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The Design and Preparation of a Model Spectrin Protein: βII-Spectrin L2079P

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Spectrin isoforms are cytoskeletal proteins that give stability to cells. Site directed mutagenesis was used to replace residue 2079 in brain spectrin βII from leucine to proline, the corresponding amino acid in red blood cell spectrin βI. We have shown previously that, in spectrin βI, the region downstream of the proline residue is unstructured, whereas the corresponding region in spectrin βII (downstream of a leucine residue) appears to be helical. This structural difference has been suggested to be responsible for binding specific proteins to each β-spectrin isoform, with G5 only to βI-spectrin and F11 only to βII-spectrin. Thus, it is possible that the mutation from leucine to proline in βII-spectrin may lead to a conformational change in βII, from helical to unstructured. In this study, a recombinant protein consisting of a fragment of βII-spectrin, with L2079P mutation, has been designed and prepared.

Introduction

Spectrin isoforms are common cytoskeletal proteins that gives the stability and the unique shape to many cells. Spectrin isoform of the brain cells (spectrin II) plays a critical role in neuronal growth and secretion. Spectrin isoform of the red blood cells (spectrin I) provides deformability in red blood cells. Both spectrin I and spectrin II consist of two subunits, α-spectrin and β-spectrin, to form $\alpha\beta$ heterodimers. Two heterodimers associate at the N-terminus of α-spectrin and the C-terminus of β-spectrin to form a functional $(\alpha\beta)_2$ tetramer.[1] In forming spectrin tetramers, the affinity between the αIIβII heterodimers is much greater than that of αIβI heterodimers. Consequently, the brain spectrin forms a stable network for complex neurological functions, and the red blood cell spectrin forms a flexible network to allow red blood cells to deform and to pass through small capillaries. In studies with tetramerization site model proteins, erythroid (red blood cell) alpha spectrin consisting of residues 1-368 (αI-N3), non erythroid (brain) alpha spectrin consisting of residues 1-359 (αII-N3), erythroid beta spectrin consisting of residues 1898-2083 (βI-C1), and non-erythroid beta spectrin consisting of residues 1906-2091 (βII-C1), the αIβI association exhibits equilibrium dissociation constants (K_d) in μM range and αIIβII association in nM range.[2,3] However, it is found that the difference in the affinity is largely due to structural differences in αI- and αII-spectrin, since the K_d values for αIβI association is about the same as those for the αIβII association.[4]

Despite their 80% sequence homology and similar affinity to α-spectrin isoforms, βI- and βII-spectrin selectively βInd to proteins G5 and F11, respectively. G5 and F11 were identified as β-spectrin interacting proteins in a study using phage display methods to screen a single-chain-variable-fragment library.[4] G5 binds to an unstructured region downstream of the residue 2071 (proline) of βI-spectrin (Figure 1A). However, the corresponding region in βII-spectrin assumes a helical conformation downstream of the corresponding residue 2079 (leucine) (Figure 1B). βII does not bind G5, instead it binds F11. In this study, βII-spectrin model protein, βII-C1, which consisted of residues 1906-2091, was used as the wild-type as well as the tempate to prepare L2079P mutant. The mutation of βII from leucine to proline at residue 2079 may disrupt the helical conformation beyond this point to give a conformation more similar to that of βI and thus function more similar to βI than βII. However, mutation at this site should not disrupt the association with αII-spectrin.

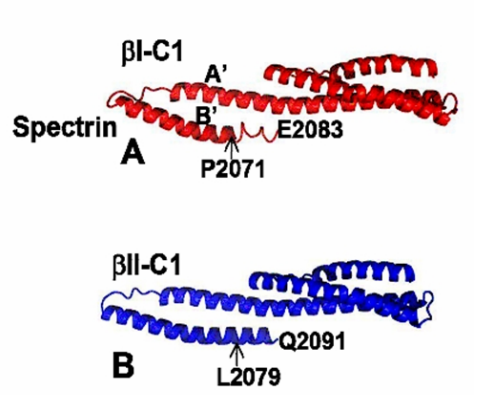

FIG. 1: Proposed C-terminal structures of βI and βII spectrin (from reference 3). **(A)** In βI spectrin the C-terminal region downstream of residue P2071 is unstructured, and **(B)** the corresponding region in βII spectrin downstream of corresponding residue L2079 is helical. Mutation L2079P may change the helix into unstructured conformation to resemble the structure of βI in this region

Materials and Methods

Standard method using primer-mediated site-directed mutagenesis procedures was used to introduce mutation L2079P. To design the primers, we used the wild type deoxyribose nucleic acid (DNA) sequence (gene code: NM 003128) for amino acid residues 2075 - 2083. The DNA sequence is 5' GCC CTG GAA AGG CTG ACT ACA TTG GAG 3', with the underlined codon as the leucine codon. A primer with the following sequence was designed to introduce the L2079P mutation - 5' GCC CTG GAA **AGG CCT** ACT ACA TTG GAG 3', with the double underlined codon as the proline codon. The nucleotide sequence in bold is the *StuI* recognition site. A specific restriction site was introduced for analysis purpose, since a successful restriction enzyme digestion indicates a successful introduction of the mutated sequence. The reverse complimentary primer was also designed. This pair of primers was then ordered from UIC Research Resources Center (RRC). A glutathione S-transferase (GST) fusion protein plasmid, pGEX-2TΔ, previously modified[4] to contain the wild type sequence for βII-spectrin consisting of residues 1906 - 2091, was used as the parent template for polymerase chain reactions (PCR) in the presence of the designed primers to generate DNA with the mutation. PCR was performed in a thermal cycler using these primers and the parent template. The PCR product was subjected to *DPN1* restriction enzyme digestion to remove the methylated parent template.

This modified pGEX-2T plasmid was transformed into DH5α competent *E. coli* cells (Clonetech, Mountain View, CA), and the cells were grown on agar plates with LB medium and ampicillin at 37°C overnight. The colonies were then used to innoculate a liquid culture (4 mL LB media) for 37°C overnight growth. The plasmid was extracted from the cells and digested with *StuI* and *BamHI* restriction enzymes. The digestion product was applied to a 1.3% agarose gel for electrophoresis analysis. The agarose gel was prepared by dissolving 2 g of agarose in 150 mL of Tris-acetate-EDTA (TAE) buffer. The mixture was heated to dissolve and poured into a gel caster to make the 1.3% agarose gel. A "Low Mass DNA standard" (NEB, Ipswich, MA) was used as a reference for DNA size (in base pairs, kilobase). Six trials were done with one negative control. The plasmid DNA was also submitted to UIC RRC for DNA sequencing.

The plasmid with correct sequence was then transformed into BL21 competent *E. coli* cells (Clonetech, Mountain View, CA) for protein expression. Protein over-expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG; from Gold Biootechnology, St. Louis, MO). Small amount of cells were first grown for whole cell electrophoresis analysis to ensure proper protein expression. Electrophoresis was performed with a 16% polyacrylamide gel in sodium dodecyl sulfate (SDS) solution. With a positive whole cell electrophoresis result, a large scale preparation of GST-βII-C1 L2079P protein was done with the BL21 cells grown in LB media (2 L) at 37°C in a flask (4 L), placed in a temperature controlled shaker (Lab line, Melrose Park, IL). After about 3 hr. growth, with optical density measured at 600 nm (OD_{600}) $\sim$ 0.3, IPTG (0.5 mM) was added, followed by another 3 hr growth at 27°C in the temperature controlled shaker. The cells were dissolved in 4 mL of 1% Triton lysis buffer, followed by centrifugation at 4600g for 20 min. The supernatant was then loaded onto a column packed with GST affinity resin (Sigma Aldrich, St. Louis, MO), pre-washed extensively with a 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 (PBS). The GST-βII-C1 L2079P fusion protein was immoβIlized on the resin while the rest of the E. coli proteins were eluted off the column with the buffer. GST βII C1 L2079P protein was then eluted using PBS containing freshly added glutathione (Sigma Aldrich, St. Louis, MO). Electrophoresis in SDS solution was performed on the fusion protein fractions. SigmaGel 1.0 Software (Jandel Scientific, San Rafael, CA) was used to analyze the gel to determine the protein purity. The protein was submitted to UIC RRC for molecular mass determination using mass spectroscopy.

The same procedure was used to obtain αII model protein consisting of residues 1 - 359 (αII-N3) and the wild type βII-C1.

Isothermal titration calorimetry (ITC) measurements were done using a VP-ITC (MicroCal, LLC, Northampton, MA) at 25°C. The proteins were dialyzed extensively in PBS buffer overnight at 4°C.

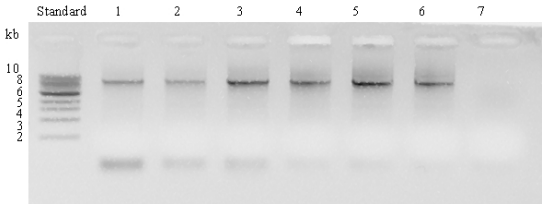

FIG. 2: Electrophoresis of PCR products after *DPN1* digestion on an agarose gel (1.3%). A DNA marker sample was loaded and labeled as Standard to show the mobility of DNA fragments, in kilobases (kb). Samples with varying DNA template-to-primer ratios were loaded to Lanes 2-6. A band at about 7 kb was observed suggesting that the DNA plasmid was amplified. Lane 7 is that of a negative control, showing no DNA amplification.

Results

The gel electrophoresis of PCR products shows modified and amplified DNA plasmid (about 7 kb) (Figure 2, Lanes 1-6). The negative control lane shows no DNA band (Lane 7). DNA sequencing results of the PCR products clearly indicate that L2079 mutation has been intro-

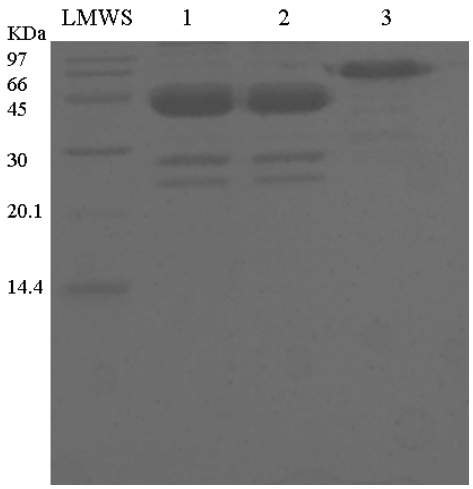

FIG. 3: Electrophoresis of protein samples in SDS buffer on a polyacrylamide gel (16%). Lane 1 is the βII-C1 L2079P with 93% purity, Lane 2 is the βII-C1 wild type with 92% purity, and Lane 3 is αII-N3 with 95% purity.

duced.

From cell growth using cells with this modified plasmid and 2 L medium, we obtained $\sim$ 1 g of cells harboring the L2079 protein, and about 27 mg of GST-βII-C1 L2079P protein at 93% purity, as shown in Figure 3, Lane 1. The purity of the GST-βII-C1 wild-type is $\sim$ 92% (Lane 2). The purity of the GST-αII-N3 is $\sim$ 95% (Lane 3). Mass spectrometry analysis indicates correct mass for the mutant protein.

ITC titration results (Figure 4) show that βII-C1 L2079P associated with αII-N3 protein with a K_d value of $\sim$ 200 nM for the complex.

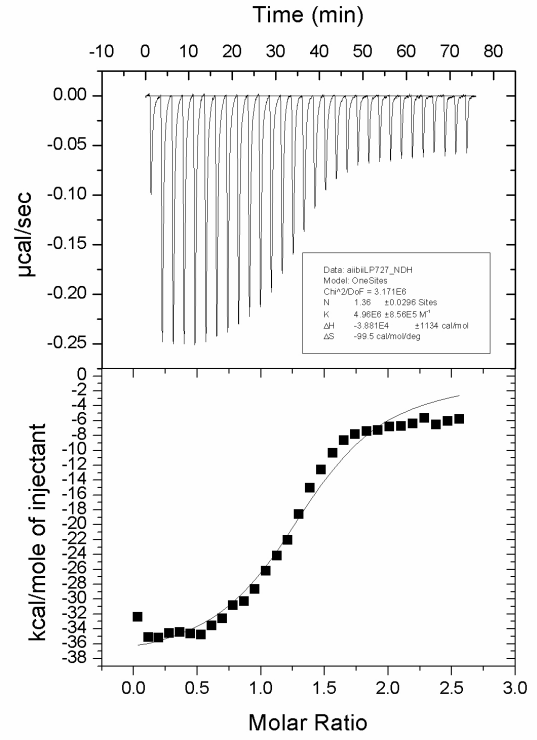

FIG. 4: ITC results indicate that the βII-C1 L2079P mutant is still functional since it associates with αII-N3, but with lower affinity than the wild type. 2.1 μM βII-C1 L2079P was used in the sample cell and 35 μM αII-N3 was used in the titrating syringe. Fusion proteins were used. The K_d from the titration was 200 nM.

Discussion

The recombinant protein βII-C1 L2079P was successfully prepared in large quantity and in high purity. ITC results of αII-N3 and βII-C1 L2079P show that the protein associates with its binding partner αII-N3. The K_d value of the complex is larger than that of the wild type complex (K_d $\sim$ 10 nM), suggesting that the mutation induces a conformational change in βII-C1 to give a reduced affinity with αII-N3. Thus, this model protein can now be used for further structural studies to determine its conformational changes and its affinity with G5 and F11 proteins.

Acknowledgements

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Abbreviations

- αI-N3 - erythroid (red blood cell) alpha spectrin consisting of residues 1-368
- αII-N3 - non-erythroid (brain) alpha spectrin consisting of residues 1-359
- βI-C1 - erythroid (red blood cell) beta spectrin consisting of residues 1898-2083
- βII-C1 - non-erythroid (brain) beta spectrin consisting of residues 1906-2091
- DNA - deoxyribose nucleic acid
- GST - glutathione S-transferase
- ITC - isothermal titration calorimetry
- kb - kilo base

- OD_{600} - optical density measured at 600 nm
- PCR - polymerase chain reaction
- PBS - 5 mM phosphate buffer with 150 mM NaCl at pH 7.4
- SDS - sodium dodecyl sulfate
- TAE - tris-acetate-EDTA

[1] D. W. Speicher, T. M. Desilva, K. D. Speicher, J. A. Ursitt, P. Hembach, and L. Weglarz, J. Biol. Chem **268**, 4227 (1993).
[2] P. A. Bignone and A. J. Baines, Biochem. J. **374**, 613 (2003).
[3] F. Long, D. McElheny, S. Jiang, S. Park, M. S. Caffrey, and L. W.-M. Fung, Protein Sci **16**, 2519 (2007).
[4] Y. Song, C. Antoniou, A. Memic, B. K. Kay, and L. W.-M. Fung (2010), manuscript in progress.