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Molecular Studies of Human Erythrocyte Spectrin: Head to-Head Interactions

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MOLECULAR STUDIES OF HUMAN ERYTHROCYTE SPECTRIN: HEAD-TO-HEAD INTERACTIONS

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LIST OF ABBREVIATIONS

A_{280}	Absorbance of the protein or peptide at 280 nm
Ab	Antibody
BSA	Bovine serum albumin
βME	β-mercaptoethanol
β-Sp	β-spectrin monomer
CD	Circular dichroism
CPM	Counts per minute
Cys167	Cysteine residue at position 167 in the α-spectrin amino acid sequence
EPR	Electron paramagnetic resonance
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared
GSH	Glutathione
GST	Glutathione-S-transferase
IPTG	Isopropyl-B-D-thio-galactoside
kD	kilo Dalton

K_a	Association (binding) constant
K_{av}	Partition coefficient
Mal6	4-Maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy
MW	Molecular weight
NBT	Nitro Blue Tetrazolium
NMR	Nuclear magnetic resonance
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl flouride
R_s	Stokes radius
SDS	Sodium dodecyl sulfate
Spx-y	α-spectrin peptide, x = the first amino acid residue in the peptide, y = the last amino acid residue in the peptide, numbers correspond to residues in intact α-spectrin
TBE	89 mM Tris, 89 mM boric acid and 2 mM EDTA
TCA	Trichloro acetic acid
V_e	Elution volume of the solute
V_o	Void volume
V_t	Total bed volume

CHAPTER I

INTRODUCTION

The spectrin superfamily is a group of proteins that have similar structural and functional features. They are present in several tissues but have different names. Spectrin is present in erythrocytes, dystrophin in muscle tissues, fodrin in brain and other tissues, and α-actinin in skeletal, cardiac and smooth muscles (Dhermy, 1991; Winkelman and Forget, 1993). It is named the spectrin superfamily since spectrin is the most studied member of the family. It is the main component of a two dimensional network of proteins called the erythrocyte membrane skeleton, that is underlying the surface of the erythrocyte membrane (Marchesi and Steers, 1968; Branton *et al.*, 1981; Gratzer, 1983; Gratzer, 1984; Bennett, 1990).

The erythrocyte membrane skeleton interacts with several integral membrane proteins thus influencing the lateral mobility of these proteins (Goodman and Branton, 1978). Beside interacting with the integral proteins, the membrane skeleton interacts with lipids in the membrane (Mombers *et al.*, 1980; Maksymiw *et al.*, 1987; Subarrao *et al.*, 1991). Through all these interactions, the membrane skeleton provides support to the lipid bilayer so it can withstand the shear pressure that erythrocytes have to endure passing through blood vessels. This support is essential for the survival of erythrocytes (Lux and Glader, 1981; Marchesi, 1985; Elgsaeter and Mikkelson, 1991).

Spectrin was purified for the first time from the erythrocyte membrane skeleton in 1968 (Marchesi and Steers, 1968). Purified spectrin was found to constitute 25-30% of the total erythrocyte membrane protein (Marchesi *et al.*, 1971; Fairbanks *et al.*, 1971; Steck, 1974). When the purified spectrin was run on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), it appeared on the gel as two bands with apparent molecular weight of 260 kD for the first band and 225 kD for the second band. Thus the two subunits of spectrin are often referred to as Band 1 and Band 2 (Steck, 1974), or as α- and β-spectrin.

Due to the large size and the shape of the spectrin molecule, it is difficult to apply nuclear magnetic resonance (NMR) or X-ray crystallography to study its structure. However, other techniques have been used to obtain structural and physical properties of spectrin. For example, electron microscopy (Shotton *et al.*, 1979) and sedimentation velocity (Ralston and Dunbar, 1979), studies of spectrin suggest that spectrin heterodimer is a 100 nm long, rod-like molecule with a diameter of 4-6 nm. Additional structural information was obtained on negatively stained spectrin in the partially expanded membrane skeleton, suggesting that the α- and β-spectrin subunits twist around each other forming a two-stranded helix with a pitch of about 104-106 Å and a diameter of about 36-52 Å (McGough and Josephs, 1990). Circular dichroism (CD) (Ralston, 1976) and Fourier transform infrared (FTIR) (LaBrake and Fung, 1993) studies suggest that spectrin contains 60-75% α-helix.

Spin labeling electron paramagnetic resonance (EPR) studies showed different types of motions (Fung *et al.*, 1979; Cassoly *et al.*, 1980; Dubreuil and Cassoly, 1983;

Fung and Johnson, 1983; Streichman *et al.*, 1991; Hensley *et al.*, 1993). The combination of conventional and saturation transfer EPR methods was used to determine the motions of these spin labels to be in the time ranges of 10^{-9}, 10^{-7}, 10^{-6} and 10^{-3} s (Fung *et al.*, 1979; Fung and Johnson, 1983). Dynamic light scattering was also used to study the segmental motion of spectrin tetramer (Budzynski *et al.*, 1992). It measured fluctuational segmental motions over a distance of 20-30 nm with relaxation times equal or less than 23 μs. These studies show that spectrin is a highly flexible molecule, which is understandable since it contributes to the deformability and elasticity properties of human erythrocytes.

The α- and β-spectrin subunits interact side-to-side in an antiparallel manner to form the spectrin dimer (Speicher *et al.*, 1982). This interaction starts at a nucleation site which is at the C-terminal end of the α-subunit and the N-terminal end of the β-spectrin followed by several interaction sites along the length of each of the two subunits (Speicher *et al.*, 1992). Two of the spectrin dimers can associate head-to-head (the N-terminal region of the α-subunit in one dimer interacting with the C-terminal region of the β-subunit on the other dimer) to form the spectrin tetramer (Ungewickell and Gratzer, 1978; DeSilva *et al.*, 1992; Speicher *et al.*, 1993), which is the predominant form in erythrocytes (Byers and Branton, 1985; Liu *et al.*, 1987). Diseases that are associated with abnormal red blood cell shape such as hereditary elliptocytosis and hereditary pyropoikilocytosis were found to have deficiency in spectrin tetramer and to be caused by mutations in the N-terminal region of the α-spectrin (Boulanger *et al.*, 1994; Gallagher *et al.*, 1992; Lecomte *et al.*, 1989) or in the C-terminal region of the β-spectrin (Gallagher *et al.*, 1995; Sahr *et al.*, 1993; Tse *et al.*, 1990) subunits. Most of the mutations in the α-spectrin

subunit were found to be within the first 150-200 amino acid residues. These mutations were suggested to destabilize the head-to-head interactions and thus the tetramer formation. Binding constants of spectrin dimers to give tetramer at 37 C was about 2 x 10^5 M^{-1} (Ungewickell and Gratzer, 1978; DeSilva *et al.*, 1992).

Recombinant α-spectrin peptides were used to study the domain structure and their binding with β-spectrin, to understand the role of spectrin in maintaining the biconcave shape of normal erythrocyte.

The amino acid sequence of the α- and β-spectrin subunits showed that both subunits contain homologous amino acid sequences of about 106 reisdues long (Sahr *et al.*, 1990; Winkelman *et al.*, 1990). The first 150-200 amino acid residues contain such homologous sequence. The presence of these homologous sequences suggested the presence of structurally stable domains and the structure of α- and β-spectrin subunits to be as a string of structural domains. Thus studies of the structure of these homologous sequences are essential in solving the structure of the part of molecule containing the first 150-200 amino acid residues, and of the entire molecule.

The presence of structural domains within the homologous sequence led to the question of where is the start of the domain structure? For example, in the sequence ABCDEFABCDEFABCDEF, a domain of 6 letters could start with the letter A and the domain would be ABCDEF, at B and the domain would be BCDEFA, or at C and the domain would be CDEFAB. Thus six different possibilities appear for a six letter domain, and about 106 different possibilities for spectrin domains. Assuming that folded domains will be digested slower that the non-folded part, various fragments of spectrin were

prepared by recombinant techniques and subjected to digestion by proteases. Protease products were separated for N-terminal sequencing to determine the start of the domain. The first amino acid residue in the structural domain was determined to be 26 amino acid residues downstream from the beginning of the sequence motif for *Drosophila* α-spectrin (Winograd *et al.*, 1991). Assuming that human α-spectrin has the same phase shift as *Drosophila* α-spectrin, the first amino acid residue in the first structural repeat would be amino acid residue number 49. Another study suggested that the start of the first structural repeat is amino acid residue number 54 (Speicher *et al.*, 1993). However, the start of human α-spectrin is not clear.

Based on the findings that spectrin is highly helical, the length of the spectrin subunits of 100 nm is approximately one third the length of an α-helix of 2,000 amino acid residues, the amino and carboxy termini of the subunits are located opposite to each other in the spectrin molecule, and spectrin is more flexible in solution than coiled-coil α-helical proteins, a triple α-helical bundle was suggested as the structure for the 106 amino acid sequence (Speicher and Marchesi, 1984). A modification of the above model showing the three helices (A, B and C), with the last helix (C) in the n^{th} repeat and the first helix (A) in $(n + 1)^{th}$ repeat forming a one long helix (CA) (Parry and Cohen, 1991; Parry *et al.*, 1992). X-ray crystallography of a folded domain from *Drosophila* α-spectrin supports this model (Yan *et al.*, 1993).

In this study, various recombinant α-spectrin peptides were used to study the N-terminal region of α-spectrin that is important in the binding of β-spectrin.

In the first part of this study, 8 peptides with different lengths were expressed and

purified. Limited protease digestion combined with amino acid sequencing were used to determine the start of the first structural domain of the α-spectrin subunit. This work, in collaboration with other members in our laboratory, was published in 1994 (see Appendix).

In the second part of the study, two different α-spectrin peptides and β-spectrin subunit were prepared for binding studies. Three different techniques--Superose 6 size exclusion gel chromatography, combined radiolabeling with western blotting and immunoprecipitation of radiolabeled peptides--were used to obtain binding constants. These binding constants were shown to be comparable with spectrin dimer-dimer binding constants, suggesting that these α-spectrin peptides are good model systems for α-spectrin subunit studies.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Equipment

All general chemicals were obtained from Sigma chemical Company (St. Louis, MO), Curtin Matheson Scientific, Inc. (Wooddale, IL), or Fisher Scientific (Pittsburgh, PA). Specific chemicals such as 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (Mal6) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI); Ampicillin and glutathione Sepharose 4B resin from Pharmacia Biotechnology (Piscataway, NJ); protein molecular weight standards from Gibco BRL Life Technologies Inc. (Gaithesburg, MD); dye reagent for protein assay from Bio-Rad (Richmond, CA); isopropyl-B-D-thiogalactoside (IPTG) from Promega Company (Madison, WI); ^{125}I from ICN (Costa Mesa, CA). cDNA of spectrin was a generous gift from Dr. B. G. Forget of Yale School of Medicine, New Haven, CT. All equipment were available in our laboratory with the exception of the gamma counter which is in the Department of Biology.

2.2 Cloning and Expression of α-Spectrin Peptides

2.2.1 DNA Preparation

Sense and antisense oligonucleotide primers were synthesized by National

Biosciences (Plymouth, MN). The primers were dissolved in deionized water by heating to 65°C for 10 minutes to give a final concentration of 100 pmole/ μl. The solution was stored in aliquot at -70°C to avoid repeated freezing and thawing. A 200 μl PCR cocktail was prepared by adding 2 μl of the sense oligonucleotide, 2 μl of the antisense oligonucleotide, 10 μl of 20X buffer (supplied by the company), 4 μl of 10 mM dTNPs, 179 μl of sterile deionized water, 2 μl of template (cDNA clone α3), and finally 1 μl of *Taq* polymerase in a 1.5 ml microfuge tube. The tube was placed in a thermal cycler (Eppendorf Microcycler) and 1 parafilm bead added to the mixture. The PCR was programmed to run 35 cycles of 30 seconds at the denaturation temperature (95°C), 45 seconds at the annealing temperature (calculated using the equation $T_m = 4 \times (G \text{ or } C) + 2 \times (A \text{ or } T)$, where G,C,A and T are the number of deoxyguanylate, deoxycytidylate, deoxyadenylate and deoxythymidylate in the primers, respectively), and 60 seconds at the polymerization temperature (72°C). 10 μl of the resulting mixture was loaded on an agarose gel to check if the expected DNA piece was made. A standard of 100 bp was loaded on the adjacent well to check if the DNA piece has the correct length. Then the Promega magic PCR prep DNA purification system (Promega, Madison, WI) was used to purify the DNA piece out of the mixture giving 50 μl of DNA solution.

2.2.2 DNA Agarose Gel Electrophoresis

To purify DNA fragments, 1% agarose gels were prepared. 0.5 g of agarose was added to 50 ml of TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and dissolved by boiling the solution. The solution was stirred until it cooled down and ethidium bromide was added to give a final concentration of 0.5 μg/ml (2.5 μl of 10 mg/ml

stock solution). After solution cooled down further, it was poured into a horizontal gel cast. After gel hardened at room temperature, the cast was moved to the gel chamber and TBE buffer added. DNA samples were prepared by adding a 2X loading buffer containing a tracing dye (orange G). Gel samples were loaded onto the gel and electrophoresis ran under constant voltage of 50 V until desired separation was reached. The separation was followed using a MINERALIGHT LAMP (UVP INC, San Gabriel, CA).

2.2.3 Spectrin Construct Preparation

Ten ml of overnight culture of cells containing the pGEX-2T vector were pelleted down and the supernatant decanted. The Promega magic PCR prep DNA purification (Promega, Madison, WI) system was used to give a 50 μl purified vector solution. 2 μl was electrophoresed on an agarose gel to check the presence of the vector.

The purified DNA pieces and the pGEX-2T vector were digested by incubating 20 μl of each with 5 μl of buffer 3 (10X, supplied by the company), 1 μl of EcoRI, 1μl of BamHI and deionized water to 50 μl in a water bath at 37°C for 2 hours. The cut DNA pieces and vector were purified on an agarose gel by excising the desired bands off the gel followed by extraction of the DNA from the excised bands. The gel bands were transferred to 0.5 ml microfuge tube containing glass wool and has a hole at the bottom. The microfuge tube was placed in a 1.5 ml microfuge tube and centrifuged at 2,000 g for 10 minutes. The 0.5 ml microfuge tube was discarded. The DNA was further purified by phenol extraction. 0.5X the DNA solution volume of phenol was added and the solution was spun for 2 minutes in a picofuge. To the phenol layer (upper layer), 0.5X the volume of chloroform was added and the tube spun for 2 minutes in a picofuge. To the aqueous

layer, o.1X the volume of 3M sodium acetate, 2X the volume of 100% ethanol and 2 μg of yeast tRNA (for vector only) were added. The mixture was incubated on dry ice for 15-20 minutes followed by spinning the tube for 20 minutes at 15,000 g. After decanting the supernatant, the pellet was washed with 200 μl of 70% ethanol and spun for 5 minutes at 15,000 g. The solution was dried by heating at 65°C for 5 minutes. Ten μl of 10 mM Tris, 0.1 mM EDTA, pH 8.0 was added to the tube and heated at 65°C for 5 minutes. 4 μl of the final solution was electrophoresed on an agarose gel to check the purity of the samples.

Two μl of the vector and comparable amount of the DNA piece were combined in a microfuge tube along with 1 μl of T4 DNA ligase (Epicenter Technologies, Madison, WI), 1.5 μl of the 10 X buffer (supplied with the ligase), 1 μl of ATP and deionized water to 15 μl. The mixture was incubated at 15 °C overnight followed by a second incubation at 65 °C for 10 minutes to deactivate the ligase.

A mixture of 100 μl HB101 competent cells and 5 μl plasmid were mixed and incubated on ice for 30 minutes. Then 300 μl of TSS (0.5% sodium chloride, 1% tryptone, 0.5% yeast extract, 10% PEG, 5% DMSO, and 50 mM magnesium chloride) buffer was added and the mixture was incubated with shaking for 60 minutes. The cells were poured and smeared on an agar plate and the agar plates were left overnight at 37°C. Colonies were tested for correct transformation as described below in section 2.3.1. Colonies producing fusion proteins were then smeared on agar plates and incubated overnight at 37°C. The agar plates were kept for up to 1 month afterwhich the cells were smeared on a new agar plate.

Cells containing plasmids of all the peptides used in this project were prepared by Dr. M.R. Kelley either while he was at Loyola Medical School or while he is at Riley Hospital for Children , University of Indiana , Indianapolis, IN. Cells prepared by Dr. M. R. Kelley include Spα1-166 (S12C) with the serine residue at position 12 replaced with cysteine.

2.3 Expression, Purification and Digestion of GST-Fusion Proteins

2.3.1 Testing Cells For The Ability to Express Fusion Proteins

Cells were inoculated (~4:30 p.m.) into two test tubes each containing 2 ml of autoclaved medium (prepared as described in section 2.3.2 below) and Ampicillin added to a final concentration of 0.1 mg/ml. The cells were incubated overnight at 37°C with shaking at 225 rpm. At 9:00 a.m. the following day, 100 μl aliquot of each of the test tubes was added to another test tube containing 1.9 ml of autoclaved medium and Ampicillin added to a final concentration of 0.1 mg/ml. At 11:30 a.m., isopropyl-β-D-thiogalactoside (IPTG, Pharmacia Biotechnologies, Piscataway, NJ) was added to one of the two test tubes to a final concentration of 0.1 mM. At 3:30 p.m., cells were harvested by removing 1.5 ml from each test tube to a microfuge tube. The microfuge tubes were centrifuged for 5 minutes at 15000 xg and the supernatant discarded. 100 μl of electrophoresis sample buffer was added to the pellet, mixed, and boiled for 5 minutes. The two samples, with and without IPTG, were ran side to side on a gel to determine whether fusion protein was induced in sample containing IPTG.

2.3.2 Cell Growth

Medium was prepared by dissolving 10g/L of trypton, 5g/L of yeast extract, and 5g/L NaCl in water and autoclaved. Cells were inoculated in autoclaved medium (4-5 p.m.) and Ampicillin added to a final concentration of 0.1 mg/ml. The cells were left to grow overnight at 37°C with shaking at 225 rpm. At 9:00 a.m. ($O.D._{600}$ = ~2) the cells were diluted 1:20 in autoclaved medium and ampicillin added to a final concentration of 0.1 mg/ml. The solution was left at 37°C with shaking at 200 rpm for 2.5 hours. At 11:30 a.m. ($O.D._{600}$ = 0.5-1, the mid-log phase), IPTG was added to a final concentration of 0.1 mM. The solution was left at 37°C with shaking at 200 rpm for 4 hours (3:30 p.m., $O.D._{600}$ = ~2). Cells were harvested by centrifuging the resulting solution at 5000 rpm for 10 minutes in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Wilmington, DE) with GSA rotor. Cells, if not used, were stored at -70°C until needed.

2.3.3 Fusion Protein Purification

Cells, if frozen were thawed at 4°C for 15-30 minutes and 5-10 ml of 1% Triton X-100 in PBS were added per gram of cells. They were lysed either by sonication or by freeze-thaw followed by sonication. Cell lysate was centrifuged at 10,000 rpm for 10 minutes in a Sorvall RC-5B centrifuge with SS34 rotor. The supernatant was collected and loaded on a glutathione affinity column pre-equilibrated with phosphate buffered saline at pH 7.4 (PBS7.4). After incubating the supernatant on the column for 5 minutes, it was eluted out of the column by free flow. The column was washed with PBS7.4 buffer until the absorbance at 280 nm decreased below 0.020. After that, the column was washed with 50 mM Tris pH 8.0 buffer containing 5 mM fresh glutathione. The peak containing the

fusion protein was collected and concentrated using Stirred Cells with PM10 Disc Membranes (Amicon, Beverly, Mass).

2.3.4 GST Purification

Same procedure as above except *E. coli* cells containing the pGEX-2T vector rather than modified vectors were used to get GST rather than GST-spectrin fusion protein.

2.4 Purification of α-Spectrin Peptides

2.4.1 Thrombin Cleavage of Fusion Proteins

In order to determine the optimum incubation time that would produce maximum digestion and minimum degradation of the spectrin peptide, a time study was performed. The concentrated protein was dialyzed against 50 mM Tris, 10 mM EDTA pH 8.65 buffer. The concentrated and dialyzed protein was loaded onto a thrombin column (thrombin covalently linked to agarose beads, CalBiochem, San Diego, CA) with up to 50 mg of fusion protein per 1 ml of resin and SDS-PAGE samples were taken every several hours. 24 hours incubation at room temperature was found to be sufficient. The mixture of GST, spectrin peptide and residual fusion protein was eluted out of the column.

2.4.2 Affinity Purification of α-Spectrin Peptides

The eluted mixture from the thrombin column was loaded onto a glutathione affinity column pre-equilibrated with PBS7.4 buffer. The spectrin peptide was eluted by washing the column with PBS7.4 buffer. GST and residual fusion protein were removed by washing the column with 50 mM Tris pH 8.0 buffer containing 5 mM fresh glutathione.

The spectrin peptide solution was concentrated using Stirred Cells and YM3 Disc Membranes.

2.4.3 FPLC Purification

The FPLC system (Pharmacia Biotechnologies, Piscataway, NJ) contains two P-500 pumps (A and B), LCC-500 controller, Single Path Monitor UV-1 optical unit, FRAC-100 fraction collector, and a chart recorder. Hemoglobin purified by ion-exchange chromatography was used as a reference sample to check column performance. A salt gradient was created by using two buffers: the first was 10 mM Tris, pH 7.8 with no salt while the second was 10 mM Tris, pH 7.8 with 1 M NaCl. Frozen hemoglobin was thawed immediately before use. It was diluted to 2 ml and filtered through 0.22 μm filter before 100 μl of the filtered sample was loaded onto the FPLC Mono Q column pre-equilibrated with 10 mM Tris, pH7.8 buffer. 10 ml of the buffer was used to allow all neutral and positively charged species to elute out of the column. The salt concentration in the buffer was increased to 500 mM (10 mM Tris and 500 mM NaCl), and 35 ml was used to elute negatively charged species, 10 ml of the starting buffer was used to re-equilibrate the column. Flow rate used was 0.5 ml/min. Five peaks were obtained. The first two peaks were the major peaks and appeared at the beginning, before the salt was passed through the column. The other relatively small three peaks eluted at salt concentrations of 96 ± 13, 129 ± 9, 154 mM NaCl, respectively (n = 8).

2.4.3.1 FPLC Purification of GST Protein

The glutathione affinity purified GST was loaded onto a Mono Q HR5/5 column pre-equilibrated with 10 mM Tris pH 7.8. A salt gradient was created using 10 mM Tris

pH 7.8 as buffer 1 and 10 mM Tris, 1 M NaCl, pH 7.8 as buffer 2. Used 10 ml of buffer 1 (salt concentration at 0 mM) to allow all neutral and positively charged species to elute out of the column. Mixed buffer 1 and buffer 2 to give 500 mM salt concentration, and maintained for 25 ml to elute the GST protein, increased to 1 M NaCl and stay there for 5 ml to elute any remaining material off the column. Returned to buffer 1 to re-equilibrate the column. The flow rate used throughout the program was 0.50 ml/min.

2.4.3.2 FPLC Purification of α-Spectrin Peptides

The glutathione affinity purified spectrin peptide was dialyzed against 40 mM Tris pH 7.5 buffer. The dialyzed spectrin peptide was loaded onto a Mono Q HR5/5 or HR10/10 column pre-equilibrated with the 40 mM Tris, pH 7.5 buffer. Two buffers were used to create a salt gradient, buffer A, 40 mM Tris pH 7.5 with no salt and buffer B, 40 mM Tris pH 7.5 containing 500 mM NaCl. All peptides except for Spα1-49, Spα49-167 and Spα1-446 were purified using the following procedure: The salt concentration remained at 0 mM for 40 ml to elute all neutral and positively charged species off the column. The gradient was increased to 125 mM NaCl after 40 ml and to 155 mM NaCl after another 50 ml to elute the spectrin peptides. The salt concentration was increased to 500 mM NaCl to clean the column before re-equilibrating it with buffer A. The flow rate used was 2 ml/min.

The following procedure was used for Spα1-49: 10 ml buffer A to elute neutral and positively charged species off the column. The gradient was increased to 300 mM NaCl after 50 ml to elute Spα1-49 before increasing it to 1 M to clean the column. The column was finally re-equilibrated with starting buffer. The flow rate used throughout the

program was 0.50 ml/min.

Affinity purified Spα49-167 contained another peptide that elute at the same position when the above program was used. Thus a slower gradient was required to purify this peptide. The following procedure was used: 40 ml of buffer A to elute all neutral and positively charged species off the column. The gradient was increased to 100 mM NaCl after 10 ml and remained at that concentration for 55 ml to elute the contaminating band. Spα49-167 eluted by increasing the salt concentration after 20 ml to 125 mM NaCl followed by another increase after 50 ml to 155 mM NaCl. The column was cleaned by increasing the salt concentration to 500 mM NaCl before re-equilibrating the column with buffer A. The flow rate used throughout was 2 ml/min.

Spα1-446 was purified using the following procedure: 40 ml buffer A to elute neutral and positively charged species. The salt concentration was increased to 135 mM NaCl and remained constant for 120 ml to elute other contaminants. Spα1-446 was eluted by increasing the salt concentration after 24 ml to 175 mM and keeping it constant for 64 ml. The column was cleaned by increasing the salt concentration to 500 mM NaCl before re-equilibrating the column with the starting buffer.

2.5 Protein Gel Electrophoresis

2.5.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was done according to the method of Laemmli (Laemmli, 1970) with modifications using the Bio-Rad (Richmond, CA) mini-protein II electrophoresis cell. The lower separating gel consisted of 380 mM Tris, 0.1% (W/V) SDS, 0.033% (W/V)

ammonium persulfate, 0.067% (V/V) TEMED(Bio-Rad, Richmond, CA), and pH 8.8 with the acrylamide varying between 3-16% (W/V) and the N,N'-methylene bisacrylamide varying between 0.08-0.43% (W/V). It was made by adding the appropriate amounts of 4X buffer (1.52 M Tris, 0.4% (W/V) SDS, pH 8.8), 10% (W/V) ammonium persulfate, 30% (W/V) acrylamide-0.8% (W/V) N,N'-methylene bisacrylamide solution, TEMED, and autoclaved deionized water. The upper stacking gel consisted of 3.9% (W/V) acrylamide, 0.1% (W/V) N,N'-methylene bisacrylamide, 125 mM Tris, 0.1% (W/V) SDS, 0.05% (W/V) ammonium persulfate, 0.1% (V/V) TEMED, and pH 6.8. It was made by adding the appropriate amounts of 4X buffer (500 mM Tris, 0.4% (W/V) SDS, pH 6.8), 10% (W/V) ammonium persulfate, 30% (W/V) acrylamide-0.8% (W/V) N,N'-methylene bisacrylamide solution, TEMED, and deionized water. Gel thickness was made 0.75 mm by using 0.75 mm spacers. Gel samples were prepared by adding equal volume of protein sample and 2X sample buffer containing 125 mM Tris, 20% glycerol, 4.1% (W/V) SDS, 2% (V/V) β-mercaptoethanol (βME) and 0.001% Bromophenol Blue, pH 6.8 followed by boiling the sample for 5 minutes. Sample size ranged between 1- 25 μl depending on the peptide concentration (0.1-10 μg of peptide). Molecular weight standards (the low molecular weight standards: ovalbumin (43.0 kD), carbonic anhydrase (29.0 kD), β-lactoglobulin (18.4 kD), lysozyme (14.3 kD), bovine trypsin inhibitor (6.2 kD) and insulin (α and β chain) (2.3 and 3.4 kD, respectively), or the high molecular weight standards: myosin (H-chain) (200 kD), phosphorylase B (97.4 kD), bovine serum albumin (68.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (29.0 kD), β-lactoglobulin (18.4 kD) and lysozyme (14.3 kD) were used. Gels were ran in a buffer consisted of 25 mM Tris, 192

mM glycine, and 0.1% SDS at a range of 120-160 V. Electrophoresis was stopped when the dye front reached ~0.5 cm from the bottom of the gel (40-75 minutes depending on the percentage of acrylamide in the gel-4%-16%).

2.5.2 Non-Denaturing Polyacrylamide Gel Electrophoresis

Bio-Rad (Richmond, CA) mini-protein II electrophoresis cell was used to run the non-denaturing PAGE. Separating gel, stacking gel, and electrophoresis buffer were the same as those for SDS-PAGE but contained no SDS. Sample buffer also contained no SDS or βME. Gel thickness was 1.5 mm. All other conditions were the same as those for the SDS-PAGE.

2.5.3 Coomassie Blue Staining

Both SDS-PAGE and non-denaturing-PAGE were stained in the same way. After running the gels, they were immediately transferred to a petri dish containing fixing solution (40% (V/V) methanol, 10% (V/V) acetic acid) and left on a rotator for 2 hours (~60 rpm). The gels were transferred to another petri dish containing the staining solution (0.05% (W/V) Coomassie Blue R-250) and left on the rotator for another 2 hours. After that the gels were transferred back to the fixing solution for destaining. The destaining solution was changed several times until destaining was complete. The number of changes and the time of destaining differed depending on the type of gel and the percentage of acrylamide. Non-denaturing gels required extensive (~ 1 day) destaining. The gels were then stored in 5% acetic acid before drying.

2.5.4 Silver Staining

Silver staining was done according to published literature (Merril *et al.*, 1983).

After running a gel with 10 - 300 μg of samples per well, the gel was placed in fixing solution (20% (w/v) trichloroacetic acid) for one hour. The gel was washed twice with washing solution (10% ethanol, 5% acetic acid), the first for 10 minutes and the second for 20 minutes. The gel was placed in a soaking solution (3.4 mM potassium dichromate, 3.2 mM nitric acid) for 5 minutes before staining it in 12 mM silver nitrate solution for 20 minutes. The gel was moved to the developing solution (0.28 M sodium carbonate, 0.05% (v/v) formaldehyde) and agitated for 2 minutes. Fresh developing solution was added and the gel agitated until slight yellow background appeared. The gel was placed in stopping solution (3% (v/v) acetic acid) for 5 minutes before it was washed twice with deionized water and stored.

2.5.5 Molecular Weight Determination

Both coomassie blue and silver stained gels were dried using either a Bio-Rad model 543 gel dryer (Richmond, CA) or by air drying. The coomassie blue stained gels were scanned on an ISCO model 1312 gel scanner and analyzed using a commercially available software (Chemresearch Chromatographic Data Management Software, ISCO, Lincoln, Nebraska). Using this program, the distance traveled by molecular weight standards were plotted against the log of their molecular weight. Using the relation:

$$d = b \log MW + c$$

where d is the distance traveled by the band, MW is the molecular weight of the standard and b and c are constants, linear fitting was used to determine the values of b and c. With b and c known, the distance of each peptide's band was plugged in the equation to give the estimated molecular weight.

2.6 Western Blotting

After running SDS-PAGE or non-denaturing-PAGE, if necessary, gels were prepared for blotting by soaking them in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 7.5). The blotting was done using blotting paper (USB, Cleveland, Ohio) at constant voltage of 22 V overnight at 4°C. After blotting, the blotting papers were soaked in TBS buffer (5 mM Tris, 150 mM NaCl, pH 7.4) containing 9% non-fat milk with shaking for 1.5 hours at room temperature. Then blotting papers were soaked in the primary antibody solution with shaking for 1.5 hours at room temperature. Two different mouse antispectrin monoclonal antibodies (SB-SP1 and SB-SP2, Sigma Chemical Company, St. Louis, MO) and a rabbit antispectrin polyclonal antibody (Sigma Chemical Company) were used throughout the study. Blotting papers were washed with TBST (TBS containing 0.05% Tween-20) buffer three times for 5 minutes each. After that blotting papers were soaked in the secondary antibody (Goat anti-mouse for SB-SP1 and SB-SP2 antibodies and Goat anti-rabbit for the polyclonal antibody, Pierce, Rockford, IL) with shaking for 1.5 hours at room temperature, and washed 4 times with TBST for 5 minutes each. After that the developing solution (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 0.4 mM 5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine Salt, 0.4 mM Nitro Blue Tetrazolium, pH 9.5) was added, and blotting papers were shaken gently until bands appeared to desired intensity, followed by washing twice with deionized water for 2 minutes each and air drying.

2.7 Molecular Weight Calculations

Molecular weights of spectrin peptides were determined from amino acid contents using the program PEPTIDESORT, Genetics Computer Group. Molecular weights of the following spectrin peptides: Spα1-49, Spα1-155, Spα1-167, Spα1-446, Spα41-155, Spα41-167, Spα41-273, Spα49-155, Spα49-167, Spα100-255 and Spα100-273 were found to be 5.8, 18.4, 19.9, 51.7, 13.6, 15.1, 27.3, 12.7, 18.3 and 20.6 kD, respectively. The molecular weight of Spα1-166 (S12C) was 19.8 kD. The molecular weight of 26 kD for GST was obtained from literature (Smith and Johnson, 1986). The molecular weights of fusion proteins were calculated by adding the molecular weight of the spectrin peptide to the molecular weight of GST. Molecular weights of α-and β-spectrin were obtained from literature to be 280 (Sahr *et al.*, 1990) and 246 kD (Winkelman *et al.*, 1990), respectively. 215, 89, 78, 72, 43 and 35 kD were used as molecular weights of bands 2.1 (ankyrin), 3, 4.1, 4.2, 5 (actin) and 6 (glyceraldehyde-3-phosphate dehydrogenase), respectively (Mathews, C.K. and Van Holde, K.E., 1990).

2.8 Amino Acid Sequencing

After running SDS-PAGE, gels were prepared for blotting by soaking in transfer buffer (10 mM CAPS, 10% methanol, pH 7.5). The blotting was done using polyvinylidine diflouride membranes (Applied Biosystems, Foster City, CA) at 0.15 Amp. for 1 hour. The membranes were stained in 0.05% Coomassie Blue solution for 5-10 minutes and destained in 40% methanol, 10% acetic acid solution for ~10 minutes. They were rinsed in deionized water for 2 minutes and left to air dry. The desired bands were excised and

sent for amino acid sequencing.

2.9 Protein Concentration Assay

Peptide concentration for spin labeled samples was determined by a dye binding assay (Bradford, 1976). The dye solution was prepared by diluting the dye reagent (Coomassie Brilliant Blue G-250, Bio-Rad, Richmond, CA) five times by adding 4 volumes of deionized water to one volume of the dye reagent followed by filtering the solution through a Whatman filter paper. Five BSA standard solutions were prepared with concentrations ranging from 0.2 - 1 mg/ml. 100 μl of protein samples (standards or spectrin) were added to 5 ml of the dye solution. These solutions were left to stand at room temperature for 5 minutes before the absorbance at 595 nm were measured, using the dye solution as blank. Absorbance of the standard solutions were plotted against their concentrations. Least square linear fit was used to obtain the best fit calibration equation. Concentrations of the spectrin peptide solutions were determined from the calibration equation.

2.10 Stability of α-Spectrin Peptides

2.10.1 Degradation

Spectrin peptides were tested for degradation by incubating the FPLC purified α-spectrin peptide at -70, -20, 4, 25 and 35°C and taking SDS gel electrophoresis samples at time zero and after 24 hours. These samples were electrophoresed as described in section 2.5.1 above. The gel was then studied for any degradation bands.

2.10.2 Aggregation

FPLC purified spectrin peptide samples were incubated at 4°C for 16-24 hours. Non-denaturing gel electrophoresis samples were taken after incubation time. Also, after incubation for 16 hours at 4°C the sample was reloaded on FPLC to check if any aggregation bands would appear.

2.11 Limited Chymotrypsin Digestion

Chymotrypsin stock solution was prepared by dissolving 10 mg of 3X crystallized bovine pancreatic α-chymotrypsin (Sigma) into 40 ml of 1 mM HCl solution. The stock solution was stored in 200 μl aliquot at -70°C. The FPLC purified spectrin peptide was concentrated using centricon micro concentrators (Amicon) to a final concentration of ~1 mg/ml by centrifugation at 2000 xg on a Sorvall RT6000B Refrigerated Centrifuge and an A500 rotor. A vial of chymotrypsin was thawed and added immediately to the reaction vial in a ratio of 1 μl of chymotrypsin stock solution per 100 μg of spectrin peptide, i.e. 1:400 weight ratio. The time of chymotrypsin addition was considered as time zero. At t= 0, 15, 30, 60, 90, 120, 150, and 180 minutes, a sample (about 50 μl) was removed, equal volume of 2X SDS loading buffer was added and the mixture boiled for 5 minutes. The samples were electrophoresed on SDS-PAGE.

2.12 Spin Labeling and Electron Paramagnetic Resonance of α-Spectrin Peptides

2.12.1 Spin Labeling

The sulfhydryl specific nitroxide spin label 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (Mal6) was used to spin label α-spectrin peptides. It was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) and used without further purification. A 10^{-2} M stock solution was prepared by dissolving 25 mg of Mal6 in 10 ml of acetonitrile and stored at -20°C.

In order to prepare α-spectrin peptides for spin labeling, βME was added to 3-5 mg of α-spectrin peptide to give a final concentration of 45 mM followed by incubation at 4°C for 30 minutes. The solution was loaded onto a Sephadex G25 gel filtration column pre-equilibrated with 5 mM phosphate buffer at pH 7.4 (5P7.4) buffer. The first peak containing the spectrin peptide was collected. It was concentrated using centricon micro concentrators to a final concentration of ~1 mg/ml. The second peak containing βME was discarded.

The required amount of Mal6 spin label to give a molar ratio of 24:1 to the spectrin peptide was transferred into a brown vial. Acetonitrile was removed by passing a very gentle stream of N_2 gas to give a thin film of the spin label on the surface of the vial. The spectrin peptide at 1 mg/ml was added to the vial and incubated at 4°C for 15 hours with gentle stirring. After that, the mixture was loaded on the Sephadex G25 column pre-equilibrated with 5P7.4 buffer. The first peak containing the spin labeled peptide was collected while the second peak containing the free spin label was discarded. The spectrin

peptide was concentrated using centricon micro concentrators to a final concentration of 10^{-4}-10^{-5} M. EPR samples were prepared by pipetting 20 μl of the spin labeled peptide into a capillary tube. One end was sealed using a flame and centrifuged using an EIC MICRO HEMATOCRIT MB CENTRIFUGE (DAMON IEC DIVISION, Needham Heights, Mass) to push all sample to the sealed end.

2.12.2 Electron Paramagnetic Resonance (EPR)

EPR was done on a Varian E-Line Century series EPR spectrometer coupled with a Zenith personal computer. A commercially available software program, Assyst (MacMillan Software) modified for EPR operation, was used for data acquisition and analysis. Parameters used were: incident microwave power, 2 mW; center of the field, 3242.5 gauss; scan range, 100 gauss; modulation amplitude, 1 gauss; time constant, 0.128; receiver gain, 1 x 10^4; scan time, 60 seconds. All experiments were done at room temperature (~21°C). The spin labeled samples prepared as above were placed inside a quartz tube containing silicon fluid situated within the cavity. Samples were left for 5 minutes in the cavity to equilibrate to room temperature before EPR spectrum were obtained. Spin label concentration was determined by double integrating the EPR spectra. Using the protein concentration assay mentioned above, the spectrin peptide concentration was determined. Finally, the spin label to peptide molar ratio was calculated.

2.12.3 Calculations of the Rotational Correlation Time

The rotational correlation time was calculated using the equation:

$$\tau_R = 6.5 \text{ x } 10\text{-}10\ W_0\ [(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}]$$

where h_0 is the amplitude of the middle peak, h_{-1} is the amplitude of the high field peak, h_{+1} is the amplitude of the low field peak and W_0 is the peak-to-trough distance of the center line (Fung and Johnson, 1984).

2.13 Purification of β-Spectrin Subunit

2.13.1 Preparation of Spectrin-Actin Network From Human Red Blood Cells

Packed red blood cells were obtained from a local blood bank (LifeSource). Up to 120 ml of the packed red blood cells was used in each experiment. In each of 50 ml centrifuge tubes, 5 ml of the packed red blood cells were placed and PBS7.4 buffer was added. The tubes were centrifuged at 2000 xg for 4 minutes in a Sorvall RT6000B centrifuge. The supernatant along with the buffy coat were aspirated, discarded and fresh PBS7.4 buffer was added. The centrifugation was repeated at least twice until a completely clear supernatant was obtained. A low ionic strength buffer (5mM phosphate, 1mM EDTA, pH 8.0) was added to the washed cells and centrifuged with SA600 rotor at 16000 rpm for 10 minutes in Sorvall RC-5B centrifuge. The supernatant along with the button at the bottom were aspirated and discarded and fresh buffer was added to the pellet. The washing was repeated until the supernatant cleared completely of hemoglobin. The white pellets from all tubes were combined and a 100 μl electrophoresis sample was taken. Then 5 - 10 volumes of 0.1 mM EDTA, 0.5 mM βME, 0.2 mM PMSF, pH 9.0 solution was added and the mixture incubated at 37°C for 30 minutes. The solution was centrifuged at 22000 rpm for 60 minutes at 4°C in an OTD65B Sorvall Ultracentrifuge (Du Pont Instruments, Wilmington, D.E.) with T865 rotor. The supernatant (spectrin-

actin network) was collected and the pellet discarded.

2.13.2 Preparation of Spectrin Dimer and Tetramer

The spectrin-actin was concentrated to ~10 ml using Stirred Cells with a YM100 Disc Membrane. A 100 μl electrophoresis sample was taken. The concentrated spectrin-actin was loaded onto a Superose 6 column pre-equilibrated with 5P7.4 buffer and ran at 20 ml/hour. The first and the second peak containing the spectrin tetramer and dimer, respectively, were collected and concentrated using Stirred Cells with a YM100 Disc Membranes.

2.13.3 Purification of β-Spectrin From Spectrin-Actin Network

2.13.3.1 Purification of β-Spectrin Using DE-52 Column

2.13.3.1.1 Purification of β-Spectrin Using Published Procedure

Published procedure was used to purify β-spectrin (Speicher *et al.*, 1992). The spectrin-actin was concentrated to ~ 100 ml at ~ 0.6 mg/ml using Stirred Cells with a YM100 Disc Membranes. A 100 μl electrophoresis sample was taken. The concentrated spectrin-actin was treated with solid urea, βME and 10X buffer (200 mM Tris, 1.5 M NaCl, 10 mM EDTA, pH 8.2) to give a final concentration of 3M urea, 150 mM NaCl, 20 mM Tris, 1 mM EDTA and 0.6 mM βME. This solution was mixed batchwise with DE-52 resin (Whatman, Fairfield, NJ) pre-equilibrated with 18 mM Tris, 3M urea, 135 mM NaCl, 0.9 mM EDTA and pH 8.2 buffer (300 ml of resin per 100 mg of spectrin-actin). The mixture was incubated for one hour at 4°C before the solution was eluted with a flow rate of 150 ml per hour. Three buffers with increasing NaCl concentrations were used to elute proteins. The first one contained 152 mM NaCl and used to elute actin and other

weakly bound contaminants. The second buffer contained 181 mM NaCl and used to elute β-spectrin. The third contained 234 mM NaCl and used to elute α-spectrin. The β-spectrin fraction was concentrated using Stirred Cells with YM100 Disc Membranes. The concentrated sample was dialyzed three times against isotonic KCl buffer (10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM βME, 30 μM PMSF, pH 7.4).

2.13.3.1.2 Purification of β-Spectrin Using Modified Procedure

The published procedure described above did not give β-spectrin with the purity anticipated. The procedure was modified by using 135 mM NaCl to elute actin and other weakly bound contaminants, 170 mM NaCl to elute β-spectrin and 500 mM NaCl to elute α-spectrin.

2.13.3.2 Purification of β-Spectrin Using FPLC and Mono Q HR10/10 Column

The spectrin-actin supernatant was concentrated to ~ 25 ml using Stirred Cells with a YM100 Disc Membranes and treated with urea buffer as above. The solution was then incubated for 60 minutes at 4°C and filtered through 0.22 μm filter and loaded onto a Mono Q HR10/10 anion exchange column of the FPLC system. The buffer system consisted of buffer A (18 mM Tris, 250 mM NaCl, 0.9 mM EDTA, 3 M urea, pH 8.2) and buffer B (18 mM Tris, 400 mM NaCl, 0.9 mM EDTA, 3M urea, pH 8.2). The following procedure was to purify β-spectrin: 50 ml buffer A to elute actin and other weakly bound proteins. The gradient was increased to 287.5 mM NaCl after 10 ml and remained at that concentration for 40 ml to elute β-spectrin. After that, buffer B was used to elute α-spectrin. The flow rate used was 2 ml/min.

The β-spectrin peak at 100 mM NaCl was collected and concentrated using Stirred

Cells with YM100 Disc Membranes and/or centricon micro concentrators. β-spectrin was dialyzed three times against isotonic KCl buffer (10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM βME, 30 μM PMSF, pH 7.4) and used immediately or stored at 4°C.

2.14 Stability of β-Spectrin

2.14.1 Degradation at 4°C

Stability of β-spectrin was done by incubating β-spectrin at 4°C for 6 days. Gel electrophoresis samples were taken at each day and stored at -20°C until all samples were collected. SDS-PAGE was done on these samples.

2.14.2 Degradation at -70°C

Stability of β-Spectrin was performed by taking a gel sample before freezing β-spectrin at -70°C for 3 months. After thawing the stored sample, another gel electrophoresis sample was taken. SDS-PAGE was done on both samples.

2.14.3 Aggregation at -70°C

This experiment was done as described above for the degradation except that non-denaturing PAGE was done.

2.15 Radiolabeling of α-Spectrin Peptides With ^{125}I

2.15.1 Time Study

The time study was done according to literature supplied by the company (Pierce, Rockford, IL) in order to determine the labeling time that would give maximum labeling and minimum degradation. 0.1 mCurie of ^{125}I (1 μl) was added to 1 bead of IODO-BEAD

(Pierce, Rockford, Il) in 100 μl of labeling buffer (100P6.5). After stirring for 5 minutes 10 μg of Spα41-273 were added. At 0, 1, 2, 4, 8, and 16 minutes, 5 μl sample was taken and added to 1 ml of PBS containing 0.5 mg/ml BSA. Each of the 6 samples was diluted 1:10 with 20 mM Tris, 1 mM EDTA, 0.5 mg/ml BSA, pH 7.4 by taking 100 μl of sample into 900 μl of buffer. To the diluted samples, 100 μl of 60% trichloroacetic acid (TCA) was added and the solution centrifuged. The supernatant from each sample was removed into scintillation tubes. Fresh 50 μl of 60% TCA was added to the pellet and the solution was again centrifuged. The supernatant was discarded and the pellets were placed into scintillation tubes. Gamma counter (the Department of Biology) was used to count the ^{125}I in pellet and supernatant samples. Table 1 shows counts per minute for the pellet samples.

The counts per minute shown in Table 1 were plotted using the origin program (Microcal, Northampton, MA) against time and shown in Figure 1. Figure 1 shows counts per minute were maximum after 2 minutes of iodination. This indicates that iodination of the spectrin peptide was maximum after 2 minutes, after which the peptide starts to degrade under the reaction conditions. Thus all iodination for immunoprecipitation experiments were done for 2 minutes to maximize iodination and minimize degradation of spectrin peptides.

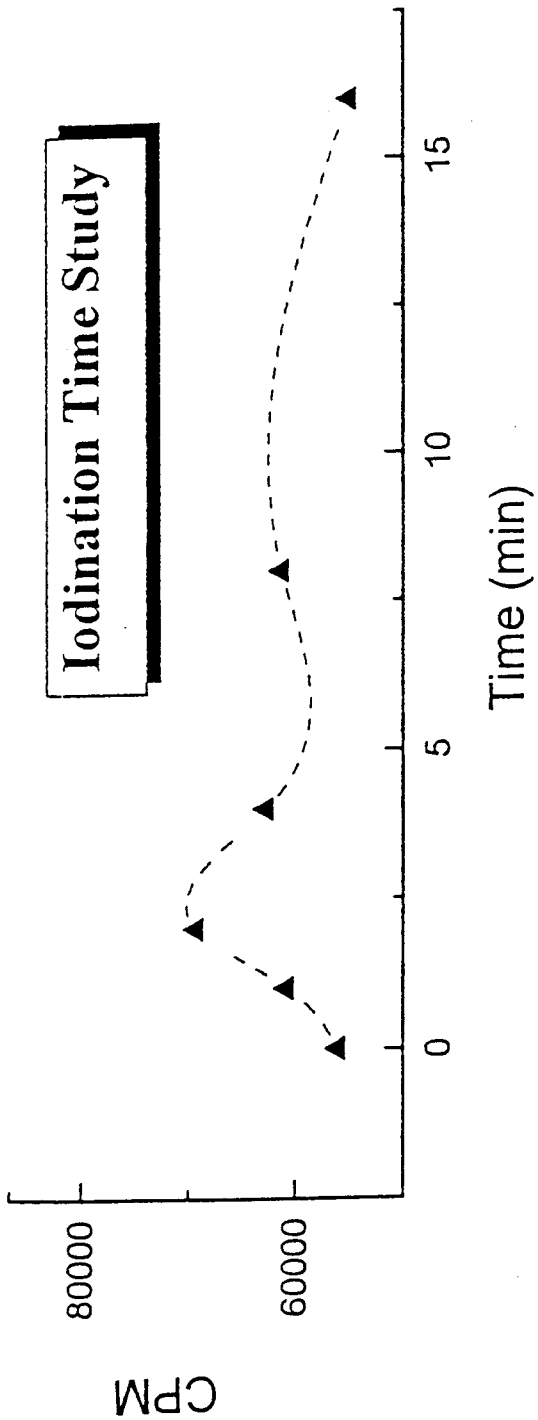
2.15.2 Radiolabeling

To activate 1 mcurie of the ^{125}I, 1 bead of IODO-BEAD was added to the solution followed by the labeling buffer (100 mM phosphate, pH 6.5) to 300 μl total volume. The solution was stirred for 5 minutes and 100 μg of the spectrin peptide was added. The

Table 1: Counts per minute for pellets for time study

TIME (min.)	**CPM** (pellet)
0	56088
1	60816
2	69294
4	62716
8	61268
16	55087

Figure 1: Iodination time study. Counts per minute of the pellets vs. minutes of iodination of the spectrin peptide Spα41-273.


Iodination Time Study
CPM
80000
60000
0
5
10
15
Time (min)

mixture was then stirred for 2 minutes at room temperature before loading it onto Sephadex G25 column pre-equilibrated with the labeling buffer. 1 ml fractions were collected and counted. These counts per minute were plotted against tube number (Figure 2). The first peak contains the radiolabeled Spα1-167 and the second one contains the excess or free iodine. Tubes containing the radiolabeled peptide were determined from the plot and grouped. The radiolabeled peptide was then dialyzed 3 times against isotonic KCl buffer to further remove free iodine. Under reaction conditions, only tyrosine residues would be labeled with iodine. According to the amino acid sequence of α-spectrin, the peptide Spα1-167 contains 5 tyrosine residues at positions: 35, 53, 81, 91, and 163.

2.16 Stokes Radius Determination

2.16.1 Column Calibration

A Superose 6 column attached to FPLC system was used to study the Stokes radii of spectrin peptides. Before conducting the study, it was necessary to calibrate the column. Table 2 below show the 6 standard proteins (Pharmacia Biotechnology, Piscataway, NJ) that were used to calibrate the column.

The Superose 6 column was pre-equilibrated with isotonic KCl buffer before loading each of the 6 standard proteins. A flow rate of 0.20 ml/min was used to pass 30 ml of buffer through the column. The elution volume for each standard protein was calculated.

2.16.2 Stokes Radius Calculations

The partition coefficients (K_{av}) for standards and samples were calculated using the

Figure 2: Radiolabeling of the spectrin peptide Spα1-167 with ^{125}I. Counts per minute vs. tube numbers are plotted.

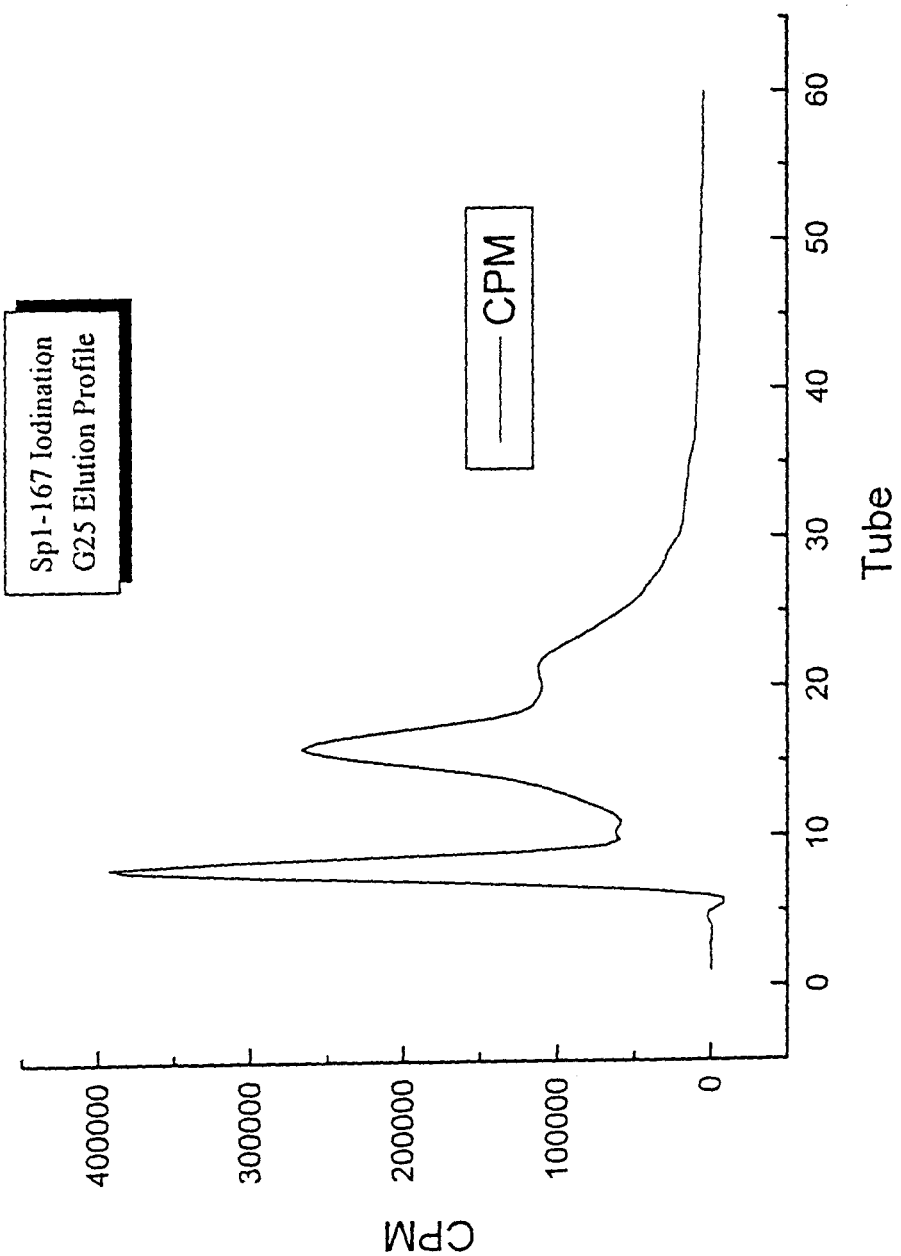

Sp1-167 Iodination
G25 Elution Profile
CPM
CPM
400000
300000
200000
100000
0
0
10
20
30
40
50
60
Tube

Table 2: Molecular weights (MW) and Stokes radii (R_s) of standards used in Superose 6 calibration

PROTEIN	MW (kD)	R_s (Å)
Thyroglobulin	669	85.0
Ferritin	440	61.0
Catalase	232	52.2
BSA	67	35.5
Ovalbumin	43	30.5
Chymotrypsinogen	25	20.9

equation $K_{av} = (V_e-V_o)/(V_t-V_o)$, where V_e is the elution volume, V_o is the void volume and V_t is the total bed volume. V_e is determined from the elution profile, V_o and V_t were supplied by the company. K_{av} values for the standards were plotted against the log of the Stokes radius (log (R_s)). Linear regression was used to obtain the best fit for the equation log (R_s) = a K_{av} + b. After determining the constants a and b from standards, the equation was used to calculate Stokes radii of spectrin samples.

2.17 Binding Assays of α-Spectrin Peptides Binding with β-Spectrin

2.17.1 Gel Filtration Assay

FPLC purified Spα1-166 (S12C), Spα1-167, spectrin-actin and β-spectrin subunit were used in this study. All samples were dialyzed in isotonic KCl buffer before starting the experiment. Spα1-166 (S12C) or Spα1-167 (1.15×10^{-6} M each) was mixed with β-spectrin (2.7×10^{-7} M) and incubated for 24 hours at 4°C. Control samples of Spα1-166 (S12C), Spα1-167 or β-spectrin were prepared by adding the appropriate volume of isotonic KCl buffer and incubated for 24 hours. After 24 hours, the incubation mixture or control samples were loaded onto the Superose 6 column pre-equilibrated with the isotonic KCl buffer. The same program used above for standards was used to obtain elution profiles for the mixture and control samples. K_{av} was calculated and the Stokes radii determined using the calibration curve. The Stokes radii were compared to check for any change between incubation and control samples.

Binding constants were calculated by using the following equations:

$$\alpha\text{-Sp} + \beta\text{-spectrin} \rightarrow \alpha\text{-Sp-}\beta\text{-spectrin (complex)}$$

with the binding constant (K_a):

$$K_a = [\alpha\text{-Sp-}\beta\text{-spectrin}]/([\alpha\text{-Sp}][\beta\text{-spectrin}])$$

The area of the spectrin peptide of control and incubated samples were calculated. The area of a peak was calculated by taking the two slopes and the base as a triangle and using the equation

$$A = 1/2 \times b \times c$$

where A is the area of the peak, b is the length of the base and c is the height of the peak (base to head of triangle). The percent of peptide remained was calculated by dividing the area of the incubated sample by the area of the control sample. This percent was multiplied by the initial concentration of the peptide to give the equilibrium concentration of the peptide. The concentration of the complex was calculated by subtracting the final from the initial concentration of the spectrin peptide. The equilibrium concentration of β-spectrin was calculated by subtracting the concentration of the complex from the initial concentration of β-spectrin.

2.17.2 Combined Western Blotting With Radiolabeling

β-spectrin was incubated with the radiolabeled peptide Spα 1-167 to give final concentrations of 9×10^{-7} and 3.5×10^{-7} M, respectively. Control samples of Spα 1-167

were prepared by adding the appropriate volume of isotonic KCl buffer. All samples were incubated for 24 hours at 4°C. After that samples were ran on 7% non-denaturing PAGE. After Western blotting with the SB-SP1 antibody, the stained bands (contained both β-spectrin and complex) and areas where the Spα1-167 usually appeared were excised and counted.

In order to determine the binding constant, the ratio of unbound to the total Spα1-167 was calculated (counts per minute of the Spα1-167 divided by the total counts per minute). The equilibrium concentration of Spα1-167 was calculated by multiplying the initial concentration of the peptide by the ratios. The concentration of the complex was calculated by subtracting the final from the initial concentration of the peptide. The concentration of β-spectrin was calculated by subtracting the concentration of the complex from the initial concentration of β-spectrin. The binding constant was then determined from the equilibrium concentrations of the three components using the binding constant equation (section 2.17.1).

2.17.3 Immunoprecipitation

β-spectrin was incubated with the radiolabeled Spα1-167 to give final concentrations of 3.3×10^{-7} M and 1.26×10^{-6} M, respectively. Control samples were prepared by adding appropriate volumes of isotonic KCl buffer to give a final volume of 5 μl. All samples were incubated for 36 hours at 4°C. Then 30 μg of the SB-SP1 antibody and 22 μl of immunoprecipitation buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.5) were added and the solution allowed to incubate for 1 hour at 4°C. Then 30 μl of Pansorbin (CalBiochem, San Diego,

CA) were added, and the mixture was incubated for 30 minutes at 4°C. The solution was centrifuged at 15000 g for 3 minutes and the supernatant collected. The pellet was washed 3 times with 1 ml immunoprecipitation buffer by centrifugation at 15000 g for 3 minutes. The ^{125}I in pellet and supernatant were counted and the counts per minute are summarized in (table 3).

The binding constant was calculated by first determining the ratio of bound Spα1-167 from the total Spα1-167. The ratio was determined by dividing the counts per minute of the pellet by the total counts per minute of both pellet and supernatant followed by subtracting the background ratio (control samples). The equilibrium concentration of the complex was calculated by multiplying the ratio by the initial concentration of Spα1-167. The concentration of the unbound Spα1-167 was caculated by subtracting the concentration of the complex from the initial concentration of the peptide. The concentration of β-spectrin was calculated by subtracting the complex concentration from the initial β-spectrin concentration. The binding constant was calculated from the equilibrium concentrations of the three components using the binding constant equation (section 2.17.1).

Table 3: Concentrations and molar ratios of Spα1-167 and β-spectrin used in immunoprecipitation experiments (Total volume, 18 μl)

	Spα1-167	**β-Spectrin**
(μ moles)	6.3×10^{-6}	1.7×10^{-6}
Concentration (M)	1.2×10^{-6}	2.7×10^{-7}
Molar Ratio	1	0.3

CHAPTER III

RESULTS

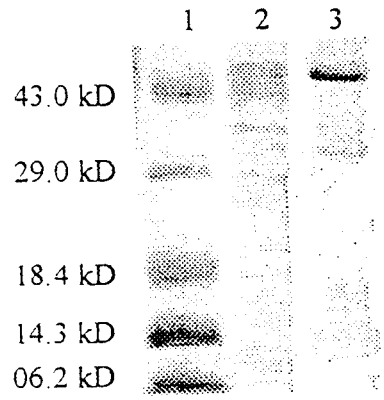
3.1 Expression, Purification and Stability of GST and α-Spectrin Peptides

3.1.1 Expression of GST and Spectrin Peptides as GST-Fusion Proteins

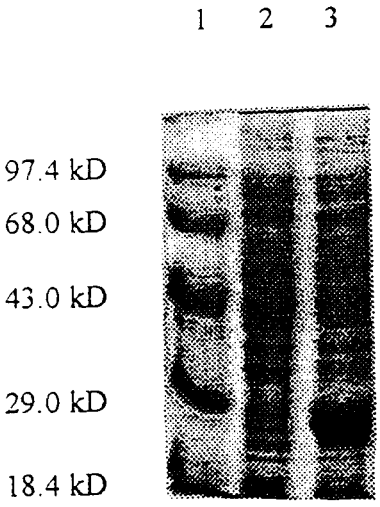
The HB101 *E. coli* cells were induced for the production of the fusion protein: GST-Spαa-b, where GST is glutathione-S-transferase, Spα is α-spectrin, a is the amino acid residue number in intact spectrin of the first amino acid in the peptide and b is the amino acid residue number in intact spectrin of the last amino acid in the peptide. Cells containing vectors of twelve different fusion proteins were prepared by Dr. Kelley: GST-Spα1-49, GST-Spα1-155, GST-Spα1-166 (S12C), GST-Spα1-167, GST-Spα1-446, GST-Spα41-155, GST-Spα41-167, GST-Spα41-273, GST-Spα49-155, GST-Spα49-167, GST-Spα100-255 and GST-Spα100-273. These fusion proteins were expressed to produce Spα1-49, Spα1-155, Spα1-166 (S12C), Spα1-167, Spα1-446, Spα41-155, Spα41-167, Spα41-273, Spα49-155, Spα49-167, Spα100-255 and Spα100-273.

SDS polyacrylamide gel electrophoresis results show that expressed proteins in cells have correct molecular weights. Figures 3a show the results for GST-Spα1-166 (S12C). Lane 1 show the molecular weight standards. Lane 3 show the whole cell extract of HB101 cells induced for the production of the fusion protein. Lane 2 show the whole

Figure 3: SDS polyacrylamide electrophoresis gels (12%) stained with 0.05% Coomassie brilliant blue R. These gels (0.75 mm thick) were run for about 40 minutes under a constant voltage of 160 V. Lane 1 in a, b and c: low molecular weight standards; lane 2: total cell extract of *E. coli* HB101 without IPTG induction; lane 3: total cell extract of *E. coli* HB101 with IPTG induction for the production of the fusion proteins (a) GST-Spα1-166 (S12C), (b) GST-Spα100-273 and (c) GST-Spα100-255.


1
2
3
43.0 kD
29.0 kD
18.4 kD
14.3 kD
06.2 kD

(a)


1
2
3
97.4 kD
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43.0 kD
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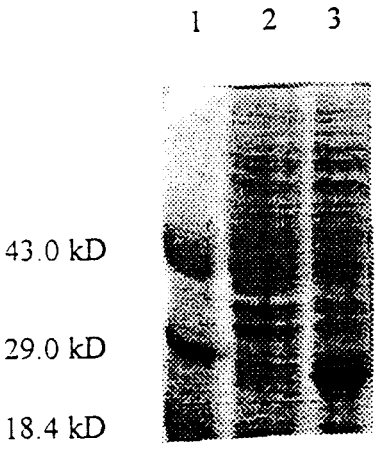
18.4 kD

(b)


1
2
3
43.0 kD
29.0 kD
18.4 kD

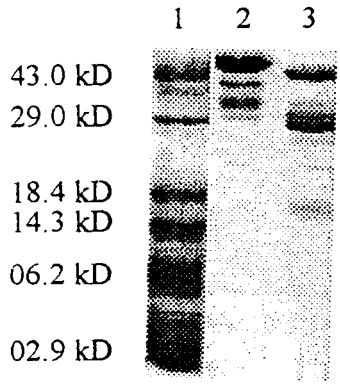
(c)

cell extract of *E. coli* without induction for the production of the fusion protein. The molecular weight of the induced band was estimated using the molecular weight standards as a reference and found to be 45 kD. Similarly, lane 3 in Figs. 3b and 3c showed induced bands for the induction of GST-Spα100-255 and GST-Spα100-273 with estimated molecular weights of 27 and 27 kD, respectively. The calculated molecular weights of these fusion proteins were: 46, 44 and 47 kD, respectively. This shows that calculated molecular weight matched the estimated value for GST-Spα1-166 (S12C) and not GST-Spα100-255 or Spα100-273. As shown above, calculated molecular weights for Spα100-255 and Spα100-273 were 44 and 47 kD while the observed molecular weights were 27 and 27 kD, respectively. This molecular weight corresponds to GST and hence indicates that DNA ligation was not successful and that the cells did not express the fusion proteins. Several other attempts to express these two fusion proteins were not successful. Nine of the proteins (GST-Spα1-49, GST-Spα1-155, GST-Spα1-167, GST-Spα1-446, GST-Spα41-155, GST-Spα41-167, GST-Spα41-273, GST-Spα49-155 and GST-Spα49-167) were done by other members in our laboratory.

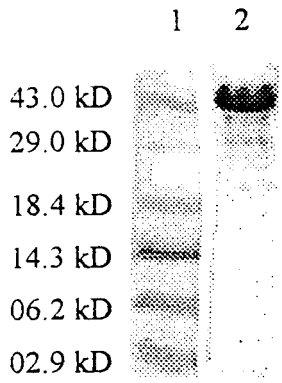
3.1.2 Purity of GST and GST-Fusion Proteins

Fusion proteins were affinity purified from cell lysate with an average yield of 0.5-7.0 O.D. values per liter of *E. coli* cell culture for all nine expressed fusion proteins except for Spα1-166 (S12C) which gave 10-15 O.D. units per liter. Some of these affinity purified fusion proteins showed however other bands of lower molecular weights. Lanes 2 and 3 in Fig. 4a show SDS-PAGE of GST-Spα1-167 and Spα41-167, respectively, while lane 1 shows molecular weight standards. Lane 2 in Figs. 4b, and 4c shows SDS-

Figure 4: SDS polyacrylamide electrophoresis gels (12%) stained with 0.05% Coomassie brilliant blue R. These gels (0.75 mm thick) were run for about 40 minutes under a constant voltage of 160 V. Lane 1 in (a), (b) and (c): molecular weight standards. Lane 2 in (a), (b) and (c): glutathione affinity purified GST-Spα1-167, GST-Spα1-166 (S12C) and GSTSpα41-273, respectively. Lane 3 in (a): glutathione affinity purified GST-Spα41-167.


1
2
3
43.0 kD
29.0 kD
18.4 kD
14.3 kD
06.2 kD
02.9 kD

(a)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2
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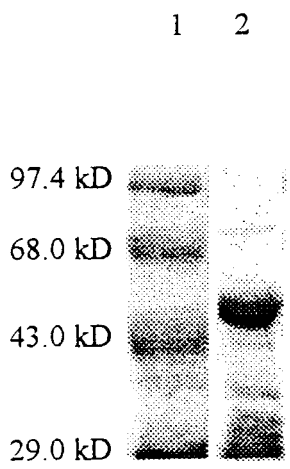
29.0 kD
18.4 kD
14.3 kD
06.2 kD
02.9 kD

(b)


1
2
97.4 kD
68.0 kD
43.0 kD
29.0 kD

(c)

PAGE of GST-Spα1-166 and GST-Spα41-273, respectively, while lane 1 shows molecular weight standards. The molecular weights of the fusion proteins: GST-Spα1-49, GST-Spα1-155, GST-Spα1-166 (S12C), GST-Spα1-167, GST-Spα1-446, GST-Spα41-155, GST-Spα41-167, GST-Spα41-273 , GST-Spα49-155 and GST-Spα49-167 were estimated to be 31 ± 1, 41 ± 2, 45 ± 2, 45 ± 2, 78 ± 4, 40 ± 2, 39 ± 1, 53 ± 2, 37 ± 2 and 39 ± 2 kD, respectively. Table 4 below show the estimated and calculated molecular weights for these peptides.

In order to determine the best time for maximum thrombin digestion without exposing the peptide to degradation fusion proteins above were loaded onto thrombin column. Electrophoresis samples were taken between 0 and 31 hours from the start of incubation and run on SDS-PAGE. It was found that after 24 hours the fusion proteins were mostly digested. Figure 5 below shows SDS-PAGE with samples at 0 and 24 hours (lanes 2 and 3, respectively). At 0 hours a major band with estimated molecular of 45 kD that matches that of the fusion protein could be seen in addition to other bands with lower molecular weight. After 24 hours the 45 kD band disappeared while a much fainter band with estimated molecular weight of 15 kD appeared indicating the digestion of the fusion protein and the production of the spectrin peptide.

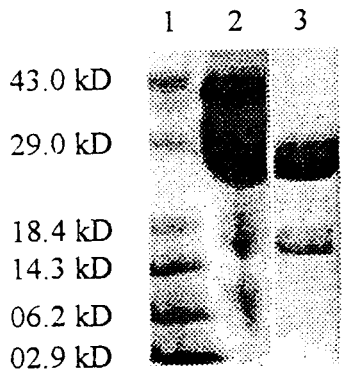
3.1.3 Purity of 9 α-Spectrin Peptides Purified With Glutathione Affinity

Nine glutathione affinity purified peptides (Spα1-155, Spα1-166 (S12C), Spα1-167, Spα1-446, Spα41-155, Spα41-167, Spα41-273, Spα49-155 and Spα49-167) were used in this study. Six were used to determine the start of the structural domain of spectrin. Spα1-166 (S12C), Spα1-167 and Spα41-273 were used in the study of the

Table 4: Estimated and Calculated molecular weights of α-spectrin peptides in kD.

FUSION PROTEINS	ESTIMATED MW	CALCULATED MW
GST-Spα1-49	31	32
GST-Spα1-155	41	44
GST-Spα1-166 (S12C)	45	46
GST-Spα1-167	45	46
GST-Spα1-446	78	78
GST-Spα41-155	40	40
GST-Spα41-167	39	41
GST-Spα41-273	53	53
GST-Spα49-155	37	39
GST-Spα49-167	39	40

Figure 5: SDS polyacrylamide electrophoresis gel (16%) stained with 0.05% Coomassie brilliant blue R. This gel (0.75 mm thick) was run for 1 hour under a constant voltage of 160 V. Lane 1: low molecular weight standard; lanes 2 and 3: GST-Spα41-167 incubated on the thrombin column for t = 0 and 24 hours, respectively.


1
2
3
43.0 kD
29.0 kD
18.4 kD
14.3 kD
06.2 kD
02.9 kD

head-to-head interactions. Glutathione affinity purified spectrin peptides were tested for their purity on SDS-PAGE. Lane 2 in Figs. 6a, 6b, 6c and 6d show SDS-PAGE of the affinity purified α-spectrin peptides Spα1-166 (S12C), Spα1-167, Spα41-167 and Spα41-273, respectively. Lane 1 in Figs 6a-6d show the molecular weight standards. All of the nine peptides purified by glutathione affinity exhibited one major band on SDS-PAGE with molecular weights matching the calculated MW for these peptides. Minor bands with estimated molecular weights that matches these of GST and corresponding fusion protein were also detected (Fig. 6). Densitometric tracing show the intensity of the major bands to be 60-95% of the total intensity in each sample. 0.5 - 5 O.D. units of affinity purified spectrin peptides were obtained per 10 O.D. units of fusion protein loaded on the thrombin column.

3.1.4 Fast Protein Liquid Chromatography (FPLC) Purified α-Spectrin Peptides

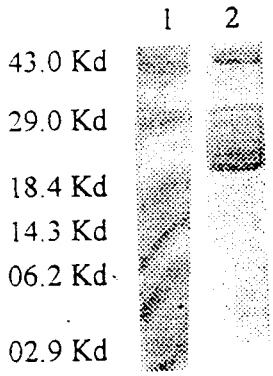
3.1.4.1 Assignment of GST Peak Using Affinity Purified GST Protein

Glutathione affinity purified GST was loaded onto Mono Q column. FPLC elution profiles show two peaks. The first peak appears at 118 ± 14 mM NaCl and could not be concentrated using a centricon-3 (3K MWCO) micro concentrator indicating molecular weight of less than 3000 kD. The second peak appears at 228 ± 4 mM NaCl and exhibited as a single band on SDS-PAGE with molecular weight of 26 kD, indicating that this was the GST peak, obtained from literature.

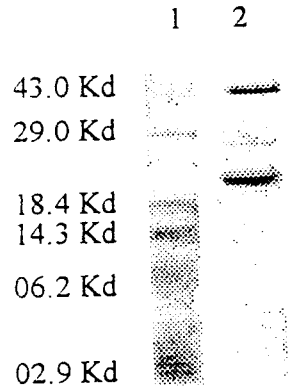
3.1.4.2 Purity of Mono Q HR10/10 Purified α-Spectrin Peptides

The net charges of the spectrin peptides Spα1-49, Spα1-166 (S12C), Spα1-167, Spα1-446, Spα41-155, Spα41-167 and Spα49-167 were determined from the program

Figure 6: SDS polyacrylamide electrophoresis gels (16%) stained with 0.05% Coomassie Brilliant Blue R. These gels (0.75 mm thick) were run for about one hour under a constant voltage of 160 V. Lane 1 in a, b, c and d show the molecular weight standards; lane 2 in a, b, c and d show the glutathione affinity purified Spα1-166 (S12C), Spα1-167, Spα41-167 and Spα41-273, respectively.


1
2
43.0 Kd
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

(a)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2
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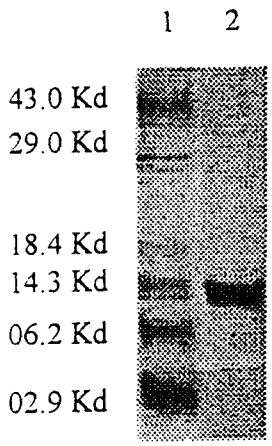
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

(b)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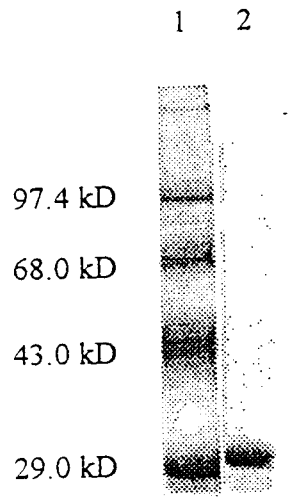
2
43.0 Kd
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

(c)


1
2
97.4 kD
68.0 kD
43.0 kD
29.0 kD

(d)

PEPTIDESORT to be $^{-}$1, $^{-}$8, $^{-}$8, $^{-}$33, $^{-}$6, $^{-}$5 and $^{-}$7, respectively. These charge values could be grouped into three groups: the first contains peptide of 1 negative charge Spα1-49; the second contains peptides of 5 to 8 negative charges Spα1-166 (S12C), Spα1-167, Spα41-155, Spα41-167 and Spα49-167 and the third contains peptide of 33 negative charges Spα1-446. Thus different salt concentrations were used for purifications. Figure 7a and 7b show FPLC elution profile for the purification of Spα1-166 (S12C) and Spα1-167. Both Spα1-166 (S12C) and Spα1-167 were eluted at about 125 mM NaCl. Lane 2 in Figs. 8a and 8b show SDS-PAGE of FPLC purified Spα1-166 (S12C) and Spα1-167 respectively, while lane 1 shows molecular weight standards. These gels show both bands with estimated molecular weights of 20 kD, as expected. Figure 9a and 9b show the densitometric tracing of FPLC purified Spα1-166 (S12C) and Spα1-167 which show one peak for each peptide.

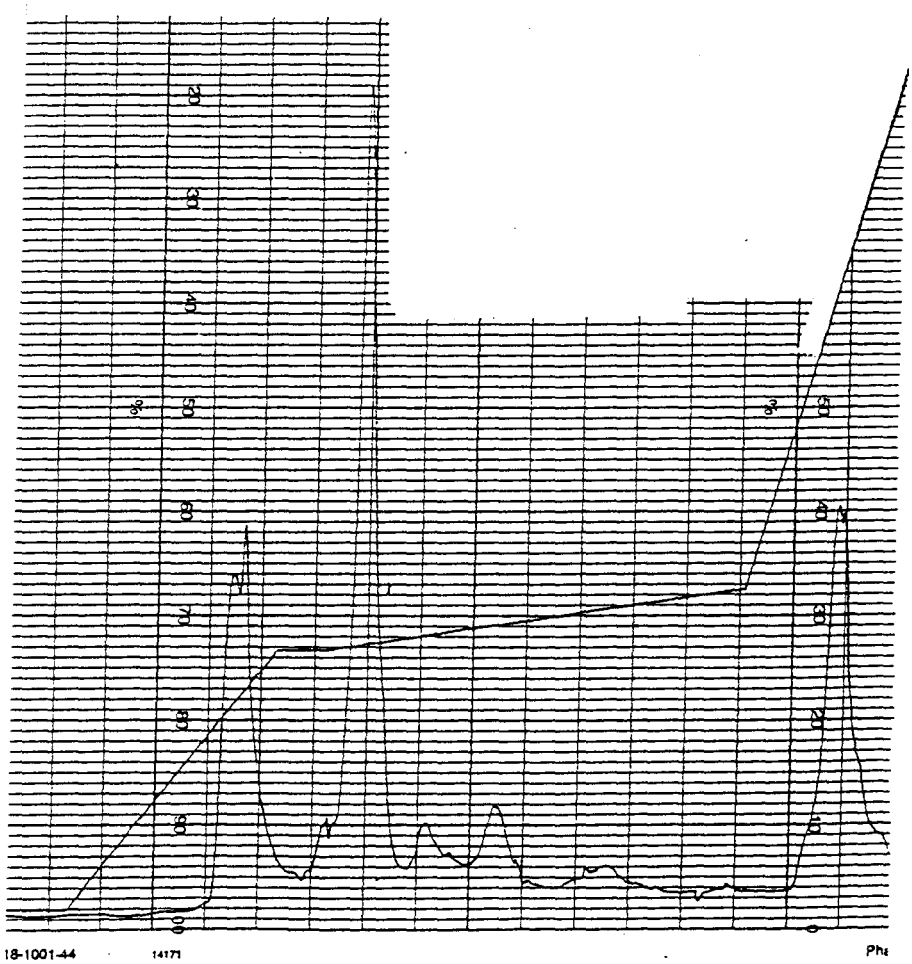
During the FPLC purification of these peptides, when the salt concentration increased sharply to 500 mM NaCl, a second peak, accounting for up to 80% of the total eluted O.D. units, appeared. SDS-PAGE revealed that this peak consisted of Spα1-166 (S12C) peptide, GST and the GST-Spα1-166 (S12C).

3.1.5 Stability of α-Spectrin Peptides

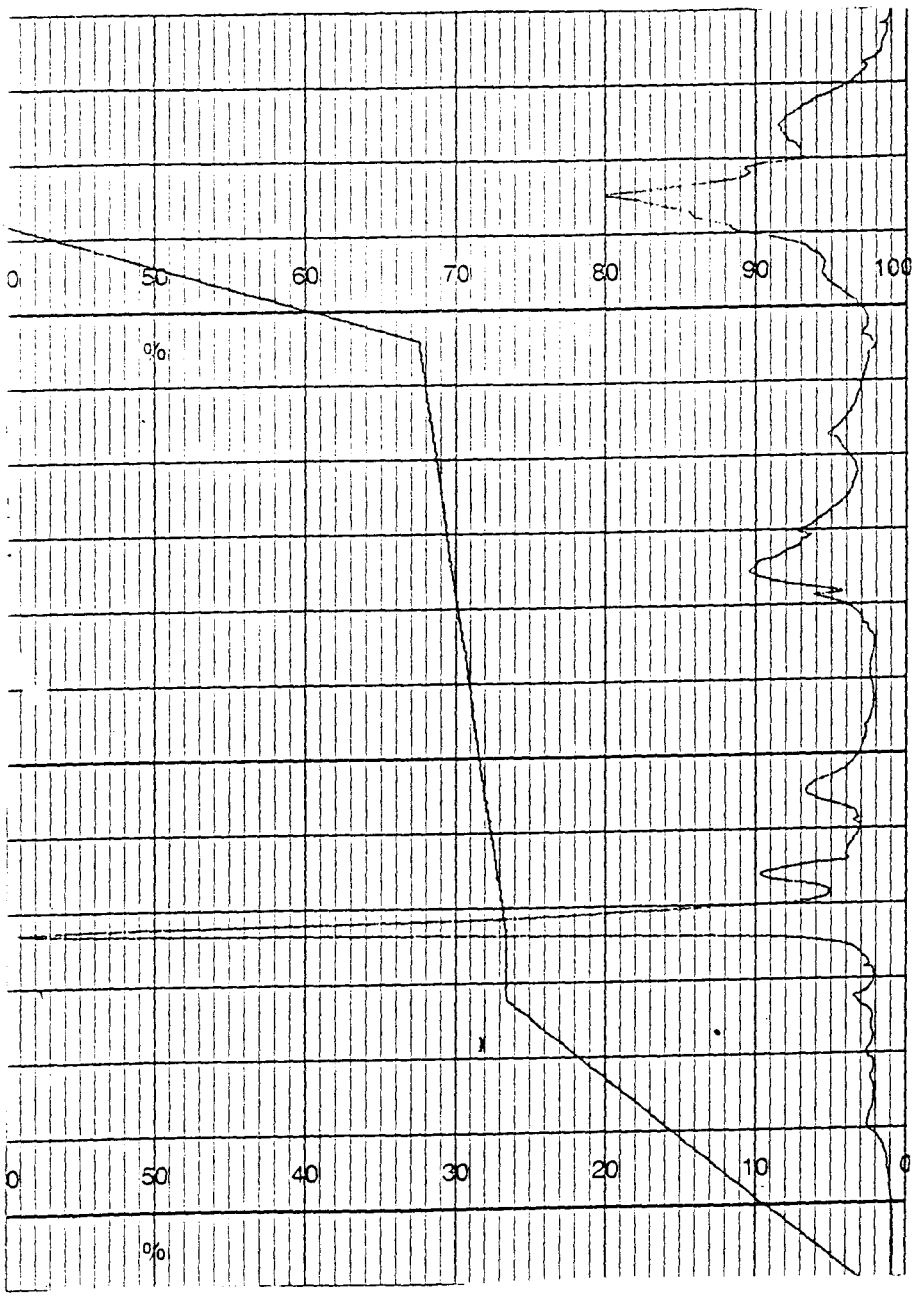
3.1.5.1 Stability of α-Spectrin Peptides From Degradation

Spα49-167 incubated at $^{-}$70, $^{-}$20, 4 and 25°C for 24 hours did not show any sign of degradation. At 35°C, a faint band of lower molecular weight appeared (molecular weight estimated to be 13 kD).

Figure 7: FPLC elution profile from Mono Q HR10/10 column using a flow rate of 2 ml/min and sensitivity of 10 mV. a: the purification of Spα1-166 (S12C); b: the purification of Spα1-167.

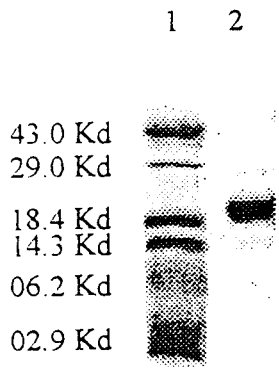


(a)

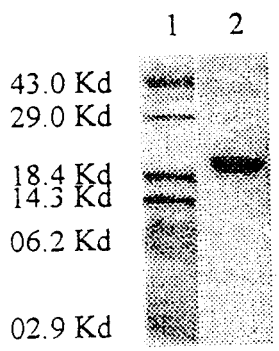


(b)

Figure 8: SDS polyacrylamide electrophoresis gels (16%) stained with 0.05% Coomasssie Brilliant Blue R. These gels (0.75 mm thick) were run for about 1 hour under a constant voltage of 160 V. Lane 1 in a and b show the molecular weight standards; lane 2 in a and b show the FPLC purified Spα1-166 (S12C) and Spα1-167.

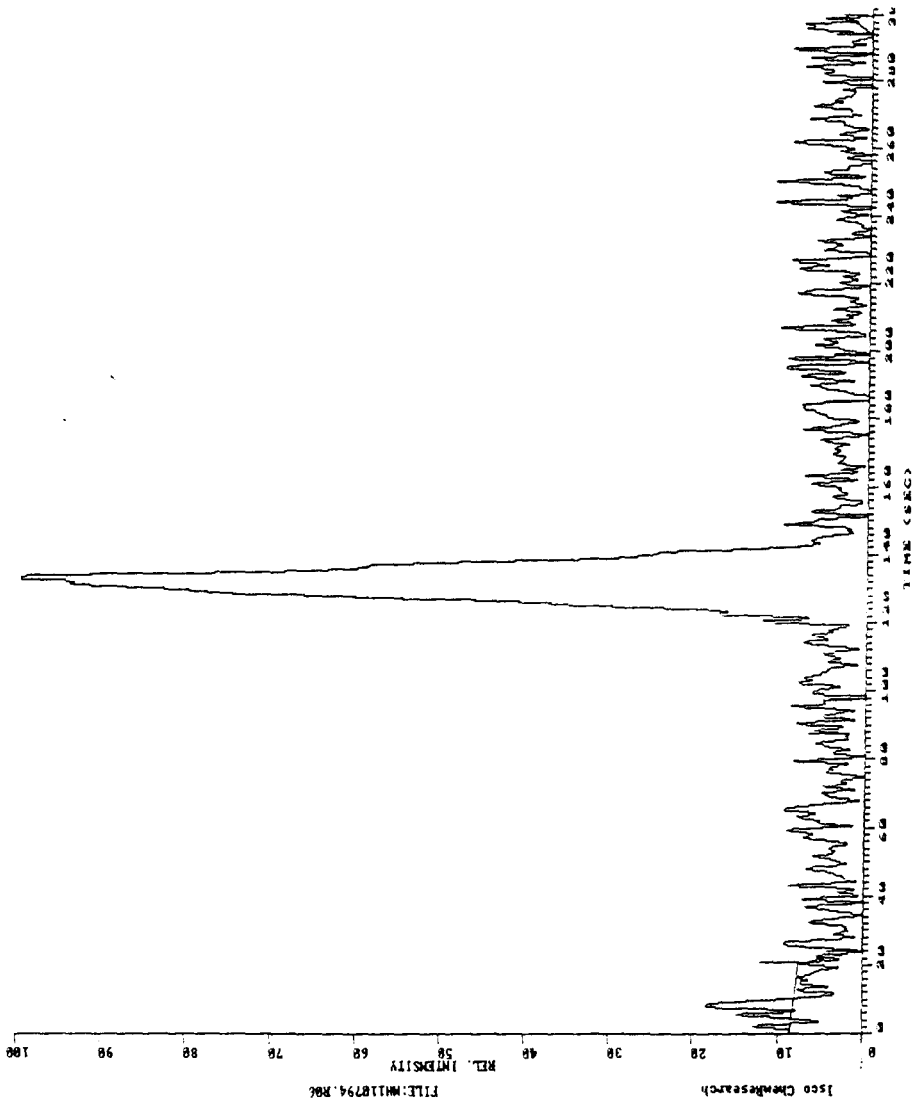

1
2
43.0 Kd
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

(a)

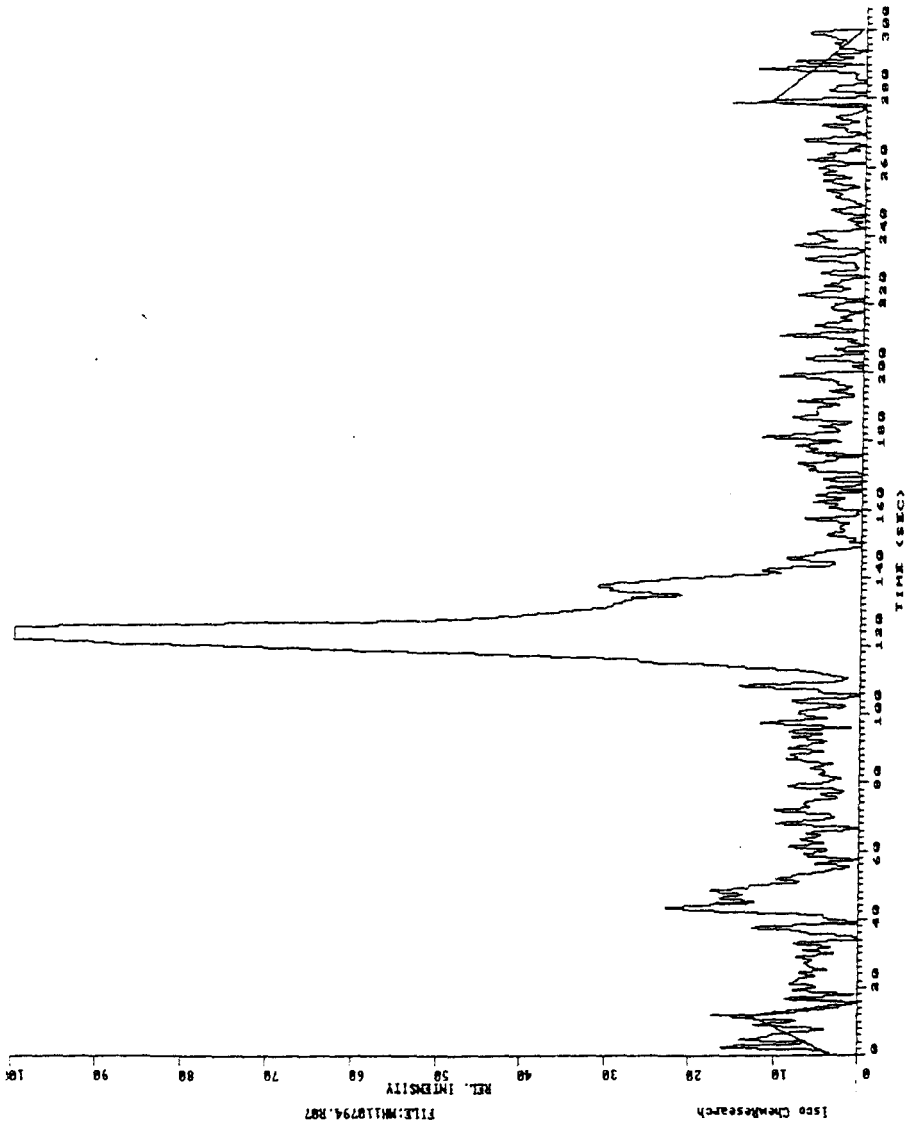

1
2
43.0 Kd
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

(b)

Figure 9: Densitometric tracing of SDS polyacrylamide electrophoresis gels stained with 0.05% Coomassie Brilliant Blue R. a: FPLC purified Spα1-166 (S12C). b: FPLC purified Spα1-167.


Isco ChemResearch
FILE:MH11B794.R06
REL. INTENSITY
TIME (SEC)

(a)


Isco ChemResearch
FILE:MH11879A.R87
REL. INTENSITY
TIME (SEC)

(b)

3.1.5.2 Stability of α-Spectrin Peptides From Aggregation

Spα49-167 incubated for 16 hours at 4°C exhibit ~ 5% aggregation, since reloading sample to FPLC Mono Q column resulted in an elution profile with a major peak and minor peaks (about 5%) eluted at 500 mM NaCl.

3.2 Purity and Stability of β-Spectrin Subunit

3.2.1 Analysis of Membrane Samples on SDS-PAGE

0.76 ± 0.1 (n = 29) ml of white membranes were obtained per ml of packed human red blood cells. Lane 1 in Fig. 10 shows SDS-Polyacrylamide gel electrophoresis of membrane sample showing 7 main bands. These 7 bands were identified from literature (Mathews, C. and Van Holde, K.E., 1990) to be 1: spectrin (α-subunit); 2: spectrin (β-subunit); 3: band 3; 4: band 4.1; 5: band 4.2; 6: actin; 7: glyceraldehyde-3-phosphate dehydrogenase. In the literature, they are usually labeled bands 1, 2, 3, 4.1, 4.2, 5 and 6.

3.2.2 Analysis of Extracted Spectrin-Actin Samples on SDS-PAGE

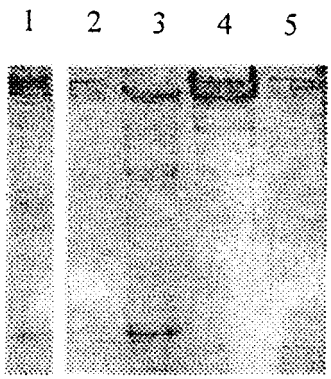
Typically 1.2 ± 0.3 (n = 29) mg of spectrin-actin network were obtained per ml of membrane or 0.8 ± 0.2 (n = 73) mg of spectrin-actin network per ml of packed red blood cells. Lane 2 in Fig. 10 shows SDS-PAGE of a spectrin-actin sample which shows that only spectrin (α- and β-subunits with approximately 1:1 ratio), band 4.1 and actin.

3.2.3 Purity of β-Spectrin Subunit

3.2.3.1 Purified on DE-52 Column

In our hands, using published salt gradient with 152 mM (to elute actin), 181 mM (to elute β-spectrin) and 234 mM (to elute α-spectrin) NaCl did not produce desirable

Figure 10: SDS polyacrylamide gel (7%) stained with 0.05% Coomassie Brilliant Blue R. This gel was run for about 40 minutes under constant voltage of 160 V. Lane 1: human red blood cell membrane; lane 2: spectrin-actin sample; lane 3: 135 mM NaCl eluted peak; lane 4: 181 mM NaCl eluted peak; lane 5: 234 mM NaCl eluted peak.


1
2
3
4
5

results. The peaks eluted with 135 or 152 mM NaCl buffer showed bands of actin, 4.1, β-spectrin and other proteins, as shown by SDS-PAGE (Figure 10). Lane 3 shows the peak at 181 mM NaCl sample which shows α-spectrin band in addition to the β-spectrin band. Densitometric tracing showed the percent area of α-spectrin to be 51 ± 10% and β-spectrin to be 16 ± 5 % with some degradation bands accounting for the rest of the area. The percent ratio of α- to β- spectrin bands was determined to be 24:76%. Lane 4 shows the 234 mM NaCl peak sample which shows one band corresponding to α-spectrin subunit. Densitometric tracing showed the percent area for α-spectrin to be 100% with no detection of β-spectrin or any other protein. Using this weak anion exchange column and under these published conditions, β-spectrin subunit could not be purified completely free of α-spectrin while α-spectrin was eluted as a pure subunit. The published method used four gel filtration columns in a series in the HPLC system.

This salt gradient was modified to 135 mM, 165 mM and 234 mM NaCl. It was found that the 135 mM NaCl buffer was sufficient to elute actin and other weakly bound proteins. Some β-spectrin eluted at this salt concentration. Densitometric tracing showed 26% of β-spectrin and 38% of actin. The peak eluted with the 165 mM buffer showed the β-spectrin as the major band (88 ± 4% (n = 5)), and α-spectrin as a very faint band (8 ± 3% (n = 5). Thus the ratio of β-spectrin to α-spectrin is about 90:10. Although this ratio is better than that obtained following the published salt gradient, β-spectrin is still not completely purified.

3.2.3.2 Purified on Mono Q HR10/10 Column

Purification using a Mono Q HR10/10 anion exchange column and a salt gradient

produced three peaks at 250 mM, 287.5 mM and 400 mM NaCl (Fig. 11). The first peak contained actin (36%), band 4.1 (4%) and other proteins as shown by SDS gel data (Fig. 12, lane 2). The second peak contained only β-spectrin (~100%) as shown by SDS gel data (Fig. 12, lane 3) and analysis of densitometric tracings (Fig. 13). The third peak contained α- (78%) and β- (16%) spectrin. Thus the ratio of the β-spectrin to the α-spectrin in peak 3 was 83:17. About 0.06 ± 0.02 (n = 40) mg of β-spectrin was extracted from 1 mg of spectrin-actin. About 0.04 ± 0.02 (n = 41) mg of β-spectrin was extracted from 1 ml of packed cells.

3.2.4 Stability of β-Spectrin

3.2.4.1 Degradation of β-Spectrin at 4°C

β-spectrin incubated in the presence of PMSF (serine protease inhibitor) at 4°C showed no significant degradation even after 6 days, as seen by SDS-gel data. However, incubating β-spectrin for 6 days without the presence of PMSF showed 70% decrease in β-spectrin.

3.2.4.2 Degradation of β-Spectrin From Freezing and Storage at -70°C

Freezing β-spectrin at -70°C, for up to 3 months, produced about 10% decrease in β-spectrin.

3.2.4.3 Aggregation of β-Spectrin at 4°C

β-spectrin was tested for aggregation at 4°C by running non-denaturing-PAGE on β-spectrin samples incubated at 4°C for 1 to 4 days. These samples were compared with spectrin-actin samples. β-spectrin appeared as one major band just below the spectrin dimer band. No decrease in the intensity of the β-spectrin band was observed.

Figure 11: FPLC elution profile from a Mono Q HR10/10 column at a flow rate of 2 ml/min and sensitivity of 10 mV. FPLC purification of β-spectrin by increasing the salt concentration from 250 to 400 mM NaCl passing by 288 mM NaCl where β-spectrin was eluted.

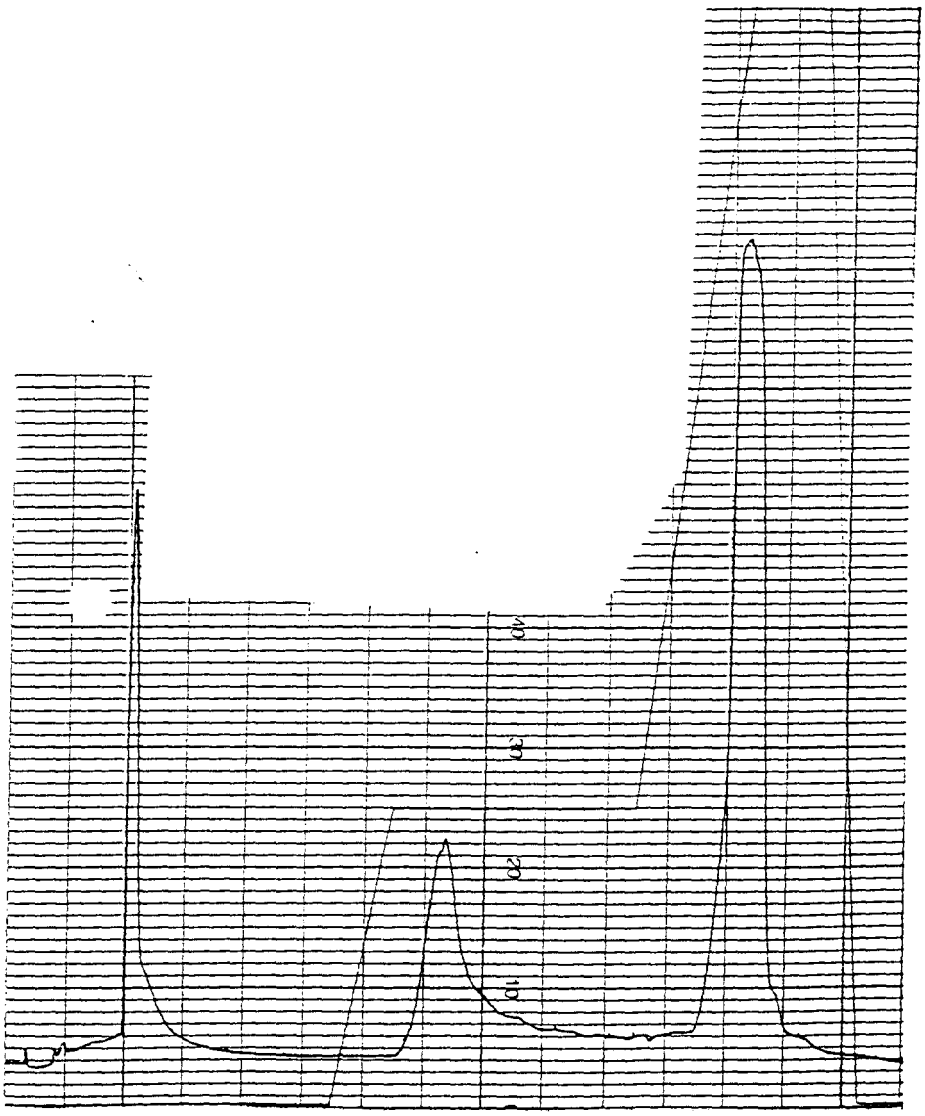

40
30
20
10

Figure 12: SDS polyacrylamide electrophoresis gel (7%) stained with 0.05% Coomassie Brilliant Blue R. The gel (0.75 mm thick) was run for about 40 minutes under a constant voltage of 160 V. Lane 1: human red blood cell membrane; lane 2: peak eluted at 250 mM NaCl; lane 3: peak eluted at 287.5 mM NaCl; lane 4: peak eluted at 400 mM NaCl.

1 2 3

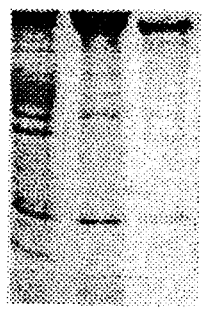
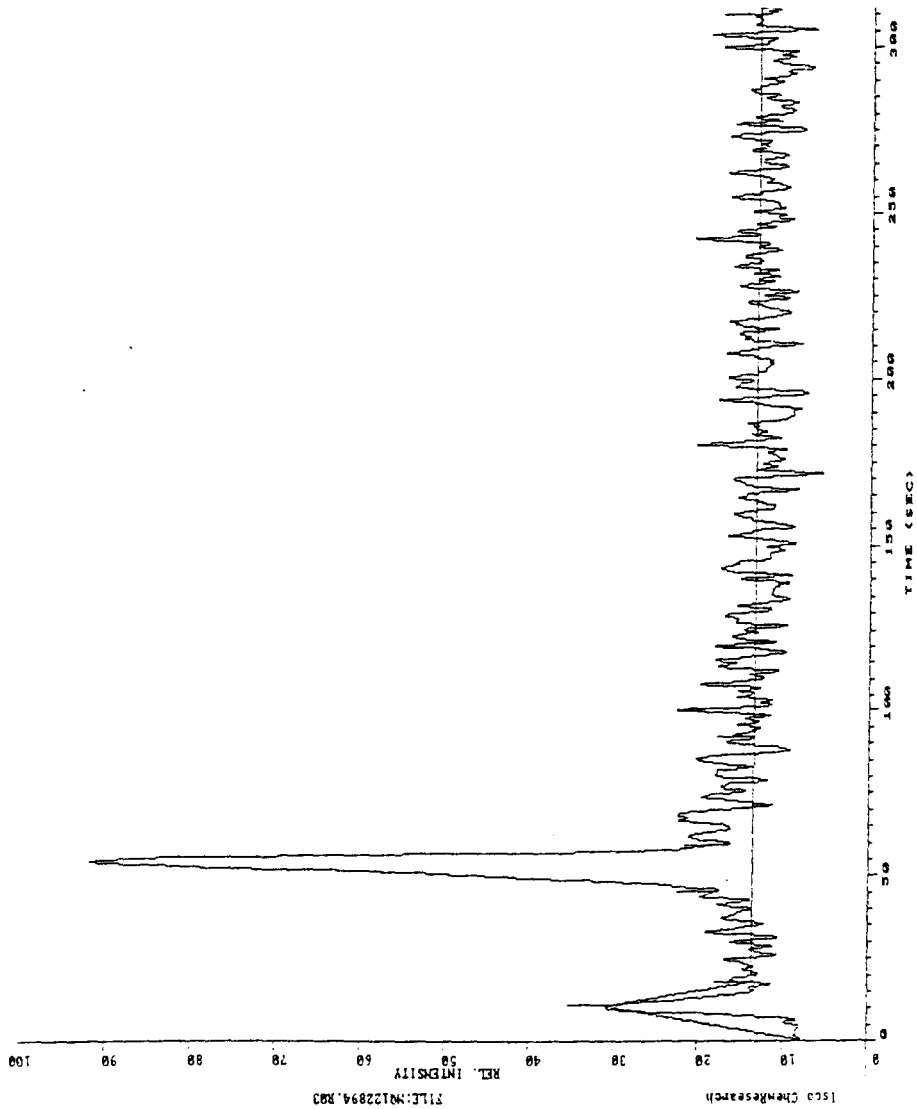


Figure 13: Densitometric tracing of SDS electrophoresis gel (7%) stained with 0.05% Coomassie Brilliant Blue R. The second peak on the FPLC elution profile eluted at 287.5 mM NaCl salt concentration.


Isco ChemResearch
FILE:NO122894.R03
REL. INTENSITY
TIME (SEC)

Thus little aggregation of FPLC purified β-spectrin was observed after 4 days at 4°C. FPLC or DE-52 purified β-spectrin were loaded and ran on the Superose 6 column. FPLC purified β-spectrin elute as one peak on the elution profile with Stokes radius (133 Å), smaller than that of spectrin dimer, and assumed to be that of β-spectrin. However, after incubating β-spectrin for five days at 4°C, a small peak appeared at the void volume. On the other hand, DE-52 purified β-spectrin elutes just after the void volume and has much higher molecular weight than that of the spectrin dimer.

3.2.4.4 Aggregation of β-Spectrin at -70°C

β-spectrin samples frozen at -70°C for about 2 months exhibit little aggregation as demonstrated by the elution profile of Superose 6, showing one peak with the same Stokes radius obtained before freezing the sample.

Thus we have developed methods to obtain stable β-spectrin and α-spectrin peptides.

3.3 Spectrin Domain Structure

The spectrin peptides Spα1-155, Spα1-167, Spα1-446, Spα41-155, Spα41-167, Spα41-273, Spα49-155 and Spα49-167 were used in limited chymotrypsin digestion study. This work was done with other members in the lab and published. Refer to the Appendix at the end of the dissertation for the published work.

3.4 Binding of α-Spectrin Peptides to β-Spectrin Subunit

3.4.1 Gel Filtration Assay

3.4.1.1 Stokes Radius

3.4.1.1.1 Stokes Radii of Spectrin monomer, dimer, tetramer, Spα1-166 (S12C) and Spα1-167

Stokes radius of spectrin dimer and tetramer were found to be 158 and 229 Å, respectively, by Superose 6 column method. The Stokes radius for β-spectrin was 133 Å. The Stokes radius for Spα1-166 (S12C) was 30 Å. The Stokes radius for Spα1-167 was either 30 or 38 Å, since two peaks appeared in the elution profile. Figure 14 includes the elution profiles for Spα1-166 (S12C), Spα1-167 and β-spectrin. Another peak appears in the β-spectrin elution profile and is probably a degradation peak.

3.4.1.1.2 Stokes Radius of β-Spectrin and Spα1-166 (S12C) Incubation Mixture

Figure 14 includes the elution profile for the incubation mixture of β-spectrin with Spα1-166 (S12C) (0.1 mg of β-spectrin was incubated with 0.012 mg of Spα1-166 (S12C)). The calculated Stokes radii are summarized in table 5. A third peak was observed with a Stokes radius of 141 Å. Residual Spα1-166 (S12C) exhibited same Stokes radius (30 Å). In addition, the area of the Spα1-166 (S12C) peak decreased by 16%.

3.4.1.1.3 Stokes Radius of β-Spectrin and Spα1-167 incubation mixture

Figure 14 includes the elution profile for the incubation mixture of β-spectrin with Spα1-167 (0.1 mg of β-spectrin and 0.012 mg of Spα1-167). The calculated Stokes

Figure 14: Superose 6 gel filtration elution profiles of samples incubated in isotonic KCl buffer for 24 hours at 4°C. (a) β-spectrin. (b) Spα1-166 (S12C). (c) mixture of β-spectrin with Spα1-166 (S12C). (d) Spα1 167. (e) mixture of β-spectrin with Spα1-167.

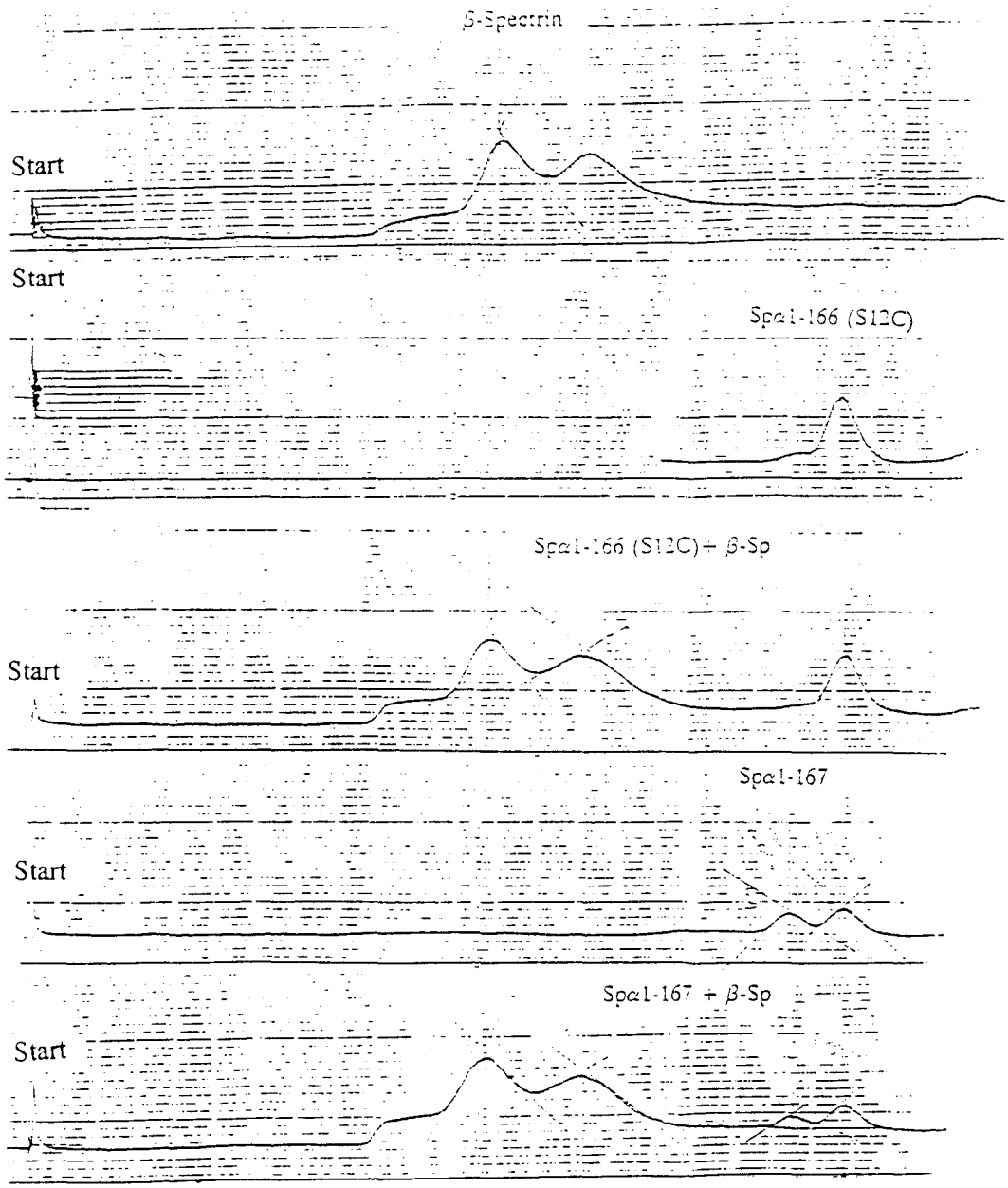

β-Spectrin
Start
Start
Spα1-166 (S12C)
Spα1-166 (S12C) + β-Sp
Start
Spα1-167
Start
Spα1-167 + β-Sp
Start

Table 5: Stokes radius of the incubation the Spα1-166 (S12C) and β-spectrin controls

SAMPLE	PEAK	STOKES RADIUS (Å)
Spα1-166 (S12C)	1	30
β-Spectrin	2	133
Spα1-166 (S12C) + β-Sp	1	30
	3	141

Table 6: Stokes radius of the incubation mixture of Spα1-167 and β-spectrin and controls

SAMPLE	PEAK	STOKES RADIUS (Å)
Spα1-167	1	30
	2	38
β-Spectrin	3	133
Spα1-167 + β-Sp	1	30
	2	38
	4	141

radii are summarized in table 6. A third peak was observed with a Stokes radius of 141 Å. Residual Spα1-167 exhibited same Stokes radius (30 and 38 Å). In addition, the area of the two Spα1-167 peaks decreased by 20%, respectively.

3.4.1.2 Binding Constants

The ratio of peak area in the incubation to that in the control sample was determined to be 0.80 and 0.84 for Spα1-167 and Spα1-166 (S12C), respectively. The equilibrium concentrations for the two mixtures were calculated (as described in section 2.17.1) and summarized in table 7. Binding constants of 6.3×10^6 and 2.1×10^6 M^{-1} were calculated for the peptides Spα1-167 and Spα1-166 (S12C), respectively.

3.4.2 Combined Western Blotting and Radiolabeling

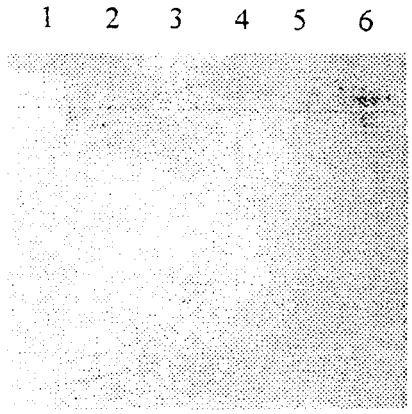
3.4.2.1 Affinity of β-Spectrin and α-Spectrin Peptides to Spectrin Antibodies

Two monoclonal antibodies (SB-SP1 and SB-SP2) and one polyclonal antibody were used in this study. The three antibodies were tested for their affinity to β-spectrin subunit and to the spectrin peptides Spα52-156, Spα1-167, Spα1-166 (S12C), Spα1-446 and Spα41-273. Figure 15 show three western blots for the three different antibodies. It could be observed that both SB-SP1 and SB-SP2 monoclonal antibodies recognized β-spectrin (to different degrees) and did not recognize any of the α-spectrin peptides. The polyclonal antibody recognized β-spectrin and very weakly the α-spectrin peptides. The monoclonal antibody SB-SP1 was chosen since it shows stronger affinity to β-spectrin subunit than SB-SP2 and since the polyclonal antibody would give some background on the Western blots. Figure 16 shows SDS-PAGE of the same samples which shows the presence and position of β-spectrin subunit and the α-spectrin peptides so it can be

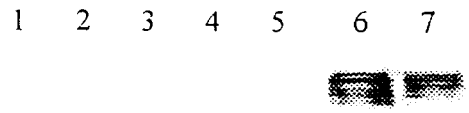
Table 7: Equilibrium concentrations for all three components in the incubation mixture of Spα1-166 (S12C) or Spα1-167 with β-spectrin.

SAMPLE	Equilibrium Concentration (M)
Spα1-166 (S12C)	9.7×10^{-7}
β-Spectrin	9.0×10^{-8}
Spα1-166 (S12C) + β-Spectrin	1.8×10^{-7}
Spα1-167	9.2×10^{-7}
β-Spectrin	4.0×10^{-8}
Spα1-167 + β-Spectrin	2.3×10^{-7}

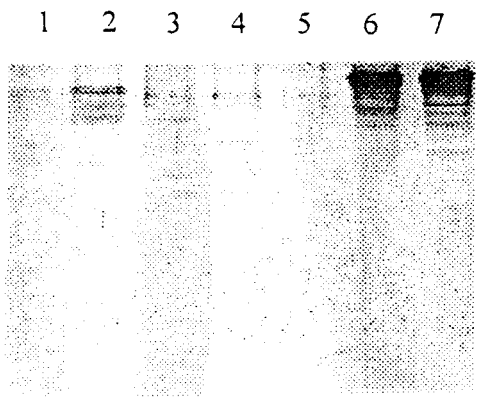
Figure 15: Western blots of spectrin peptides and β-spectrin using the monoclonal antibodies SB-SP1 (a) and SB-SP2 (b) and the polyclonal antibody (c). Lanes 1, 2, 3, 4, 5 and 6 in (a), (b) and (c): the spectrin peptides Spα41-273, Spα1-446, Spα1-166 (S12C), Spα1-167, Spα52-156 and β-spectrin monomer, respectively. Lane 7 in (b) and (c): spectrin actin.



(a)

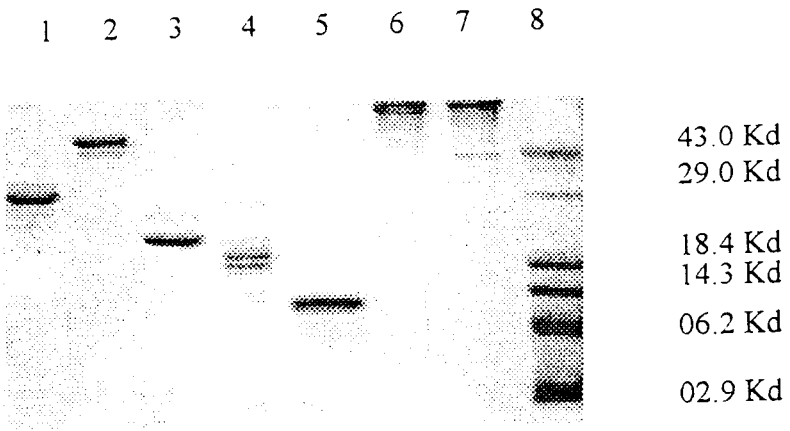


(b)



(c)

Figure 16: SDS polyacrylamide electrophoresis gel (16%) stained with 0.05% Coomassie brilliant blue R. This gel was run for about 1 hour under a constant voltage of 160 V. Lanes 1, 2, 3, 4 and 5: glutathione affinity purified Spα41-273, Spα1-446, Spα1-166 (S12C), Spα1-167 and Spα52-156, respectively. Lane 6: low molecular weight standards.


1
2
3
4
5
6
7
8
43.0 Kd
29.0 Kd
18.4 Kd
14.3 Kd
06.2 Kd
02.9 Kd

compared to the western blot.

3.4.2.2 Binding Between β-Spectrin and ^{125}I labeled Spα1-167

Figure 17 shows a Western blot of incubation mixture from two samples. The two rectangles containing dark bands (top rectangles) are detected by the antibody SB-SP1 and thus contain β-spectrin. β-spectrin is present in these areas either as free β-spectrin or as a complex with radiolabeled Spα1-167. These areas were excised and counted. The two bottom rectangles are the radiolabeled Spα1-167 (free Spα1-167) area and was also excised and counted. Counts per minute for the β-spectrin area (combined β-spectrin and complex) and for the Spα1-167 area are summarized in table 8. The ratios of unbound Spα1-167 to the total Spα1-167 for the two samples were calculated for samples 1 and 2 and found to be 0.37 and 0.42. The equilibrium concentrations of the three components were calculated (section 2.17.2) and summarized in table 9. Binding constant of $2.3 \pm 0.3 \times 10^6 M^{-1}$ (n = 2) was calculated.

3.4.3 Immunoprecipitation

The binding constant was calculated as described in section 2.17.3. The ratio of bound Spα1-167 to the total Spα1-167 was calculated for both samples and found to be 0.24 and 0.16 after subtracting the background (table 10). Equilibrium concentrations were calculated using these ratios and are summarized in table 11. Finally, binding constants were calculated from the equilibrium concentrations and found to be $5.7 \pm 4.3 \times 10^6 M^{-1}$ (n = 2).

Figure 17: Western blot of incubation mixture of radiolabeled Spα1-167 with β-spectrin from two samples. The top two rectangles show the stained β-spectrin and complex while the bottom rectangles show the area where radiolabeled Spα1-167 usually appears. These four areas were excised and counted.

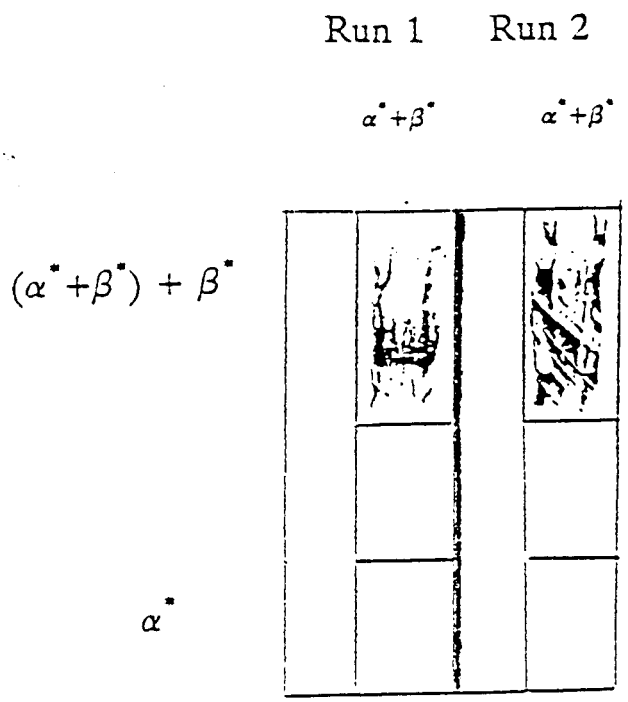

Run 1
Run 2
$\alpha^{*}+\beta^{*}$
$\alpha^{*}+\beta^{*}$
$(\alpha^{*}+\beta^{*}) + \beta^{*}$
α^{*}

Table 8: Counts per minute obtained for β-spectrin with complex bands and the Spα1-167 bands excised from Western blot.

SAMPLE	BAND	CPM
1	β-Spectrin	551
	Spα1-167	326
2	β-Spectrin	229
	Spα1-167	166

Table 9: Equilibrium concentrations of all three components of mixtures of radiolabeled Spα1-167 with β-spectrin.

Number	Sample	Equilibrium Concentration (M)
1	Spα1-167	1.3×10^{-7}
	β-spectrin	6.8×10^{-7}
	Spα1-167 + β-spectrin	2.2×10^{-7}
2	Spα1-167	1.5×10^{-7}
	β-spectrin	7.0×10^{-7}
	Spα1-167 + β-spectrin	2.0×10^{-7}

Table 10: Counts per minute for pellet and supernatant samples and calculated ratios of the pellet to the total counts per minute.

SAMPLE	**Number**	**CPM (PELLET)**	**CPM (SUP.)**	**RATIO** Pellet/ (pellet + Sup.)
Spα1-167	1	2830	5413	0.34
	2	1676	4650	0.26
Spα1-167 + β-spectrin	1	581	5236	0.1
	2	921	8272	0.1

Table 11: Equilibrium concentration of all three components of the mixture of radiolabeled Spα1-167 with β-spectrin

Number	Sample	Equilibrium Concentration (M)
1	Spα1-167	9.6×10^{-7}
	β-spectrin	3.0×10^{-8}
	Spα1-167 + β-spectrin	3.0×10^{-7}
2	Spα1-167	1.06×10^{-6}
	β-spectrin	1.3×10^{-7}
	Spα1-167 + β-spectrin	2.0×10^{-7}

3.5 Spin Labeling

3.5.1 Purity of spin labeled Spα41-167, Spα1-166 (S12C) and Spα41-273

Purified Spα41-167, Spα41-273 and Spα1-166 (S12C) were spin labeled. SDS-PAGE of these spin labeled peptides showed single band for each with estimated molecular weight of 15 kD, 26 kD and 20 kD which matches those of the calculated values. Densitometric analysis showed the percent area of the peptide band of all to be about 100%.

3.5.2 Molar Ratio of Spin Labeled Spα41-167, Spα41-273 and Spα1-166 (S12C)

Spα41-167 contains one cysteine residue at position 167 which is the last residue in the spectrin peptide. Conditions such as spin labeling incubation time and the amount of spin label used were changed to obtain maximum spin labeling of this peptide. Maximum spin label to Spα41-167 molar ratio of 0.5-0.8 was obtained for this peptide.

Spα41-273 contains two cysteine residues at positions 167 and 224. Spin label to Spα41-273 molar ratio was found to be 1.2, which is about double the value obtained for Spα41-167. Further, EPR spectrum shows two different populations of spin labels. This indicated that both cysteine residues were being spin labeled.

Spα1-166 (S12C) contains one cysteine residue that was introduced by site directed mutagenesis at position 12. Spin label to Spα1-166 (S12C) molar ratio was determined to be 0.5 ± 0.03 ($n = 3$).

3.5.3 Motional Comparison of Spα41-167, Spα41-273 and Spα1-166 (S12C)

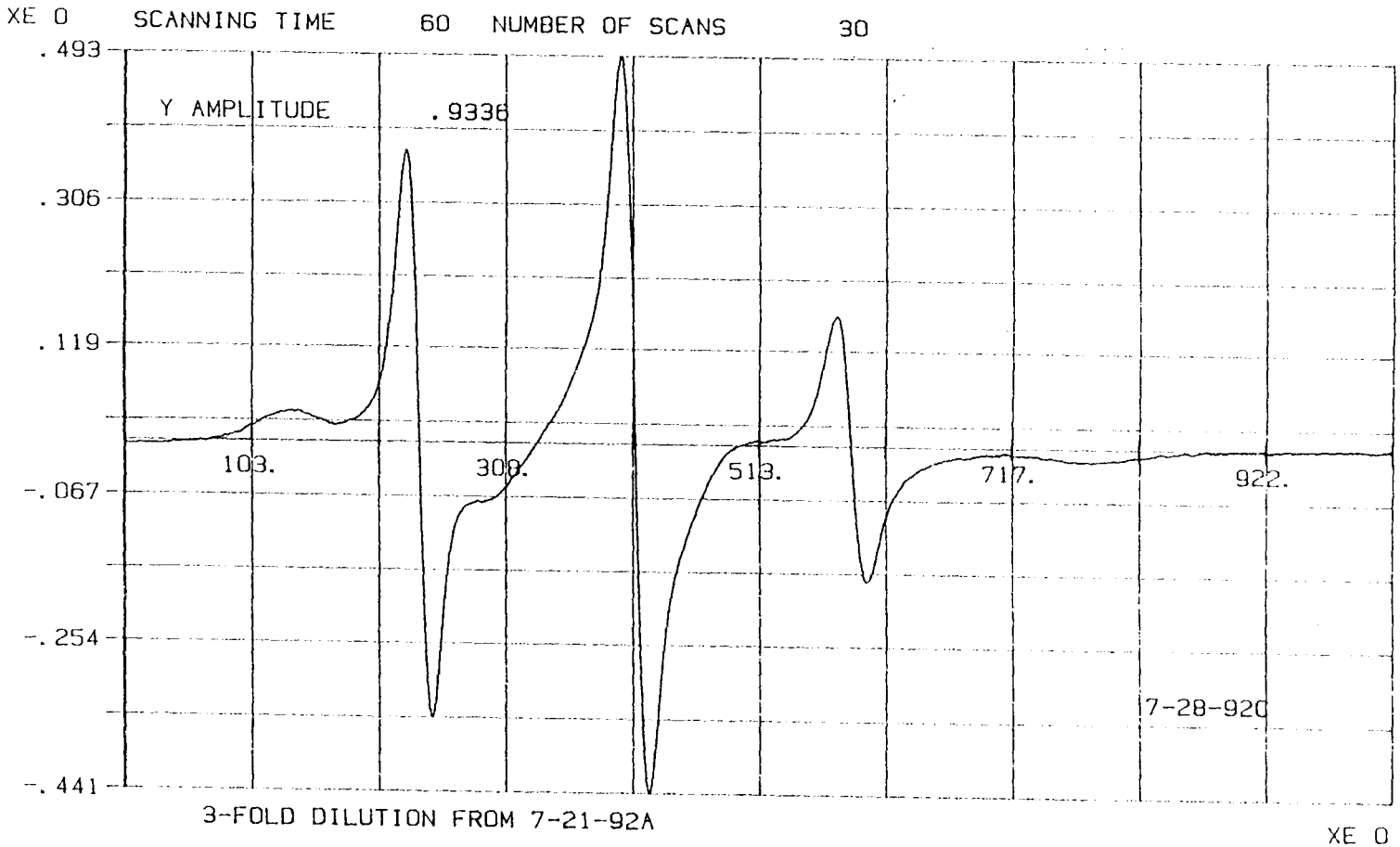
EPR spectrum of Spα41-273 show two populations of spin labels, one with slow

motion and the other with fast motion (Fig. 18a). EPR spectrum of both Spα41-167 and Spα1-166 (S12C) show one population of fast motion (Figs. 18b and 18c).

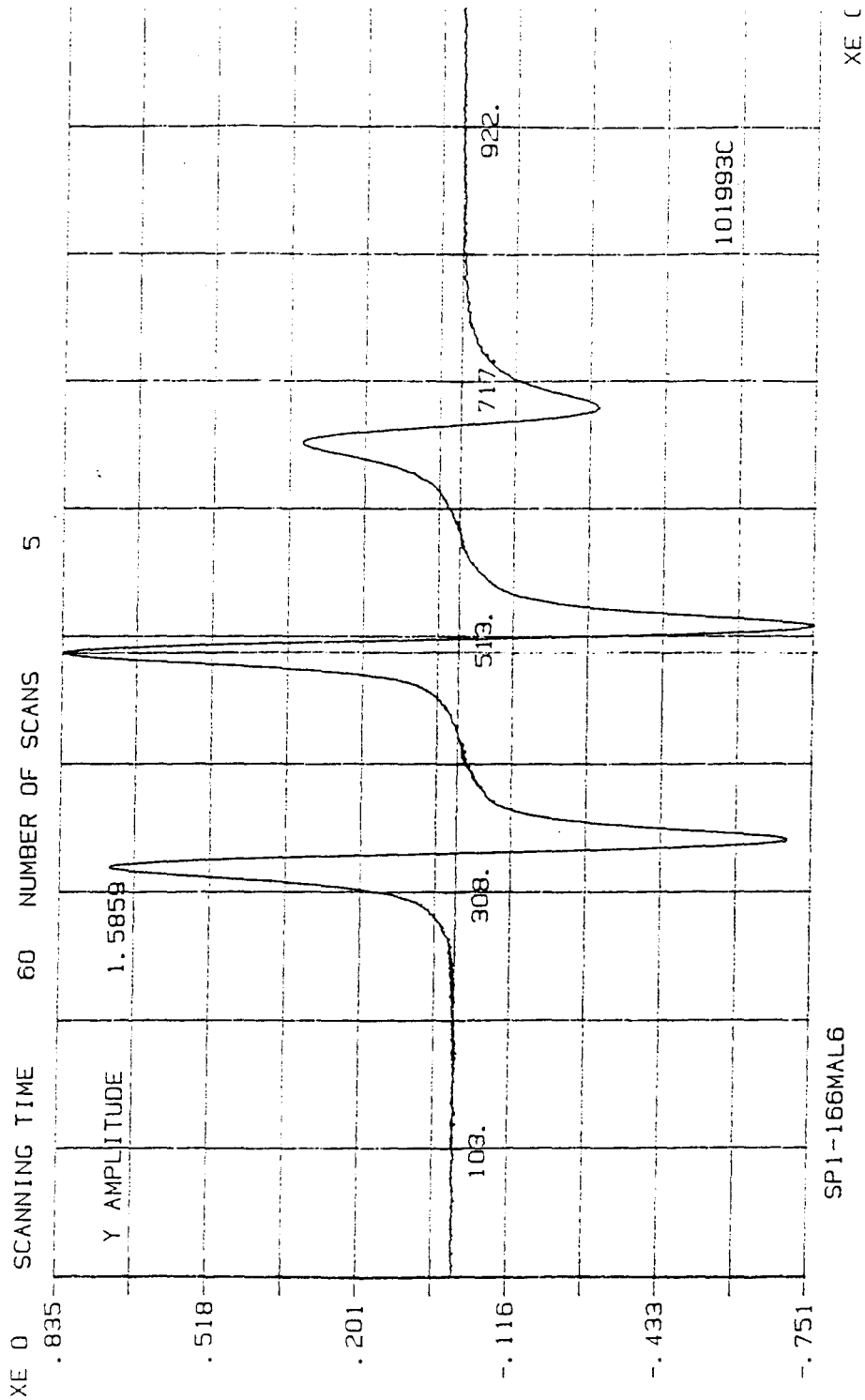
EPR spectrum of the Spα1-166 (S12C) peptide was compared quantitatively to Spα41-167 and Spα41-273 by taking the ratio of the peak to peak height of the high field peak to the peak to peak height of the middle peak. The lower the ratio, the slower the spin label motion. Table 12 summarizes the ratios obtained for the three peptides.

Rotational correlation time was calculated for the three peptides Spα1-166 (S12C), Spα41-167 and Spα41-273 and found to be 1.8×10^{-10}, 1.7×10^{-10} and 2.3×10^{-10} s, respectively. These values agree with the ratio of the peak-to-peak height of the high field to middle field and summarized in table 12. It could be seen from table 12 that the ratio of Spα1-166 (S12C) is higher than that of Spα41-273 and lower than that of Spα41-167. The rotational correlation time of Spα1-166 (S12C) is also higher than the rotational correlation time of Spα41-167 but less than that of Spα41-273. This indicates a faster motion for Spα1-166 (S12C) than that of Spα41-273 and slower than Spα41-167.

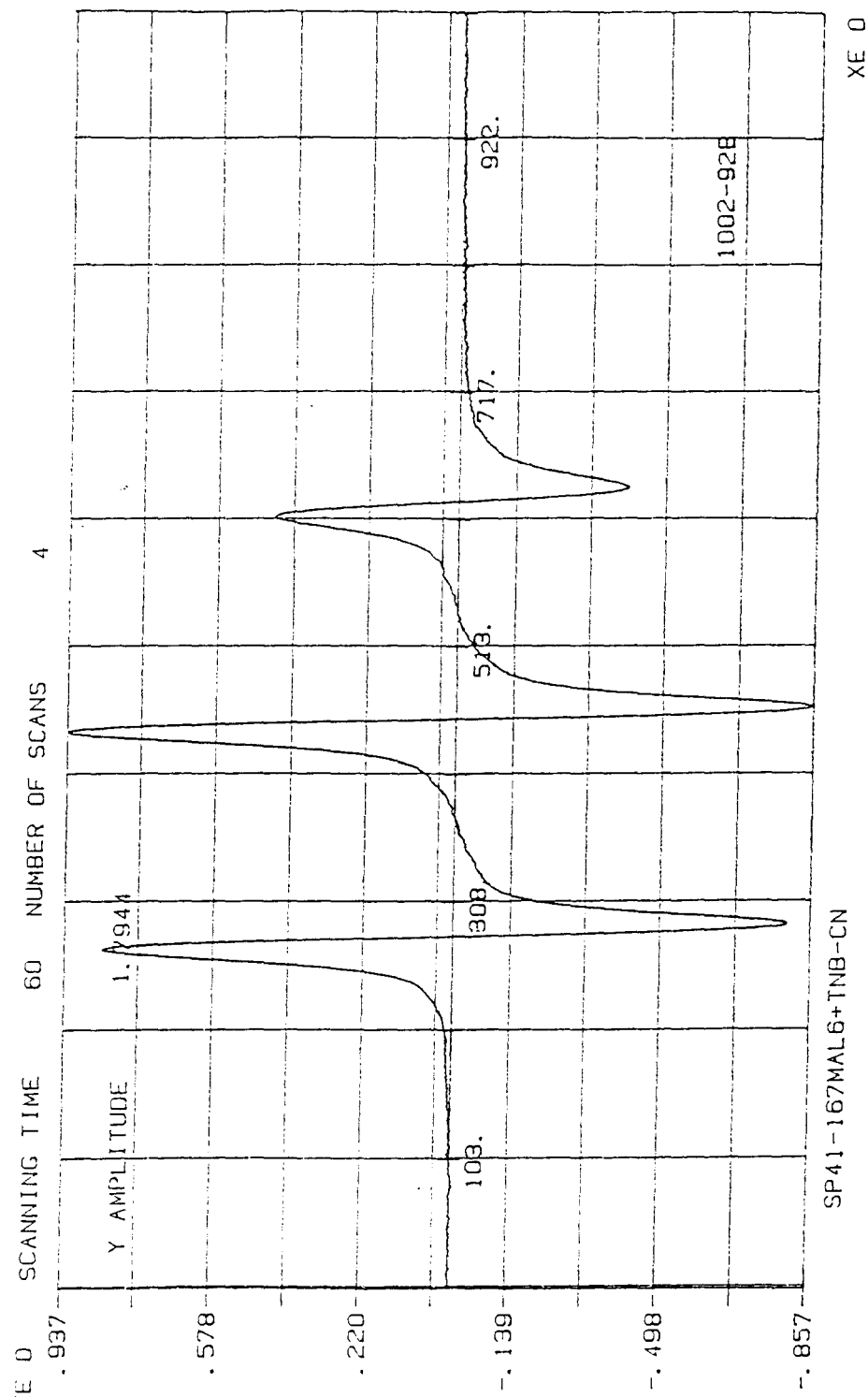
Figure 18: EPR spectra obtained with 2 mW incident microwave power and a scan time of 60 seconds. a: EPR spectrum of Spα41-273. b: EPR spectrum of Spα1-166 (S12C). c: EPR spectrum of Spα41-167.


XE 0
SCANNING TIME 60
NUMBER OF SCANS 30
.493
.306
.119
-.067
-.254
-.441
Y AMPLITUDE .9336
103.
300.
513.
717.
922.
7-28-92C
3-FOLD DILUTION FROM 7-21-92A
XE 0

(a)


XE 0
SCANNING TIME 60 NUMBER OF SCANS 5
Y AMPLITUDE 1.5859
.835
.518
.201
-.116
-.433
-.751
108.
308.
513.
717
922.
101993C
SP1-166MAL6
XE (

(b)


XE 0
SCANNING TIME 60 NUMBER OF SCANS 4
Y AMPLITUDE
1.944
308
103.
513.
717.
922.
1002-928
.937
.578
.220
-.139
-.498
-.857
SP41-167MAL6+TNB-CN
XE 0

(c)

Table 12: Ratio of the peak-to-peak height of the third to the middle peaks in the EPR spectrum

PEPTIDE	RATIO
Spα1-166 (S12C)	0.41
Sp41-167	0.48
Spα41-273	0.32

CHAPTER IV

DISCUSSION

4.1 The First Human α-Spectrin Structural Domain Begins with Serine

(From *J. Biol. Chem.* (1994) **269**, 25955-25958. See Appendix.)

"The 106 amino acid sequence motifs of spectrin have been suggested to fold into stable structural domains, consisting mostly of coiled coils of triple helices. With the advent of molecular biology and biophysical techniques, structural studies of these spectrin 106-amino acid structural domains became approachable. However, one of the difficulties in such an approach is determination of the correct phasing of the structural domains, which may or may not coincide with the phasing of the sequence motifs. Proper identification of the domain phasing is vital to the construction of stable spectrin domains for molecular studies. A previously published phasing shift for Drosophila α-spectrin indicated a downstream phase-shift of 26 amino acids for the structural domains (Winograd *et al.*, 1991). Using this phase-shift, we prepared recombinant spectrin peptide with the sequence from residue 49 to residue 155 of human erythrocyte α-spectrin and found this peptide to be unstable relative to other peptides that we prepared. Using several other recombinant α-spectrin peptides and following the protease digestion approach, we digested spectrin peptides with elastase and chymotrypsin and analyzed the

amino acid sequence of the digestive products. We provide the first experimental evidence in identifying the first amino acid residue of the first spectrin domain in human erythrocyte α-spectrin as residue 52 (Ser)".

4.2 Binding of α-Spectrin Peptides to β-Spectrin Subunit

Two spectrin peptides were used in this part of the study, Spα1-167 and Spα1-166 (S12C). The segment containing amino acid residues 52-156 are folded into triple helical bundle. The N-terminal region (residues 1-51), though its structure is not clear, interacts with β-spectrin directly.

Three different methods were used to detect the binding between the β-spectrin subunit and Spα1-166 (S12C) or Spα1-167 in 10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM βME, 30 μM PMSF, pH 7.4 at 4°C. These methods included the use of the gel filtration method (Superose 6 column on the FPLC system), combined Western blotting with radiolabeling, and immunoprecipitation.

4.2.1 Gel Filtration

The partial disappearance of the α-spectrin peak in the presence of β-spectrin was used as an indication of α -peptide-β-spectrin complex formation. Furthermore, the peak areas of the α-peptide in the presence and absence of β-spectrin were determined quantitatively to obtain equilibrium concentrations of the α-peptide and of the complex. The binding constants were found to be 2.1 x 10^6 M^{-1} for Spα1-166 (S12C) and 6.3 x 10^6 M^{-1} for Spα1-167, both at 4 C.

Gel filtration method was used to determine binding between the αI fragment

(residues 7-639, which contains 5 domains with a molecular weight of about 80 kD) and β-spectrin (Speicher *et al.*, 1992). Binding constant was determined to be 4.5 x 10^6 M^{-1} at 0°C.

4.2.2 Combined Western Blotting With Radiolabeling

In the combined Western blotting with radiolabeling method, the radiolabeled peptide concentrations in both free form and bound to β-spectrin were detected quantitatively. The average binding constant was determined to be 2.3 ± 0.3 x 10^6 M^{-1} (n = 2). This value is comparable to those obtained from gel filtration and to the published value mentioned above.

4.2.3 Immunoprecipitation

This method has been used qualitatively in binding studies of different peptides from α- and β-subunits of *Drosophila* spectrin (Viel and Branton, 1994). We have used this method quantitatively on purified α-spectrin peptides and β-spectrin subunit. As with the combined Western blotting with radiolabeling method, the concentrations of the complex were measured quantitatively from a mixture of the β-spectrin and the complex in the pellet. The unbound peptide concentration was measured in the supernatant. Binding constants, at 4°C, were found to be 5.7 ± 4.3 x 10^6 M^{-1} (n = 2).

4.2.4 Comparison of the Three Binding Methods

Although these three methods provide similar binding constants, there are differences between these methods.

The gel filtration method requires the measure of the difference in the α-peptide concentrations, obtained from peak area, in samples with and without β-spectrin. These

two samples cannot be run simultaneously. Thus a great care is required in loading the control and mixture samples to ensure identical experimental conditions.

In the combined Western blotting with radiolabeling, two components are being detected thus eliminating the need for using controls in calculating binding constants. However, this method is very laborious and requires a long time of processing samples including blotting overnight. Also, it involves excising the spectrin peptide bands, which are not stained and thus care is necessary in excising these areas of the Western blot. This problem could be solved, however, using additional polyclonal antibody which recognizes the α-spectrin peptides (although to a lesser extent) in addition to β-spectrin.

In the immunoprecipitation method both the unbound peptide and the complex are being detected. However the presence of background binding of unbound peptide to the antibody presents a source of error. The control sample is needed for calculating binding constants. This method is the fastest of the three, and many samples can be processed at the same time.

Although the three methods are different, the calculated binding constants, appear to be in good agreement with each other, ranged between 2-6 x 10^6 M^{-1} at 4 C. These values are also in close agreement with the published value of 4.5 x 10^6 M^{-1} for the αI binding to the β-spectrin subunit. Thus the binding of these two peptides to β-spectrin resembles the binding of the αI fragment to the β-spectrin. These peptides appear to be a good model system for studying association properties of αβ spectrin. Other workers have found that β-spectrin-αI equilibrium at the 37 C gave a binding constant of 1.4 x 10^5 M^{-1} (DeSilva *et al.*, 1992). Binding was found to increase with decreasing temperature.

The binding constant was found to be about 1 x 10^6 M^{-1} at 30°C and further increased to 4.5 x 10^6 M^{-1} at 0°C. In addition, a cloned peptide with the first 158 N-terminal amino acid residues gave a binding constant of 6.6 x 10^5 M^{-1} at 30°C (Kotula *et al.*, 1993).

Since the αI domain does not contain the first 6 amino acid residues, it was determined that the removal of these residues did not affect the head-to-head interactions. The removal of the next 10 amino acid residues decreased the binding by 50% (Speicher *et al.*, 1993). Cloned peptides that did not contain the first 27 amino acid residues did not show any binding to the β-spectrin (Kotula *et al.*, 1993). Peptides including the N-terminal amino acid residues of α-spectrin without the folding domain did not bind to β-spectrin. It was suggested that the first folding domain acts as a template for the folding of the less ordered N-terminal residues and its presence is thus necessary for any binding study.

Thus the use of the peptides used in this study as well as other modified peptides may provide insights toward binding sites of head-to-head association in spectrin. These peptides can be used as a model to represent the spectrin molecule. The head-to-head interaction region could be studied in a more detailed way. Deseases associated with this area could be more understood with these models in hand. Our preliminary spin label EPR results on the α-peptides indicate that EPR will be useful in obtaining both binding and structural information on the N-terminal region of α-spectrin.

Conclusion

In summary, we have prepared spectrin peptides Spα1-166 (S12C) and Spα1-167

to high purity while preserving their stabilities under experimental conditions. The N-terminal portion of these peptides (amino acid residues 1-51) are involved in binding with the β-spectrin. The central portion (amino acid residues 52-156) fold into triple helical bundles, while the remaining part (amino acid residues 157 - 166/167) is assumed to be loosely folded. We demonstrate their binding abilities with the β-spectrin with three independent methods. The binding constants were 2-6 x 10^6 M^{-1} in isotonic KCl buffer at 4°C. Thus the two peptides can serve as a good model for α-spectrin, they include a folding unit and have similar binding affinity to the β-spectrin subunit.

Now this system is poised for comprehensive studies to obtain structural information of normal spectrin and to understand abnormal binding properties in spectrin mutants.

APPENDIX

Communication

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The First Human α-Spectrin Structural Domain Begins with Serine*

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The 106-amino acid sequence motifs of spectrin have been suggested to fold into stable structural domains, consisting mostly of coiled coils of triple helices. With the advent of molecular biology and biophysical techniques, structural studies of these spectrin 106-amino acid structural domains became approachable. However, one of the difficulties in such an approach is determination of the correct phasing of the structural domains, which may or may not coincide with the phasing of the sequence motifs. Proper identification of the domain phasing is vital to the construction of stable spectrin domains for molecular studies. A previously published phasing shift for *Drosophila* α-spectrin indicated a downstream phase-shift of 26 amino acids for the structural domain (Winograd, E., Hume, D., and Branton, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 10788–10791). Using this phase-shift, we prepared a recombinant spectrin peptide with the sequence from residue 49 to residue 155 of human erythrocyte α-spectrin and found this peptide to be unstable relative to other peptides that we prepared. Using several other recombinant α-spectrin peptides and following the protease digestion approach, we digested spectrin peptides with elastase and chymotrypsin and analyzed the amino acid sequence of the digestive products. We provide the first experimental evidence in identifying the first amino acid residue of the first spectrin domain in human erythrocyte α-spectrin as residue 52 (Ser).

Underlying the cytoplasmic surface of the human erythrocyte membrane is a dense, two-dimensional network of spectrin and other proteins that provides support to the lipid bilayer and maintains the erythrocyte flexibility (1). Spectrin, which comprises α- and β-subunits, plays a critical role in maintaining the architecture and therefore the integrity of the red cell membrane. Many hereditary hemolytic anemias involve spectrin mutations (2–7). In addition, several other proteins, such as dystrophin, α-actinin, fibrin, etc., share many properties similar to those of erythrocyte spectrin and are grouped as the spectrin superfamily (1). Thus, it is important to understand the structural properties of spectrin. However, little experimental information is available to describe the detailed three-dimensional structure of spectrin. Amino acid (8) and cDNA (9, 10) sequence analyses of human erythrocyte spectrin (will be referred to as human spectrin hereafter; other types of spectrin will be clearly stated) reveal that most parts of both subunits consist of homologous sequence motifs. Each motif consists of about 106 amino acids in length. There are 22 sequence motifs in tandem in the α-subunit and 17 sequence motifs in the β-subunit. These sequence motifs have been suggested to fold into stable structural domains, consisting mostly of coiled coils of triple helices (11, 12). With the advent of molecular biology and biophysical techniques, structural studies of these spectrin 106-amino acid structural domains (spectrin domains) became approachable. Recently, the x-ray structure of a homodimer of a 106-amino acid fragment in segment 14 of *Drosophila* α-spectrin has been published (13). However, one of the difficulties in such an approach is to determine the correct phasing of the structural domains, which may or may not coincide with the phasing of the sequence motifs.

Winograd *et al.* (14) provided experimental evidence from *Drosophila* α-spectrin fragments to suggest a downstream phase-shift of 26 amino acids for the structural domain such that the first amino acid in a folded unit is residue 26 in the 106-residue sequence motif. Assuming human α-spectrin has the same phase-shift as *Drosophila* α-spectrin, the first amino acid at the N-terminal end of the first structural domain (AA1) would be residue 49 (Leu), since residue 24 (Ile) is the first amino acid of the first sequence motif in human α-spectrin (9). Based on information obtained from protease protection analysis (12), Speicher *et al.* (15) suggest that structural domains begin and end around residue 30 of the sequence motif of α-spectrin. These phase-shifts have been used to construct structural models or recombinant peptides. The Winograd phase-shift (14) was used to construct recombinant peptides either of α-spectrin for x-ray studies (13) or folding studies (16) or of β-spectrin for supra-motif studies (17). The phase-shift of dystrophin was compared with Winograd's phase-shift in spectrin and was found to be different by an upstream shift of 9 amino acids (18). The Speicher phase-shift (12) was used to construct recombinant peptides for spectrin tetramer binding site studies (15). Kennedy *et al.* (19) stated that the phasing of the structural domain in β-spectrin is shifted approximately 20–30 residues toward the C terminus compared with the phasing of the sequence motifs.

Not only do different published results suggest different amino acids as AA1 for the structural domain, different numbering systems for the amino acids in the spectrin sequence are also used. Before the publication of α-spectrin cDNA results, Glu was residue 1 according to Speicher and Marchesi (20). The human spectrin cDNA results detected 6 additional amino acids located upstream of Glu to give MEQFPKE as the first 7

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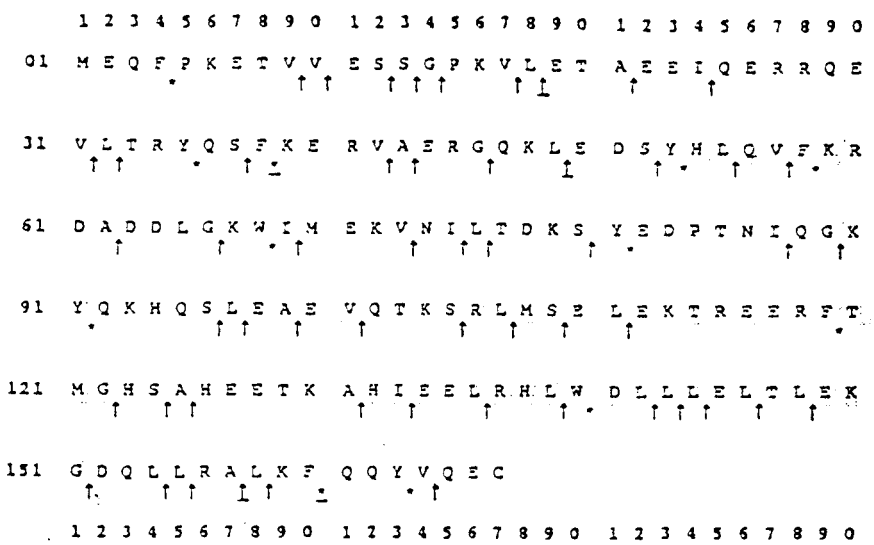

FIG. 1. Amino acid sequence of human erythrocyte α-spectrin from residue 1 to 167. The cDNA numbering system is shown and used throughout this presentation. Elastase (↑) and chymotrypsin (*) recognition sites are included. *Underlined arrows/asterisks* indicate sites of enzyme cleavage as reported here. The amino acid residues in the first α-spectrin structural domain are *shaded*.

amino acids in α-spectrin (9). Thus residue 1 (Glu) in the previous numbering system (20) is now residue 7 in the cDNA numbering system (9). Both numbering systems are used in the literature, although not necessarily clearly referenced, for all types of spectrin. Parry and co-workers (21) identify the starting position of the first structural motif as residue 46, using the numbering system of Speicher and Marchesi (20). Yan and co-workers (13) in their work on segment 14 of *Drosophila* α-spectrin use the cDNA numbering system. We follow the cDNA numbering system in this report (Fig. 1).

Using several recombinant human α-spectrin peptides and following the protease digestion approach used by Winograd and co-workers (14), we provide the first experimental evidence in identifying the first amino acid residue of the first spectrin domain in α-spectrin. Proper identification of domain phasing is vital to the construction of stable spectrin domains for molecular studies. Our results show that the first spectrin structural domain starts with residue 32 (Ser) (Fig. 1).

MATERIALS AND METHODS

Spectrin Recombinant Peptides—The cDNA clone α3 that contains the N-terminal region of the α-subunit (9), a generous gift of Dr. B. G. Forget (Yale University School of Medicine, New Haven, CT), was linearized with *Sac*II and used as a template. Oligonucleotide primer containing sequence corresponding to residues 1–9 of α-spectrin, in a sense orientation, and primer containing sequence corresponding to residues 159–167, in an antisense orientation, were synthesized by National Biosciences (Plymouth, MN) and used to make the spectrin construct of a peptide with the sequence of α-spectrin from residue 1 to residue 167 (Spα1–167).[1] The published α-spectrin sequence (9) was used. Similarly, spectrin constructs of Spα49–155 and Spα49–167 were also prepared. Some of the oligonucleotide primers were synthesized by the Wells Center Oligonucleotide Synthesizer Laboratory. The spectrin constructs were ligated into the *Bam*HI and *Eco*RI sites of the glutathione *S*-transferase (GST) expression vector pGEX-2T (Pharmacia Biotech Inc.). These ligations were in-frame with the GST protein. The pGEX-2T vector contains a thrombin cleavage site between GST and the inserted gene, *i.e.* spectrin construct. The spectrin peptides were located immediately following the thrombin cleavage site. The expression of fusion protein was under the control of a *lac* promoter. Thus, high, inducible levels of fusion proteins were produced.

The overproduced fusion protein (GST-thrombin cleavage site-spectrin peptides) was recovered from bacterial lysate and passed over a glutathione affinity column (*e.g.* glutathione-Sepharose 4B from Pharmacia), using published methods (22). The column, with the bound fusion protein, was extensively washed, followed by elution with buffer containing fresh glutathione. The spectrin peptide was cleaved from the GST by immobilized thrombin gel (Calbiochem) and eluted with GST and residual uncleaved fusion protein from the gel. The mixture was passed over the glutathione affinity column again to remove GST and residual fusion protein to give relatively pure spectrin peptides. If necessary, the peptides were further purified by fast protein liquid chromatography (FPLC) with a Mono Q column (Pharmacia), using 40 mM Tris at pH 7.5 and an NaCl gradient.

Enzyme Digestion and Peptide Sequencing—Elastase from porcine pancreas (Sigma) in 5 mM Tris at pH 5.0 was mixed with a spectrin peptide at a weight ratio of 1:12.5, in 50 mM Tris at pH 8.8 and room temperature. Samples were taken from the mixture at various time points for gel electrophoresis analysis. Similarly, chymotrypsin from bovine pancreas (Sigma) in 1 mM HCl was mixed with spectrin peptide at a weight ratio of 1:400 (14), in 40 mM Tris with 125 mM NaCl at pH 7.5 and room temperature. 15% polyacrylamide gel electrophoresis was performed using electrophoresis buffer containing 0.5% SDS. Low range protein molecular mass standards (Life Technologies, Inc.) at 43.0, 29.0, 18.4, 14.3, 8.2, and 3.0 kDa were used to guide the molecular mass calculation. For amino acid sequence analysis, polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA) were used for electroblotting. The amino acid sequencing of the digestive product was done by the University of Illinois Biotechnology Center.

RESULTS AND DISCUSSION

Using Winograd's downstream phase-shift of 26 amino acids, we designed Spα49–155, a peptide with 107 amino acid residues and with AA1 at position 49, to be a peptide that included the first stable structural domain of human α-spectrin. However, Spα49–155 was found to be unstable when compared to the other Spα peptides that will be discussed below. The electrophoretic gel of Spα49–155 purified by FPLC techniques exhibited several bands, with the predominating band at 12.7 kDa and two minor bands at about 9.8 and 8.7 kDa (Fig. 2A,

[1] The abbreviations used are: Spα1–167, a peptide with the sequence of α-spectrin from residue 1 to residue 167 (similar abbreviation system was used for other peptides); GST, glutathione *S*-transferase; FPLC, fast protein liquid chromatography; AA1, the first amino acid at the N-terminal end of the first structural domain.

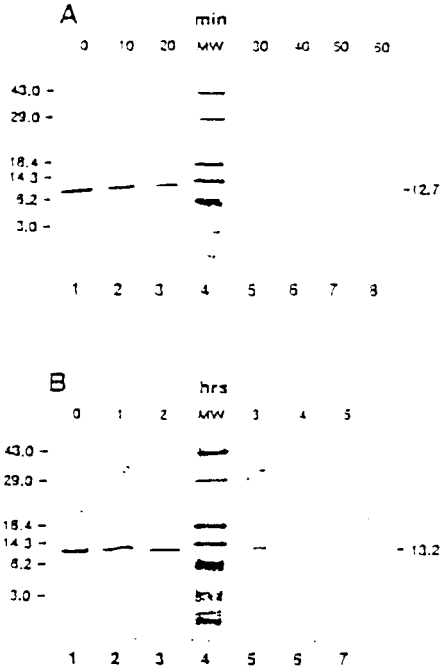

FIG. 2. SDS-PAGE (18% gel) of elastase digestion products of α-spectrin peptides, stained with Coomassie Brilliant Blue. *A*: *lane 1*, FPLC-purified Spα49–155 (no elastase present); *lane 2*, 10 min after the introduction of elastase, at a peptide to enzyme weight ratio of 12.5:1; *lane 3*, 20 min; *lane 5*, 30 min; *lane 6*, 40 min; *lane 7*, 50 min; *lane 8*, 60 min. *Lane 4*, molecular mass standards. *B*: *lane 1*, FPLC-purified Spα49–167 (no elastase present); *lane 2*, 1 h; *lane 3*, 2 h; *lane 5*, 3 h; *lane 6*, 4 h; and *lane 7*, 5 h. *lane 4*, molecular mass standards.

lane 1). The molecular mass for Spα49–155 calculated from the sequence was 12.7 kDa. Thus, we believe that the major band was that of Spα49–155 and the minor bands were from degradation products of Spα49–155. In the presence of elastase, at a weight ratio of 1:12.5, Spα49–155 was quickly digested into smaller fragments. At t = 10 min, bands below 12.7 kDa appeared at low intensities with concurrent decrease in the intensity of the 12.7-kDa band (*lane 2*). As the digestion reaction with elastase proceeded further, a steady decrease in the intensity of the 12.7-kDa band and an increase in the intensities of the smaller bands were observed (*lanes 3* and *5–7*). At t = 60 min, the band at 12.7 kDa was no longer detectable but faint bands below 12.7 kDa were visible (*lane 8*). In contrast, under similar conditions, the peptide Spα49–167 was quite stable. Spα49–167, with a molecular mass of 14.2 kDa calculated from the sequence, appeared as a single band at 13.2 kDa on the gel (Fig. 2*B*, *lane 1*). After a 60-min digestion with elastase under the conditions listed above for Spα49–155, the Spα49–167 digestion product appeared as a single band at 13.2 kDa (*lane 2*). This band persisted at t = 2 h (*lane 3*). The intensity of this band decreased at t = 3 h (*lane 5*), appeared as only a faint band at t = 4 h (*lane 6*), and finally became not detectable at t = 5 h (*lane 7*). Thus these digestion results suggest Spα49–167 is folded into a more stable configuration than Spα49–155 and the folded structure resists elastase digestion. The amino acid residues beyond 155 in Spα49–167 provide elements to fold the peptide into a more stable structure.

The FPLC-purified peptide Spα1–167, with a molecular mass of 19.9 kDa calculated from the sequence, appeared as a

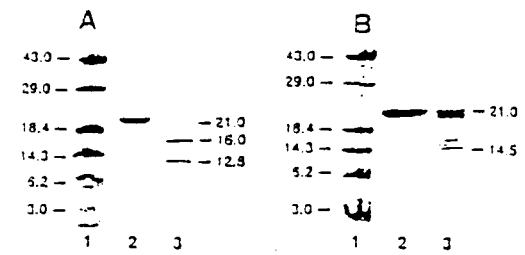

FIG. 3. SDS-PAGE (18% gel) of proteolytic digestion of Spα1–167, stained with Coomassie Brilliant Blue. *A*, elastase digestion of Spα1–167. *Lane 1*, molecular mass standards; *lane 2*, Spα1–167 (no elastase present); *lane 3*, 1 h after the introduction of elastase, at a peptide to enzyme weight ratio of 12.5:1. Bands at 16.0 and 12.3 kDa were used for amino acid sequence analysis. *B*, chymotrypsin digestion of Spα1–167. *Lane 1*, molecular mass standards; *lane 2*, Spα1–167 (no chymotrypsin); *lane 3*, 90 min after the introduction of chymotrypsin at a peptide to enzyme weight ratio of 400:1. Band at 14.5 was used for N-terminal amino acid sequence analysis.

single band at about 21.0 kDa (Fig. 3*A*, *lane 2*). Elastase digestion of Spα1–167 (at weight ratio of 1:12.5) resulted in two bands at 16.0 and 12.3 kDa at t = 90 min. These two bands persisted in the digestion mixture at t = 4 h (Fig. 3*A*, *lane 3*). The band intensity became undetectable at t = 5 h (not shown). These data suggested that the digestion products were very stable peptides when compared with Spα49–155 and had similar stability to the digestion product of Spα49–167, which also withstood elastase digestion beyond 4 h.

The N-terminal sequence analysis of the 12.3-kDa fragment was found to be EDSYHLQVFK, which aligned perfectly with 50–59 in the α-spectrin sequence (Fig. 1). The elastase cleavage sites around this region of α-spectrin consist of residues 46 (Gly), 49 (Leu), and 52 (Ser), and cleavage sites at the C-terminal end of the peptide consist of residues 151 (Gly), 154 (Leu), 155 (Leu), 157 (Ala), 158 (Leu), and 164 (Val). We suggest that elastase cleaved at 49 (Leu) and at 157 (Ala) to give the peptide Spα50–157, which has a calculated molecular mass of 12.3 kDa. The first amino acid at the N terminus of this digestion product was clearly 50 (Glu), as indicated by the sequence analysis data. However, the last amino acid at the C-terminal end was less certain and was suggested by matching gel electrophoresis molecular masses with calculated molecular masses. It was also possible that the digestion product was Spα50–154, Spα50–155, or Spα50–158, with calculated molecular masses of 12.5, 12.6, or 12.9 kDa, respectively. Thus the N-terminal sequence analysis of the elastase digestion product (12.3 kDa) of Spα1–167 suggests that AA1 is either 50, 51, or 52. If AA1 is 50 or 51, then the first stable structural domain of human α-spectrin is Spα50–154 or Spα51–155, since the first motif of α-spectrin comprises 105 amino acid residues (20), with a molecular mass of 12.5 or 12.6 kDa, respectively. However, we found that the elastase digestion product of Spα49–155, a peptide that encompasses both 50–154 and 51–155, did not produce any peptides with stability similar to those found in the digestion products of the other peptides that we studied. This piece of evidence suggested that the C-terminal residue of the first structural domain was beyond 155. We conclude that the C-terminal residue is 156 and AA1 is 52, giving a stable structural domain of Spα52–156. Based on this conclusion, we suggest that Spα1–167 contains a fully folded structural domain which is able to resist elastase digestion. Elastase will only digest Spα1–167 at sites prior to 52 and after 156 to give the digestion product Spα50–157, with a molecular mass of 12.3 kDa. As discussed above, the digestion product of Spα1–167 appeared as a band at 12.3 kDa (Fig. 2*A*, *lane 3*).

The N-terminal sequence of the 16.0-kDa fragment was found to be ETASSIQERR, which aligned perfectly with residues 19–28 in the α-spectrin sequence. The elastase cleavage sites around this region of α-spectrin consist of 17 (Val), 18 (Leu), and 21 (Ala). The possible peptides were Spα19–154, Spα19–155, Spα19–157, or Spα19–158, with calculated molecular masses of 16.3, 16.4, 16.6, or 16.7 kDa, respectively. We suggest that the peptide is either Spα19–157 or Spα19–158. This peptide probably contains Spα19–51, the so-called "helix 3" (12), which is also called "helix A" (11), plus the first stable structural domain Spα52–156 and residue 157.

We also performed chymotrypsin digestion to obtain independent digestion products from Spα1–167. Chymotrypsin has cleavage sites different from those of elastase. After 90 min of chymotrypsin digestion (at a weight ratio of 1:400), the single band of Spα1–167 at 21.0 kDa (Fig. 3*B*, *lane 2*) resulted in 6 bands at or below 21.0 kDa, with the smallest one at about 14.5 kDa (*lane 3*). The top band at 21.0 kDa was assumed to be undigested Spα1–167, while the other five were chymotrypsin digestion products of Spα1–167. The N-terminal sequence analysis of the smallest band at 14.5 kDa revealed an amino acid sequence of KERVAE, which aligned perfectly with positions 39 (Lys) through 44 (Glu) of α-spectrin. The chymotrypsin cleavage sites around this region consisted of 35 (Tyr), 38 (Phe), and 53 (Tyr), and the cleavage sites at the C-terminal end of this peptide consisted of 140 (Trp), 160 (Phe), and 163 (Tyr). We suggested that the chymotrypsin digestion product at 14.5 kDa was Spα39–160, which has a calculated molecular mass of 14.5 kDa. It is also possible that the digestion product was Spα39–163, with a calculated molecular mass of 14.9 kDa. The chymotrypsin digestion data indicate that AA1 is between residues 39 and 53, which supports the conclusion obtained from the elastase digestion data to indicate that AA1 is 52. Thus our experimental results indicate that the first amino acid in the human α-spectrin structural domain is 52 (Ser), which corresponds to a downstream phase-shift of 29 amino acid residues from the sequence motif reported by Speicher and Marchesi (20).

It is interesting to note that when we carefully studied the sequences published in the literature on work that reportedly used the Winograd phase-shift (13, 16, 17) to prepare recombinant peptides, we found that MacDonald *et al.* (16) had used a phase-shift corresponding to residue 49 (Leu) as AA1, whereas Byers *et al.* (17) and Yan *et al.* (13) had used a phase-shift corresponding to 52 (Ser). We had mentioned earlier that in a modeling study, Parry *et al.* (21) identify the starting position of the first structural motif as residue 46, with their numbering system according to Speicher and Marchesi (20). Since this numbering system starts with amino acid 7 (Glu) as 1, this modeling study has also placed AA1 as 52. Thus, AA1 being 52 might have been suggested earlier without experimental evidence in the literature. However, since the phase-shifts were not clearly stated as 52 (Ser) in some of these studies (13, 17), different phase-shifts have been used to prepare recombinant spectrin peptides (16, 18, 19).

Our experimental studies show that amino acids 52–156 in human α-spectrin are folded as a stable structural domain. Preparation of this and related peptides for biophysical studies will provide us with detailed structural information on spectrin.

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VITA

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given the final approval by the committee with reference to content and form.

This dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dec. 4, 1995
Date

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