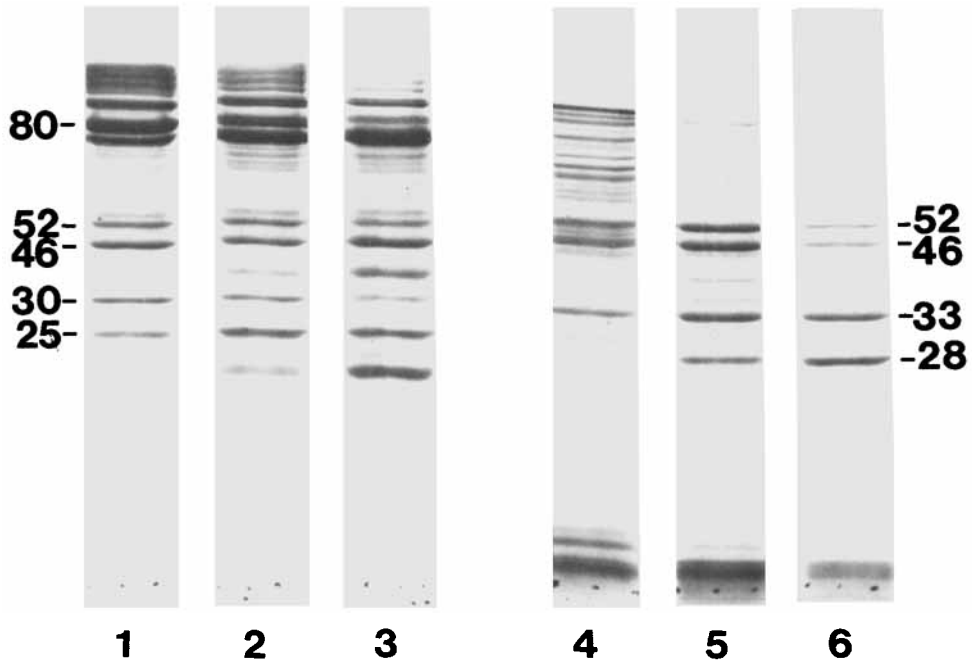

Fig. 3. Effect of varying enzyme:substrate ratios on trypsin digestion of isolated spectrin monomers. Purified spectrin monomers (200 μg aliquots, 1 mg/ml) in 10 mM sodium phosphate/40 mM NaCl, pH 8.0 were incubated with trypsin at the indicated enzyme:substrate ratios for 20 hr at 0°C. Reaction was terminated by addition of an equal volume of $NaDodSO_4$-gel solubilizing buffer and the sample was immediately boiled for 2 min. Aliquots (60 μg) were electrophoresed on 11% polyacrylamide gels (anode at bottom). Enzyme-substrate ratios were: α subunit: lane 1, 1:400, lane 2, 1:100, lane 3, 1:25; β subunit: lane 4, 1:400, lane 5, 1:100, lane 6, 1:25. Gels were stained with Coomassie blue dye. Apparent molecular masses are indicated as $M_r \times 10^{-3}$.

The stoichiometric yield of all peptide fragments was always less than 1:1 with respect to the starting protein since proteolytic resistance is a relative term (if enzymatic cleavage is sufficiently vigorous *all* lysine and arginine bonds will be cleaved) describing predominant intermediates in a dynamic process. The 80,000-dalton peptide from the α-I domain illustrates this point. Using milder enzymatic conditions than those used in Figure 3 the ratio of 80,000 dalton peptide to starting material was nearly 1:1. In Figure 3 some of the 80,000-dalton peptide had been further proteolysed to a 74,000-dalton peptide (band immediately below 80,000 daltons) and the ratio of 80,000-dalton peptide to starting material was less than 1:1. Further digestion would eventually lead to complete conversion to smaller peptides.

The effects of varying enzyme concentrations upon peptide patterns produced by proteolysis of the β subunit were more marked. The β subunit was in general more sensitive to proteolytic digestion and its domains were less clearly defined, with the exception of a 33,000-dalton peptide. This peptide was the most protease-resistant β subunit domain, comparable in resistance to the 80,000-dalton peptide from the α subunit. Peptides were observed at 52,000 and 46,000 daltons for the β

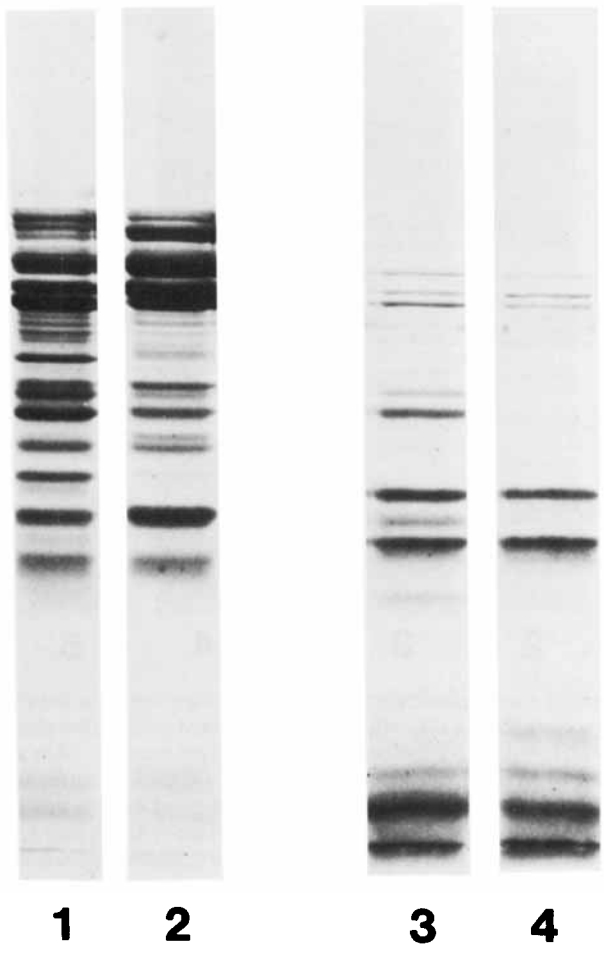

Fig. 4. Effect of temperature on proteolytic resistance of spectrin. Purified spectrin monomers (200 μg aliquots, 1 mg/ml) in 10 mM sodium phosphate/40 mM NaCl, pH 8.0 were incubated with trypsin for 1 hr. Reaction was terminated and 60 μg aliquots were electrophoresed as described in Figure 3. Experimental conditions were: α subunit: lane 1, 0°C, E:S = 1:25, lane 2, 25°C, E:S = 1:500; β subunit: lane 3, 0°C, E:S = 1:25, lane 4, 25°C, E:S = 1:250.

subunit but these peptides were not as proteolytically resistant as the peptides of the same molecular weight from the α subunit. As indicated in Figure 3, the only intermediate-size peptides that persist in significant amounts after digestion at high enzyme concentrations are the 33,000- and 28,000-dalton peptides.

Time course digestions at a single enzyme concentration produce peptide patterns similar to those in Figure 3. The same peptides persist and similar intermediate forms were observed.

Temperature effects on proteolytic resistance of spectrin are more complicated since they have at least two components, temperature effects on enzymatic activity of trypsin and the effect of increased thermal motion of the substrate molecule that influences accessibility of sites to the enzyme. To attempt a meaningful evaluation of the latter, an appropriate adjustment in the enzyme concentration was necessary

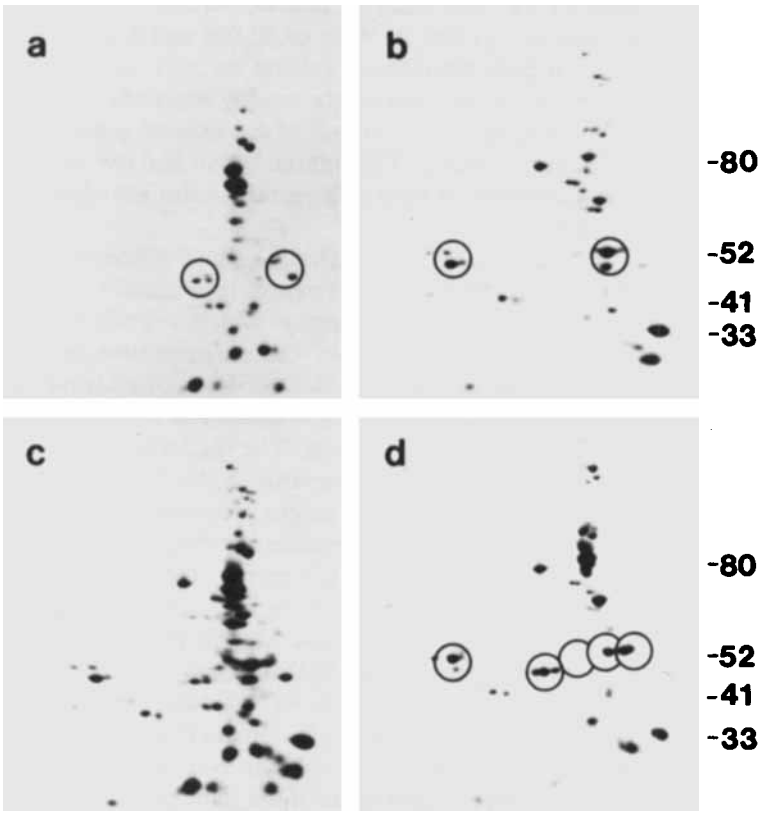

Fig. 5. Two dimensional polyacrylamide gels of tryptic peptides. Two-dimensional gels were prepared as described in Methods. Samples were incubated with trypsin as described in Figure 3 with an enzyme-substrate ratio of 1:100 for 20 hr at 0°C. Isoelectric separation (first dimension) was in the horizontal direction; the portion of the resulting pH gradient represented on these gels was linear and ranged from approximately pH 6.5 (left side) to pH 4.7 (right side). The anode for the second dimension was at the bottom. Molecular weights are indicated as $M_r \times 10^{-3}$. Gels a and b contained 60 μg of tryptic peptides of isolated α subunit and β subunit, respectively. Gels c and d contained 100 μg of tryptic peptides from $NaDodSO_4$-purified spectrin dimer and native spectrin dimer, respectively. Peptides from specific spectrin domains [21,22] in the 46,000 to 52,000 dalton size range have been circled. The origins of these peptides, using the nomenclature of Speicher et al [22] and going from left to right are: (a) αIV and αIII, (b) βIV and βII, (d) βIV, αII, αIV, βII, αIII.

to compensate for the temperature effect on activity. When enzyme concentration was decreased 20-fold, approximately the same amount of proteolysis occurred at 23°C (Fig. 4) as at 0°C. The most proteolytically resistant peptides at 0°C were also resistant at 23°C (e.g., 80,000-dalton peptide from α subunit; 33,000- and 28,000-dalton peptides from the β subunit) but the other intermediate-size peptides from the β subunit that were more proteolytically sensitive than most α subunit peptides at 0°C were not generated in significant amounts at 23°C for any trypsin concentration. Most α subunit intermediate-size peptides observed at 0°C were also observed at 23°C.

A more informative analysis of the tryptic peptide patterns of the subunits is possible using two-dimensional polyacrylamide gels (Fig. 5). Two-dimensional gels

also facilitate the analysis of the more complex peptide patterns obtained from tryptic digestions of both subunits together. Peptides of 52,000 and 46,000 daltons from several different segments of both subunits are evident on the two-dimensional gels. The subunit origin of most major peptides can be readily established by comparison of two-dimensional gels containing tryptic digests of the isolated subunits with gels from tryptic digests of subunit mixtures. The subunit origin and the unique nature of peptides from different domains of both polypeptide chains have been confirmed by cellulose peptide mapping.

A comparison of tryptic digests from $NaDodSO_4$-purified spectrin and untreated (native) spectrin indicates that some differences in proteolytic susceptibility do exist (Fig. 5). The differences, with one exception which is detailed below, appear to be quantitative rather than qualitative. These quantitative differences give a first impression of gross differences between $NaDodSO_4$-purified spectrin and untreated spectrin but careful examination of the gels illustrated in Figure 5 reveals the presence of the same peptide species in both cases. The $NaDodSO_4$-purified spectrin produces larger amounts of smaller peptides primarily in the 25,000–30,000-dalton range at the expense of their higher molecular weight precursors. These same peptides are observed in tryptic digests of native spectrin but they are minor components. In several cases the same peptide domain is represented by both 46,000- and 52,000-dalton peptides and the 46,000-dalton peptide is usually the predominant fragment in digests from $NaDodSO_4$-purified material. The predominant species from tryptic digestion of native spectrin is the 52,000-dalton species in each case. The only major peptide that is not common to both $NaDodSO_4$-purified and native spectrin is the 46,000-dalton peptide from the α-II domain (Fig. 5c,d) that is not observed in digests of spectrin which were pretreated with $NaDodSO_4$. It is apparent that $NaDodSO_4$-purified material contains most of the same peptide cleavage points but is generally more protease sensitive than native spectrin. Tryptic digests of native spectrin, which look quantitatively as well as qualitatively similar to $NaDodSO_4$-purified material, can be produced by using more vigorous enzymatic conditions.

DISCUSSION

Despite its great size spectrin has the ability to refold to a nearly normal conformation after denaturation with $NaDodSO_4$. Refolding is demonstated by recovery of α helix conformation [25], functional activities [24], at least some noncovalent associations between subunits and almost complete recovery of resistance to proteolysis [29]. Of these criteria proteolytic resistance is probably the most sensitive indication of structural integrity of the entire molecule since it is likey to reflect the most subtle differences between native and refolded molecules. The two most significant differences between native and refolded spectrin are in the α-II region (Fig. 5) and the general increased proteolytic susceptibility of $NaDodSO_4$-purified material. The largest unique peptide from the α-II region is 46,000 daltons from native spectrin. This peptide is completely absent from tryptic peptides of refolded spectrin over a wide range of enzymatic conditions and only smaller peptides from the domain (35,000, 30,000, 25,000, and 16,000 daltons) are observed. Since the α-helical conformation appears to be almost completely restored, the difference in proteolytic resistance may be due to some deficiencies in the tertiary structure

and/or subtle structural variations caused by residual binding of $NaDodSO_4$ to the dialyzed, refolded protein. These deficiencies appear most pronounced in the α-II region as evidenced by the complete lack of a peptide. However, the general increased proteolytic susceptibility of $NaDodSO_4$-purified spectrin would suggest minor deficiencies in refolding throughout the molecule. This less than perfect fidelity may be due to incomplete removal of $NaDodSO_4$.

The preservation of most molecular functions has permitted the use of individual subunits and peptides purified in the presence of $NaDodSO_4$ in numerous earlier structural and functional studies [14,20–25]. In this report the availability of isolated subunits simplified the one- and two-dimensional gel patterns obtained from tryptic digests and permitted identification of subunit origin for most peptides. It is also possible to recombine subunits to form functional dimer complexes after selectively modifying one subunit. This technique is not limited to radiolabeling and analysis of peptide patterns as used here but is potentially a useful method to selectively introduce a variety of chemical probes into spectrin.

The use of proteolytic digestion has proven to be a useful tool for studying the substructure of spectrin, as well as providing a method of dividing this mammoth molecule into smaller more manageable units for further analysis. The resistance of large regions of the molecule to proteolytic degradation under mild conditions has permitted this dissection and may also provide useful insight to potential structural repeats. Analysis of the peptide patterns on one- and two-dimensional gels as presented in this report, coupled with cellulose peptide map analysis [21–23] have enabled us to construct a comprehensive low resolution topographical map of the spectrin molecule (Fig. 6). In this model the α subunit is represented as being composed of five proteolytically resistant domains while the β subunit is composed of four similar segments. The domains are defined by their response to mild trypsin digestions. It should be noted that the domain sizes were selected to represent as much of the molecule as possible rather than the sizes of major components produced by any single set of experimental conditions.

Enzymatic digestion under mild conditions may provide some limited insight into the folding of large molecules such as spectrin where no sequence or X-ray crystallographic data is available. Based upon the amino acid composition of spec-

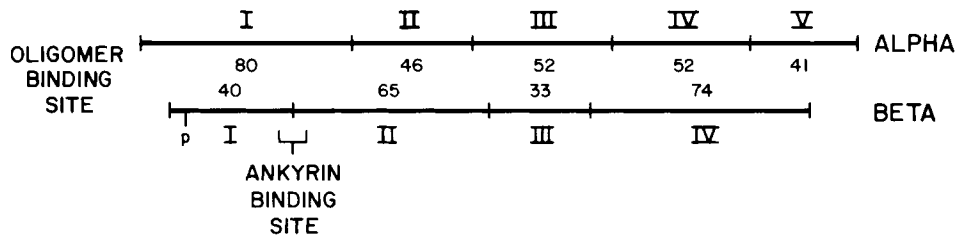

Fig. 6. The substructure of the spectrin dimer. Analysis of tryptic peptides by two-dimensional polyacrylamide gels and two-dimensional cellulose–peptide maps is consistent with this substructure of spectrin. Domains are identified (Roman numerals) using the nomenclature of Speicher et al [22]. Numbers indicate approximate molecular weights of domains ($M_r \times 10^{-3}$). The phosphorylation sites on the β subunit and the oligomer and ankyrin binding sites are also indicated.

trin, one out of every 7.4 residues is a lysine or arginine. The striking propensity for trypsin to chose a very small number of the total possible cleavage sites under the mild conditions used herein must be attributed to relative inaccessibility of the majority of cleavage sites in the native molecule and hence reflect in a crude sense something about the refolding of the polypeptide chain. The most readily cleaved peptide bonds would be those that protrude away from the surface of tightly folded regions, possibly at the segments connecting different domains. The possibility that at least some of the peptides observed by restricted tryptic digestion are due to large regions of the molecule devoid of lysine and arginine can be discounted since complete hydrolysis of spectrin (37°C, 24 hr, E:S, 1:100) does not produce peptides larger than approximately 3,000 daltons. Spectrin has a very high α-helix content [20,25], and since α helices are relatively resistant to proteolytic attack, it is likely that spectrin is composed of multiple, tightly folded α helical domains oriented along the long axis of the 1000 Å molecule. Further evidence that the susceptibility of spectrin to proteolysis is due to folding of the protein is provided by the rapid loss of intermediate-sized peptides when the molecular structure is perturbed with denaturants [29].

Two general features of protein domain structure have emerged from the analysis of sequence and crystallographic data of a large number of proteins [30,31]. Many large proteins seem to have evolved from much smaller peptides via gene duplication and fusion. The genes that fuse might be completely or partially replicated a number of times or different genes might fuse producing a new protein from two distinctly different precursors. A second striking feature is that polypeptide chain folding is even more strongly conserved than amino acid sequence. Given the large sizes of spectrin's subunits, it is likely that both polypeptides evolved via gene fusion from much smaller components. While only amino acid sequence and/or crystallographic data can definitively establish the size and nature of such ancestral components, a reasonable hypothesis can be made based upon current knowledge. Since the major factor restricting trypsin cleavage under mild conditions is polypeptide chain folding, ie, domain structure, the peptides produced might to some extent reflect similarities of chain folding. The frequency of occurrence of tryptic peptides of both 46,000 and 52,000 daltons from different segments of the molecule provides preliminary evidence of a repeating domain structure within spectrin. Figure 7 illustrates the locations within the spectrin molecule of all tryptic peptides in this size range that have been identified. The location and size (approximately 50,000 daltons) of the suggested repeating structure also is represented. An obvious feature of this hypothetical model is that the four terminal regions of the dimeric molecule would be smaller and could be either structurally unrelated to the repetitive structure hypothesized here or a partial homologous repeat. It is interesting to note that the mass of the questionable areas in the center of the β subunit is approximately 50,000 daltons and could represent a complete copy of the major repeating structure that has developed sufficient differences to produce different peptide products. Two feasible alternatives for the origin of this segment of the molecule include two partial copies of the major repeat and two smaller regions different from the major repeat but related in structure to the terminal region of spectrin. The minimum size of the major structural repeat might be much smaller than the suggested 50,000 daltons; perhaps one-half, one-third, or even smaller.

Other evidence supports the hypothesis that multiple repeats of a single ancestral peptide make up all or a large portion of the spectrin molecule. Amino acid

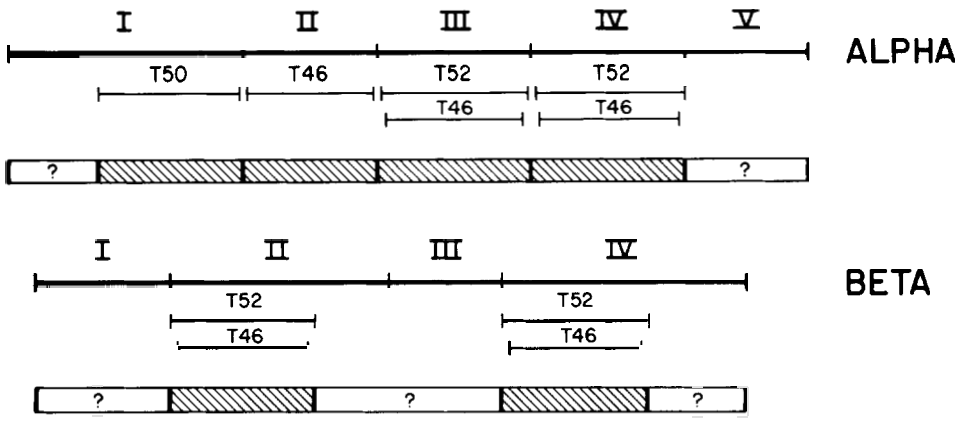

Fig. 7. Hypothetical repeating structure of spectrin. Localization of tryptic peptides in the 46,000–52,000 dalton size range based upon analysis of cellulose peptide maps. With the exception of the T50 peptide from the αI domain these peptides correspond to the circled peptides in Figure 5. The T50 peptide is a minor cleavage product that has been identified using monoclonal antibodies to the αI domain. A hypothetical repetitive structure is represented by the bar diagrams immediately below the tryptic peptides. The repeat units are represented by hatched sections. Sections of more ambiguous origin occur at the ends of both subunits and in the center of the β subunit.

compositions of several spectrin peptides larger than 20,000 daltons are similar to each other and to the amino acid compositions of both subunits. Most peptides 20,000 daltons or larger have similar isoelectric points deviating from the pH 5.2 isoelectric point of the parent by less than one-half of a pH unit. Furthermore, several different segments of each subunit produce an extensive cascade of peptides and the sizes of peptides throughout the cascade are identical. This would be consistent with homologous domains (primary cleavage sites) with similar chain fold and hence similar subdomains (secondary cleavage sites).

A repeating structure of 8,000 daltons has previously been suggested by Anderson [32] based primarily upon the peptide pattern produced by mild trypsin treatment of spectrin at low salt concentrations. A series of peptides were produced ranging in size from the intact monomers to 150,000 daltons. We have duplicated his results and mapped some of the peptides obtained (data not given, [21,22]). Our results are not consistent with sequential removal of 8,000-dalton units from both subunits, but the possibility of an 8,000-dalton repeat can not be discounted.

A clearer understanding of the underlying substructure of the molecule must await more detailed structural analysis. In the interim, the domain structure as defined in Figure 6 remains a useful model since the molecule is almost completely represented even if these operationally defined domains do not reflect the underlying repetitive structure with complete fidelity.

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