Short communication

YEAST TWO-HYBRID AND ITC STUDIES OF ALPHA AND BETA SPECTRIN INTERACTION AT THE TETRAMERIZATION SITE

AKIN SEVINC, MARTA A. WITEK and LESLIE W.-M. FUNG*
Department of Chemistry, University of Illinois at Chicago,
845 W. Taylor Street, MC 111, Chicago, IL 60607, USA

Abstract: Yeast two-hybrid (Y2H) and isothermal titration calorimetry (ITC) methods were used to further study the mutational effect of non-erythroid alpha spectrin (αII) at position 22 in tetramer formation with beta spectrin (βII). Four mutants, αII-V22D, V22F, V22M and V22W, were studied. For the Y2H system, we used plasmids pGBKT7, consisting of the cDNA of the first 359 residues at the N-terminal region of αII, and pGADT7, consisting of the cDNA of residues 1697-2145 at the C-terminal region of βII. Strain AH109 yeast cells were used for colony growth assays and strain Y187 was used for β-galactosidase activity assays. Y2H results showed that the C-terminal region of βII interacts with the N-terminal region of αII, either the wild type, or those with V22F, V22M or V22W mutations. The V22D mutant did not interact with βII. For ITC studies, we used recombinant proteins of the αII N-terminal fragment and of the erythroid beta spectrin (βI) C-terminal fragment; results showed that the K_d values for V22F were similar to those for the wild-type (about 7 nM), whereas the K_d values were about 35 nM for V22M and about 90 nM for V22W. We were not able to detect any binding for V22D with ITC methods. This study clearly demonstrates that the single mutation at position 22 of αII, a region critical to the function of non-erythroid α spectrin, may lead to a reduced level of spectrin tetramers and

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

abnormal spectrin-based membrane skeleton. These abnormalities could cause abnormal neural activities in cells.

Key words: Spectrin tetramerization subunit interactions, Yeast two-hybrid, Isothermal titration calorimetry

INTRODUCTION

Spectrin, a prominent cytoskeletal protein, exerts its fundamental role in cells by forming a sub-membrane filamentous network. An essential aspect of the spectrin network formation is the tetramerization of spectrin $\alpha\beta$ heterodimers. We have previously used the yeast two-hybrid system and random mutagenesis to investigate the effects of amino acid mutations on the tetramerization of non-erythroid (brain) spectrin (fodrin) [1]. The Y2H techniques have been developed as convenient and useful methods to screen for protein interactors [2-4], particularly when libraries of vectors containing protein cDNAs are commercially available. We have used such methods to identify some interactors of non-erythroid alpha spectrin (αII) [5]. These studies are often qualitative in nature - a protein either interacts or does not interact with another protein. However, several studies report quantitative results from Y2H studies. For example, colonies of Y2H system with common polymorphisms of *BRCA1* from cancer predisposing mutations were considerably smaller than controls [6], colony growth rates (cell viability) correlate with the strengths of interactions [7, 8], the levels of transcription activation correlate with the strength of the binding interaction in a "small colony phenotype", a growth phenotype discovered serendipitously [9], and β-galactosidase activities correlate with protein-protein interaction affinities [10, 11]. Yet, some authors indicate that, "our results emphasize the difficulty of attempting to quantitate differences in affinity from two-hybrid experiments alone" [7]. Others show that Y2H results do not correlate with protein affinities [12-14]. Since protein expression, structures and nature of interaction may vary from system to system in Y2H systems, many studies have focused on studying single mutation effects on protein-protein interactions [*e.g.*, 9].

In our study, we used both the Y2H system and ITC methods to further study the mutational effect of αII at position 22 on tetramer formation. Previously we have used ITC methods to determine K_d values of $\alpha\beta$ heterodimer association to form tetramers in model systems [*e.g.,* 15-19]. Recently, we found that mutation of αII at position 37 increases the K_d value from about 9 nM for αII with beta I spectrin (βI) to 10 μM for the R37P mutation [15]. Residue 22 in αII corresponds to a "*d*" position in the heptad repeat and is in the interface of the triple helical bundle in $\alpha\beta$ tetramers [15]. In this study, we found that the mutation effect was most severe for V22D, followed by V22W and V22M, whereas little effect was observed for V22F.

METHODS

Yeast two-hybrid assays

The Y2H system with colony growth and β-galactosidase detection methods were used to determine the interaction between βII and αII, wild-type or its mutants, at the tetramerization region. The Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) was used. The yeast strain Y187, which is auxotrophic for leucine and tryptophan with Gal4-inducible *lac*Z gene, or strain AH109, which is auxotrophic for adenine, histidine, leucine, lysine, tryptophan and uracil and with Gal4-inducible *lac*Z genes was used. Plasmids pGBKT7 (pBD) with the cDNA of the non-erythroid alpha spectrin (αII) consisting of the first 359 residues at the N-terminal region (αII-N) (pBD-αII-N) and pGADT7 (pAD) with the cDNA of beta-spectrin consisting of residues 1697-2145 at the C-terminal region (βII-C) (pAD-βII-C) were previously prepared [1]. Plasmids of two mutations at position 22 of αII-N, V22W and V22M, prepared by standard methods [20] as well as two previously prepared mutants (V22D and V22F) [1] (pBD-αII-N-V22Δ) were also used.

For the colony growth assay, AH109 cells with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22Δ, were grown at 30°C on agar plates with a growth medium containing all essential amino acids but tryptophan, leucine and histidine, and lacking adenine (SD/-W/-L/-H/-A with SD Minimal Agar Base and -Leu/-Trp/-His/-adenine DO Supplement, both from Clontech) for three days before photography. Under this high-stringency growth condition, cells with strongly interacting protein pairs grow and form colonies, whereas colonies with proteins with low-affinity interactions may be missed (Clontech user manual). We also prepared pAD-βI-C, with βI-C consisting of residues 1898-2083 of βI and performed colony growth assay with pBD-αII-N or pBD-αII-N-V22D.

For β-Galactosidase assay via colony lift method, strain Y187 cells with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22Δ, were grown at 30°C on agar plates with a growth medium containing all essential amino acids but leucine and tryptophan (SD/-Leu/-Trp with SD Minimal Agar Base and -Leu/-Trp DO Supplement; both from Clontech) for three days before colony lifting steps, as described in the manufacturer user manual. Cells with interacting protein pairs produce β-galactosidase to give a blue color on filter papers when soaked with a solution consisting of its substrate, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal soaking solution, see Clontech user manual).

Isothermal titration calorimetry

Recombinant proteins αII-N, αII-N-V22Δ (V22D, V22F, V22M and V22W) and βI-C were prepared, following standard laboratory techniques [15]. Briefly, protein expression vector pGEX-2T was used to express glutathione S-transferase fusion protein, and purified with affinity column chromatography, with thrombin cleavage of fusion protein. DNA sequence analysis and protein mass spectrometry analysis results were obtained (Research Resources Center,

University of Illinois at Chicago). Protein purity was checked with gel electrophoresis, using 16% polyacrylamide gel with 0.1% SDS. Helical contents of the proteins were determined using circular dichroism spectra [20]. We have found that βI-C and βII-C proteins exhibit similar affinities for αII-N [15]. However, βII-C recombinant protein is more difficult to prepare than βI-C protein due to its low expression level. Thus, βI-C was used for ITC experiments. ITC measurements were performed at 25°C using an isothermal titration calorimeter (VP ITC, MicroCal, LLC, Northampton, MA) [15]. Protein pairs (βI-C with αII-N, or αII-N-V22Δ) were dialyzed overnight in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4 (PBS) to ensure identical solution conditions in titrating protein pairs. In addition, all samples were thoroughly degassed prior to calorimetry titration. Each αII-N or αII-N-V22Δ sample (30 μM) was titrated into the sample cell containing βI-C protein (3 μM). Titrations of βI-C (30-100 μM) into αII-N or αII-N-V22Δ (3 μM) were also performed. Titration isotherms were analyzed with a single binding site assumption, as before [15], to obtain dissociation constants, K_d.

RESULTS

Yeast two-hybrid assays
For colony growth assay, cells with either βI-C or βII-C and with αII-N or αII-N-V22F, -V22M, or -V22W formed well separated colonies with diameters of 2-5 mm after 3 days, with no specific colony size associated with cells of a particular mutant (Fig. 1). However, cells with αII-N-V22D, with either βI-C (data not shown) or βII-C (Fig. 1) did not show any growth after 3 days (Fig. 1). For the β-galactosidase activity (colony-lift) assay, Y187 cells with αII-N or αII-N-V22F, -V22M, or -V22W showed a distinct blue color, but without a consistent color variation associated with cells with a particular mutation (Fig. 2).

Isothermal titration calorimetry assay
Recombinant protein analysis
The SDS gel electrophoresis data showed that all αII-N (wild type and mutants) and βI-C proteins were ~90% pure. Electrophoretic masses were ~42 kDa for αII-N proteins and ~22 kDa for βI-C. Mass spectrometric results showed 42,241.0 Da for αII-N (expected mass is 42,242.5 Da), 42,258.6 Da for αII-N-V22D (expected mass is 42,258.5 Da), 42,289.0 Da for αII-N-V22F (expected mass is 42,290.6 Da), 42,274.8 Da for αII-N-V22M (expected mass is 42,274.6 Da), 42,329.8 Da for αII-N-V22W (expected mass is 42,329.6 Da) and 22,036.9 Da for βI-C (expected mass is 22,036.9 Da). The CD spectra of αII-N, αII-N-V22Δ and βI-C exhibited characteristic features of similar spectrin recombinant proteins [21], with minima at 222 and 208 nm. Helical contents were ~75%, in good agreement with published results [21].
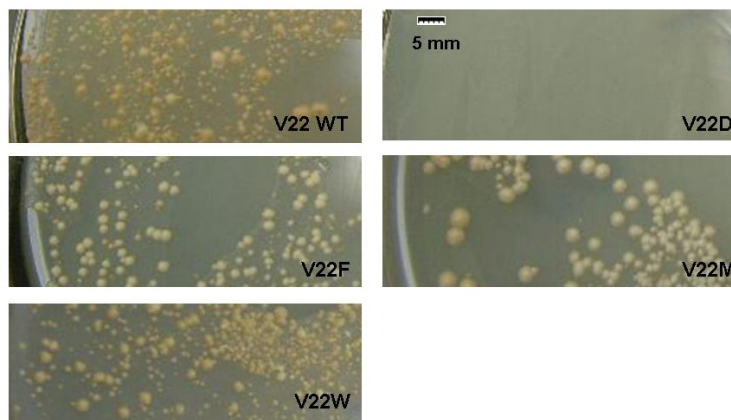
Fig. 1. Colony Growth Assay. AH109 cells co-transformed with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22D, -V22F, -V22M, or -V22W, were grown for 3 days at 30°C, following procedures from the manufacturer (Clontech). Colonies, 2-5 mm in diameter, were found for cells expressing αII-N (marked as V22 WT above), αII-N-V22F (V22F), αII-N-V22M (V22M), or αII-N-V22W (V22W), whereas cells expressing αII-N-V22D (V22D) did not show any growth. The scale bar is shown in top right panel.
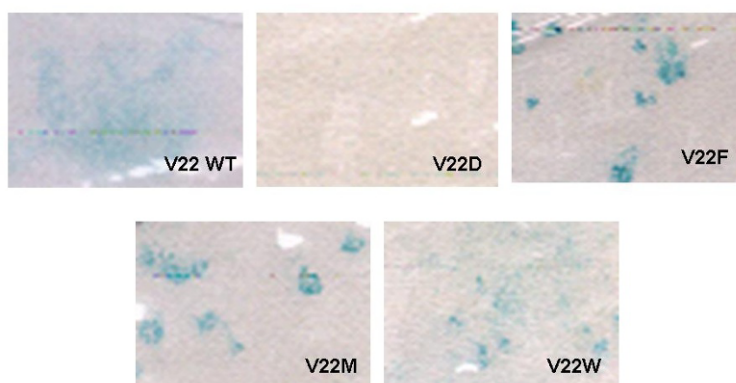

Fig. 2. β-Galactosidase Activity Assay via Colony Lift Method. Y187 cells co-transformed with pAD-βII-C and pBD-αII-N, -αII-N-V22D, -αII-N-V22F, -αII-N-V22M, or -αII-N-V22W were grown for 3 days at 30°C following procedures from the manufacturer (Clontech). Colonies were transferred onto filter papers, subjected to freeze-thaw cycles, and incubated on a second set of filter papers pre-soaked with β-galactosidase substrate (X-gal) for 30 min. Filter papers for colonies with αII-N, αII-N-V22F, αII-N-V22M and αII-N-V22W all showed blue color, but those with αII-N-V22D did not show blue color.

ITC results

The ITC isotherm of βI-C/αII-N system at 25°C showed that sufficient heat (-0.45 μcal/sec) was released during titration of αII-N into βI-C (Fig. 3), with an average K_d value of 6.9 ± 0.5 nM (n = 3), in good agreement with previous findings of a similar system (with βI-C) [19], and the values are similar to that

with βII-C [18]. The K_d value was 6.7 $\pm$ 0.3 nM for βI-C/αII-N-V22F, 35 $\pm$ 4 nM for βI-C/αII-N-V22M and 93 $\pm$ 28 nM for βI-C/αII-N-V22W. However, for βI-C/αII-N-V22D system, there was insufficient heat released either when αII-N-V22D (30 μM) was titrated with βI-C (3 μM) (Fig. 3, Tab. 1), or when βI (30-100 μM) was titrated with αII-N-V22D (3 μM), indicating that the K_d value for this system is larger than 100 μM.
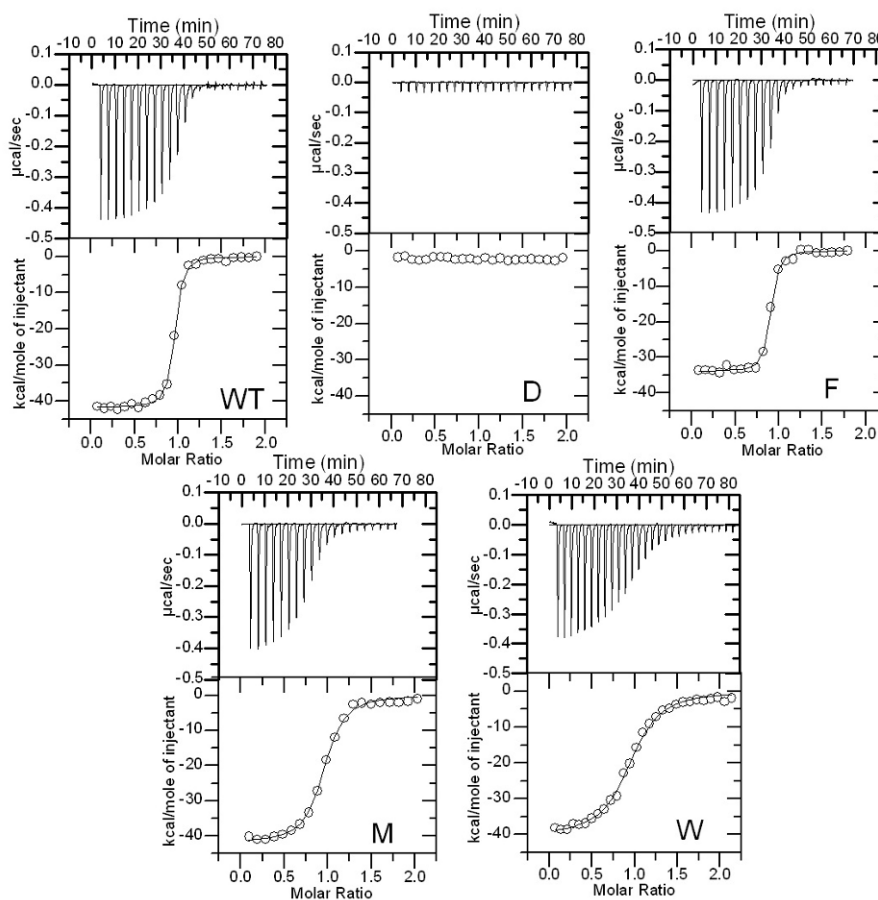

Fig. 3. ITC Measurements. Recombinant protein samples of βI-C, αII-N (marked as WT above), αII-N-V22D (D), αII-N-V22F (F), αII-N-V22M (M) and αII-N-V22W (W) were dialyzed together in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4 and degassed thoroughly prior to ITC measurements. αII-N proteins (29-35 μM) were each individually titrated into the sample cell containing βI-C protein (3 μM). Typical ITC titration isotherms and fitted curves are shown. The average K_d values (n = 3), determined from the fitted curves using a single-binding site model of the manufacturer (MicroCal) software, were 6.9 nM for βI-C/αII-N and 6.7 nM for βI-C/αII-N-V22F, 35 nM for βI-C/αII-N-V22M and 93 nM for βI-C/αII-N-V22W. Little heat was released for βI-C/αII-N-V22D titration and no K_d was obtained.

Tab. 1. Y2H and ITC results of alpha and beta spectrin model proteins interaction.

pBD Plasmid[a]	Colony Growth[b]	β-Galactosidase Activity[c]	K_d (nM) [d]
αII-N	Yes	Blue color	6.9
αII-N-V22D	No	No color	Not detectable
αII-N-V22F	Yes	Blue color	6.7
αII-N-V22M	Yes	Blue color	35
αII-N-V22W	Yes	Blue color	93

[a]pAD-βII-C with different pBD-αII-N plasmids in the Y2H experiment; we also used pAD-βI-C with pBD-αII-N or pBD-αII-N-V22D, and the results were the same as those with pAD-βII-C; [b]yeast AH109 cells were grown in a medium containing all essential amino acids but tryptophan, leucine and histidine, and lacking adenine; [c]yeast Y187 cells were grown in medium containing all essential amino acids but leucine and tryptophan for the colony lift assay; [d]ITC experiments using recombinant proteins of αII-N and mutants listed and of βI-C were carried out at 25°C in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4.

DISCUSSION

The Y2H systems have been widely used to study protein-protein interactions. In this study, both colony growth and β-galactosidase activity detection results showed that αII spectrin with mutations V22F, V22M or V22W interacted with βII spectrin at the tetramerization site (N-terminal region of αII and C-terminal region of βII). However, αII-N-V22D did not interact with βII-C. With those αII mutants that interacted with βII-C, we were not able to detect any differences in interactions between V22F, V22M or V22W with βII-C. Both colony growth rate and colony size, as well as the blue color indication for β-galactosidase activity did not show detectable differences between V22, V22F, V22M and V22W. The ITC methods require not only the preparation of recombinant proteins but also the characterization of these proteins for proper functional analysis. In our systems, we characterized the protein systems with high resolution mass spectrometry analysis as well as by circular dichroism analysis. We have found that, for both α and β spectrin recombinant proteins used for tetramerization studies, it is important to obtain their CD results to demonstrate that the proteins are folded properly before ITC experiments. The ITC results show that the K_d values for V22F and the wild type with βI-C were about the same, with a K_d of about 7 nM. However, V22M and V22W both exhibited lower affinity than the wild type, with K_d values of 35 nM and 93 nM, respectively. The ITC results of V22D titration with βI-C showed little interaction, with K_d values larger than 100 μM. As indicated in Methods section, we have found that βI-C and βII-C proteins exhibit similar affinities for αII-N [15], and in this study we showed that results similar to those of βII-C were obtained when βI-C was used with αII-N wild type or with V22D.

Spectrin tetramer formation involves the bundling of three helices, one from α (Helix C') and two from β (Helix A' and Helix B'), forming a triple helical bundle [15, 21]. Mutations that affect the triple helical bundling lead to lower

affinity. Previous studies reveal that the V22 position of αII is critical for its tetramerization with βII [1]. Sequence alignment shows that αII V22 corresponds to V31 in erythroid α spectrin (αI). αI V31 has been identified as a hot spot that leads to severe clinical symptoms [22]. In triple helical bundling of αII and βII helices, an N-terminal hydrophobic cluster [18] involves three residues in the αII Helix C' (I15, V22, and L23) and two residues in the βII Helix A' (V2019 and F2022), and one residue in the βII Helix B' (F2073) [15]. Thus, it is not surprising that mutations at the V22 position may affect non-erythroid spectrin tetramer formation. Since V22 is involved in a hydrophobic cluster during helical bundling to form tetramers, a mutation from V to a charged residue D clearly weakens the hydrophobic cluster and thus severely reduces the ability of V22D to interact with Helices A' and B' in βII-C. Mutation of V22 to other hydrophobic residues such as V22F did not affect its interaction with βII-C. The mutations of V22M and V22W lowered the affinity by about 5 times and 10 times, respectively. Hydrophobicity of individual side chains, and the properties of the interacting clusters also affected the triple helical bundling. The K_d values determined by ITC represented a ΔG value of about -46.6 kJ/mol (11.1 kcal/mol) for βI-C with either αII-N or αII-N-V22F, -42.6 kJ/mol (-10.1 kcal/mol) with αII-N-V22M, and -40.1 kJ/mol (-9.6 kcal/mol) with αII-N-V22W. Thus, the tetramers of these αII mutants and β spectrin exhibit slightly differing stabilities from each other. As discussed previously [18], αII spectrin has recently been reported to be essential for stabilizing nascent sodium channel clusters [23], assembling the mature node of Ranvier [23], and regulating endothelial cell-cell contacts [24]. The tetramer formation of αII-βII spectrin is also essential in the regulatory step for neuritogenesis [25]. Tetramerization is clearly important for spectrin function. At present, no clinical mutations in αII spectrin, including the tetramerization region, have been identified. A reduced level of spectrin tetramers and abnormal spectrin-based membrane skeleton could cause abnormal neural activities in cells.

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