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### Short communication

## IMPORTANT RESIDUE (G46) IN ERYTHROID SPECTRIN TETRAMER FORMATION

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**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  $\alpha$ -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  $\alpha$ -spectrin model protein to give G46A, G46R or G46E, respectively. We found that their association affinities with a  $\beta$ -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

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Abbreviations used:  $\alpha$ I-N1 –  $\alpha$ I-spectrin fragment of residues 1-156;  $\beta$ I-C1 –  $\beta$ I-spectrin fragment of residues 1898-2083; G46A –  $\alpha$ I-N1 variant with the G46 residue replaced by Ala; G46E –  $\alpha$ I-N1 variant with the G46 residue replaced by Glu; G46R –  $\alpha$ I-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; Sp $\alpha$ I – erythroid  $\alpha$ -spectrin; Sp $\beta$ I – erythroid  $\beta$ -spectrin; Sp $\alpha$ II – non-erythroid  $\alpha$ -spectrin;  $T_m$  – temperature with 50% thermal unfolding;  $U_{mid}$  – urea concentration with 50% unfolding

the partial domain single helix in  $\alpha$ -spectrin, Helix C', to allow a more stable helical bundling in the  $\alpha\beta$  complex in spectrin tetramers. These results not only showed the importance of residue G46 in erythroid  $\alpha$ -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  $\alpha$ - (Sp $\alpha$ I) and the N-terminal region of the  $\beta$ - (Sp $\beta$ I) subunits associate to form an  $\alpha\beta$  hetero-dimer [4, 5]. Two such dimers associate at the opposite end, the N-terminal region of Sp $\alpha$ I and the C-terminal region of Sp $\beta$ I, 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 Sp $\alpha$ I ( $\alpha$ I-N1) and the fragment consisting of residues 1898-2083 of Sp $\beta$ I ( $\beta$ I-C1) have been used for these studies [6]. The N-terminal region of Sp $\alpha$ I 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  $\alpha$ I-N1 show that the region commonly referred to as the N-terminal partial domain of Sp $\alpha$ I 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  $\beta$ I-C1, with a dissociation constant of about 1  $\mu$ M [6], and that the junction region undergoes conformational change upon association with  $\beta$ I-C1 [10, 11]. We have suggested that this junction region plays an important role in spectrin tetramerization [10, 11].

Non-erythroid  $\alpha$ -spectrin (Sp $\alpha$ II), with its sequence homologous to that of Sp $\alpha$ I, exhibits a tetramer to dimer dissociation constant value 15 times lower than that of Sp $\alpha$ I [12]. Small angle X-ray scattering [6] and spin label EPR [13] studies show that the Sp $\alpha$ II 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 Sp $\alpha$ I and Sp $\alpha$ II, which is G46 in Sp $\alpha$ I and R37 in Sp $\alpha$ II [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  $\alpha$ I-N1 (WT) with Ala (G46A), Glu (G46E), or Arg (G46R), and determined dissociation equilibrium constants ( $K_d$ ) of their complexes with  $\beta$ I-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  $\mu$ M, and we suggest that a salt bridge may be involved in stabilizing the Helix C', allowing for a higher affinity in its association with Sp $\beta$ I.

## MATERIALS AND METHODS

### Protein preparation and characterization

$\alpha$ I-N1 and  $\beta$ I-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  $\mu$ M) 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  $\beta$ I-C1, were dialyzed in the same PBS buffer overnight. ITC experiment was performed as before [6]. Briefly, after degassing the dialyzed protein samples,  $\beta$ I-C1 (16 - 22  $\mu$ M) was titrated with 160 - 220  $\mu$ M of WT, G46A, G46E or G46R at 25°C, using a 7  $\mu$ l 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  $\pm$  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  $\beta$ I-C1 was 56%, again similar to reported values [6, 11]. The  $T_m$  value obtained was 56 °C for WT, G46A and G46E and 55 °C 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 $\beta$ I-C1

The  $K_d$  value for WT (0.6  $\mu$ M, 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  $\mu$ M) was 10 times less than that for WT. G46A titration resulted in a  $K_d$  value (0.4  $\mu$ M) very similar to that of WT. G46E titration yielded a much lower binding affinity ( $K_d = 9.8 \mu$ M), about 16 times weaker than that of WT. The enthalpy values ranged from -32 to -35 kcal/mol, and the entropy ( $T\Delta S$ ) values ranged from -24 to -27 kcal/mol for these associations. Our results clearly showed the importance of Gly residue at position 46 of Sp $\alpha$ I 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 Sp $\beta$ I associate with Helix C' of the N-terminal region of Sp $\alpha$ I 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 Sp $\beta$ I has been published. Experimentally determined spectrin structural domains, for example, the 14<sup>th</sup> structural domain in *Drosophila* spectrin [16], the 16<sup>th</sup> domain in chicken brain  $\alpha$ -spectrin [17] and the 1<sup>st</sup> structural domain in human erythrocyte  $\alpha$ -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 Sp $\alpha$ I, 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 Sp $\beta$ I. 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).

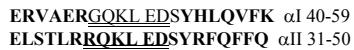
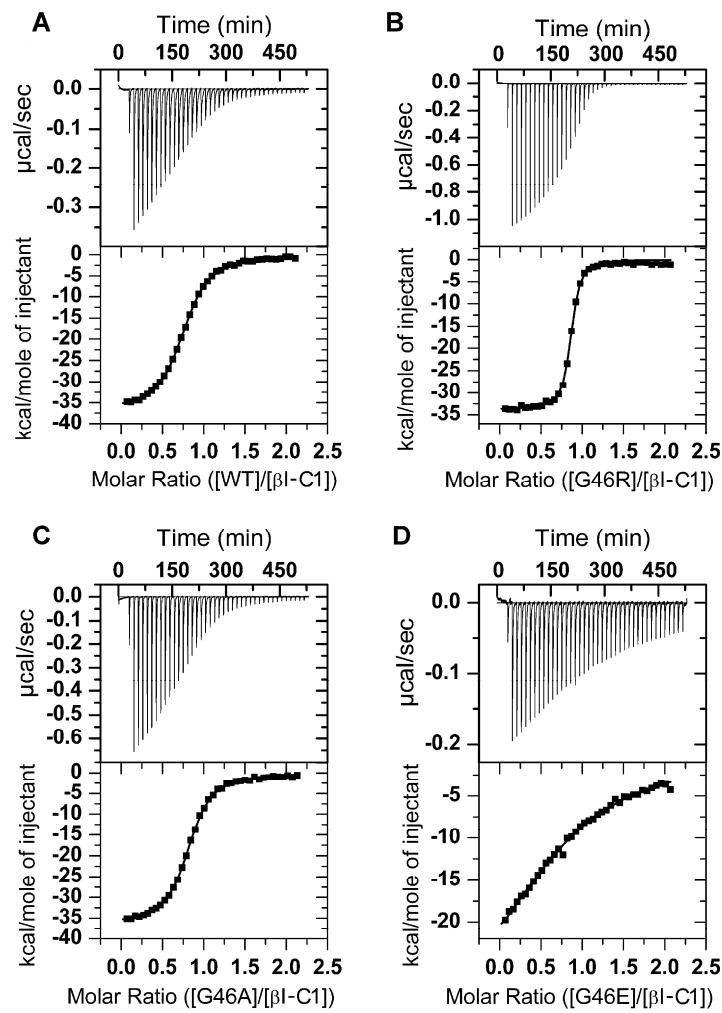


Fig 1. ITC analysis of the association of αI-N1 WT and its variants with βI-C1. βI-C1 (about 16 μM) in the calorimetric cell was titrated with WT (about 160 μM) (A), G46R (B), G46A (C) or G46E (D), with 7 μl for each titration injection. All samples were dialyzed in the same buffer of 5 mM phosphate 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  $\alpha$ I-N1 WT and its variants with  $\beta$ I-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.

	$K_d$ ( $\mu$ M)	N
WT	$0.56 \pm 0.01$	$0.72 \pm 0.05$
G46R	$0.05 \pm 0.01$	$0.78 \pm 0.06$
G46A	$0.41 \pm 0.00$	$0.74 \pm 0.09$
G46E	$9.77 \pm 0.06$	$0.78 \pm 0.06$

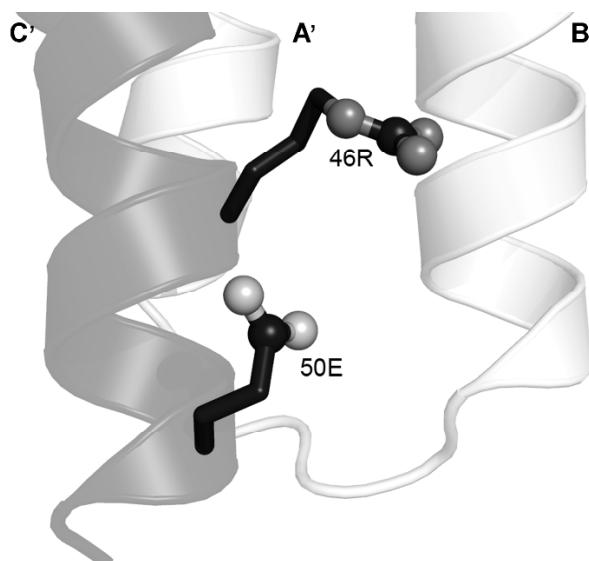


Fig 2. Model structure of Helix C' (dark gray) in the presence of  $\beta$ -spectrin partial domain Helix A' and Helix B' (light gray) (Song *et al.*, 2009, *Protein Sci.*, in press) in the region consists of residues 46R and 50E to indicate their potential interaction. Positive guanidinium group and 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  $\beta$ I-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  $\text{Sp}\alpha\text{II}$ , since corresponding to G46 in  $\text{Sp}\alpha\text{I}$  is R37 in  $\text{Sp}\alpha\text{II}$ . The  $K_d$  value of  $\alpha\text{II-N1}$  with  $\beta\text{I-C1}$  is 12 nM [6], which is a lower value than that of G46R (0.05  $\mu\text{M}$ ). 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  $\text{Sp}\alpha\text{I}$ , G46C and G46V, have been studied previously, with  $K_d$  values of 2  $\mu\text{M}$  for G46C [11], and of 3.7  $\mu\text{M}$  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  $\text{Sp}\beta\text{I}$ , 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  $\alpha\text{I-N1}$ . 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  $\alpha\text{I}$ -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  $\alpha\text{I}$ -spectrin is important for spectrin tetramerization. Our study implies that in  $\alpha\text{II}$ -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  $\alpha\text{II}$ -spectrin [21].

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