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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 11 June 2009
Final form accepted: 21 August 2009
Published online: 08 September 2009

Volume 15 (2010) pp 46-54
DOI: 10.2478/s11658-009-0031-3

Short communication

IMPORTANT RESIDUE (G46) IN ERYTHROID SPECTRIN TETRAMER FORMATION

JIANXIA KANG, YUANLI SONG, AKIN SEVINC and LESLIE W.-M. FUNG*
Department of Chemistry, University of Illinois at Chicago,
845 W. Taylor Street, MC 111, Chicago, IL 60607, USA

Abstract: Spectrin tetramerization is important for the erythrocyte to maintain its unique shape, elasticity and deformability. We used recombinant model proteins to show the importance of one residue (G46) in the erythroid α-spectrin junction region that affects spectrin tetramer formation. The G46 residue in the erythroid spectrin N-terminal junction region is the only residue that differs from that in non-erythroid spectrin. The corresponding residue is R37. We believe that this difference may be, at least in part, responsible for the 15-fold difference in the equilibrium constants of erythroid and non-erythroid tetramer formation. In this study, we replaced the Gly residue with Ala, Arg or Glu residues in an erythroid α-spectrin model protein to give G46A, G46R or G46E, respectively. We found that their association affinities with a β-spectrin model protein were quite different from each other. G46R exhibited a 10-fold increase and G46E exhibited a 16-fold decrease, whereas G46A showed little difference, when compared with the wild type. The thermal and urea denaturation experiments showed insignificant structural change in G46R. Thus, the differences in affinity were due to differences in local, specific interactions, rather than conformational differences in these variants. An intra-helical salt bridge in G46R may stabilize

* Author for correspondence. e-mail: lfung@uic.edu, tel.: (312) 355-5516, fax: (312) 996-0431

Abbreviations used: αI-N1 – αI-spectrin fragment of residues 1-156; βI-C1 – βI-spectrin fragment of residues 1898-2083; G46A – αI-N1 variant with the G46 residue replaced by Ala; G46E – αI-N1 variant with the G46 residue replaced by Glu; G46R – αI-N1 variant with the G46 residue replaced by Arg; ITC – isothermal titration calorimetry; PBS – 5 mM phosphate buffer at pH 7.4 with 150 mM sodium chloride; SpαI – erythroid α-spectrin; SpβI – erythroid β-spectrin; SpαII – non-erythroid α-spectrin; T_m – temperature with 50% thermal unfolding; U_{mid} – urea concentration with 50% unfolding

the partial domain single helix in α-spectrin, Helix C', to allow a more stable helical bundling in the αβ complex in spectrin tetramers. These results not only showed the importance of residue G46 in erythroid α-spectrin, but also provided insights toward the differences in association affinity between erythroid and non-erythroid spectrin to form spectrin tetramers.

Key words: Erythroid spectrin, Tetramerization, G46, mutation, ITC

INTRODUCTION

Erythroid spectrin, a major skeleton protein in the red blood cell membrane, plays a crucial role in maintaining the unique shape, elasticity and deformability of erythrocytes [1-3]. The C-terminal region of the erythroid α- (SpαI) and the N-terminal region of the β- (SpβI) subunits associate to form an αβ hetero-dimer [4, 5]. Two such dimers associate at the opposite end, the N-terminal region of SpαI and the C-terminal region of SpβI, to form a functional tetramer. The tetramerization sites have been studied with recombinant model proteins. For example, the fragment consisting of the first 156 residues of SpαI (αI-N1) and the fragment consisting of residues 1898-2083 of SpβI (βI-C1) have been used for these studies [6]. The N-terminal region of SpαI harbors several clinically important mutations. Several hereditary hemolytic anemia diseases, such as hereditary elliptocytosis and hereditary pyropoikilocytosis, are found to be related to mutations in this region to give lower levels of spectrin tetramers in erythrocytes [7, 8]. High resolution solution NMR studies of αI-N1 show that the region commonly referred to as the N-terminal partial domain of SpαI consists of an unstructured region (residues 1-20) and Helix C' (residue 21-45) [9]. The first triple helical bundle structural domain consists of residues 52-156. Interestingly, NMR results also show that the junction region, residue 46-51, is unstructured. Residue 52 in the first structural domain is also unstructured. Thus, Helix C' is connected to the first helix of the first structural domain by a seven-residue unstructured fragment, resulting in independent motions of Helix C' with respect to the first structural domain. It has been shown that Helix C' associates with helices in the C-terminal region of βI-C1, with a dissociation constant of about 1 μM [6], and that the junction region undergoes conformational change upon association with βI-C1 [10, 11]. We have suggested that this junction region plays an important role in spectrin tetramerization [10, 11].

Non-erythroid α-spectrin (SpαII), with its sequence homologous to that of SpαI, exhibits a tetramer to dimer dissociation constant value 15 times lower than that of SpαI [12]. Small angle X-ray scattering [6] and spin label EPR [13] studies show that the SpαII Helix C' is connected to the first structural domain by a helical junction region. Sequence alignment shows that there is only a single residue difference in the junction region between SpαI and SpαII, which is G46 in SpαI and R37 in SpαII [6]. We suggest that this residue may play a role in

spectrin tetramer formation. It has been reported that the sequences in this region are very strongly conserved over great evolutionary distances (7).

In this study, we replaced Gly of residue 46 in αI-N1 (WT) with Ala (G46A), Glu (G46E), or Arg (G46R), and determined dissociation equilibrium constants (K_d) of their complexes with βI-C1, using isothermal titration calorimetry (ITC) methods. The WT and its variants exhibited a wide range of K_d values, from 0.05 to 10 μM, and we suggest that a salt bridge may be involved in stabilizing the Helix C', allowing for a higher affinity in its association with SpβI.

MATERIALS AND METHODS

Protein preparation and characterization

αI-N1 and βI-C1 were prepared as before [6]. G46A, G46E and G46R plasmids were generated by site directed mutagenesis [11]. The sequences of all plasmids were confirmed by DNA sequencing (DNA Services Facility, Research Resources Center, University of Illinois at Chicago). Recombinant proteins were expressed and purified as before [6, 9]. The protein molecular masses were determined with high resolution LTQ-FT mass spectrometry (Proteomics and Informatics Services Facility, Research Resources Center, University of Illinois at Chicago). The purity was determined by SDS gel electrophoresis. The helical contents of the proteins were determined using circular dichroism methods [6], with a JASCO-810 spectrophotometer.

Protein denaturation

Circular dichroism spectra (190-260 nm) of WT and G46R (5 - 10 μM) in 5 mM phosphate buffer at pH 7.4 with 150 mM sodium chloride (PBS) were collected from 20.0 - 85.0°C with a 5.0°C increment. A 10-min equilibration time was used for each temperature before data collection. The normalized ellipticity at 222 nm as a function of temperature was used to obtain the temperature with 50% thermal unfolding (T_m), as before [14]. Urea induced denaturation was also followed by monitoring ellipticity at 222 nm. Samples with urea (0.0 to 9.0 M with 0.5 M increment), were prepared by adding stock solution of urea in PBS, while keeping the total volume and protein concentration constant. Samples were incubated overnight at room temperature before measurements. The urea concentration with 50% unfolding (U_{mid}) was determined as before [14].

Affinity measurement

All protein samples, WT, G46A, G46E, G46R and βI-C1, were dialyzed in the same PBS buffer overnight. ITC experiment was performed as before [6]. Briefly, after degassing the dialyzed protein samples, βI-C1 (16 - 22 μM) was titrated with 160 - 220 μM of WT, G46A, G46E or G46R at 25°C, using a 7 μl volume and a 750 s interval for each titration injection and a total of 40 injections. The data were fit with a single binding site model to give K_d values.

RESULTS AND DISCUSSION

Protein characterization

The molecular masses of the proteins were within ± 1.0 Da of the expected values. All proteins were at least 90% pure. The helical contents were about 55% for WT and its variants, similar to reported values for WT [6, 11]. The helical content for βI-C1 was 56%, again similar to reported values [6, 11]. The T_m value obtained was 56 ^oC for WT, G46A and G46E and 55 ^oC for G46R. The U_{mid} values were 2.9 M for WT and 3.3 M for G46R. Similar denaturation properties for wild type and mutant proteins indicated that the replacement of Gly to Ala, Arg and Glu did not alter the global conformation of the protein.

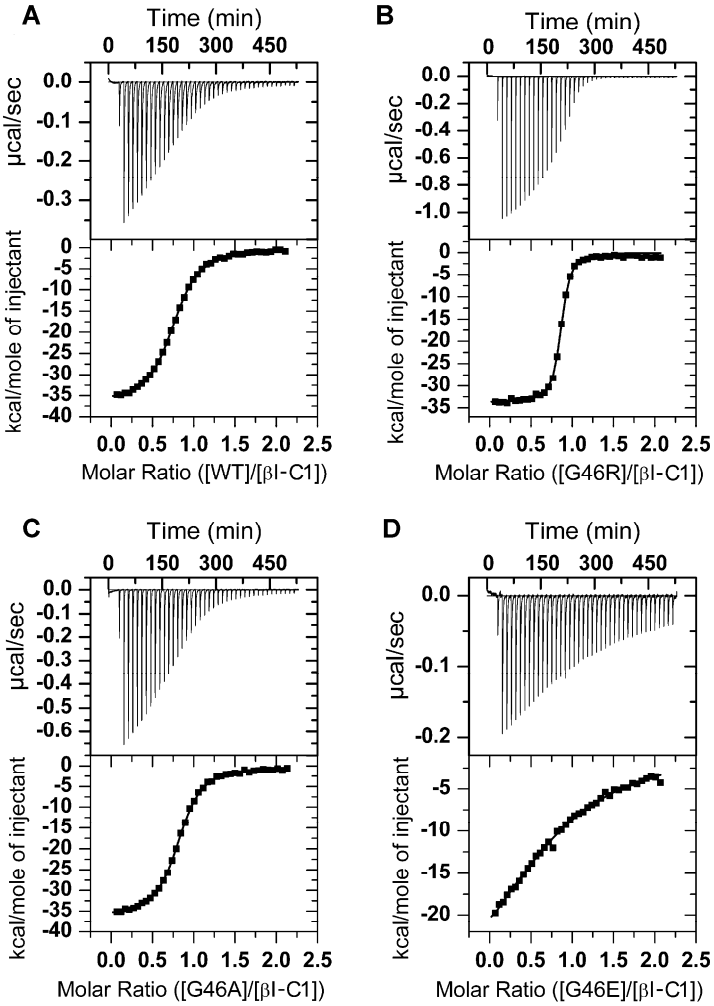
WT, G46R, G46A and G46E exhibited different association affinities toward βI-C1

The K_d value for WT (0.6 μM, Fig. 1 and Tab. 1) was similar to published values [6]. G46R titration yielded a much steeper binding curve, and the K_d value (0.05 μM) was 10 times less than that for WT. G46A titration resulted in a K_d value (0.4 μM) very similar to that of WT. G46E titration yielded a much lower binding affinity (K_d = 9.8 μM), about 16 times weaker than that of WT. The enthalpy values ranged from -32 to -35 kcal/mol, and the entropy (TΔS) values ranged from -24 to -27 kcal/mol for these associations. Our results clearly showed the importance of Gly residue at position 46 of SpαI in maintaining the normal tetramer levels in erythrocytes.

Previous work has demonstrated that the helices, presumably Helices A' and B' after the last structural domain, of the C-terminal region of SpβI associate with Helix C' of the N-terminal region of SpαI to form a triple helical bundle, similar to the structural domains in spectrin [5, 6]. No NMR or X-ray crystallographic structure of the C-terminal region of SpβI has been published. Experimentally determined spectrin structural domains, for example, the 14th structural domain in *Drosophila* spectrin [16], the 16th domain in chicken brain α-spectrin [17] and the 1st structural domain in human erythrocyte α-spectrin [10], indicated the presence of significant side chain interactions in helix bundling.

The non-polar side chains at "*a*" and "*d*" heptad sequence positions allow for hydrophobic effect to give a coiled-coil packing [17]. The conformational change of the junction region (residues 46-52) of SpαI, from unstructured to helical, suggested that residues with side chains that promote helical conformation in the region to allow better coiled-coil packing may increase its association affinity with SpβI. Ala, Arg and Glu all exhibit helical propensities larger than Gly, with values 0, 0.21, and 0.40 kcal/mol, respectively, compared to 1 kcal/mol for Gly [18]. Although Ala is a strong helix-forming amino acid, the mutation of G46 to G46A did not affect the association suggesting that the increased affinity in G46R was not likely due to increased helical propensity in Arg side chain.

The significant increase in the association affinity of G46R, and the significant decrease in that of G46E were most likely due to the negatively charged Glu residue at position 50 (Fig. 2).



ERVAERGQKL EDSYHLQVFK αI 40-59
ELSTLRRQKL EDSYRFQFFQ αII 31-50

Fig 1. ITC analysis of the association of αI-N1 WT and its variants with βI-C1. βI-C1 (about 16 μM) in the calorimetric cell was titrated with WT (about 160 μM) (A), G46R (B), G46A (C) or G46E (D), with 7 μl for each titration injection. All samples were dialyzed in the same buffer of 5 mM phosphate at pH 7.4 with 150 mM sodium chloride. The sequence of residues 40-59 of αI, including G46, is given under (C) and (D). The helical regions are in bold and the junction regions are double underlined. The aligned αII sequence of residues 31-50 is also given. The junction region in αII is helical (Ref. 13).

Tab 1. K_d values from ITC results of the association of αI-N1 WT and its variants with βI-C1 at 25 °C and pH 7.4. The values were the mean values of a triplicate titration. The ITC data were fitted with a single binding site model to give K_d values. The mean values for the binding site (N) from curve fitting are also shown.

	K_d (μM)	N
WT	0.56 ± 0.01	0.72 ± 0.05
G46R	0.05 ± 0.01	0.78 ± 0.06
G46A	0.41 ± 0.00	0.74 ± 0.09
G46E	9.77 ± 0.06	0.78 ± 0.06

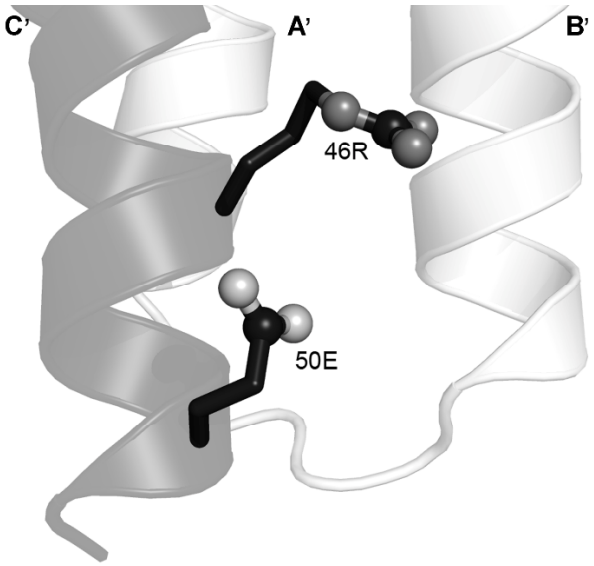


Fig 2. Model structure of Helix C' (dark gray) in the presence of β-spectrin partial domain Helix A' and Helix B' (light gray) (Song *et al*., 2009, **Protein Sci.**, in press) in the region consists of residues 46R and 50E to indicate their potential interaction. Positive guanidinium group and negative carboxylic group were shown with N as dark gray sphere, and O as light gray sphere. Black spheres are C atoms.

When the junction region, which includes residues 46 - 52, becomes helical upon binding βI-C1 [12], residue 50 is the $(i+4)^{th}$ residue, with residue i at position 46. Thus, in G46R, the Arg residue at position 46 probably forms an intra-chain salt bridge with Glu at position 50 to stabilize Helix C' and therefore the complex structure. However, in G46E, the Glu residue at position 46 probably destabilizes Helix C' due to electrostatic repulsion with another Glu at position 50. In general, salt bridges can contribute 0.2-0.5 kcal/mol for surface-exposed residues and 3-5 kcal/mol for buried residues in the hydrophobic core of a globular protein [19].

These results would also predict a higher affinity in SpαII, since corresponding to G46 in SpαI is R37 in SpαII. The K_d value of αII-N1 with βI-C1 is 12 nM [6], which is a lower value than that of G46R (0.05 μM). Thus, other factors are also involved to provide higher affinity for tetramer formation in non-erythroid spectrin than in erythroid spectrin.

Two single point mutations of SpαI, G46C and G46V, have been studied previously, with K_d values of 2 μM for G46C [11], and of 3.7 μM for G46V [7]. G46V is a pathogenic mutation responsible for mild hereditary elliptocytosis. These results show a decreased affinity by the replacement of the Gly residue with non-ionic amino acid residues.

Two clinical mutations related to mild and severe elliptocytosis, R45T and R45S, respectively, were studied before [20]. Although R45T and R45S showed moderately and significantly decreased affinity with SpβI, respectively, NMR studies of both proteins demonstrated only minor conformational changes in the local region flanking residue 45, and there was no global conformational change of Helix C' or of the first structural domain. Consistent with these results, the G46R mutation also showed no global conformational change on αI-N1. Thus, the binding affinity variation most likely originates in the variation of local interactions in the complex, due to a change in molecular recognition and association. Recently, a similar conclusion was drawn for other clinical mutations [8].

CONCLUSION

We identified position 46 in the junction region of αI-spectrin in this work as being crucial to spectrin tetramerization. In G46R, the positively charged side-chain at position 46 (Arg residue) produced an increased affinity, a 10-fold decrease in K_d value, without altering the thermal stability of the protein, probably due to a specific electrostatic interaction to stabilize Helix C'. In G46E, the negatively charged side-chain residue (Glu) consequently exhibited a reduced affinity, a 16-fold increase in K_d value. We concluded that residue 46 in the N-terminal junction region of αI-spectrin is important for spectrin tetramerization. Our study implies that in αII-spectrin tetramerization, R37 is also an important residue. Mutation at this position, for example, to a residue with negatively charged side chain, or even with a neutral side chain, may result in neurological disorders and may affect interactions of other proteins with αII-spectrin [21].

Acknowledgments. We would like to thank Dr. B. G. Forget of the Yale University School of Medicine for the cDNA of erythrocyte spectrin. This work was supported by a grant from NIH to LWMF (GM68621). The high-resolution/high-mass accuracy LTQ-FT mass spectrometer was supported by grants from the Searle Funds at the Chicago Community Trust to the Chicago Biomedical Consortium and the University of Illinois at Chicago Research Resources Center.

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