Initiation of Spectrin Dimerization Involves Complementary Electrostatic Interactions between Paired Triple-helical Bundles*

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The spectrin heterodimer is formed by the antiparallel lateral association of an α and a β subunit, each of which comprises largely a series of homologous triple-helical motifs. Initiation of dimer assembly involves strong binding between complementary motifs near the actin-binding end of the dimer. In this study, the mechanism of lateral spectrin association at this dimer nucleation site was investigated using the analytical ultracentrifuge to analyze heterodimers formed from recombinant peptides containing two or four homologous motifs from each subunit (α20–21/β1–2; α18–21/β1–4). Both the two-motif and four-motif dimer associations were weakened substantially with increasing salt concentration, indicating that electrostatic interactions are important for the dimer initiation process. Modeling of the electrostatic potential on the surface of the α20 and β2 motifs showed that the side of the motifs comprising the A and B helices is the most favorable for association, with an area of positive electrostatic potential on the AB face of the β2 motif opposite negative potential on the AB face of the α20 motif and vise versa. Protease protection analysis of the α20–21/β1–2 dimer showed that multiple trypsin and proteinase K sites in the A helices of the β2 and α21 motifs become buried upon dimer formation. Together, these data support a model where complementary long range electrostatic interactions on the AB faces of the triple-helical motifs in the dimer nucleation site initiate the correct pairing of motifs, i.e. α21–β1 and α20–β2. After initial docking of these complementary triple-helical motifs, this association is probably stabilized by subsequent formation of stronger hydrophobic interactions in a complex involving the A helices of both subunits and possibly most of the AB faces. The β subunit A helix in particular appears to be buried in the dimer interface.

Members of the spectrin family of membrane skeleton proteins are widely expressed in vertebrates as well as in lower organisms. In erythrocytes, spectrin is the major component of the membrane skeleton, a network of spectrin oligomers cross-linked with short actin filaments that is bound to the membrane and provides cell membrane stability. The most common form of spectrin on intact cell membranes is the tetramer, which is formed by the “head-to-head” association of two elongated heterodimers, each comprising an α subunit and a β subunit with molecular masses of 280 kDa (1) and 246 kDa (2), respectively. Both the α and β subunits consist largely of a series of homologous motifs, each approximately 106 residues in length (3). The tertiary structure of these motifs is a triple-helical bundle, as determined by x-ray crystallography (4) and NMR spectroscopy (5). Head-to-head tetramers form by binding of complementary partial motifs at the ends of the α and β subunits to form a complete triple-helical bundle (6–8).

The antiparallel lateral association of the α and β subunits to form a heterodimer is initiated near the actin-binding end of the molecule at a dimer nucleation site, where complementary motifs from each subunit form a high affinity association (9–11). A minimum of two motifs from each monomer (α20–21 and β1–2) is thought to be necessary for association (11), although it has been proposed that small sections of the non-homologous domains at the tail end of the dimer are also required (10). Between β1 and β2, and between α20 and α21, are 8-residue inserts that are not part of the typical 106-residue motif. Increasing or decreasing the length of these inserts abolished binding, indicating that the relative register of the two motifs from each subunit is important for association (10). Larger recombinant peptides containing additional homologous motifs exhibited stronger binding, indicating that each additional laterally associated pair of motifs contributed to the overall binding affinity between the subunits (11). This finding is consistent with an earlier study (9), which proposed that spectrin dimers assemble in a zipper-like mechanism, in which the initial binding at the nucleation site is followed by weaker binding along the length of the dimer.

The nature of the molecular forces that contribute to the lateral association of α and β subunits is unknown. Although the motifs required for binding at the nucleation site have been identified, the specific regions on the surfaces of these triple-helical motifs that bind to the complementary subunit and the nature of the molecular interactions has not been elucidated. Similarly, the associations between non-nucleation motifs further along the subunit that moderately increase dimer affinity are poorly understood. Determining the nature of lateral αβ associations should contribute to our understanding of spectrin’s structural and functional properties, including the basis of spectrin’s elasticity, the apparent differences in the morphology and self-association of erythroid and nonerythroid isoforms, and the possible effects of the subunits on each other during interactions with other molecules. For example, lateral interactions between the subunits may be involved in the down-regulation of brain spectrin self-association by calpain I and Ca2+/calmodulin (12).

The ionic strength-dependent dissociation of erythroid and brain spectrin oligomers to dimers and then to monomers (13, 14) indicates that electrostatic interactions play an important role in the lateral association of the intact subunits. However,
it is not known whether electrostatic interactions are directly involved in lateral associations, or indirectly involved via control of subunit flexibility, possibly via electrostatic interactions between consecutive motifs. Furthermore, it is not known whether electrostatic interactions are important for the initial association at the dimer nucleation site or for a subsequent step in dimer assembly, such as the weaker non-nucleation site lateral interactions or closing the hairpin loop at the head end of the molecule. Spectrin subunits can also be dissociated at pH > 9.5, though this dissociation is accompanied by ~10% unfolding of the secondary structure (15).

In this study, we investigate the role of electrostatic interactions during spectrin dimer nucleation and identify the regions of the triple-helical motifs that contact the complementary motif on the paired subunit. The contribution of electrostatic interactions to the dimer nucleation site association was determined using sedimentation equilibrium analyses. Modeling of the electrostatic potential on the surface of two laterally opposed nucleation site motifs, as well as two opposed non-nucleation motifs, revealed regions of complementary electrostatic potential on the AB faces of the triple-helical bundles that were predicted to be the dimer interface region. Finally, this model was tested using protease protection analyses to probe the spectrin dimer nucleation site region before and after lateral association, in order to experimentally identify regions that are buried upon dimer formation.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—The following recombinant peptides were used in this study: a20–21 (motifs 20 and 21 of the subunit, comprising residues 2033–2259), α18–21 (motifs 18–21; residues 1818–2259), β1–2 (motifs 1 and 2 of the β subunit; residues 293–528), and β1–4 (motifs 1–4; residues 293–743). The nomenclature for spectrin motifs is that described by Winkelmann et al. (2), where β1 refers to the first homologous motif of the β subunit. The motifs described are structural motifs with the phasing of Yan association, in order to experimentally identify regions that are predicted to be the dimer interface region. Finally, this model was tested using protease protection analyses to probe the spectrin dimer nucleation site region before and after lateral association, in order to experimentally identify regions that are buried upon dimer formation.

**Expression and Purification of Recombinant Peptides**—The spectrin-GST molecules were expressed and purified as described except that β1–2, β1–4, and α18–21 were expressed at 30 °C, α20–21 was expressed at 18 °C, and all of the proteins were purified from the soluble fraction after cell lysis. Peptides were cleaved from the GST molecule using bovine thrombin (Sigma) at 37 °C for 4 h. The optimal thrombin cleavage conditions were: α20–21 and α18–21, 2 units/mg protein; β1–4, 17 units/mg; and β1–2, 8 units/mg. NaCl was added to a final concentration of 150 mM (β1–4) and 500 mM (β1–2) to decrease the formation of secondary cleavage products. The cleaved spectrin peptides were purified by rechromatography on a glutathione-Sepharose column. The α20–21 peptide was then separated from residual GST by anion-exchange chromatography on a 1-ml HiTrap-Q column (Amersham Pharmacia Biotech). The α20–21 was bound to the column in Buffer A (65 mM NaCl, 5 mM sodium phosphate, 2.5 mM EDTA, 75 μM PMSF, pH 7.3), washed with Buffer A, and eluted with Buffer B (1 mM NaCl, 5 mM sodium phosphate, 2.5 mM EDTA, 75 μM PMSF, 0.05% sodium azide, pH 7.3). The final purification step for all spectrin peptides was HPLC gel filtration on two preparative (21.5 × 600 mm) TSK-gel columns (G3000SW + G2000SW) in series (Toso Haas) equilibrated with Tris-buffered saline (20 mM Tris-Cl, 130 mM NaCl, 1 mM TCEP, pH 7.5). Fractions at the leading edge of the dimer peak (to ensure no contamination with monomers) were pooled and dialyzed for 24 h at 4 °C against Tris-buffered saline containing 0.3–1.0 M NaCl. Sedimentation equilibrium experiments were performed at 4 °C in an Oris Ultracentrifuge, using the weight-averaged molecular weight of the two peptides (21). At least three data sets from different loading concentrations and/or rotor speeds were fitted simultaneously.

1 The abbreviations used are: GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Tricine, N-tris(hydroxymethyl)methylglycine; MALDI, matrix-assisted laser desorption/ionization.

2 The program SEDNTERP was written by T. Laue, J. Hayes, and J. Philo and is available on the RASMB web site. NONLIN, MATCH, and REEDIT are available from the Analytical Ultracentrifugation Facility at the University of Connecticut via the FTP site.
All of the experimental data were fitted well by either an ideal single species model, or an ideal monomer-dimer model. Goodness of fit was determined by examination of the residuals and minimization of the variance. The association constants returned by NONLIN from the monomer-dimer fits were converted to the molar scale using either the calculated molar extinction coefficient of the peptide, for the absorbance data, or the sequence molecular weight of the peptide and a specific fringe displacement of 3.26 fringes (liters/g) for interference data (21).

**Molecular Modeling**—Models of the Drosophila a8, a20, and b2 motifs were constructed using the atomic coordinates of the a14 crystal structure. The structure of the monomer of the Drosophila a14 motif (Dx14) was derived from the crystal structure of the Drosophila a14 dimer as described by Yan et al. (4). The Dx14 model was then refined by constrained energy minimization with Discover® software (Biosym Technologies) using the all-atom constant valence force field as described above.

Calculations of electrostatic potential mapped to the accessible surface of the motif were performed using the program GRASP (23). The program maps a molecule onto a three-dimensional cubic grid, and calculates the electrostatic potential at each grid point using a finite difference solution to the nonlinear Poisson-Boltzmann equation (24, 25). The accessible surface area of the motifs was defined as the surface mapped out by the center of a probe of radius 1.4 Å rolled around the van der Waals surface of the protein. The ionic strength was 130 mM, and an ionic radius of 2.0 Å was used. The dielectric was 2 for the protein interior and 80 for the surrounding solvent. Charges were assigned to the ionized groups of Asp and Glu (−1) and Lys and Arg (+1). Histidine and termini residues were neutral.

**RESULTS**

*Salt Dependence of α/β Lateral Association*—The salt dependence of the lateral association of two- and four-motif nucleation site recombinants was investigated using sedimentation equilibrium. Each of the four recombinants was tested alone, and in all cases the sedimentation equilibrium data were fitted well by a single ideal species model with an estimated molecular weight within 4% of the calculated sequence mass, demonstrating that each recombinant is stable and monomeric (data not shown). Experiments were then performed on the a20–21/β1–2 or α18–21/β1–4 heterodimers, in salt concentrations of 0.1–1.0 M. For all heterodimer samples, the data were described well by a model describing an ideal monomer-dimer reaction, enabling estimation of the association constant. Representative data are shown in Fig. 1. The salt dependence of each association is shown in Fig. 2 and compared with previously published data on the association of whole spectrin subunits (13). For both the two-motif and four-motif nucleation peptides, there is a marked weakening of the association with increasing salt that is substantially greater than the effect on whole erythroid spectrin subunits. The salt dependence of the lateral association is the same for the two-motif and four-motif heterodimers, although the strength of the association increases with the number of motifs. At all salt concentrations, the association of α18–21/β1–4 was about 3-fold stronger than that of α20–21/β1–2, consistent with the hypothesis that motifs other than those in the minimum nucleation site (α20–21/β1–2) make slight contributions to the strength of dimer assembly.

**Molecular Modeling of the Drosophila a14 Motif**—Models of the Drosophila a14 motif (Dx14) were derived from the crystal structure of the Drosophila a14 dimer as described by Yan et al. (4). The Dx14 model was then refined by constrained energy minimization with Discover® software (Biosym Technologies) using the all-atom constant valence force field as described above.

The electrostatic potential of a protein can be calculated using

![Graph](http://www.jbc.org/Downloaded from http://www.jbc.org/)
a numerical procedure to solve the Poisson-Boltzmann equation, such as that contained in the programs Delphi (24, 25) and GRASP (23). The weakening of lateral association between intact spectrin subunits and the nucleation site peptides with increasing ionic strength indicates that attractive electrostatic interactions between the subunits are likely to play an important role in lateral association. Identification of areas of complementary electrostatic potential on the surface of opposing motifs should help to identify the faces of the triple-helical motifs involved in subunit-subunit association.

In the present study, models of four Drosophila motifs that are laterally paired in the dimer, α20 and β2 (in the dimer nucleation site) and α14 and β8 (11), were constructed. Drosophila motif sequences were used because the models were based on the crystal structure of the Drosophila α14 motif (4). Although the nucleation motifs show reduced homology to the general spectrin motifs, and are closer to the motifs of α-actinin, alignment of the nucleation motif sequences with that of α14 shows that the homology of α20 and β2 to typical spectrin motifs is greater than that of the other nucleation motifs, α21 and β1 (10, 11). The hydrophobic residues in the a and d helix positions in the a-helices of α14, which form the core of the bundle (4), are conserved in α20 and β2. Furthermore, there are no insertions or deletions other than the 8-residue insert that occurs before the start of the predicted A helix of the motifs. The α20 motif contains 3 proline residues, but these are found at the beginning or end of the α-helices and should not greatly disturb the secondary structure. Thus, it is reasonable to assume that the α20 and β2 motifs will fold into a helical bundle similar to that seen in α14.

The electrostatic potential of the modeled motifs was calculated and mapped to the solvent accessible surface of the motifs using the GRASP program (23). As the spectrin motif structure is a bundle of three α-helices, with each helix aligned roughly parallel or antiparallel to the others, the surfaces of the motif can be divided into three faces, AB, BC, and CA, according to the helices that make up the face, as shown in Fig. 3. Fig. 4A shows the electrostatic potential on the three faces of the α20 and β2 motifs. The BC faces of both motifs (Fig. 4A, middle panels) have a predominantly negative charge and would be expected to repel each other. The potential on the CA faces is also not very complementary, as both motifs have negative potential in the center of the face and positive potential at the edges (Fig. 4A, bottom panels). In contrast, the AB face of the β2 motif has an area of strong positive potential that is complementary to an area of strong negative potential on the AB face of the α20 motif. The positive and negative potentials are directly opposite one another when the two motifs are in the antiparallel orientation (Fig. 4A, top panels). Similarly, the negative potential on the AB face of β2 is complementary and lies opposite to the positive residues on the α20 AB face. Hence, the most compatible pairing of faces on these two motifs is obtained by docking the AB face of α20 with the AB face of β2.

In order to examine a pair of motifs that is not part of the nucleation site, a model was constructed of the Drosophila β8 motif, which is predicted to be laterally paired to the α14 motif in the dimer (11). The electrostatic potential on the α14 and β8 motifs showed a similar trend to α20/β2 (Fig. 4B). In these motifs, both the BC and CA faces were strongly negative, making interaction between these faces unlikely. However, the AB face of α14 shows a strikingly different electrostatic potential, with a large area of positive potential in the middle of the face. Since all faces of β8 are negatively charged, the AB face of α14 is the only likely face of this motif to interact with the β subunit. While all faces of β8 are negative and complementary to this positive face, it is most likely that the interaction face of all laterally paired motifs would be consistent throughout the length of the subunit. Together, these modeling experiments predict that the most likely dimer interface involves association of the AB faces on both subunits.

**Protease Protection Experiments**—To experimentally test this model of the dimer interface region, limited proteolysis of α20–21 and β1–4 was performed before and after formation of the 1:1 αβ dimer complex to investigate changes in protease susceptibility upon association. Initially, pilot digests were performed with the proteases trypsin, chymotrypsin, proteinase K, elastase, and endoproteinase Glu-C, using various enzyme:substrate ratios between 1:5 and 1:200. Aliquots were taken at intervals between 5 and 90 min for analysis by Tricine SDS-PAGE. All enzymes could cleave the complex and individual components, and the more extreme digestion conditions for
most enzymes extensively degraded the proteins. However, usually only the first several cleavages yielded informative data concerning protected sites, since it rapidly became impossible to distinguish between alternative cleavage pathways and sites as cleavages accumulated. The trypsin and proteinase K digests showed the greatest differences between monomers and the dimer, and were selected for further analysis. Trypsin cleaves at the carboxyl side of lysine and arginine residues, while proteinase K has a broad specificity toward aliphatic, aromatic and other hydrophobic residues. For both proteases, the digestion pattern as well as the time dependence of the digestion observed by SDS-PAGE were highly reproducible between different sample preparations (data not shown). The digests were then scaled up in order to obtain sufficient quantities of digest products for analysis by reverse-phase HPLC, N-terminal sequencing, and MALDI mass spectrometry.

SDS-PAGE of representative preparative \( \alpha_{20} - 21 \)/\( \beta_{1} - 2 \) trypsin and proteinase K digests are shown in Fig. 5. Reverse-phase HPLC was also used to separate and quantify the proteolytic products. Changes in peptide yields of at least 2-fold were considered significant. Fig. 6 shows the HPLC chromatographs of \( \alpha_{20} - 21 \)/\( \beta_{1} - 2 \) dimers and the individual monomers digested with proteinase K. The HPLC fractions were analyzed by SDS-PAGE in order to correlate the HPLC peaks with the bands observed in Fig. 5. The N- and C-terminal boundaries of the proteolytic products were then identified. The N termini were determined by Edman sequencing of the fragments after SDS-PAGE followed by electroblotting to polyvinylidene difluoride membranes, or by directly sequencing the HPLC fractions. The molecular masses were determined by MALDI mass spectrometry of the HPLC fractions. The N-terminal sequence of each fragment combined with its molecular mass allows unambiguous identification of the C terminus, since the error in mass determination is less than one residue mass. All of the major proteolytic fragments were identified and are listed in Table I.

Fig. 7 maps the fragments onto the predicted secondary structure of \( \alpha_{20} - 21 \) and \( \beta_{1} - 2 \), based on their sequence homology with a typical spectrin motif, for which the structure has been solved (4). In both the tryptic and proteinase K digests, \( \alpha_{20} - 21 \) is fairly resistant to proteolysis under the conditions shown, both as a monomer and in a heterodimer complex, although a few informative \( \alpha \) fragments were observed. The \( \beta_{1} - 2 \) is readily cleaved into several major fragments, almost all of which begin at the N terminus of \( \beta_{1} \) and end at protease cleavage sites in the predicted A helix of the \( \beta_{2} \) motif, thus encompassing all of \( \beta_{1} \). The other two protease-resistant \( \beta_{1} - 2 \) fragments begin in the BC loop and the C helix of \( \beta_{1} \), respectively, and end near the C-terminal end of the peptide, thus including most of the \( \beta_{2} \) motif (Fig. 7A).

The change in the digestion pattern upon dimer formation was investigated qualitatively by comparing the SDS-PAGE bands and quantitatively by comparing the HPLC peak areas of the monomer digests with those of the dimer digest. The most prominent tryptic fragment of \( \alpha_{20} - 21 \), \( \alpha_{T1} \), involves a cleavage on the CA face of the \( \alpha_{21} \) A helix. This site is not protected upon dimer formation since it is produced in similar...
Cleavage sites occur in the A helix of the K, the cleavage pattern of changes substantially upon dimer assembly. With proteinase K digestion of the dimer. Fig. 7 shows the location of these protected products, P1 and P2, and the partial A helix is destabilized leading to this minor fragment. The susceptibility of both trypsin and proteinase K yield from monomers and the dimer complex. In contrast, the most prominent proteinase K peptides, aP1a and aP1b, are partially protected in the dimer, and these two cleavage sites map to the AB face of the a21 A helix (Fig. 7B).

The susceptibility of β1–2 to both trypsin and proteinase K changes substantially upon dimer assembly. With proteinase K, the cleavage pattern of β1–2 is qualitatively reproduced in the dimer digest; however, the decrease in cleavage products and corresponding increase in uncleaved β1–2 in the dimer demonstrates that β1–2 is more protease-resistant when associated with a20–21 due to protection of several cleavage sites in the dimer. Fig. 7 shows the location of these protected proteinase K cleavage sites in β1–2. In all cases the protected cleavage sites occur in the A helix of the β2 motif with most of the sites located on the AB face. One cleavage site, P4, is actually located in the interior of the triple helix motif. However, this fragment is a minor peptide in the digest of the β1–2 motif (see Fig. 5B, lane 4) that is likely to represent a secondary cleavage after the peptide is first cleaved to one of the major products, P1 and P2, and the partial A helix is destabilized leading to this minor fragment.

In the case of trypsin, the digestion pattern of β1–2 alone and in the dimer are again qualitatively similar, but there are both increases and decreases in the amounts of the various cleavage products after association. Before dimer formation, the major protease-resistant fragment of β1–2 is βT4, which comprises the whole β1 motif and ends in the A helix of β2, similar to the proteinase K fragments. After dimer association, there is a substantial reduction in the amount of βT4, while βT2 and βT3 become the major protease-resistant fragments. As Fig. 7A shows, βT4 and βT2/βT3 are mutually exclusive fragments, i.e., a single β1–2 molecule cannot give rise to both fragments. Thus, by analysis of the HPLC peak areas, it is possible to determine the number of β1–2 molecules giving rise to βT4 (which is the lower limit of molecules cut at this site), as well as the number of molecules that were definitely not cut at the βT4 site (by adding the numbers of whole β1–2, βT1, and βT2/βT3). Similarly, the number of molecules that were not cut at the βT2/βT3 sites can be estimated by adding the numbers of whole β1–2, βT1, and βT4. In this way it was possible to determine that the proportion of β1–2 molecules cut at the βT4 site is at least 51–60% in the monomer digest, and no more than 24% in the heterodimer digest. Conversely, the proportion of molecules cut at the βT2/βT3 sites is no more than 18–24% in the monomer digest and at least 43% in the dimer digest. This analysis shows that in the dimer, cleavage at the βT4 site is reduced, while the βT2 and βT3 sites become more sensitive to trypsin digestion. Fig. 7B and C maps the protected and exposed trypsin and proteinase K sites onto a model of the triple-helical motif.

**DISCUSSION**

Previous studies have demonstrated the importance of electrostatic interactions in the stability of both erythroid and nonerythroid spectrin oligomers (13, 14). Both studies showed a salt-dependent dissociation of spectrin dimers into their component subunits. However, the mechanism for this dissociation was unknown. Spectrin subunits are highly elongated and flexible charged molecules; thus, there are a number of ways in which increased ionic strength could reduce dimer affinity. For example, the high ionic strength could weaken interactions between consecutive motifs, resulting in increased flexibility that may in turn weaken lateral association or could destabilize the head end closed hairpin loop (6). Alternatively, high ionic strength could destabilize laterally paired motifs along the length of the molecule and/or the laterally associated motifs near the actin-binding end of the molecule that initiate dimer formation.

The aim of the present study was to investigate the mechanism of lateral association of spectrin α and β motifs in the dimer nucleation site. The role of electrostatic interactions in lateral association was investigated by perturbing this interaction with increasing ionic strength, and the electrostatic potential on the surface of four motifs was modeled in order to determine the most likely faces of the triple-helical motifs for attractive electrostatic interactions. Protease protection experiments were then used to identify the regions of spectrin motifs that become protected or exposed upon dimer formation. The primary focus of this work was on the dimer nucleation recombinants a20–21 and β1–2, which comprise the minimum nucleation site for lateral association.³

The importance of electrostatic interactions to the lateral association at the dimer nucleation site was investigated using both two-motif and four-motif heterodimers, a20–21/β1–2 and α18–21/β1–4, respectively. As shown in Fig. 2, the association of each heterodimer was substantially weakened in salt concentrations above 130 mM. It is possible that the effect of salt on this association is an indirect result of a salt-induced confor-

³ S. L. Harper, G. E. Begg, and D. W. Speicher, manuscript in preparation.
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The cleavages at residues 230 and 231 are common to T1, T2, T3, and small complementary peptides to T4 and T5. These latter peptides were not identified and quantitated. Hence, the overall degree of cleavage of this site was not determined.

| Peptide* | N terminus* | Observed mass | Sequence mass | Residues* | Cleavage location* | Yield dimer/monomer* |
|----------|-------------|---------------|---------------|-----------|--------------------|---------------------|
| αT1      | QLPQKL      | 16,855        | 16,862        | 6–148     | Helix A, e/f       | Same                |
| αP1a     | GSPGKQLP    | 15,195        | 15,190        | 1–131     | Helix A, b/c       | ↓                   |
| αP1b     | GSVDHAI     | 15,318        | 15,319        | 1–132     | Helix A, e/d       | ND↓                 |
| βT1a     | VYTPEDGK    | 16,849        | 16,859        | 90–230    | Helix C, d/e       | ND↓                 |
| βT2a     | 17,035      | 17,015        | 90–231        |           | BC loop*           | ↑ ↑↑                 |
| βT3a     | LVSVDINRA   | 15,983        | 15,961        | 98–230    | Helix A, b/c       | ↓                    |
| βT3b     | 16,147      | 16,117        | 98–231        |           | Helix A, e/d       | ↓                    |
| βT4a     | GSVDHAI     | 15,671        | 15,681        | 1–135     |                   |                     |
| βT4b     | 15,840      | 15,837        | 1–136         |           |                   |                     |
| βT5a     | GSVDH       | 9,348         | 9,317         | 1–82      | BC loop            | Same                |
| βT5b     | 9,640       | 9,604         | 1–84          |           | BC loop            | ↓                    |
| βP1      | GSVDHAI     | 18,358        | 18,396        | 1–157     | Helix A, e/d       | ↓                    |
| βP2      | GSVDHAI     | 17,034        | 17,043        | 1–146     | Helix A, f/g       | ↓                    |
| βP3      | GSVDHAI     | 16,516        | 16,526        | 1–142     | Helix A, b/c       | ↓                    |
| βP4      | GSVDHAI     | 15,986        | 15,984        | 1–137     | Helix A, d/e       | ↓                    |

* Fragments of α20–21 (α) or β1–2 (β) digested with trypsin (T) or proteinase K (P).

Mass determined by Edman sequencing of reverse-phase HPLC fractions and/or SDS-PAGE bands. The GS residues at the N terminus are part of the thrombin cleavage site that remains after digestion of the fusion protein.

Locations of the pertinent proteolytic cleavage sites within the triple helical motif of Yan et al. (4) are indicated. Cleavages within helices are further defined by their locations within the heptad repeats typically seen for paired helices where amino acid positions are labeled a, b, c, d, e, f, and g, with the a and d positions primarily hydrophobic and buried in the interior of the triple helical bundle. Cleavage locations are indicated by the "↑" that separates the two heptad positions indicating the flanking amino acid residues. See Yan et al. (4) and Fig. 7 for further details.

Relative yield of the fragment in a dimer relative to its yield in a parallel monomer digest.

The cleavages at residues 230 and 231 are common to T1, T2, T3, and small complementary peptides to T4 and T5. These latter peptides were not identified and quantitated. Hence, the overall degree of cleavage of this site was not determined.

Data refer to the N-terminal cleavage site for this fragment.

Analysis of electrostatic potential has been used to help identify the binding sites of several other proteins (22, 31, 32). Fig. 4 shows the electrostatic potential on the three faces (AB, BC, and CA) of the Drosophila α20, β2, α14, and β8 motifs, each face comprising the surfaces of two adjacent α-helices. The dimer interface could involve interaction of a single α-helix from each motif in the heterodimer, two adjacent helices from each motif, or one helix from one subunit and two helices from the other subunit. In other proteins involving the self-association of α-helical bundles, such as apoferritin (33), cytochrome c (34), and Salmonella typhimurium aspartate receptor (35), the interface involves interaction between two helices from each protomer. In addition, homodimers usually interact via the same site on each subunit (36). The spectrin subunits and respective paired motifs are not identical, but are homologous, and have evolved from a common gene. Hence, it is reasonable to predict that the lateral interaction of the subunits might occur via the same region of each paired motif, as experimentally supported by the observed protease protection of A helices from both subunits. As shown in Fig. 4A, the AB face shows striking charge complementarity in the nucleation motif pair.

![Fig. 7. Location of protease-sensitive sites.](image-url)
\(\alpha 20/\beta 2\) when the motifs are aligned antiparallel, as they are in the dimer, and is therefore distinctly favored as the dimer nucleation interface site. The pattern of electrostatic potential on the surface of Drosophila \(\alpha 20\) and \(\beta 2\) is expected to be highly conserved in the corresponding erythrocyte motifs, since the proportion of charged residues in the Drosophila motifs that are identical or substituted with the same charge in the erythrocyte motifs is 76% and 92% for \(\alpha 20\) and \(\beta 2\), respectively.

The electrostatic potential of the \(\alpha 14\) and \(\beta 8\) motifs also favors lateral interaction of the AB faces, although the complementarity is less pronounced than for the modeled nucleation site motifs. As shown in Fig. 4B, the strikingly positive potential of the AB face of \(\alpha 14\), among the strongly negative potential of all other faces on \(\alpha 14\) or \(\beta 8\), clearly indicates that this face will be favored in lateral association with the \(\beta\) motif. In this motif pair, the \(\alpha\) motif AB face has the potential to interact favorably with any region of the \(\beta\) motif based on electrostatic complementarity alone, and in fact it is possible that this potential flexibility has some role in the formation of less specific interactions between motifs outside the nucleation site.

The use of limited proteolysis to identify binding surfaces has been previously described for several protein interactions (37–39). In this study, the protease protection analyses provide interesting experimental data on the location of the dimer lateral interface site. When \(\beta 1–2\) is digested with either trypsin or proteinase K, the major protease-resistant fragments start at the N terminus of \(\beta 1\) and end in the \(\beta 2\) motif, leaving the \(\beta 1\) motif intact. After association with \(\alpha 20–21\), there are two changes in the protease susceptibility of \(\beta 1–2\); there is a marked decrease in proteolytic cleavage of \(\beta 2\), and, in the case of trypsin, there is an increase in cleavage at two sites in the \(\beta 1\) motif. As shown in Fig. 7, the trypsin sites on \(\beta 1–2\) exposed in the dimer occur in the BC loop and the C helix of the \(\beta 1\) motif, which as noted above is very protease-resistant in the \(\beta 1–2\) monomer. The increased proteolysis of these sites after dimer formation must be due to a conformational change of this motif induced by association with \(\alpha 20–21\). The accessibility of the C helix in the dimer shows that it cannot be part of the dimer association interface, and its location on the opposite side of the motif from the AB face supports our hypothesis that the AB faces form the subunit-subunit interaction site.

All of the trypsin and proteinase K cleavage sites identified in these experiments as protected in the dimer (Fig. 7 and Table I) are located in the A helices of the \(\alpha 20\) and \(\beta 21\) motifs. This indicates that these A helices are largely buried in the dimer, and location of the invariant tryptophan in the middle of the AB face is expected to be highly conserved for both subunits resulted in increased exposure of the \(\alpha\) subunit tryptophans to both hydrophilic and hydrophobic quenchers.

Complementary electrostatic interactions in the spectrin dimer nucleation site are likely to play a pivotal role in dimer initiation. The proteins barnase and barstar have been shown to associate initially via long range electrostatic interactions (43), which assist in bringing the proteins together rapidly in the correct orientation, prior to the final high affinity association involving precise short range interactions (43). Thus, a reasonable model for initiation of spectrin dimerization is that the electrostatic complementarity and other unique structural features of the nucleation motifs (\(\alpha 20–21\) and \(\beta 1–2\)) ensure proper docking of the correct lateral pairs. This is a critical initial step in dimerization, since each subunit comprises a large number of very similar motifs that must be correctly paired with their complementary partner. The long range electrostatic recognition of the appropriate complementary subunit motif should ensure that dimer assembly proceeds without formation of incorrectly paired intermediates that would slow overall assembly. In addition to influencing orientation and pairing of correct motifs in the nucleation site, the electrostatic interactions would be expected to increase the reaction on-rate and hence increase dimer affinity at lower ionic strengths, as demonstrated in Fig. 2.

It is likely that the interacting face of the motifs is consistent along the length of the subunits, such that initial interaction of AB faces in the dimer nucleation region would favor interaction of AB faces along the entire subunit as dimer assembly is completed. This hypothesis is consistent with the complementary electrostatic potential we have observed between the AB faces of \(\alpha 14\) and \(\beta 8\). However, the greater salt dependence of the nucleation site association compared with that of the entire subunits (Fig. 2) indicates that electrostatic interactions are most critical for initial pairing of the dimer nucleation region. This supports our model where the primary role of the electrostatic interactions is to increase the rate of subunit docking in the correct register at the dimer nucleation site.

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