Spin label EPR structural studies of the N-terminus of α-spectrin

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Abstract Spectrin, a vital component in human erythrocyte, is composed of α- and β-subunits, which associate to form $(\alpha\beta)_2$ tetramers. The tetramerization site is believed to involve the α-spectrin N-terminus and the β-spectrin C-terminus. Abnormal interactions in this region may lead to blood disorders. It has been proposed that both termini consist of partial structural domains and that tetramerization involves the association of these partial domains. We have studied the N-terminal region of a model peptide for α-spectrin by making a series of double spin-labeled peptides and studying their dipolar interaction by electron paramagnetic resonance methods. Our results indicate that residues 21–42 of the N-terminus region exhibit an α-helical conformation, even in the absence of β-spectrin.

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Key words: Spectrin; Electron paramagnetic resonance; Dipolar broadening; α-Helix

1. Introduction

Members of the spectrin super-family are found in many different cells. Human erythrocyte spectrin is responsible for cell flexibility and deformability [1], and is composed of two subunits, α-spectrin (280 kDa) and β-spectrin (246 kDa), which associate laterally to form heterodimers, which further associate to form the biologically significant tetramer [2,3]. Impaired tetramer formation leads to blood disorders [4,5].

The α- and β-spectrin subunits consist largely of sequence homologous units of approximately 106 amino acids [6,7], folded into triple α-helical bundles [8]. X-ray [9,10] and NMR [11] studies of various types of recombinant peptides of spectrin fragments show that these helices are slightly distorted, coiled coil α-helices. It has been suggested that the N-terminus of α-spectrin and the C-terminus of β-spectrin consist of partial domains, and these partial domains associate during spectrin tetramerization [12]. We have recently shown that deletion of a small region (residues 1–21) at the N-terminus of an α-spectrin peptide does not significantly affect its association with β-spectrin [13]. However, deletion of residues 1–36 significantly reduces the association, with affinity diminished by more than two orders of magnitude [13]. According to the phase information we obtained earlier [14], the segment containing residues 22–51 corresponds to the partial domain region in α-spectrin that has been suggested to be the β-spectrin binding site. However, synthetic or recombinant peptides consisting of residues 1–50 [15] or of residues 7–28 or 7–53 [16] do not associate with β-spectrin and do not appear to fold properly. It is possible that the proposed partial domain in α-spectrin assumes a helical structure only in the presence of interacting β-spectrin and/or in the presence of other tandemly linked structural domain(s). It is also possible that this region does not fold into an α-helical conformation at all. No experimental evidence is currently available on the conformation of spectrin around the tetramerization region.

We prepared a peptide consisting of the first 368 residues of α-spectrin (Spα1–368) with the three native cysteines substituted by alanines. This peptide, a fragment of the N-terminus of α-spectrin, was used since it binds β-spectrin [13]. We have found that recombinant peptides with more than one structural domain exhibit stability similar to that of intact spectrin [17], and Spα1–368 consists of three structural domains, as well as the N-terminal partial domain. This cysteine-less Spα1–368 peptide was used for cysteine replacement, with either one cysteine or two cysteines at specific positions, within the first 20–42 residues, for spin label attachment. The labeled peptides were used for dipolar coupling electron paramagnetic resonance (EPR) studies [18]. Our results provide evidence for an α-helical structure in the region spanning residues 21–42 in the Spα1–368 peptide, even in the absence of β-spectrin.

2. Materials and methods

2.1. Spin-labeled α-spectrin peptides

Introduction of point mutations into the α-spectrin peptide gene located in the pGEX-2T vector was done by employing the QuickChange Mutagenesis Procedure (Stratagene, La Jolla, CA, USA). The parent spectrin fragment was Spα1–368 [13]. We first replaced all the endogenous cysteine residues in this peptide with alanine residues. A cysteine-less peptide, Spα1–368 C167A C224A C325A, was produced by three sequential rounds of mutagenesis, and served as the parent peptide for subsequent reintroduction of a single cysteine residue at positions 21, 28, 35 or 42 (A21C, R28C, Y35C or V42C, respectively). Spα1–368 A21C, a single cysteine peptide, then served as the parent peptide for additional cysteine replacements, at positions two, three, four or five residues away from the first (i.e. A21C/E23C, A21C/I24C, A21C/Q25C, A21C/E26C). In addition, three more two-cysteine peptides (R28C/L32C, Y35C/K39C and V42C/G46C) were prepared. These replacements were confirmed by DNA sequencing and protein mass spectroscopy. The peptides were obtained from *Escherichia coli* cell extracts and purified as before [17]. Circular dichroism studies [17] indicated that the helical contents for Spα1–368 and cysteine-less Spα1–368 were both 76%. For the peptides containing a single cysteine residue, the helical content was 71% for A21C, 70% for R28C, 69% for Y35C and 76% for V42C. These values suggested that the peptides were well folded, and that the cysteine replacement did not affect the general conformation of the peptides. Binding studies [13] showed that all peptides except R28C exhibited similar affinities to

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Abbreviations: EPR, electron paramagnetic resonance; MTSSL, 3-methylthiosulfonyl-1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline; Spα1–368, a recombinant peptide with the sequence of α-spectrin from residue 1 to residue 368

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β-spectrin (data not shown). Mutations at position 28 are known to cause decreased levels of spectrin tetramers [4,5]. Thus, it was within expectation that peptide R28C did not bind β-spectrin. Dithiothreitol was incubated with α-spectrin peptides, and free dithiothreitol was removed prior to spin labeling. Excess (20-fold) 3-methylthiosulfonyl-1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline (MTSSL) (from either Reanal, Budapest, Hungary, or Toronto Research Chemicals, Ont., Canada) was used to interact with the peptides for at least 2 h. Excess MTSSL was removed by gel filtration.

2.2. EPR

X-band EPR spectra were obtained on a Varian E-109E spectrometer with a TM_{102} cavity and a fitted liquid N_2 Dewar flask. Samples of approximately 30–70 μM in 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 were immersed in liquid N_2 in the Dewar flask. EPR spectra were taken at a microwave power of 0.1 mW. A magnetic field sweep width of 400 G was used to ensure a good baseline. The modulation was set at 4 G.

2.3. Determination of distances by the Fourier deconvolution method

The distance between two interacting spin labels was determined from the extent of broadening due to spin-spin coupling, a method developed by Rabenstein and Shin [18]. The method treated the EPR spectrum of the two interacting spins in a doubly labeled species, D(B), as a convolution of the spectrum of one spin in the singly labeled species, S(B), with a dipolar broadening function, M(B), with B as the magnetic field. In the analysis, we only considered the values below a threshold for $B^* > 0.1\ G^{-1}$, with B^* as the Fourier conjugate of B [18]. In this analysis, M was expected to be a Pake-type doublet [18]. However, numerical errors due to the discrete nature of the data, instrumental limitations and actual variation in the distance between the two spin systems of the sample cause the observed signal to deviate from a Pake pattern. Thus, approximations were used. Firstly, an average splitting value ($\langle 2B \rangle$) [18] for each doubly labeled peptide was calculated from the function M(B). Secondly, the splitting was assumed to be axially symmetric (i.e. the individual spin systems can be considered as point dipoles). With these assumptions, the average distance between two interacting spins ($\langle r \rangle$, in Å) was then determined from $\langle 2B \rangle$ (in G), since $\langle r \rangle = [(0.75)(3/2g_e\beta\mu_0/4\pi)/\langle 2B \rangle]^{1/3}$, where g_e (= 2.002) was the isotropic g value for the electrons, β ($= 9.274 \times 10^{-24}$ J T^{-1}) was the electron Bohr magneton, μ_η ($= 4\pi\ 10^{-7}$ T^2 J^{-1} m^3) was the permeability of a vacuum and thus $3/2g_e\beta\mu_\eta/4\pi = 27.8$ G nm^3 [19]. The factor 0.75 in the equation was introduced due to the assumption of axial symmetry [18].

This method [18] also allowed us to estimate the amounts of D(B) and S(B) in doubly labeled samples contaminated with singly labeled species, due to incomplete labeling.

All data were analyzed with Origin (Microcal, Northampton, MA, USA).

2.4. Determination of distances between residues in a theoretical cylinder

The α-helices in structural domains of different types of spectrin are coiled coils [9–11], which have 3.5 residues per turn [20,21]. Such conformation has been found most commonly in coiled coils, but also in a single (monomeric) coil [21]. Thus it is reasonable to assume that if the partial domain consisting residues 21–49 is an α-helix, then it may be a coiled coil. However, for simplicity, in our estimation of distances between neighboring amino acid residues in an α-helix, we used parameters for a straight (ideal) α-helix with a cylinder of radius 6.7 Å [18], a pitch of 1.5 Å and 3.6 residues per turn [20]. The distances obtained from our EPR spectra would not allow us to distinguish between the ideal α-helix and the coiled coil α-helix conformations.

3. Results

EPR spectra, at 77 K, of doubly labeled peptides with one cysteine at position 21 and another cysteine at a position two,

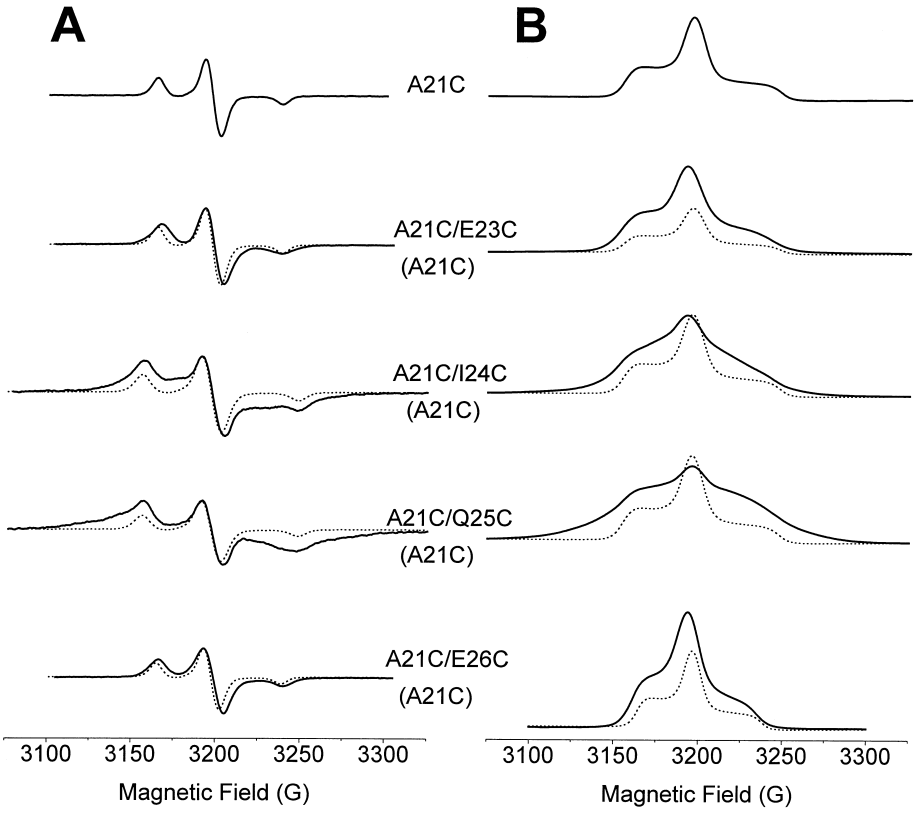

Fig. 1. Conventional first derivative (A) and absorption (B) EPR spectra of MTSSL spin-labeled single cysteine (A21C) and double cysteine (A21C/E23C, A21C/I24C, A21C/Q25C and A21C/E26C) peptides, all derived from a cysteine-less Spα1–368 peptide, in 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 and at 77 K. Various line broadenings were observed in the spectra of A21C/E23C, A21C/I24C, A21C/Q25C and A21C/E26C (solid lines), when compared with that of A21C (dotted lines).

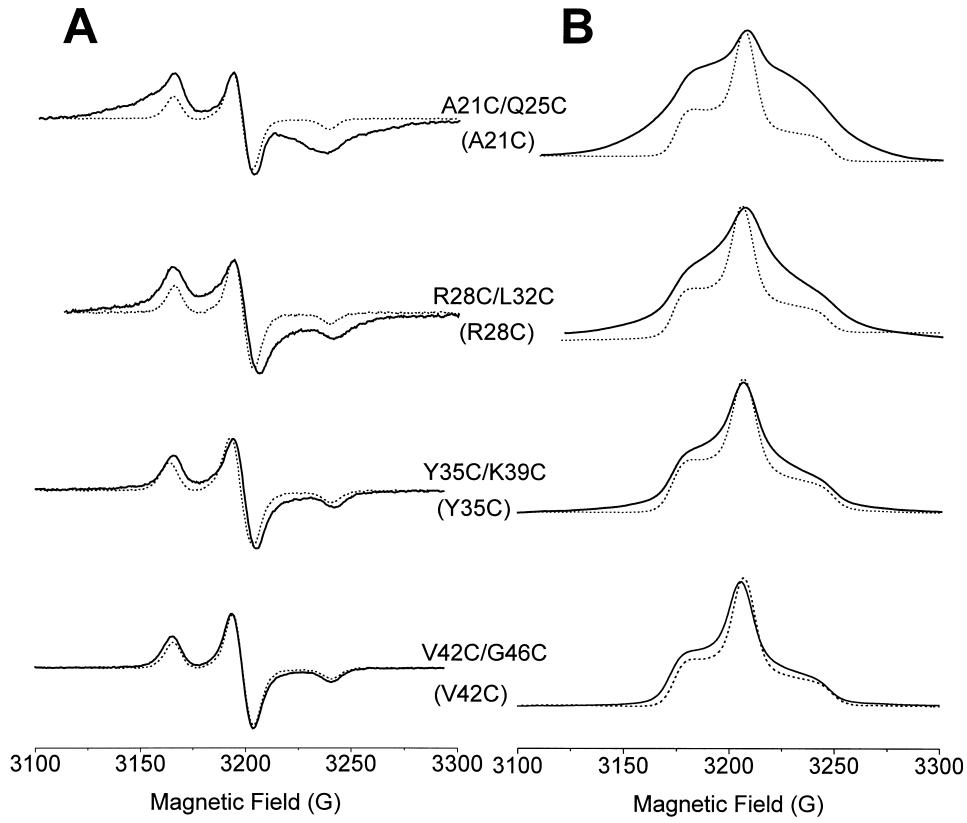

Fig. 2. Conventional first derivative (A) and absorption (B) EPR spectra of MTSSL spin-labeled single cysteine (A21C, R28C, Y35C and V42C, dotted line) and double cysteine (A21C/Q25C, R28C/L32C, Y35C/L32C and V42C/G46C, solid line) peptides, all derived from a cysteine-less Spα1–368 peptide, in 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 and at 77 K.

three, four or five residues away from position 21 (A21C/E23C, A21C/I24C, A21C/Q25C or A21C/E26C, respectively) are shown in Fig. 1 (the first derivative mode in Fig. 1A and the absorption mode in Fig. 1B). These spectra exhibited a various extent of line broadening when compared with the spectrum of the peptide singly labeled at position 21 (A21C). The broadening features were more prominent in the absorption mode (Fig. 1B). The spectra of A21C/E23C, with labels at the *i* and *i*+2 positions, and of A21C/E26C, with labels at the *i* and *i*+5 positions, exhibited some broadening, but less than the spectra of other doubly labeled samples. The spectra of the A21C/I24C peptide, with labels at the *i* and *i*+3 positions, and of the A21C/Q25C peptides, with labels at the *i* and *i*+4 positions, were significantly broader than the spectrum of A21C. The spectrum of A21C/Q25C was the broadest in this family of spectra. The broadening observed in these spectra was most likely due to the dipolar broadening of the spins of two adjacent labels, since we also obtained EPR spectra of these doubly labeled samples in the presence of 6 M urea and observed no broadening (data not shown). It is likely that the spin label pairs in the unfolded peptides were far apart and experienced only weak dipolar interactions that were not detectable by EPR.

In the coiled coil α-helical segment of a peptide, the amino acid residues follow the heptad (*abcdefg*) sequence and can be shown to arrange in a helical wheel pattern [20]. Thus, if the segment containing residues 21–51 folded into a α-helical structure, residues 21, 28, 35 and 42 would then be in the '*d*' position in the heptad. The '*i*+4' residues (residues 25, 32, 39 and 46) would then be in the '*a*' position. Consequently, the spectra for R28C/L32C, Y35C/K39C and V42C/G46C would be broader than those for R28C, Y35C and V42C, but would be similar to those for A21C/Q25C. The spectra of these peptides demonstrated line broadening, when compared with the spectra of R28C, Y35C and V42C, respectively (Fig. 2). These spectra were generally similar to the spectrum of A21C/Q25C. The spectrum of R28C/L32C was quite similar to that of A21C/Q25C. However, the spectra of Y35C/K39C and V42C/G46C were only slightly broadened, suggesting either that the distances between the cysteine pairs at positions 35/39 and 42/46 were larger than the cysteine pairs at positions 21/25 and 28/32, or that there were significant amounts of non-broadened (singly labeled) signals in the spectra of these two peptides. Quantitative analysis of the spectra indicated that these two peptides contained only about 35–45% of signals from doubly labeled species, D(*B*) (dipolar broadened) (45.5 ± 6.4% for Y35C/K39C and 37.0 ± 8.5% for V42C/G46C). Thus simple examination of line width to obtain distance information would not be reliable in these cases. The average amount of D(*B*) was 75.5 ± 14.8% (2–3 experimental runs) for A21C/E23C, 74.5 ± 6.4% for A21C/I24C, 81.7 ± 5.5% for A21C/Q25C, 79.0 ± 22.6% for A21C/E26C and 87.5 ± 2.1% for R28C/L32C.

A more quantitative analysis of the spectra provided values of the average splitting (⟨*B*⟩) and of the average distance (⟨*r*⟩). The ⟨2*B*⟩ and ⟨*r*⟩ values are shown in Table 1. For the A21C family peptides, the peptide A21C/Q25C with the two cysteines at positions 21 and 25 exhibited the largest coupling, and the peptide A21C/E26C with the two cysteines at positions 21 and 26 exhibited the least coupling. These results

Table 1
Values of average splitting ($\langle 2B \rangle$) and average distance ($\langle r \rangle$) for Spα1–368 peptides containing two spin-labeled cysteines at various positions

Peptide	$\langle 2B \rangle$ (G)	r (Å)
A21C/E23C	17.96 ± 0.30	10.5 ± 0.1
A21C/I24C	24.95 ± 1.82	9.4 ± 0.2
A21C/Q25C	36.83 ± 0.99	8.3 ± 0.1
A21C/E26C	12.97 ± 0.21	11.7 ± 0.1
R28C/L32C	36.83 ± 0.99	8.3 ± 0.1
Y35C/K39C	37.02 ± 9.53	8.3 ± 0.7
V42C/G46C	27.35 ± 1.86	9.1 ± 0.2

The $\langle 2B \rangle$ S.E.M. measurements varied, as a result of numerical errors due to the discrete nature of the data and instrumental limitations. Additionally, since the values were only an average, as described in Section 2, actual variation in the distance of the sample distorted the observed signal as also did any anisotropy of the sample.

strongly suggested that the backbone of residues 21–25 was not in a random coil structure, but in a folded structure.

In a theoretical, ideal α-helical cylinder, the calculated distance between two cysteines at the i and i+2 positions was 13.5 Å, at the i and i+3 positions 8.1 Å, at the i to i+4 positions 7.6 Å and at the i to i+5 positions 14.7 Å. These values supported our interpretation that the backbone of residues 21–25 was folded into an α-helix.

The $\langle 2B \rangle$ values for R28C/L32C, Y35C/K39C and V42C/G46C were quite similar to those obtained for A21C/Q25C (Table 1). The $\langle r \rangle$ values were also similar. These values suggest that the region consisting of residues 28–42 also exhibited a helical character. The slight variation in distance values of this series (A21C/Q25C, R28C/L32C, Y35C/K39C and V42C/G46C) probably reflects intrinsic uncertainties in the methods used for data analysis.

4. Discussion

Since it has been shown that the α-spectrin terminus is vital for spectrin tetramerization, the structure of this region is of particular interest for understanding its role in the tetramerization process and in blood disorders resulting from deficiency in spectrin tetramer level. It has been suggested, based on sequence homology and secondary structure algorithms [12], that a partial domain (single α-helix) in the α-spectrin terminus interacts with a complementary partial domain (two α-helices) of the β-spectrin C-terminus to form a complete triple helical bundle. The exact region of the partial domain in either α- or β-spectrin is not known. Based on our phasing information [14], the partial domain in the α-spectrin terminus begins around residue 22 and extends to residue 51. Based on a secondary structural prediction, the partial domain begins around residue 17 [12]. Experimental evidence is needed to provide detailed information on these partial domains. Recombinant peptides with just the first 50 residues do not appear to be well folded. It has been shown that peptides consisting of residues 7–28 (Spα7–28) and 7–53 (Spα7–53) do not fold well and do not bind β-spectrin [16]. Furthermore, peptide Spα1–50 also does not fold well [15]. We have shown that model peptides with two or three domains exhibit stability properties more similar to those of spectrin than peptides with a single domain [17]. It is possible that the first 50 residue region, consisting of the sequence of the proposed partial domain, is unstable as an independent region and is dependent on the presence of at least one or two structural domains [17]. Thus, we used a peptide with three additional domains, Spα1–368, for our studies of the α-spectrin terminus partial domain.

Since the first domain begins at residue 52 [14], we hypothesized that the partial domain consisted of residues 21–51. If the backbone of this region was folded as an α-helix, then residues 21, 28, 35 and 42 should be at position '*d*' in the helical wheel heptad notation, and these residues should be close to residues 25, 32, 39 and 46, respectively (all in the '*a*' position). Our EPR data indicate significant dipolar broadening in these cysteine pairs. Furthermore, we showed that the distances between four cysteine pairs exhibited the following trend: A21C/Q25C < A21C/I24C < A21C/E23C < A21C/E26C, in good agreement with our assumption that the backbone of this region was folded as an α-helix. It should be noted that an assumption was made in the $\langle r \rangle$ equation that the coupling between the two spins was axially symmetric [18]. Many diradicals, however, have been shown to be more anisotropic, with a substantial rhombic component ($E > 0$) [22]. The degree of rhombicity is related to the relative orientation of the electron orbits in each of the spin systems. This relative orientation is unknown, and the distortions in the Pake function [19] caused by this unknown rhombicity will affect the relationship between the distance and the average coupling/splitting.

In conclusion, our EPR data provided strong evidence that the peptide backbone in the region consisting of residues 21–42 in Spα1–368 was folded as an α-helix.

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