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Structural and Dynamic Study of the Tetramerization Region of Non-Erythroid α -Spectrin: A Frayed Helix Revealed by Site-Directed Spin Labeling EPR[†]

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Abstract

The N-terminal region of α -spectrin is responsible for its association with β -spectrin in a heterodimer to form functional tetramers. Non-erythroid alpha spectrin (α II-spectrin) has significantly higher association affinity with β -spectrin than the homologous erythroid alpha spectrin (α I-spectrin). We have previously determined the solution structure of the N-terminal region of α I-spectrin by NMR methods, but currently no structural information is available for α II-spectrin. We have now used cysteine scanning, spin labeling EPR and isothermal titration calorimetry (ITC) methods to study the tetramerization region of α II-spectrin. EPR data clearly showed that, in α II-spectrin, the first 9 N-terminal residues were unstructured, followed by an irregular helix (Helix C'), frayed at the N-terminal end, but rigid at the C-terminal end, which merges into the putative triple helical structural domain. The region corresponding to the important unstructured junction region linking Helix C' to the first structural domain in α I-spectrin was clearly structured. Based on the published model for aligning Helices A', B' and C', important interactions amongst residues in Helix C' of α I/II-spectrin and Helices A' and B' of β I/II-spectrin are identified, suggesting similar coiled coil helical bundling for spectrin I and II in forming tetramers. The differences in affinities are likely due to the differences in the conformation of the junction regions. Equilibrium dissociation constants of spin labeled α II and β I complexes from ITC measurements indicate that residues 15, 19, 37 and 40 are functionally important residues in α II-spectrin. Interestingly, all four corresponding homologous residues in α I-spectrin (residues 24, 28, 46 and 49) have been reported to be clinically significant residues involved in hematological diseases.

Spectrin, consisting of α -spectrin and β -spectrin subunits, is a major component of the cytoskeleton in cells. Two α -spectrin isoforms (I and II) and five β -spectrin isoforms (I, II, III, IV and H) have been identified in humans (1). The C-terminal end of α -spectrin and the N-terminal end of β -spectrin associate to form an $\alpha\beta$ heterodimer (2). Two heterodimers associate at the other ends of the dimers, the N-terminal end of α -spectrin of one dimer and the C-terminal end of β -spectrin of the other dimer, to form a functional tetramer (3).

Erythroid spectrin (spectrin I) was first identified in red blood cells and is responsible for cell flexibility and deformability (4). Mutations that impair the formation of erythroid tetramers lead to hematological diseases (5,6). Non-erythroid spectrin is found in brain cells as well as other cells (1). Non-erythroid α -spectrin (α II-spectrin) associates with non-erythroid β -spectrin

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(β II-spectrin) to form tetramers in a manner similar to erythroid spectrin, except with higher affinity (7). α II-spectrin also associates with erythroid β -spectrin (β I-spectrin) with high affinity, as well as with functionally important proteins in the nucleus (8) and in brain (9). Measurements of the breakdown products of α II-spectrin have been suggested to be clinically relevant as a quantitative marker to measure traumatic brain injury (10–12). α II-spectrin has recently been reported to be essential for stabilizing nascent sodium-channel clusters (13), assembling the mature node of Ranvier (13), and regulating endothelial cell-cell contacts (14). The tetramer formation of α II- and β II-spectrin is also essential in the regulatory step for neuritogenesis (15).

Tetramerization is clearly important for spectrin function, and yet currently very little structural information is available for understanding the detailed mechanism of tetramerization as well as the differences in affinities between erythroid and non-erythroid α - and β -spectrin association. We have previously obtained a solution structure for the first 156 amino acid residues at the N-terminal region of human α I-spectrin (Fig 1A;16,17). The solution structure shows that the first 20 residues are unstructured, followed by α -helix consisting of residues 21 – 45. The most unexpected and interesting finding is that this helix (Helix C', often referred to as the N-terminal partial domain) is followed by a 7-residue (residues 46 – 52) unstructured junction region. Residue 53 is the first residue in Helix A₁. Helix A₁, Helix B₁ and Helix C₁ bundle to form the first structural domain of α I-spectrin. The triple helical structural domain is similar to other (*Drosophila* and chicken brain) spectrin structural domains (18–22), although the helices and loops connecting the helices differ in lengths (16). It is interesting to note that conformations of other loop/junction/linker regions between spectrin structural domains have been speculated to be important in spectrin flexibility (21) and domain stability or folding/unfolding (23–27). The importance of the particular unstructured junction region between Helix C' and the first structural domain in α I-spectrin, as revealed by NMR studies, has recently been recognized for its clinical significance (28,29).

Based on this NMR structure, we refer to the entire region prior to the first triple helical bundle, and not just Helix C', as the “tetramerization region” since its interaction with the β -spectrin C-terminal region is responsible for the formation of spectrin tetramers. For α I-spectrin, the tetramerization region consists of residues 1 – 52. The corresponding tetramerization region of α II-spectrin, through sequence alignment, is thus residues 1 – 43 (Fig 1B); these two sequences exhibit 79 % similarity and 72 % identity (30). Yet, the association of α II-spectrin with β -spectrin exhibits an affinity that differs from that of α I-spectrin with β -spectrin by approximately two orders of magnitude. Our isothermal titration calorimetric (ITC) studies of an α II-spectrin model protein (residues 1 – 149) show a K_d value of 0.012 μ M with a C-terminal β I-spectrin model protein (β I, consisting of residues 1898 – 2083 of β I), and a K_d value of 1.1 μ M for the corresponding α I-spectrin protein (residues 1 – 156) with the same β I-spectrin modelprotein (30). Kinetic studies of an immobilized GST fusion protein of α II-spectrin (residues 1 – 145, a system similar but not identical to our system) associating with His-tag β I-spectrin model protein (residues 1898 – 2137) show a k_{on} of 2,900 $M^{-1} S^{-1}$ and k_{off} of $8 \times 10^{-4} S^{-1}$ ($K_d \sim 0.3 \mu$ M) whereas similar measurements for α I-spectrin protein (residues 1 – 154) with the same β I-spectrin model protein show a k_{on} of 59 $M^{-1} S^{-1}$ and k_{off} of $5 \times 10^{-5} S^{-1}$ ($K_d \sim 0.9 \mu$ M) (7).

Small angle X-ray scattering studies show that the recombinant α II-spectrin protein (149 residues) exhibits a more extended conformation than the α I-spectrin protein (156 residues) (30). We interpret this difference in molecular shape to be the consequence of a more rigid junction region in the α II protein. This suggestion is supported by molecular dynamics simulations (31) and by energetic considerations (32). However, high resolution structural information is not yet available for this region of α II-spectrin.

We used site-directed spin labeling methods to label 39 N-terminal residues (residues 9 – 47), one at a time, of a recombinant protein that consists of the first 359 residues (the tetramerization region plus three structural domains) of α II-spectrin, and used electron paramagnetic resonance (EPR) techniques to monitor the motion and accessibility of the attached labels to obtain structural and dynamic information for the scanned region. Site-directed spin labeling EPR has been shown to be a powerful method to monitor the dynamic and structural features of protein systems at a residue level, especially when X-ray and NMR data are not available (33). The first 359 residues in α II-spectrin are homologous to the first 368 residues in α I-spectrin, which have been studied extensively (34–37). In this study, we found that the N-terminal Helix C' of α II-spectrin is an irregular helix. This helix was frayed at the N-terminal end, and rigid with restricted motions at the C-terminal end, with the helix merging into the putative triple helical structural domain. The region corresponding to the unstructured junction region in α I-spectrin was clearly not unstructured in α II-spectrin. It was interesting to note that there was a phase shift in the mobility periodicity, suggesting the helix to overwind around residues 18 and 19, and underwind around residues 37 and 38. We also identified residues 15, 19, 37 and 40 as functionally important residues in α II-spectrin.

MATERIALS AND METHODS

Spectrin recombinant proteins

A DNA fragment coding for the first 359 residues of α II-spectrin was previously constructed (38) in a yeast MatchMaker vector pBD- α II with *Bam*HI and *Eco*RI restriction sites matching those of the *E. coli* expression vector pGEX-2T. The spectrin insert was simply cut and ligated into pGEX-2T vector for protein expression to give α II-spectrin consisting of residues 1–359 (abbreviated as α II). Two native cysteine residues at positions 158 and 315 were replaced with alanine residues by site-directed mutagenesis methods following standard procedures (36) to give a cysteine-less α II, and used as the parent protein for single cysteine replacement. A total of 39 plasmids, each with a single cysteine residue, scanning positions 9 to 47 (Fig 1B) were prepared. DNA sequences were analyzed at the DNA Sequencing Facility in the Research Resources Center at the University of Illinois at Chicago (RRC). Confirmed plasmids were transformed into BL21-CodonPlus(DE3)-RIPL competent cells (Stratagene, La Jolla, CA) for protein expression. Proteins of the α II family, including wild type (WT), cysteine-less and single cysteine proteins (α II Δ), were expressed and purified following standard procedures (36), except that 2 mM β -mercaptoethanol was included in thrombin cleavage buffer for α II Δ proteins to prevent disulfide bond formation and maintain the efficiency of thrombin cleavage. A model protein of erythroid C-terminal β I-spectrin fragment consisting of residues 1898 – 2083 was prepared as described previously (35).

Molecular masses of all proteins were determined by RRC with high resolution LTQ-FT mass spectrometry methods; proteins with masses that deviated from expected values by 3 Da or more were rejected. The purity of protein from each preparation was determined by 16 % SDS-PAGE. Each protein was further analyzed by circular dichroism methods at 20 °C to provide helical contents (35). Concentrations of proteins in 5 mM phosphate with 150 mM NaCl at pH 7.4 (PBS7.4) were determined from spectroscopic absorbance values at 280 nm, using the extinction coefficients of 38,960 cm⁻¹ M⁻¹ for all α II Δ proteins except for Y26C and Y44C (37,470 cm⁻¹ M⁻¹) and 31,130 cm⁻¹ M⁻¹ for β I (with extinction coefficients calculated from sequence, including two extra GS residues remaining after thrombin cleavage).

The α II Δ proteins were spin labeled with the well studied spin label (1-oxy-2,2,5,5,-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate (Toronto Research Chemicals, Toronto, ON) following standard procedures (36). Each spin labeled protein was designated by its native amino acid residue, its position in sequence and “R1”, such as L9R1 or F46R1, following the published notation of R1 as the labeled cysteine residue (39). As a group, they

were referred to as α IIAR1. To determine non-specific background labeling, the α II cysteine-less protein was labeled in parallel.

The hydrodynamic radii (R_h) of a few α IIAR1 samples (50 μ M, 100 μ L in PBS7.4) were measured by dynamic light scattering methods, as before (32), using a PD2000 system (Precision Detectors Inc, Franklin, MA) with an HPLC YMC-Pack Diol 200 size-exclusion column (Waters Corporation) and a flow rate of 0.7 – 0.8 mL/min.

Isothermal titration calorimetry (ITC)

The association/dissociation equilibrium constant of each of the thirty nine α IIAR1 proteins as well as WT and cysteine-less proteins with β I were determined from ITC measurements at 25 °C with a VP-ITC unit (MicroCal, LLC, Northampton, MA), following standard procedures (32). Each α IIAR1 protein sample was co-dialyzed with β I (3 ~ 8 μ M). The α IIAR1 proteins were the titrant proteins with a concentration of 50 ~ 100 μ M except I15R1, R19R1 and L40R1, which were at 120 ~ 200 μ M) Titration isotherms were analyzed using the MicroCal software to obtain the association constant (K_a) assuming a single binding site model.

EPR Studies

α IIAR1 samples (~100 μ M) in PBS7.4 were used for EPR studies. Since most published studies using similar approaches were done in the presence of 30 % (w/w) sucrose (39), we also included 30 % sucrose in our samples, for ease of comparing side-chain mobility with published results. Spectra at 20 °C were acquired with a Bruker (Bruker BioSpin Corp., Billerica, MA) EMX spectrometer at 9.45 GHz equipped with an HS cavity and a variable temperature unit. Incident microwave power was set at 2 mW, modulation amplitude at 1 G, time constant 20.48 msec, conversion time 40.96 msec and scan width 100 Gauss. Spin label concentrations of labeled proteins were determined by double integration of EPR spectra using the WinEPR software from Bruker. For cysteine-less protein samples, we observed weak EPR signals, and found about 0.17 spin label per protein molecule (see Results section). This signal was subtracted from each EPR spectrum of α IIAR1 proteins to give an experimental spectrum for that protein.

Accessibility experiments were done with a Varian E-109E spectrometer at 9.35 GHz, equipped with a loop-gap resonator (Molecular Specialties Inc., Milwaukee, WI) at room temperature (about 22 °C). Spin labeled protein samples (about 100 μ M) in PBS7.4, with and without 5 mM nickel ethylenediamine-di(o-hydroxyphenyl acetic acid) (Ni-EDDA), were placed in gas permeable TPX sample tubes (Molecular Specialties Inc., Milwaukee, WI). Ni-EDDA was synthesized as described previously (40). Nitrogen gas was continuously purged around the sample tube during each measurement. The peak-to-peak amplitudes of the central line were monitored with a microwave power of 2 mW, and measurements, as a function of microwave power, began only after the complete removal of oxygen gas from the sample, as indicated by no further decrease in signal amplitude. The amplitude values at microwave power level of 0.5 to 40 mW were analyzed following established methods (40) to give accessibility values (with 5 mM Ni-EDDA) for all 39 α IIAR1 protein samples.

Spectral Analysis, Spectral Simulation and Spectral Fitting

A combination of spectral subtraction, spectral fit and spectral simulation were used to analyze the experimental spectra.

For simulation, we used the published program “NLSL” with the “microscopic order; macrosopic disorder (MOMD)” model to describe the protein-bound nitroxide spin label dynamics in a magnetic field (41–46). The MOMD model has been frequently used to analyze X-band EPR spectra of proteins (43,44), although it is more appropriate for 250 GHz spectra

than for 9 GHz spectra (45). The program requires the input of multiple parameters (including the values of the spin quantum number of the interacting nuclear spin, magnetic field strength, magnetic tensor, *etc.*), and allows a maximum of 10 parameters (rotational correlation time, τ_c ; motional asymmetry, N ; ordering, c_{20} , *etc.*) to vary until the simulated spectrum fits the targeted experimental spectrum. A detailed analysis of the interplay between the fit obtained and the motional parameters by manually varying these parameters shows that the program simulated many of the experimental spectra well, although some of the errors are quite large (for example, for diffusional rates, errors of 11 % for R_{xx} , 23 % for R_{yy} and 11 % for R_{zz}) (46). We used published magnetic tensor values ($g_{xx} = 2.0076$, $g_{yy} = 2.0050$, $g_{zz} = 2.0023$; $A_{xx} = 6.2$, $A_{yy} = 5.9$, $A_{zz} = 37$ from ref 46 for all but strongly immobilized component, discussed below), which were improved values over those used in our earlier publication (47), and varied the four system-specific parameters ($gib0$ for inhomogeneous line broadening, $rbar$ for rotational correlation time with $1/(6 \times 10^{rbar}) = \tau_c$, N for motional asymmetry and c_{20} for ordering potential) (46).

For experimental spectra fitted with multiple spectral components, linear regression routine (Minitab, State College, PA) was used to determine the relative percentage of each component to best fit the experimental spectra.

RESULTS

Protein Characterization

The SDS gel electrophoresis data showed that all αII and βI proteins were 85 to 96 % pure, with an average value of 90 ± 4 % ($n = 42$). Most of the molecular masses of proteins used in this study were within 1 Da of the expected values, and none differed more than 2.5 Da. The expected mass of the WT protein was 42,242.5 Da. Three randomly selected spin labeled proteins (at positions 15, 23 and 26) were also analyzed, and their masses were also as expected.

A representative CD spectrum of each $\alpha II\Delta R1$ as well as the cysteine-less protein was shown in Fig 2. The CD data showed that the helical contents for αII proteins (WT, cysteine-less and $\alpha II\Delta R1$ proteins) were around 75 % (Fig 2).

The average hydrodynamic radius from dynamic light scattering measurements of WT, L9R1 and R28R1 was 3.9 ± 0.06 nm ($n = 3$), which was larger than that of BSA (3.6 nm, a larger “spherical” protein of 67 kDa), indicating $\alpha II\Delta R1$ proteins (about 42 kDa) were highly asymmetric.

For spin labeled proteins, we found non-specific (background) labeling, similar to those observed in αI -spectrin model systems (36,51). The spin label-to-protein ratio for cysteine-less αII protein was 0.17 ± 0.04 ($n = 3$). This background labeling seemed high, although it was similar to that of αI -spectrin (36). About 5 % of this signal came from spin labeled glutathione trapped in the protein (51). About 10 % signal remained after the samples were treated with urea (6 M), following by dialysis and column chromatography. The background signals, both in amounts and in spectral features, were similar for all $\alpha II\Delta R1$ samples.

The average value for spin label-to-protein ratios for all 39 $\alpha II\Delta R1$ samples, after subtracting background label signals, was 0.8 ± 0.2 ($n = 39$).

Association of $\alpha II\Delta R1$ proteins with βI

ITC measurements showed that the K_d values of WT and cysteine-less αII proteins with βI were about 12 ± 2 nM, similar to the published value of a smaller αII -spectrin protein consisting of the first 147 residues (30). The K_d values for most of the $\alpha II\Delta R1$ samples ranged from 6 – 50 nM (Fig 3C). For example, for L9R1 (Fig 3A) the K_d value was 28 nM. The values for

F29R1, L32R1, K39R1, S43R1 and Y44R1 were slightly higher, ranging from 50 – 80 nM (Fig 3C). However, four proteins exhibited much weaker affinity than the rest, with K_d values of 237 nM for I15R1, 1.14 μ M for R19R1, 177 nM for R37R1 and 729 nM for L40R1 (Fig 3B). The replacement of arginine with R1 at position 19 introduced the largest perturbation (about a 100-fold reduction in K_d), followed by lysine at position 40 (60-fold reduction), isoleucine at position 15 (20-fold reduction) and arginine at position 37 (15-fold reduction) (Fig 3D). However, in general, the effects of spin labeled cysteine scanning of 39 residues at the N-terminal region of α II introduced little perturbation with respect to its functional properties, and thus presumably little structural perturbation, except for residues at positions 15, 19, 37 and 40.

EPR Studies

The experimental EPR spectra, with the background signal subtracted, of all α II Δ R1 proteins (scanning from position 9 to position 47) showed both similarities and variations (Fig 4). Initial analysis of these spectra for line width, second moment and hyperfine separation, *etc.* did not reveal any meaningful structural information.

Close examination of the spectra indicated that most spectra consisted of multiple motional components. Various spectral simulation and spectral fitting analyses led us to conclude that most of these spectra consisted of three components (Components I, II and III), each component with specific spectral characters (for example, line-width and hyperfine separation) substantially different from the other two.

The spectrum of L9R1 appeared to be of a single component (Fig 4) and was easily simulated with a single set of parameters ($r_{\text{bar}} = 8.09$ or $\tau_c = 1.4$ ns; $\text{gib0} = 0.02$; $N = 0.80$ and $c_{20} = 0.06$ or $S_{20} = 0.01$) to give a spectrum (Fig 5A, top spectrum) that fit the experimental spectrum very well (Fig 4). Calculating τ_c values with spectral amplitudes of the three signal peaks (49), a rotational correlation time of 1.3 ns was obtained, which agreed well with the value obtained from the simulation. This spectrum represented a relatively fast anisotropic motion and was presumably from the free χ_4/χ_5 rotation of the spin label moiety (see 46 for the χ_4 and χ_5 definitions) without any structural constraints, and was similar, for example, to the fast component (1.2 – 1.7 ns) of spectra from the cellular retinol-binding protein (52).

This component was relatively easy to identify in multi-component spectra, with both its narrow hyperfine separation ($2A_{zz} = 30.9$ G) and its narrow line width (2.2 G for the low-field peak). Most α II Δ R1 spectra, except spectra 39 – 46, clearly consisted of this component (Fig 4). We referred to this component as Component I. We subtracted the L9R1 spectrum from spectra 10 – 47, guided by its distinct sharp line and narrow hyperfine separation, until the sharp signals were removed. Under-subtraction showed spectra with reduced sharp signals, and over-subtraction showed distorted spectra. Subtractions for spectra 39 – 46 resulted in spectral distortion indicating that these spectra contained only insignificant amounts of Component I.

Spectra 39 – 46, particularly spectrum 46, showed substantial amounts of a strongly immobilized component, with a large hyperfine separation ($2A_{zz} = 67.6$ G). We simulated the spectrum of Y46R1 with two components and obtained an excellent fit (Fig 4). For the slower component, we used the following magnetic tensor values $g_{xx} = 2.0077$, $g_{yy} = 2.0059$, $g_{zz} = 2.0022$; $A_{xx} = 5.5$, $A_{yy} = 5.6$, $A_{zz} = 35.5$ (48), and we fixed $r_{\text{bar}} = 6.50$ or $\tau_c = 53$ ns; $c_{20} = 5.50$ or $S_{20} = 0.80$, and varied only gib0 and N . The values of $\text{gib0} = 3.79$ and $N = 0.58$ gave the best fitted spectrum. The parameters for the faster component were $\text{gib0} = 0.01$; $r_{\text{bar}} = 7.63$ or $\tau_c = 3.91$ ns; $c_{20} = 1.14$ or $S_{20} = 0.32$; $N = 0.39$.

We fixed the slower component τ_c to 53 ns since the calculated rotational correlation time was 53 ns using an A_{zz} value of 33.8 G from the F46R1 spectrum and an empirical formula (48). It is also interesting to note that the rotational correlation time calculated for our protein was 58 ns for rotation about the major axis assuming it to be a prolate ellipsoid (32) in 30 % sucrose, with a R_h value of 3.9 nm from dynamic light scattering (see above, **Protein Characterization**). This slower component in F46R1, referred to as Component III (Fig 5A), was thus probably from the labeled side-chain that was strongly immobilized, with the rotational motion of the protein as the observed motion for the label. It should also be noted that both the experimental detection and the NLSL simulation was not as sensitive to motions slower than 20 ns and therefore the simulated/experimental spectrum for τ_c of 20 ns does not differ much from that of 53 or 58 ns (50).

Each experimental spectrum of α II Δ R1, with the sharp signals (L9R1 spectrum) already removed, had Component III subtracted to give a “difference spectrum”. The amounts of Component III removed from spectra were determined when the immobilized signal ($A_{zz} = 33.8$ G) was removed without distorting the spectra. The difference spectrum of each was then simulated. The parameters $\text{gib0} = 0.02$; $N = 0.80$ as used for L9R1 were used, and r_{bar} and c_{20} were varied to give a simulated spectrum. We also varied all four parameters (gib0 , N , r_{bar} and c_{20}) for best fit and obtained τ_c values very similar to those obtained by holding gib0 and N identical to those obtained by varying only r_{bar} and c_{20} . This component was called Component II.

Fig 5 shows a typical operation. The spectrum of L9R1 (Component I, 29 %) and Component III (11 %) were subtracted from the experimental spectrum of Y26R1 to give a difference spectrum of Y26R1, which was used to simulate Component II of Y26R1 (Fig 5A middle spectrum). The sum of the three components, in their proper proportions (Fig 5B), then gave the fitted spectrum of Y26R1 (Fig 5C). The fitted spectra for all proteins agreed well with their corresponding experimental spectra (Fig 4).

Since a visual inspection of Y26R1 spectrum suggested at least two components. We also used the program to simulate with two components, and obtained one component with $\tau_c = 1.2$ ns, 36 % and another with 6.3 ns, 64 %. However, this “best fit” spectrum (Fig 5D) did not fit the experimental spectrum as well as the three component fit (Fig 5C), indicating that the three-component fit was necessary and unique.

The relative amounts (percentages) of each component that best fit the experimental spectra was obtained by linear regression routine and showed different amounts of each component for each protein (Fig 6). The amounts of Component III for the proteins were generally less than 20 %, except for 41R1 (26 %), 46R1 (40 %) and 47R1 (41 %) (Fig 6). The amounts of Component I decreased from 100 % at position 9 to about zero at positions 39 – 46. Despite our best effort to attend to details in our spectral analysis, these values all carried certain uncertainties. Thus, only the general patterns of these values were considered. As shown in Fig 6, a general “decreasing” trend was observed for the amounts of Component I as we moved down the sequence, whereas the amounts of Component II exhibited a general “increasing” trend, in going from positions 9 – 40.

The τ_c values of Component II (τ_c^{II}) ranged from 2 to 4.5 ns for the α II Δ R1 proteins with labels at positions 10 – 47 (no Component II for L9R1) (Fig 6), and were residue-position specific, suggesting that the Component II motions were those of the backbone motions of the scanned residues. Similar τ_c values have been reported for the backbone motions of noninteracting residues on the helix surface of GCN4-58 bZip (44). The τ_c^{II} values exhibited a regular periodic pattern dependence on residue positions (Fig 7), with the values for residues 38 – 45 less regular than the rest. A nonlinear least square fit of all data to a sine wave with a periodicity of 3.5

showed a good fit between the data and the sine wave (sine wave 1) except for those in the middle region. When we fit just the data of the middle region (positions 19 – 37), a new sine wave with a different phase (sine wave 2) resulted. Interestingly, the sine wave 1 (all positions) was shifted relative to the sine wave 2 (positions 19 – 37), by one residue toward the N-terminal end, or one residue deletion (Fig 7). The τ_c^{II} values for residues 46 and 47 did not fit either of the two sine waves.

Accessibility values with 5 mM Ni-EDDA varied from 0.35 to 0.55 for all positions except for position 46, which was 0.23. Values larger than 0.25 are considered to be from residues on the surface of a helix, whereas those smaller than 0.25 are from those buried by tertiary contacts (53).

Discussion

Conformation of the N-Terminus of Human α II-Spectrin

The sequence for the scanned region (residues 9 – 47) in α II-spectrin is 85 % similar and 69 % identical to that in α I-spectrin (*residues 18 – 56, using italic font for α I-spectrin residue numbers to reduce potential confusion*) (Fig 1). The alignment also shows that residues corresponding to residues 2 – 10 in α I-spectrin are missing in α II-spectrin (38). Based on sequence alignment with α I-spectrin and the NMR structure of the first 156 residues of α I-spectrin (17), the putative structure of residues 1 – 47 in α II consists of an unstructured region (residues 1 – 11), Helix C' (residues 12 – 36), the unstructured junction region (residues 37 – 43), and a part of Helix A₁ of the first structural domain (residues 44 – 47). However, our current studies show that the conformation of α II, at least of the scanned region, differs from this predicted conformation, in spite of high sequence homology with α I-spectrin.

The oscillating periodicity of 3.5 for τ_c^{II} (of Component II) as a function of residue position suggested a helical conformation, which we discuss below. First, we discuss the motional components that we observed for residues 9 – 47. Component I was the fast, unconstrained motional component (about 1.4 ns) and thus was associated with unstructured conformation. Component II was from the labeled side chain being immobilized due to restricted χ_4/χ_5 rotation and reflecting backbone motions, or backbone conformations. Component III was strongly immobilized ($\tau_c = 53$ ns, or at least larger than 20 ns) with little χ_4/χ_5 rotation and reflecting an overall protein motion. The amount of Component III was fairly constant for most of the residues. For each residue, Components I and II coexisted, but the amounts of each component varied as a function of the residue position. It is interesting to note that other EPR studies have identified multiple motional components for a single labeled site. For example, residue 11 in a helix of spin labeled phospholamban exhibits 25 % of a fast motional component (0.63 ns) and 75 % of a slow motional component (4.2 ns) and were attributed to two conformations of helix in equilibrium (54). However, we are not aware of other publications with varying amounts of a particular motional component (such as our Component I) as a function of residue position.

Residue 9, with Component I spectrum, was indeed unstructured, as predicted. Component II results showed that the conformation of residues 10 and 11, and subsequent residues, were not unstructured, contrasting with that of residue 9. Thus, residue 10 was at the start of a helix, with τ_c^{II} values at 3.7 ns. The periodic variation of τ_c^{II} values as a function of residue position, in the region consisting of residues 10 – 47, suggested that this region folded into a curved (coiled), amphiphilic helix (36). As we have shown before, residues on the smaller curvature face (hydrophobic side) exhibited less mobility as compared to residues on the larger curvature face (hydrophilic side). A good correlation between the mobility and sequence heptad pattern was observed for residues 19 – 37. A similar correlation was also observed previously (36). The residues with large τ_c values (such as those at positions 19, 22, 25, 26, 28, 29, 30, 33 and

36, in bold shown below) correlated very well with the *a* and *d* positions in the heptad pattern in this region (the residues with τ_c fitted by sine wave 2 in Fig 7).

1		1		3
0		9		7
ETAEDI QERR QQVDLRYH RF KELSTLRRQK LEDSYRFQ				
fg abc defga bcdefg abcd efg abc defg abc defga				

However, for the regions prior to residue 19 and after residue 37 (the residues with τ_c fitted by sine wave 1 in Fig 7), the immobile residues were at the hydrophilic positions (heptad positions *b*, *e*, and *f*). This change in the correlation between τ_c and heptad position indicated an irregular coiled helix. Interestingly, an examination of the sequence of this region, for example by the Marcoil program (55), shows one heptad residue deletion after residue 30, with K30 at “*e*” and E31 at “*g*”. This one-residue deletion in the heptad pattern in the sequence corresponds to two successive stammers in coiled coil helices (56), and is able to tighten up a coiled coil and shorten the local pitch length (57). The existence of a stammer is usually responsible for local flexibility of the helix (56). These structural features may enhance the interface interactions when associating with its binding partner (57) or may disrupt the interactions leading to diseases, such as an in-frame deletion in the desmin gene that leads to skeletal or cardioskeletal myopathy (58). Since the α II-spectrin association affinity with β -spectrin was higher than that of α I-spectrin, we examined the effect of this irregularity in α II partial domain on interactions with β -spectrin, which will be discussed below.

Residues 10 and 11 exhibited a large portion (50 – 60 %) of unstructured (Component I) motion. The proportion of Component I in the subsequent residues decreases gradually as we moved down the helix. Finally, no Component I was observed at residues 39 – 46. Thus, the N-terminal end of the helix was frayed and more flexible. The residues in the helix gradually became less frayed and less flexible, and more rigid with restricted motion, probably due to tertiary contacts. The helix merged into the putative triple helical structural domain. Residue 46 was the only residue not on the exposed surface of a helix, with low accessibility to Ni-EDDA, and therefore was buried, or facing the core of the putative helical bundle. Logically, the following residue, residue 47, was also a part of the triple helical structural domain in Helix A₁, with relatively rigid side chain mobility, but exposed to solvent with high accessibility to Ni-EDDA. If residues 44 and 45 were part of Helix A₁, as predicted, but on the hydrophilic side, its EPR signals would be similar to those on a single helix. We observed a gradual increase in τ_c^{II} values from residue 42 to residue 47, with no clear ending of Helix C' and starting of Helix A₁.

In α I, Helix C' has clear boundaries (residues 21 – 45) and is connected to the first structural domain via an unstructured junction region (residues 46 – 52) (17). Our prior work has suggested that the junction region in α II is not flexible, as in α I, but more rigid, and probably helical (36). The EPR spectra of these residues in α II (residues 37 – 43) differed from those of corresponding residues (residues 46 – 52) in α I-spectrin, which exhibit relatively fast side-chain motions (51). As mentioned above, in α II, residues 39 – 46 showed no Component I motion, and showed mostly Component II motions, with τ_c around 2 – 3 ns, except for residue 41 (about 4.4 ns). These results are in good agreement with previous speculations (36) that residues in this region exhibit restricted motions. The periodicity in the region was less regular than the upstream region, although some oscillation was observed in both τ_c^{II} values and in amounts of Component II. We suggest that the conformation of this region is rigid, irregular, and possibly helical, but definitely not unstructured.

The existence of the immobilized Component III in residues 10 – 47 further indicated that the motion of Helix C' was rigidly coupled with that of the structural domain, exhibiting no independent motion, but reflecting the motion of the entire protein molecule, in strong contrast

with Helix C' in α I-spectrin, which exhibits motions independent of the structural domain (17).

In brief, there is no clear junction region between the partial domain Helix C' and Helix A₁ of the first structural domain in α II-spectrin. The Ni-EDDA accessibility results put residue 46 in Helix A₁, but it is not clear where Helix C' ends and where Helix A₁ starts. From our EPR data, we suggest the following conformation for the α II-spectrin N-terminal region. Residues 1 – 9: unstructured; residues 10 – 37/38: frayed helix (Helix C'); residues 38/39 – 43/44/45: rigid and possibly helical, and residues 44/45/46: the start of the first triple helical bundle structural domain of α II-spectrin.

Potential Interactions

Based on our NMR structure of the N-terminal region of α I-spectrin for helical register to predict the partial domain interactions at the tetramerization region as well as on other published work on spectrin structural domains, we have previously suggested that the interactions between Helix C' of α I-spectrin and Helices A' and B' of β I-spectrin involve hydrophobic core interactions and salt bridges between residues in the three helices (17). It is interesting to note that recent publications correlate our solution structure of α I-spectrin with some common mutations associated with hereditary elliptocytosis (28,29). Using the same model for helical register (17), we found that all the residues involved in the interactions are conserved between α I- and α II-spectrin. Therefore, similar to the interactions listed in Table II of our previous paper for α I-spectrin (17), we suggest the following hydrophobic clusters: *2014F-15I-2072T* (*italic* for residues from Helices A' and B' of β I-spectrin partial domain, and **bold** for residues from Helix C' of α II-spectrin) and *2024W-26Y-2061W*. In addition, two additional hydrophobic clusters were found in this model: *2021A-22V-2065F* and *2032L-33S-2054F*. The β I-spectrin residues were within 10 Å radius (from C _{β} of each residue) of the α II-spectrin residues. The salt-bridges between Helices A', B' and C' that we identified were **19R-2069E** and **25R-2022E**. These residues were within 3.8 Å (a distance used as a limit for salt bridge interaction, see 59) of each other. These potential interactions correlated well with our current ITC data, showing that residues 15 and 19 in α II-spectrin were critical for the association with C-terminal β I-spectrin protein. Residues 22, 25, 26 and 33 were at local maxima of τ_c^{II} (Fig 7), and thus on the hydrophobic surface of Helix C', and positioned for interaction with β -spectrin. In our previous findings with random mutagenesis and yeast-two-hybrid methods, we identified residues E10D, I15F/N, R18G, V22D, R25P, Y26N, R28P and R37P as significant mutations, but not D2Y, G5V, V6D and V8M, for interaction with β II-spectrin (38). The residues 15, 22, 25 and 26 (underlined in previous sentence) were identified in our model in the current studies as important interaction sites. Previously we identified that residues 10 and 37 were important for binding with β II-spectrin and speculated that Helix C' in α II-spectrin may be longer than Helix C' in α I-spectrin (38). This EPR study suggested that Helix C' in α II-spectrin starts at residue 10, rather than residue 12 (the corresponding position to which α I-spectrin Helix C' starts) and with no clear end residue, but it merges into the helix in the first structural domain.

Currently, no clinical mutations in the tetramerization region of α II-spectrin have been identified. Based on both ITC and EPR data, we suggest that mutations at positions 15, 19, 37 or 40 may lead to reduced level of spectrin tetramers and abnormal spectrin-based membrane skeleton, which may cause abnormal neuro-activities in cells. It is interesting to note that all four homologous positions 24, 28, 46 and 49 in α I-spectrin have been identified as clinical hot spots (4). In our previous speculation, we also indicated that the common charge-charge interactions between Arg-Lys at the end of the triple helical bundle was lacking in the helical bundling model (17) since the lysine residue in most C helices in full structural domains (*Drosophila* and chicken brain, for example) is replaced by an arginine residue in α I-spectrin

Helix C' (*R45*). Interestingly, in α II-spectrin, there are R36, which aligns with *R45* in α I, and R37, which is more similar to *R45* if Helix C' in α II is "overwound" by one residue. Thus, according to our model, the common salt bridge at the "bottom" (C-terminal end) of many spectrin structural domains is lacking when the helices of the partial domains come together to form tetramers in both α I- and α II-spectrin.

It is also interesting to note that the selected interacting residues in β I-spectrin are also conserved in β II-spectrin. Thus, based on these predictions, the high degree of sequence homology among α I-, α II-, β I- and β II-spectrin at the tetramerization site (Helices A', B' and C') suggest similar coiled-coil helical bundling for spectrin I and spectrin II to form tetramers. The differences in affinities are likely due to the differences in the junction regions, with the α I-spectrin junction region consisting of 7 unstructured residues and with α II-spectrin having Helix C' merging into the first structural domain, as indicated in this study. We suggest that the limited independent motion for Helix C' in α II-spectrin and the relatively free motion for Helix C' in α I-spectrin contribute, at least in part, to the differences in association affinity with β I to give a K_d of about 0.01 μ M for α II (359 residues) protein with β I model protein (this work) and a K_d of about 1 μ M for a corresponding α I-spectrin model protein (368 residues) with the same β I model protein (51).

Abbreviation

α II	recombinant protein with first 359 amino acids of non-erythroid α -spectrin
α IIA	recombinant proteins of α II with single cysteine residue in the scanned region
α IIA^{R1}	spin labeled α IIA
β I	recombinant protein of erythroid β -spectrin with residues 1898 – 2083
α I-spectrin	the full length erythroid alpha spectrin
α II-spectrin	the full length non-erythroid alpha spectrin
β I-spectrin	the full length erythroid beta spectrin
βII-spectrin	the full length non-erythroid beta spectrin
EPR	electron paramagnetic resonance
Helix A'	the first of the two C-terminal partial domain helices of β -spectrin
Helix A₁	the first helix in the first structural domain of α -spectrin
Helix B'	the second of the two C-terminal partial domain helices of β -spectrin

Helix B₁	the second helix in the first structural domain of α -spectrin
Helix C'	the N-terminal partial domain helix of α -spectrin
Helix C₁	the third helix in the first structural domain of α -spectrin
ITC	isothermal titration calorimetry
Ni-EDDA	nickel ethylenediamine-di(o-hydroxyphenyl acetic acid)
PBS7.4	5 mM sodium phosphate buffer with 150 mM NaCl at pH 7.4
R_h	hydrodynamic radius
τ_c	rotational correlation time
τ_c^{II}	τ_c values of Component II

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Figure 1.

NMR solution structure of the first 156 residues of erythroid α I-spectrin (ref. 17) (A) and the proposed structure for the first 149 residues of α II-spectrin (ref. 30) (B). The NMR structure identifies the first helix (Helix C') consisting of residues 21 – 45 and the first structural domain of triple helical bundle (Helices A₁, B₁ and C₁) consisting of residues 53 – 154. The junction region between Helix C' and Helix A₁ is unstructured and consists of residues 46 – 52. Through sequence homology, the corresponding residues for α II are also labeled, with residues 37 – 43 corresponding to the unstructured junction region in α I.

Figure 2.

Circular dichroism (CD) spectra for all α II proteins (wild type, cysteine-less and 39 α II Δ R1). The α II cysteine-less protein was shown as a thick black line; all other spectra were shown in gray. Spectra were recorded at 20 °C with sample $\sim 10 \mu\text{M}$ in PBS7.4. Raw ellipticity was normalized by protein concentration to give molar ellipticity. Helical content was calculated from molar ellipticity at 222 nm using $36,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ as 100 %. The helical content of each α II Δ R1 is shown in the inset.

Figure 3.

Representative ITC titration curves and analyzed data for α II Δ R1 samples with typical affinities for association with β I, such as that of L9R1 (at a concentration of 85 μ M; with β I at 5.5 μ M in PBS7.4) with a K_d value of 28 nM (A). The titration curve and analyzed data of L40R1 (at 126 μ M; with β I at 7.8 μ M) represent those with relatively low association affinities with β I, with a K_d value of 730 nM at 25 $^{\circ}$ C (B). The K_d values for α II Δ R1 proteins scanning from position 9 to position 47 (C) show that the affinity in association with β I for most of the proteins are similar to each other and to that of the WT parent protein (12 nM), except for I15R1 (237 nM), R19R1 (1.14 μ M), R37R1 (177 nM) and L40R1 (730 nM). The values for the proteins with R1 at positions 29, 32, 39, 43 and 44 were slightly higher than that of WT,

ranging from 50 – 80 nM. The K_d values of αIIAR1 normalized by the K_d value of WT, $K_d/K_d(\text{WT})$, (**D**) shows that replacing the native residues with R1 caused the largest perturbation at position 19, followed by position 40 and then positions 15 and 37.

Figure 4.

EPR spectra of $\alpha\text{II}\Delta\text{R1}$ family scanning residues 9 – 47 (thin solid line) at 20 °C, with background signals removed. All samples were in PBS7.4 with 30 % (w/w) sucrose. The protein concentrations were generally about 100 μM , and the spin label to protein ratios were about 0.8. A total of 64 scans were acquired for each spectrum. The corresponding simulated spectra (thick dotted lines) were also plotted. See Figure 5 for fitting and simulation details.

Figure 5.

Simulated spectra of L9R1 (Component I) (top spectrum in **A**) and of Component III (bottom spectrum in **A**). L9R1 spectrum (29 %) and Component III (11 %) were subtracted from the experimental spectrum of Y26R1 to give the Y26R1 difference spectrum, which was used to simulate Component II of Y26R1 (middle spectrum in **A**). The sum of these three components, in their proper proportion (**B**), gave the fitted spectrum (dash line) of Y26R1 (**C**), which matched the experimental spectrum (thin solid line) well. (**D**) The sum of two simulated spectra generated by the simulation program directly, with a $\tau_c = 1.2$ ns for one component (36 %) and a $\tau_c = 6.3$ ns for another component (dash line) (64 %). This fit was not used since the spectrum did not fit the experimental spectrum well (thin solid line), but is shown to indicate the uniqueness of the three-component fit. Components I and III were the same for all α IIAR1 spectra, whereas Component II varies as a function of residue position. Component II for each protein was obtained and used for fitting to give best fit of experimental spectra in Fig 2. The parameters used in simulation were as follow: gib0 = 0.02, N = 0.80, rbar = 8.08 and $c_{20} = 0.06$ for L9R1 (Component I); gib0 = 3.79, N = 0.58, rbar = 6.50 and $c_{20} = 5.50$ for Component III, and gib0 = 0.02, N = 0.80, rbar = 7.69 and $c_{20} = 0.01$ for Component II of Y26R1.

Figure 6.

Relative amounts (%) of Component I (○) (top), Component II (■) (middle) and Component III (Δ) (bottom) were used to give best fit spectra for α IIAR1 proteins. Component I and Component III for all α IIAR1 proteins were the same as those shown in Fig 5A. Component II spectrum for each α IIAR1 protein was individualized. See text for details. The amounts of Component III were generally less than 20 % for all proteins, except for E41R1, Y46R1 and R47R1. The amounts of Component I exhibited a general “decreasing” trend, whereas the amounts of Component II exhibited a general “increasing” trend, from positions 9 – 47. There were no Components I for positions 39 – 46. The uncertainties were 9 % for Component I, 8 % for Component II and 4 % for Component III.

Figure 7.

Rotational correlation time (τ_c) used to simulate Component II spectrum of each α II Δ R1 protein, except that at position 9, which had no Component II. The values exhibited a periodicity in labeled residue positions in α II. A nonlinear least square sine wave fit with a periodicity of 3.5 ($y = \sin(x-A)/3.5 * 2\pi$, where A is the “phase”), using all data points (lower dotted line, sine wave 1), matched the data at positions 10 – 18 and 38 – 45. However, for the data of positions 19 – 37, the lower dotted line did not fit well. A separately fitted sine wave, using just the data set of positions 19 – 37 gave the upper dotted line (sine wave 2). The two dotted lines were shifted by 102° , or the periodicity of positions 20 – 37 was shifted by one residue toward the N-terminal end (one residue deletion) when compared with the lower dotted line. Also shown (top x-axis) are the sequence of residues 9 – 47 and the corresponding heptad assignment.