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Ankyrin Recognizes Both Surface Character and Shape of the 14-15 di-repeat of β -Spectrin

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Abstract

The spectrin-based cytoskeleton is critical for cell stability, membrane organization and membrane protein trafficking. At its core is the high-affinity complex between β -spectrin and ankyrin. Defects in either of these proteins may cause hemolytic disease, developmental disorders, neurologic disease, and cancer. Crystal structures of the minimal recognition motifs of ankyrin and β -spectrin have been determined and distinct recognition mechanisms proposed. One focused on the complementary surface charges of the minimal recognition motifs, whereas the other identified an unusual kink between β -spectrin repeats and suggested a conformation-sensitive binding surface. Using isothermal titration calorimetry and site-directed mutagenesis, we demonstrate the primacy of the inter-repeat kink as the critical determinant underlying spectrin's ankyrin affinity. The clinical implications of this are discussed in light of recognized linker mutations and polymorphisms in the β -spectrins.

Keywords

spectrin; ankyrin; erythrocyte membrane; isothermal titration calorimetry

Introduction

Ankyrin, a membrane-associated adaptor protein, forms a high-affinity complex with spectrin thereby creating an essential link between membrane proteins and the spectrin-based

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Authorship contributions:

Penelope J. La-Borde – made 5 β I 14-15 mutants, expressed and purified proteins, performed ITC measurements, and analyzed the data.

Paul Stabach – made other β I 14-15 mutants, reviewed the manuscript.

Ivana Simonovic – assisted in site-directed mutagenesis, protein purification and ITC measurements; reviewed manuscript.

Jon S. Morrow – designed parts of the research, analyzed data, and wrote parts of the manuscript.

Miljan Simonovic – designed experiments, analyzed data, wrote parts of the manuscript, and prepared figures and tables.

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cytoskeleton. Disruption of this complex affects membrane stability, lateral distribution of membrane proteins, and membrane protein trafficking and anchoring, leading to a growing array of recognized pathologies including hemolytic anemias, cardiac arrhythmias, and neurodegenerative disease. The molecular basis of these disorders stem from a failure of the ankyrin-spectrin linkage confined within a region spanning repeats 14 and 15 of β -spectrin (β 14-15) [1,2]. This region is conserved in β -spectrin isoforms I–IV, all of which bind ankyrin with high affinity. Ankyrin, conversely, binds spectrin within its highly conserved ZU5 domain [2,3]. Recently, crystal structures of both β 14-15 and ZU5 have been determined, and distinct mechanisms to explain the specificity of ankyrin-binding have been proposed [4,5]. Ipsaro *et al.* [4] suggested that complementary surface charges between acidic residues in β I14 and a positive face of ZU5 were the critical determinants of complex formation, while Stabach *et al.* [5] suggested that an unusually large kink between repeats β I14 and β I15 created the ankyrin-binding site. In this latter model, residues from helix C of β I14 (h14C) and the β I14-15 linker were critical for ankyrin recognition. Given that single nucleotide polymorphisms leading to frame-shift and missense mutations exist in or about these regions in various spectrins, as well as the established association of several linker mutations with hemolytic disease, it was of interest to further refine the boundaries and mechanism of ankyrin binding under physiologic conditions in solution. We prepared single-site mutants that span both putative ankyrin-binding sites, and measured their affinity for ankyrin in solution by isothermal titration calorimetry (ITC). Our results highlight the importance of the conformation between spectrin di-repeats as a critical determinant of ligand specificity, and suggest that seemingly innocuous mutations/polymorphisms in the various spectrin linker/repeats may have physiologic consequences.

Materials and Methods

Protein purification and ITC

The β I14-15 constructs were expressed and purified as previously described [5]. ZU5 was expressed at 17°C in BL21 (DE3) cells. Upon lysis in 20mM Tris-HCl pH 8.0, 0.3M NaCl, 10% glycerol, the cleared lysate was loaded onto a Ni-affinity column and ZU5 was eluted with 300mM imidazole. The final purification step for all the proteins was done on a S75 size-exclusion column in 50mM Tris-HCl pH 8.0, 200mM NaCl. Spectrin samples were concentrated to 400 μ M, whereas ZU5 was concentrated to 40 μ M. The pure proteins were flash-frozen in liquid nitrogen and stored at –80°C. ITC was carried out at 25°C using a Microcal VP-ITC calorimeter. The heat change was measured by titrating β I14-15 into the cell containing ZU5. The binding parameters were calculated in Microcal Origin 5.0 [6].

Results and Discussion

Two distinct sites on β I14-15 have been proposed to mediate ankyrin recognition (Figure 1A, B). The first putative site is composed of D₁₇₀₀, D₁₇₀₁, E₁₇₀₃, E₁₇₀₈, E₁₇₁₀, E₁₇₇₀, D₁₇₇₃, E₁₇₇₇, D₁₇₈₁ and E₁₇₈₄ (Figure 1A) [4]. These residues are located in helices A (h14A) and C (h14C) of β I14 and form a negatively charged patch on the surface of β I14-15 that is not found in other spectrin di-repeats (Figure 1A). The second putative site is located at the C-terminus of h14C, the 14-15 linker and the loop 15B-C and comprises D₁₇₈₁, E₁₇₈₄, L₁₇₉₂, T₁₇₈₈, Y₁₈₆₆ and A₁₈₆₇ (Figure 1B) [5]. To determine which site is important for ankyrin recognition, we have analyzed the ZU5 binding ability by ITC of ten single-site mutants that span both putative binding regions of the β 14-15 di-repeat. The ZU5 domain was used because it binds spectrin with the same affinity as the full-length ankyrin [2]. All proteins were purified by size-exclusion chromatography before ITC measurement (see: Materials and Methods). The mobility of all mutant β I 14-15 constructs in the size-exclusion chromatography was indistinguishable from the WT protein, indicating that they were compact and monodisperse,

and no tendency to aggregation was detected (that might have affected the ITC measurements, data not shown). When wild type (WT) β I 14-15 was titrated into the ZU5 sample, a curve indicative of strong single-site binding was obtained (Figure 2A). Regardless of the β I 14-15 construct, the binding was endothermic with a large positive entropic component suggesting that ankyrin-spectrin complex formation is spontaneous under physiological conditions (Table 1, Figures 2 and 3). The affinity of WT β I 14-15 for ZU5 was 194nM (Table 1, Figure 2A). To ensure that the observed heat change was a consequence of a specific protein-protein interaction and not due to the heat of solvation, three control experiments were performed: (i) buffer was titrated into the ZU5 sample, (ii) the 9–10 di-repeat of α II spectrin was titrated into ankyrin, and (iii) the WT β I 14-15 construct was titrated into the buffer alone. No significant heat change was observed in these control experiments (Figure 2G, H, and data not shown), confirming that the heat change observed on titration of the WT β I 14-15 into ankyrin was due to spectrin-ankyrin complex formation.

In the next set of measurements the contribution of the ankyrin-binding site as proposed by Ipsaro et al. [4] (Figure 1A) was tested. In particular, the negatively charged residues of h14A and the C-terminal part of h14C were analyzed. Residues from the N-terminal of h14C (E₁₇₇₀, D₁₇₇₃, and E₁₇₇₇) were not examined herein because we have previously shown that replacing any of these residues with alanine did not affect interaction with ankyrin [5]. Our ITC results revealed that most of the mutants of h14A (D1700A, D1701A, E1703A and E1708A) did not significantly affect ankyrin binding, retaining K_d values of 320–440nM (Table 1, Figure 2B–E). However, the E1710A mutant exhibited an ~9-fold reduction in affinity (1.7 μ M) compared to the WT protein, suggesting that E₁₇₁₀ might be directly involved in binding to ankyrin (Table 1, Figure 2F). Inspection of the three-dimensional structure of β I14-15 reveals that the side-chain of E₁₇₁₀ is spatially very close to the C-terminal of h14C and the 14-15 linker (Figure 1B), residues previously implicated in ankyrin-binding [5]. This observation led us to speculate that E₁₇₁₀, D₁₇₈₁, E₁₇₈₄, L₁₇₉₂, and T₁₇₈₈ might form the complete ankyrin-binding site (Fig. 1B). In agreement with this proposal, mutations D1781A and T1788A decreased the binding affinity by 10- (2.1 μ M) and 30-fold (5.8 μ M), respectively (Table 1, Figure 3A, C). Moreover, the E1784A mutation decreased the strength of the interaction by more than two orders of magnitude (43.4 μ M) (Table 1, Figure 3B). These results confirm that polar and acidic residues at the C-terminus of h14C and the 14-15 linker interact with ankyrin directly. In general, these results demonstrate that the closer a residue is to the linker region, the stronger is its involvement in ankyrin recognition. Another region of β I-spectrin that is sequentially distant but spatially adjacent to the linker region is the loop that connects helices B and C in the repeat 15. This loop is also known as loop 15B-C. We have previously speculated that the Y₁₈₆₆-A₁₈₆₇ couple in the 15B-C loop stabilizes the unusual kink between repeats 14-15, that in turn might promote formation of the ankyrin-binding site [5]. This proposal is supported by a comparison of the three published crystal structures of the β I14-15 di-repeat. Two crystals derived from β I-spectrin display nearly identical 14-15 di-repeat structures [4,5], whereas the third contains the 14–16 tri-repeat from β II-spectrin [7]. Despite differences in crystallization conditions, crystal packing, and in the actual molecule crystallized, the inter-repeat angle between β I4 and β I5 is indistinguishable between them (Figure 4). This structural conservation further suggests that the angle between repeats 14 and 15 is important for function, especially when compared to chicken α -spectrin repeats where the inter-repeat angle deviates significantly in four crystal forms [8].

To test if the inter-repeat angle and the conformation of the 15B-C loop are critical for ankyrin binding, the single-site mutants of the Y₁₈₆₆-A₁₈₆₇ couple were tested for their ability to bind ankyrin. The Y₁₈₆₆ side-chain forms hydrogen bonds with the backbone atoms of β I14 and presumably stabilizes the inter-repeat kink [5], whereas A₁₈₆₇ probably plays a role in stabilizing the overall conformation of the 15B-C loop. When Y₁₈₆₆ was replaced by alanine, binding affinity decreased by a hundred fold (22 μ M) (Table 1, Figure 3D). It is likely that the

replacement of the bulky tyrosine side-chain with a small hydrophobic alanine removed hydrogen bonds and hydrophobic interactions that stabilized both the inter-repeat angle and the overlying ankyrin-binding site. Similarly, when A₁₈₆₇ was substituted with proline, ankyrin affinity declined 70-fold (14.9 μ M) (Table 1, Figure 3E). Proline presumably introduces additional constraints to the 15B-C loop that perturb both the orientation of the Y₁₈₆₆ side-chain and the inter-repeat angle. Collectively, these results indicate that ankyrin recognition is mediated by solvent-exposed residues in and around the linker region and by the distinct shape of the 14-15 di-repeat, and not by the negatively charged surface of the 14th repeat.

Conclusions

Ankyrin binding is uniquely conferred on the 14-15 di-repeat of the β -spectrins by C-terminal polar residues in h14C and the 14-15 linker, in concert with the 14-15 kink and the conformation of the 15B-C loop (Figures 1B and 1C). The results rule out a mechanism based primarily on complementary surface charges.

These findings have several potential clinical implications. Review of the SNP databases (dbSNP, www.ncbi.nlm.nih.gov/snp) reveals polymorphisms (e.g. frame shift, missense) in the β -spectrins clustered in or about regions analogous to the ankyrin binding region (highlighted in Figure 1D). A particular hot spot for these mutations is in the B-C loop of the downstream repeat, a site corresponding to the critical 15B-C loop in β I14-15. The finding of so many polymorphisms in this region implies that either this region is functionally unimportant, or that variation can bestow subtle but meaningful functional differences in the ability of spectrin to bind ankyrin and presumably other ligands (5).

Our results exclude the possibility that this region is not important, and in fact demonstrate that even minor variation within this critical linker-loop region may have major effects on spectrin's ligand-binding properties. Indeed, many mutations associated with hereditary hemolytic anemia occur in the inter-repeat links [9]. Thus, while hemolytic phenotypes or neurodegenerative disease may define the most severe of the spectrin-ankyrin binding deficiencies, we postulate that additional, perhaps more subtle, pathologies or functional distinctions are likely to accompany polymorphisms in this site or in similar regions of another di-repeat units that may interact with different ligands [5]. It will thus be important as genome-wide association screens evolve to be alert to the potential pathologic consequences of polymorphisms in this critical region of spectrin.

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Figure 1. ITC measurements revealed that ankyrin interacts with the surface residues of β II14 and that it recognizes the distinct shape of the β II14-15 di-repeat

(A) Cartoon representation of the β I-spectrin 14-15 di-repeat structure with one putative ankyrin-binding site highlighted in red. The electrostatic surface potential map reveals a large acidic patch on β II14-15 comprised of negatively charged residues from h14A and h14C. This patch has been proposed to interact directly with the positively charged surface of the ZU5 domain of ankyrin (4). (B) The alternative putative ankyrin-binding site is located near the 14-15 linker region and includes residues from both β -spectrin repeats. Cartoon representation of β I 14-15 is displayed on the left for orientation. The view is rotated 90° clockwise around the vertical axis relative to A. The surface potential map reveals a less pronounced acidic

character of this site. This site also includes residues in the B–C loop of β I15 (5). **(C)** Surface representation of the ankyrin-binding site determined by ITC. The ankyrin-binding site includes E1710 from h14A and is almost identical to the one proposed by Stabach *et al.* (5). The site is shown in two orientations: the view on the left is the same as in **A**, whereas the view on the right is the same as in **B**. The di-repeat is beige and the residues critical for ankyrin binding are red. **(D)** SNP database analysis reveals that missense mutations in β I–IV spectrin repeats cluster in and around the linker region. Mutations (green) are mapped onto the surface of the β I 14–15 di-repeat depicted in the same orientations as in **C**.

Figure 2. With the exception of D1710, no other residues in h14A interact with ankyrin (A) ITC data for the wild-type (WT) β I 14-15 reveal high-affinity binding. (B–F) The binding curves for D1700A, D1701A, E1703A, E1708A and E1710A β I14-15 mutant constructs respectively. Regardless of the β I-spectrin construct tested, the interaction event is enthalpically unfavorable (endothermic). However, a large positive entropic component facilitates the complex formation (see: Table 1). (G) When the buffer solution was titrated into the ZU5 sample, no significant heat change was recorded. (H) Titration of the 9–10 di-repeat of α II-spectrin into ZU5 did not produce significant heat change.

Figure 3. C-terminal residues of h14C, the linker, and the B–C loop of β I 15 are critical for ankyrin binding

(A–E) ITC data for the D1781A, E1784A, T1788A, Y1866A and A1867P mutants respectively reveal impaired binding to the ZU5 domain. Complex formation remained endothermic with a large positive entropic value.

Figure 4. The inter-repeat angle between repeats 14 and 15 in β -spectrin is conserved in three crystal forms

The 14-15 di-repeat structures of β I-spectrin [4,5] were superimposed onto the corresponding di-repeat of the β II-spectrin [7]. Only the main-chain atoms were used in calculation. The structure reported by Stabach et al. [5] is colored in red, that of Ipsaro et al. [4] is in olive, and that of Davies et al. [7] is in blue.

Table 1

Thermodynamic parameters for complex formation between various β I14-15 constructs and the ZU5 domain of ankyrin. All the data were calculated based on a single-site binding. (± 2 SD).

β I14-15	ΔH (cal/mol)	ΔS (cal/mol/K)	K_d (μM)
WT	$5,976 \pm 56$	50.76	0.19 ± 0.03
D1700A	$4,423 \pm 48$	43.90	0.44 ± 0.06
D1701A	$4,639 \pm 42$	45.01	0.36 ± 0.04
E1703A	$4,818 \pm 42$	45.89	0.32 ± 0.04
E1708A	$3,270 \pm 76$	40.10	0.43 ± 0.12
E1710A	$3,733 \pm 50$	38.92	1.70 ± 0.21
D1781A	$5,519 \pm 171$	44.45	2.10 ± 0.39
E1784A	$5,213 \pm 229$	37.45	43.40 ± 4.25
T1788A	$2,076 \pm 65$	30.95	5.73 ± 0.91
Y1866A	$2,523 \pm 99$	29.77	22.00 ± 0.29
A1867P	904 ± 61	25.11	14.90 ± 4.00