Mapping the Human Erythrocyte \( \beta \)-Spectrin Dimer Initiation Site Using Recombinant Peptides and Correlation of Its Phasing with the \( \alpha \)-Actinin Dimer Site*

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Jeanine A. Ursitti†, Leszek Kotulaś, Tara M. DeSilva, Peter J. Curtis, and David W. Speicher‡

From The Wistar Institute, Philadelphia, Pennsylvania 19104

Human erythroid spectrin dimer assembly is initiated by the association of a specific region near the N-terminal of \( \beta \)-spectrin with a complementary region near the C-terminal of \( \alpha \)-spectrin (Speicher, D. W., Weglarz, L., and DeSilva, T. M. (1992) J. Biol. Chem. 267, 14775-14782). Both spectrin subunits consist primarily of tandem, 106-residue-long, homologous, triple-helical motifs. In this study, the minimal region of dem,106-residue-long,homologous,triple-helicalmotifs.

14782. Both spectrin subunits consist primarily of tandem, 106-residue-long, homologous, triple-helical motifs. In this study, the minimal region of \( \beta \)-spectrin required for association with \( \alpha \)-spectrin was determined using recombinant peptides. The start site (phasing) for construction of dimerization competent \( \beta \)-spectrin peptides was particularly critical. The beginning of the first homologous motif for both \( \beta \)-spectrin and the related dimerization site of \( \alpha \)-actinin is approximately 8 residues earlier than most spectrin motifs. A four-motif \( \beta \)-spectrin peptide (\( \beta \)-1-4) with this earlier starting point bound to full-length \( \alpha \)-spectrin with a \( K_d \) of about 10 nM, while deletion of these first 8 residues reduced binding nearly 10-fold. N- and C-terminal truncations of one or more motifs from \( \beta \)-1-4 showed that the first motif was essential for dimerization since its deletion abolished binding, but \( \beta \)-1 alone could not associate with \( \alpha \)-monomers. The first two motifs (\( \beta \)-1-2) represented the minimum lateral dimer assembly site with a \( K_d \) of about 230 nM for interaction with full-length \( \alpha \)-spectrin or an \( \alpha \)-spectrin nucleation site recombinant peptide, \( \alpha \)18-21. Each additional motif increased the dimerization affinity by approximately 5-fold. In addition to this strong inter-subunit dimer association, interactions between the helices of a single triple-helical motif are frequently strong enough to maintain a non-covalent complex after internal protease cleavage similar to the interactions thought to be involved in tetramer formation. Analysis of hydrodynamic radii of recombinant peptides containing differing numbers of motifs showed that a single motif had a Stokes radius of 2.35 nm, while each additional motif added only 0.85 nm to the Stokes radius. This is the first direct demonstration that spectrin's flexibility arises from regions between each triple helical motif rather than from within the segment itself and suggests that current models of inter-motif connections may need to be revised.

The membrane skeleton of the human erythrocyte consists of a network of proteins that associates with the inner surface of the cell membrane and imparts remarkable structural integrity and flexibility to circulating erythrocytes. Spectrin is the major structural component of this specialized submembranous protein network. The basic functional unit of spectrin is a heterodimer formed by side-to-side, antiparallel association of a 280-kDa \( \alpha \) subunit with a 240-kDa \( \beta \) subunit. Spectrin dimers associate head-to-head to form tetramers, the predominant form of spectrin in the membrane skeleton. These tetramers cross-link short actin oligomers, an association modulated by band 4.1, to form a dynamic two-dimensional submembrane latticework. Other associated proteins include: ankyrin, adducin, calmodulin, tropomyosin, tropomodulin, and band 4.9 (for reviews, see Bennett and Gilligan (1993), Delau-ney and Dhermy (1993), Luna and Hitt (1993), Lunch and Heit (1992), Winkelmann and Forget (1993), and Lux and Palek (1995)).

Electron microscopy of spectrin dimers shows flexibility 100-nm long rod-like molecules with strong lateral association of the subunits near the physical ends of the rods and weak associations in the central region (Shotton et al. 1979). In contrast, dimers in situ are only about 30 nm in length (Ursitti et al., 1991). This ability of the spectrin molecule to shorten and extend as well as its flexibility are attributed to the series of homologous 106-residue segments or motifs initially identified by partial peptide sequence (Speicher and Marchesi, 1984) and confirmed by complete sequencing of cDNAs for the \( \alpha \) subunit (Sahr et al., 1990) and \( \beta \) subunit (Winkelmann et al., 1990). Analysis of spectrin motif conformational phasing (the boundaries for complete folding units) using recombinant proteins established the starting point of properly folded spectrin motifs at approximately positions 25-30 of the original sequence alignment (Winograd et al., 1991). Yan et al. (1993) recently determined the crystal structure of the 14th segment of Drosophila \( \alpha \)-spectrin, which directly confirmed the phasing predicted from the recombinant peptide phasing experiments as well as the triple helical conformation of the basic 106-residue spectrin motif.

Previous studies of lateral association of \( \alpha \) and \( \beta \) subunits (Morrow et al., 1980; Sears et al., 1986; Yoshino and Minari, 1991; Speicher et al., 1992) used mild trypsin digestion to dissect spectrin into a reproducible pattern of intermediate-sized peptides. The latter study used a HPLC1 gel filtration

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†Present address: University of Maryland School of Medicine, Dept. of Physiology, 655 W. Baltimore St., Baltimore, MD 21201.
‡Present address: Laboratory of Molecular Neurobiology, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY 10314.
§To whom correspondence should be addressed: The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Tel.: 215-898-3972; Fax: 215-898-0664.

1 The abbreviations used are: HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI, matrix-assisted laser desorption/ionization.
analyzed to analyze association of tryptic peptides with complementary spectrin subunits. These analyses showed that dimer assembly occurs very rapidly (within seconds) and that dimerization requires a specific region at the tail end of the subunits represented by the tryptic αV and βV domains (Speicher et al., 1992). These tryptic domains include most or all of the repetitive segments α19–21 and β1–4. Interaction of these regions is apparently the initial step of dimer assembly, which is followed by subsequent lateral association of additional α and β motifs. A mutation in α-spectrin in this region has been identified, αEEL, that affects dimer assembly (Alloisio et al., 1991; Wilmatte et al., 1993; Randon et al., 1994). When this mutation is present along with an elliptocytosis mutation on the same chain, symptoms of the elliptocytosis mutation are often silent or mitigated since the αEEL mutation will decrease incorporation of the mutated chain onto the membrane. In contrast, elliptocytosis mutations on the opposite allele from the LELY mutation will decrease incorporation of the dimer nucleation region recombinant peptides for proper polypeptide chain folding, dimer binding affinity, and hydrodynamic properties. These analyses show that the minimum β peptide for dimer assembly contained the first two homologous motifs, but not the actin binding domain, and each additional motif further increased dimer binding affinity. Analysis of hydrodynamic radii of these recombinant peptides provided the first direct demonstration that spectrin’s flexibility apparently resides in the connecting region between triple helical motifs rather than within the segment itself.

MATERIALS AND METHODS

Isolation of α-Spectrin Monomers—Spectrin was extracted from fresh human red cells within 24 h of collection and α-monomers were purified as described previously (Speicher et al., 1992) using a modification of the ion exchange purification initially developed by Yoshino and Marchesi (1984).

Design and Construction of β-Spectrin Expression Plasmids—Oligonucleotide primers were designed to amplify specific regions of the β-spectrin nucleation site from the cDNA by the polymerase chain reaction using Vent polymerase (New England Biolabs). Primers contained regions within 5 to 15 bases upstream and 5 to 15 bases downstream of the sequences for the G1 and G2 sites, respectively, to allow directional cloning of the insert into the pGEX-2T expression vector (Pharmacia Biotech Inc.).

Five β-spectrin nucleation site clones and one α-spectrin nucleation site clone were produced for this study. The specific oligonucleotide primers used are listed in Table I. Initially, the start site (phasing) for a clone encompassing the first four homologous β motifs, β1–4, used the codon for amino acid residue 301, which corresponds to the phasing reported by Winograd et al. (1991). Further analysis of the potential start site of this β1–4 peptide led to the production of another clone, β1–4*, which contains eight additional codons at the N terminus of the expressed peptide. Subsequent truncations of full motifs on the C-terminal and N-terminal ends of the β1–4 recombinant were prepared as described in Table I.

The entire α-spectrin nucleation site, encompassing repetitive motifs α18–21, was designed essentially as described above with the exception that the nucleotide sequence contained a BamHI restriction enzyme site. Therefore, the oligonucleotides used for polymerase chain reaction were designed to contain a BamHI site both at the 5’ and 3’ ends. This restriction enzyme creates the same overhang as BamHI thus allowing cloning into the BamHI site of pGEX-2T. A BamHI site near the 3’ end of the region to be amplified was apparently not changed by altering the spectrin sequence in the 3’ primer. This resulted in utilization of the stop codon of the pGEX-2T vector. A single motif α recombinant, α1 (residues 50–158), was prepared as described previously (Kotula et al., 1993).

All expression plasmids were transformed into the DH5α strain of Escherichia coli. Each construct was completely sequenced to verify the integrity of the recombinant vectors. The α- and β-spectrin cDNA clones were kindly provided by Dr. Bernard Forget (Yale University, New Haven, CT).

Expression and Purification of Recombinant Peptides—Overnight cultures of the cells were diluted 1:20 in LB medium containing 50 μg/ml ampicillin. Cells were grown to an optical density of 0.5–0.7 at 37°C before induction with 0.1 mM isopropyl β-D-thiogalactopyranoside. After induction, the cells were harvested by centrifugation. Cell pellets were stored at −80°C and thawed on ice just before use. Fusion proteins were purified as described previously (Kennedy et al., 1991) with minor modifications. Briefly, each 600 ml cell pellet was resuspended in 15 ml of lysis buffer (50 mM Tris, 50 mM diisopropyl fluorophosphosphate, 0.15 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1% Triton X-100, pH 8.0) and sonicated to lyse the cells. The supernatant of the lysed cells was collected by centrifugation. The β1–2 fusion protein was released into the supernatant after lysis and this supernatant could be loaded directly onto a reduced glutathione-Sepharose 4B column (Pharmacia). The fusion proteins of α18–21, β1–3, β1–4, β2–4, and β2–5 were primarily in inclusion bodies and were extracted in 25 ml of urea buffer (5 M urea, 50 mM Tris, 5 mM β-mercaptoethanol, 5 mM EDTA, 1 mM diisopropyl fluorophosphophate, 0.15 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 20% glycerol, 1% Triton X-100, pH 7.3) for 2–3 h followed by an overnight dialysis against 2 liters of PBS (126 mM NaCl, 10 mM sodium phosphate, 5 mM EDTA, 0.15 mM PMSF, 1% Triton X-100, pH 7.3). The recombinant fusion proteins were then purified on a glutathione-Sepharose 4B column. Peptides were cleaved from the GST molecule in the elution buffer (50 mM Tris, 10 mM reduced glutathione, pH 8.0) using bovine thrombin (Sigma). NaCl (final concentration 150 mM) was added to the elution buffer prior to thrombin digestion for the β1–4 and β1–3 fusion products to decrease the formation of secondary cleavage products. The ratio of thrombin to fusion protein and the potential advantage of rapid chain conformational changes determined empirical fusion protein concentration. The optimal thrombin cleavage conditions were: β1–4, 20 units/mg; β1–4*, 10 units/mg; β1–3, 2 units/mg; β1–2, 4 units/mg; β2–4, 1 unit/mg; and α18–21, 1 unit/mg for 3 h at 37°C. Cleaved peptides were purified by rechromatography on a glutathione-Sepharose column followed by HPLC gel filtration on two preparative (21.5 × 600 mm) TSK-gel columns (G3000SW + G2000SW) in series (Toso-Haas) in phosphate-buffered saline (126 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 0.15 mM PMSF, 0.05% sodium azide, pH 7.3). Analytical HPLC Gel Filtration Binding Assay—Spectrin α-monomers or the α18–21 peptides were mixed with purified recombinant nucleation domain of α- and β-spectrin at a ratio of 1:1 at 0°C for different times ranging from 5 min to 15 h. Under most conditions equilibrium was reached at 25 min for most binding assays. For most binding assays to determine Stokes’ radius, the proteins and peptides were separated on two analytical (7.8 × 300 mm) TSK-gel columns (G3000SW + G2000SW) at 4°C with a flow rate of either 0.4 or 0.8 ml/min. Binding experiments used only the G3000SW column at 1.0 ml/min at 4°C in phosphate-buffered saline buffer. Eluted proteins were detected by absorbance at 280 nm and intrinsic tryptophan fluorescence (excitation 280 nm, emission filter 370 nm) and were quantified on a data acquisition system (PE Nelson Analytical) using peak area. Response factors for each protein were determined by replicate injections of known quantities (determined by quantitative amino acid analysis) for each component. Molecular weights used for calculations of purity were: α-monomer, 280,000; α1–4, 52,058; β1–2, 52,964; β1–3, 40,374; β1–2, 27,998; β1–3, 15,681; β2–3, 38,924; α18–21, 51,938. Association (Kd) and dissociation constants (Kd) for binding of β-spectrin nucleation region peptides with intact α-spectrin monomers were determined by calculating the amount of unbound peptide relative to control samples under the same conditions.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed using 7 and 10% slab gels (1.5 × 100 mm) as described previously (Laemmli, 1970). For low molecular weight components, electrophoresis was performed using 7.5% slab gels (18% acrylamide, 0.375% agarose) with a 2-mm thick stacking gel. After separation by SDS-polyacrylamide gel electrophoresis, proteins were detected by absorbance at 280 nm and intrinsic tryptophan fluorescence (excitation 280 nm, emission filter 370 nm) and were quantified on a data acquisition system (PE Nelson Analytical) using peak area. Response factors for each protein were determined by replicate injections of known quantities (determined by quantitative amino acid analysis) for each component. Molecular weights used for calculations of purity were: monomer, 280,000; α1–4, 52,058; β1–2, 52,964; β1–3, 40,374; β1–2, 27,998; β1–3, 15,681; β2–3, 38,924; α18–21, 51,938. Association (Kd) and dissociation constants (Kd) for binding of β-spectrin nucleation region peptides with intact α-spectrin monomers were determined by calculating the amount of unbound peptide relative to control samples under the same conditions.
three helices represent turn regions. The recombinant peptides are
unit defined by crystallography (Yan et al., 1993). Spaces between the
spectrin dimer). Non-spectrin amino acids introduced at the ends of
the right margin (see above, "and"

Design and Characterization of β-Spectrin Nucleation Site
Recombinant Peptides—As noted above, previous results using
spectrin peptides from mild protease cleavage of purified spectrin
monomer mapped the spectrin dimer nucleation site to
approximately the last three to four homologous motifs of the α
subunit and the first four homologous motifs of the β subunit
(Speicher et al., 1992). To determine the minimal nucleation
site requirements of β-spectrin as well as physical properties of
this region, a series of recombinant peptides were produced by
truncating the previously defined β-spectrin nucleation region
(β1–4) at either the N terminus or the C terminus. In addition,
an α-spectrin nucleation site peptide was constructed that in-
cluded the entire putative nucleation site region on the α sub-
unit, α18–21. The sequence content of each peptide is shown
diagrammatically in Fig. 1A and their relationship to the over-
al motif structure of a spectrin dimer is shown in Fig. 1C. As
illustrated, the phasing of each recombinant peptide correlates
with the boundaries of homologous triple helical motifs as
defined by the high resolution structure of a single motif de-
termined by Yan et al. (1993) with the exception of the "+" series of β-peptides, which begin 8 residues before the pre-
dicted repetitive segment (see below).

In addition to the recombinant constructs shown in Table I
and Fig. 1A, the β1+ peptide was isolated as a proteolytic
by-product of the β1–2* construct. During purification of the
β1–2* peptide, a highly specific cleavage product was observed
due to cleavage at residue 425 (see Fig. 1A) as defined using
MALDI mass spectrometry which yielded an experimental
mass after removing the GST moiety by thrombin cleavage
of 15,688 Da (data not shown). This proteolytically produced
peptide contained the entire β1+ motif with only a few additional
residues on the C-terminal end (see Fig. 1A), was native as
shown by circular dichroism and gel filtration, and was easily
separated from the parent β1–2* peptide by gel filtration.

All recombinant peptides were expressed in bacterial cells as
fusion proteins with glutathione S-transferase (GST), which
was cleaved and separated from the recombinant proteins prior
to use for functional studies. The purity of all peptides was
>95% (Fig. 1B). The N termini of the cleaved peptides were
confirmed by N-terminal sequence analysis and masses were
confirmed by MALDI mass spectrometry (data not shown). The
β1–2*, respectively, on a 10% Laemmli gel; and lane 8 (2 μg/lane), β1+
on a 5–15% linear gradient Tricine gel. C, arrangement of the structural
motifs in an anti-parallel spectrin dimer. The β subunit is comprised of
an actin binding domain (ABD), 17 homologous motifs (numbered rec-
tangles), and a small non-homologous phosphorylated C-terminal do-
main (solid squiggle). The α subunit is comprised of an N-terminal
partial and 20 full homologous motifs (motifs 1–9 and 11–21), an SH-3
type motif (motif 10), and a non-homologous C-terminal region consist-
ing primarily of two EF-hand type motifs (diamonds).
The β1-4 construct was difficult to cleave from the GST moiety and a substantial amount of a secondary cleavage product was produced (lane 4, Fig. 1B). N-terminal sequence analysis of this smaller peptide showed that it resulted from a secondary cleavage after Arg\(^{374}\), which is located in the turn between helices B and C of motif β1. The full-length β1-4 peptide and its cleavage product could not be separated by either ion exchange or gel filtration chromatography due to their similar size, charge, and other physical properties.

Proper Phasing Results in a Less Constrained Conformation of the β1-4 Peptide—The unusual difficulty encountered with thrombin cleavage of the GST-β1-4 construct relative to other GST fusion proteins containing complete spectrin motifs (see below) led to further evaluation of the β1 start point. To examine the relationship between the longer α-actinin and spectrin nucleation region motifs relative to the more common 106-residue motif, the last four α-spectrin (α18-21), the first four β-spectrin (β1-4), and the four spectrin-type motifs of human cytoskeletal α-actinin were aligned against numerous 106-residue spectrin motif sequences using the computer program ALIGN to perform optimized pairwise alignments. Gaps and insertions of the longer motifs relative to the 106-residue motif and its associated crystallographic structure were placed (Fig. 2A) in the most frequently aligned position from the individual pairwise comparisons. Two observations emerged from this alignment. First, all insertions larger than a single residue mapped to two regions, an 8-residue segment near the beginning of the motif that probably extends the A helix as previously suggested (Viel and Branton, 1994), and variable length insertions that map to the turn region between helices B and C. Second, a number of prolines are relocated in positions that were previously suggested (Viel and Branton, 1994), and variable length insertions of the longermotif relativetothe106-residuemotif were analyzed in parallel with the most typical 106-residue motif, since the three C-terminal α-actinin motifs (A2, A3, and A4) and their most homologous spectrin counterparts (β2, α20, and α21, respectively) all contained an extra 8 residues in this region. This prediction of a start site for the first motif that is 8 residues earlier than the initial β1-4 recombinant compares favorably with the observed mild protease cleavage sites for both α-actinin and β-spectrin as shown in Fig. 2B. In addition, although various start sites have been reported for the α-actinin motif based on alignments of sequences, the revised start site presented here for the first motif of spectrin and α-actinin agrees with the recent phasing analysis of α-actinin using recombinant peptides reported by Gilmore et al. (1994).

To further evaluate the phasing of the first β motif, a β1-4 recombinant peptide, which started at residue 293 compared with residue 301 for the β1-4 peptide, was produced and analyzed in parallel with the β1-4 peptide. Thrombin cleavage of the β1-4 and β1-4 fusion proteins differed markedly at physiological ionic strength. The β1-4 fusion protein was efficiently cleaved without production of secondary cleavage products. In contrast, under the same conditions, <50% of the β1-4 protein was cleaved and a prominent secondary cleavage product was formed (data not shown).

### N-Terminal Phasing for the β1-4 Peptide

| Peptide  | Codons   | Amino acids | 5’-Primer | 3’-Primer |
|----------|----------|-------------|-----------|-----------|
| β1-4     | 996–2324 | 301–743     | AGAGGATCCTCATTGCCGACAGGAAGG | GATGAATCCTTACCTGACATCTGGAG |
| β1-4     | 972–2324 | 293–743     | TGCGTACGCTTATGGACATGCCATTG | GATGAATCCTTACCTGACATCTGGAG |
| β1-3     | 972–2006 | 285–637     | TGCGTACGCTTATGGACATGCCATTG | GATGAATCCTTACCTGACATCTGGAG |
| β1-2     | 972–1679 | 293–528     | TGCGTACGCTTATGGACATGCCATTG | GATGAATCCTTACCTGACATCTGGAG |
| β2-4     | 1338–2324| 414–743     | AGAGGATCCTCATTGCCGACAGGAAGG | GATGAATCCTTACCTGACATCTGGAG |
| α18-21   | 5638–6963| 1818–2259   | GAAGATCTCTAGAATCTGCATTC | TGAAGATCTAGACCTAGTTGTGTCCTCAG |

### N-Terminal Phasing for the β1-4 Peptide

- For functional analysis of recombinant peptides from the β-Spectrin Dimer Nucleation Site Region—In order to identify the minimal requirements for the β-spectrin nucleation site and the effects of additional motifs on binding affinity, the recombinant β peptides were evaluated in solution binding assays with either purified native α-spectrin monomers or recombinant α18-21. Time course experiments showed that binding equilibrium was usually reached within 5 min or less under most concentrations and molar ratios evaluated. Therefore, protein mixtures were routinely incubated for 25 min prior to measurement of complex formation using a rapid HPLC gel filtration separation as shown in Fig. 5. Free α-monomers could not be resolved from complexes due to the small change in size when the complex was formed. Therefore, carefully quantified amounts of α-monomers and recombinant peptides were combined and association constants were determined by measuring the loss of recombinant peptide from its normally eluting position relative to an identical control without α-monomer. Control experiments showed that only equimolar binding occurred and that any dissociation of complex that occurred during the analysis did not increase the area of the unbound recombinant peptide peak. As shown in Fig. 5, no detectable binding was observed for the β1+ β2-4 peptides.

### N-Terminal Phasing for the β1-4 Peptide

- In addition, no binding to α18-21 monomers was detected for these two proteins when as much as a 3- or 5-fold molar excess of recombinant peptide was used. The β1+ peptide, although required for nucleation site binding, is apparently not sufficient.

### Binding Affinities of the Recombinant β Peptides with α-Monomers

The binding affinities of the recombinant β peptides with α-monomers are summarized in Table II. The importance of N-terminal phasing for the first β motif is illustrated by the observation that the β1-4 peptide has nearly a 10-fold lower affinity for α-monomers compared with the 8-residue longer β1-4 peptide. The minimum dimerization site contains the...
first two motifs, which has a $K_d$ of about 230 nM. Each additional motif contributes to the affinity of the complex apparently through low affinity lateral pairing of additional motifs and a 4-motif nucleation site peptide has a $K_d$ of about 10 nM.

The lateral association of recombinant peptides with $\alpha$-monomers is readily reversible. As shown in Fig. 6, both the high affinity $\beta_1$-$4$ peptide and the lower affinity $\beta_1$-$2$ peptides can compete with each other for binding to $\alpha$-spectrin monomers. $\beta_1$-$2$ was also able to compete with $\beta_1$-$3$ for binding to $\alpha$-spectrin (data not shown).

The Actin Binding Domain Is Not Required for Dimer Assembly and Does Not Substantially Contribute to Dimer Affinity—As illustrated above, high affinity dimers can readily form without the presence of the N-terminal actin binding domain. To further evaluate whether the actin binding domain may contribute positively or negatively to dimer assembly, binding measurement of intact $\alpha$ subunit to the $\alpha_1$-$2$ recombinant peptide was performed. The $K_d$ for this interaction between an intact $\beta$ subunit and a 4-motif $\alpha$ peptide is about 15 nM which compares favorably with the $K_d$ of 10 nM observed for a 4-motif $\beta$ peptide interaction with intact $\alpha$-monomers.

Noncovalent Associations between Helices within a Single Motif Are High Affinity Interactions That Frequently Maintain Functional Complexes—As described above, the dramatic reduction in affinity of the $\beta_1$-$4$ peptide (8-residue shorter N-terminal) compared with $\beta_1$-$4$ suggested that additional truncation of the $\beta_1$ motif would further reduce or abolish binding. In this context, an apparently inconsistent observation was that the 43-kDa peptide, the secondary cleavage product of the $\beta_1$-$4$ recombinant, did show detectable binding to $\alpha$-monomers. In the experiment shown in Fig. 5 B (lane 3), a faint 43-kDa band was observed on the original gel and some preparations of the $\beta_1$-$4$ peptide showed even more extensive binding of the 43-kDa band to $\alpha$-monomer than the illustrated experiment. As noted above, N-terminal sequence analysis of this peptide showed that it was cleaved at residue 374 and...
lacked helices A and B of the β1 motif. It was therefore quite surprising that this truncated form of the molecule could effectively compete with a larger amount of intact β1-4, which was always present in these preparations, for association with α-monomers.

Further analysis of these samples by Tricine gel electrophoresis detected a band at about 8 kDa. Similarly, samples that were initially separated by HPLC gel filtration still contained the 8-kDa peptide. MALDI mass spectrometry of the peptide mixture confirmed that the 8-kDa (observed mass = 8,733.8 Da versus expected mass for GS 501–374 = 8,725 Da) and 43-kDa fragments (observed mass = 43,398.3 Da versus expected mass for 375–743 = 43,379 Da) were produced by a single protease cleavage at residue 374 and that the 43-kDa fragment had an intact C-terminal. Since the expected 0.1% error of this technique is less than a single amino acid residue mass, this method reliably defines the C-terminal boundary of proteins with known sequences when the N-terminal has been determined by sequence analysis.

FIG. 3. CD spectra of α-monomer and representative recombinant peptides. —, α-monomer (0.47 mg/ml); —, β1-4 (0.33 mg/ml); - - - - , β1-3 (0.47 mg/ml); and — —, β1-2 (0.38 mg/ml). All proteins were dialyzed into isotonic buffer and protein concentrations were determined by quantitative amino acid analysis prior to CD measurements. Mean residue ellipticity [θ]_200 is expressed in degree cm²/dmol.

FIG. 4. Hydrodynamic properties of recombinant β-spectrin nucleation site peptides. The Stokes’ radii of β-spectrin recombinant peptides were determined by HPLC gel filtration. Peptides include β1-4 (▲), β1-4* (+), β1-3* (+), β2-4 (●), β1-2* (+), β1* (+), and α1 (○) with duplicate determinations shown for β1-4*. The α1 motif peptide (residues 50–158) is described in Kotula et al. (1993). The more compact β1-4 peptide was not included in the linear regression plot. Slope = 0.85, y intercept = 1.5, R² = 0.9994.

A previous report from our laboratory using peptides produced by mild proteolysis showed that spectrin dimers assembled like a zipper with initiation of the process occurring near the tail end (actin binding end) of the molecule (Speicher et al., 1992). In the present study, we further characterized dimer nucleation using β recombinant peptides. The primary structure and conformational integrity of these recombinant peptides were confirmed by full-length DNA sequencing, N-terminal sequencing of the cleaved peptide, mass spectrometry, and circular dichroism measurements to ensure that the peptides were free of polymerase chain reaction-based mutations and properly folded. Therefore, the observed differences in dimer assembly properties of the N-terminal and C-terminal truncations of the β-spectrin nucleation site represent functionally important findings.

The minimum β peptide capable of dimerizing to the α subunit contains the first two homologous motifs (β1 and β2), and each additional motif (β3 and β4) increases the binding affinity approximately 5-fold, apparently by forming additional lower affinity lateral associations with a complementary motif in the α-monomer (Table II). Although direct binding affinity measurements have only been made here on recombinant peptides with lengths up to 4 motifs, dimer affinity continues to increase with each additional motif as it laterally pairs with its complementary partner throughout the length of the two subunits (Speicher et al., 1992). This size dependent increase in dimerization affinity is apparently due to formation of additional lateral associations outside the first 4 motifs since preferential dimerization of larger peptides is not observed when either nucleation site 4-motif recombinant fusion protein (GST-β1-4* or GST-α18–21) is used instead of the complementary monomer (Speicher et al., 1992) and data not shown).

The β1 motif is required for dimerization, but is insufficient for high affinity association as shown by the loss of dimer formation capacity of the native β2-4 and the β1-3 recombinant peptides (Fig. 5). In addition, these experiments showed that the precise phasing (starting point) of the β1 motif had a critical effect on both binding affinity (Fig. 5 and Table II) and molecular shape (Fig. 4). The appropriate N-terminal boundary of this motif is different from the phasing that applies to the more common 106-residue spectrin-type motif.

This altered start site for the β1 motif and its structural and functional importance had not been previously identified. The
presence of an additional 8 amino acids relative to the 106-residue motifs had been proposed near the beginning of erythrocyte spectrin motifs a20, a21, and b2 (Sahr et al., 1990; Winkelmann et al., 1990) and near the beginning of Drosophila spectrin motifs a20, a21, and b2 (Vieland Branton, 1994). The corresponding start site for the closely related first α-actinin motif had not been clearly defined, with reported possibilities ranging from α-actinin residue 245 to 266 (Baron et al., 1987; Imamura et al., 1988; Blanchard et al., 1989). The difficulty encountered in cleaving a b1–4 fusion protein using the phasing defined for 106-residue spectrin-type motifs and further consideration of the locations for mild protease cleavage sites for b2-spectrin and α-actinin (Fig. 2B) suggested that both the first b motif and the first α-actinin motif may have an extra 8 residues in the first helix as suggested by the alignment in Fig. 2A. The resulting improved thrombin cleavage of the fusion protein, loss of secondary cleavage site, larger solution molecular shape, and nearly 10-fold higher binding affinity of a recombinant protein with an additional 8 residues on the N-terminal supported this hypothesis. It is particularly striking that the dimerization affinity of the 8-residue shorter b1–4 peptide has an order of magnitude lower binding affinity for α-monomers compared with the b1–4*1 peptide and its affinity is even lower than the b1–3*1 peptide. The motif phasing determined experimentally for the β1 motif and inferred for the first α-actinin motif in this study is consistent with the α-acti-
nin motif start site recently determined by Gilmore et al. (1994) using recombinant peptides.

The nomenclature for spectrin motifs used in this study is as described by Winkelmann et al. (1990) where only homologous \( \beta \) motifs are numbered from 1 to 17 (see Fig. 1C). A recent publication describing dimer assembly of Drosophila spectrin (Viel and Branton, 1994) uses an alternate nomenclature where the actin binding domain is designated as the first segment of \( \beta \)-spectrin. Although each nomenclature has its merits, the nomenclature used by Winkelmann et al. (1990) for human erythroid spectrin has been more frequently cited and is used here.

The largest discrepancy between the present study and the qualitative evaluation of Drosophila spectrin dimer assembly (Viel and Branton, 1994) is that the non-homologous regions after the \( \alpha 21 \) motif (EF-hand motifs) and before the \( \beta 1 \) motif (actin binding motif) of Drosophila spectrin were found to be required for high affinity dimer assembly. The corresponding regions of human erythroid spectrin are clearly not required for dimer assembly since the \( \beta 1-4 \) recombinant could associate with either \( \alpha \)-spectrin monomers or an \( \alpha 18-21 \) recombinant protein with a \( K_d \) of approximately 10 nM and intact \( \beta \)-monomers bind to the \( \alpha 18-21 \) recombinant with a similar affinity. Although these experiments do not rule out a direct interaction of the \( \alpha \) EF-hand motifs with the \( \beta \)-spectrin actin binding domain, this potential interaction is clearly not required for initiation of dimer assembly as demonstrated in this study. In addition, the possible interaction between the EF-hand motifs and the actin binding domain would be expected to be a low affinity interaction since the actin binding domain is quickly cleaved from dimers with trypsin and does not remain covalently bound to the intact \( \alpha \) subunit (Speicher et al. (1992) and data not shown). It is particularly surprising that the Drosophila \( \beta 1-4 \Delta 288 \) peptide is inactive since it corresponds closely to our \( \beta 1-3 \) recombinant, which has a \( K_d \) of about 38 nM. These differences could reflect a species and/or tissue-specific isoform difference since Drosophila spectrin is more closely related to the human brain spectrin (fodrin) isoform than to erythroid spectrin. Also, the Drosophila recombinant peptides, which were produced in a reticulocyte lysate system and not purified to homogeneity, had unknown conformational integrity. Some nonfunctional Drosophila peptides may not have folded properly or were not stable enough to retain binding activity in the presence of SDS used in the immunoprecipitation buffer. It should be noted that Lombardo et al. (1994) cited substantial difficulties in preparing stable, native peptides of brain \( \beta \)-fodrin that contain only a portion of the actin binding domain. Since the present study shows that the phasing of the \( \beta 1 \) motif has a dramatic effect on dimer binding affinity, it is most likely that some Drosophila \( \beta \) peptides were too short and other peptides may not have correctly folded as suggested by the observations of Lombardo et al. (1994).

Thrombin cleavage of the improperly phased \( \beta 1-4 \) fusion protein led to the interesting observation that interactions between helices within a single conformational motif are strong enough to retain noncovalent complexes during extensive dialysis or gel filtration chromatography ("Results"). Similar noncovalent associations within a triple helical motif were observed between adjacent peptides produced by mild trypsin treatment from both the \( \alpha \) (DiPaolo et al., 1993) and \( \beta \) subunits (Speicher et al., 1992) suggesting that, as a general rule, inter-helix interactions within triple helical spectrin-type motifs are very high affinity interactions. These helix A-B \( \leftrightarrow \) C interactions may be similar to the interaction between incomplete motifs of the \( \alpha \) and \( \beta \) subunits that form the tetramer binding site (Tse et al., 1990; Speicher et al., 1993; Kotula et al., 1993; Parquet et al., 1994; Kennedy et al., 1994).

Comparison of Stokes radii of recombinant proteins used in this study provided a unique opportunity to evaluate the relative contributions of individual motifs and the connecting regions between motifs to the molecular shape in solution. This comparison is of particular interest since the recent crystallographic model of a single spectrin motif predicts that adjacent motifs are linked by a single long helix formed by continuing the C helix of the first motif into the A helix of the next motif (Yan et al., 1993). In this model, the substantial molecular flexibility associated with spectrin might arise from dynamic rearrangements within the triple helical bundle and/or could involve a non-apparent disruption of the helix between motifs. Alternatively, adjacent motifs might be connected by a flexible, non-helical linking region as suggested by some earlier models. Measurement of the Stoke's radii of two single motif peptides, the non-nucleation site \( \alpha 1 \) peptide and the nucleation site \( \beta 1 \) peptide, showed a disproportionately high contribution of the first motif to the molecular shape (2.35 nm), while each additional motif contributes only about 0.85 nm to the molecular size. These results strongly suggest that individual motifs are relatively rigid in solution as might be expected for triple helical bundles with strong inter-helix interactions (see above). The substantially smaller and uniform incremental increase with each additional motif suggests that there is substantial flexibility in the connection or interface between motifs that allow the motifs to pleat and fold as suggested by models (Bloch and Pumplin, 1992) derived from electron microscopic evidence (Shotton et al., 1979; Byers and Branton, 1985; Shen et al., 1986; Liu et al., 1987; Ursitti et al., 1991). These data suggest that the current model of spectrin structure where adjacent segments are connected by a long helix should be re-evaluated.

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Jeanine A. Ursitti, Leszek Kotula, Tara M. DeSilva, Peter J. Curtis and David W. Speicher

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