Solution structure of
α-syntrophin PH-PDZ tandem domain

by

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Solution structure of
\(\alpha\)-syntrophin PH-PDZ tandem domain

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Abstract

The focus of this dissertation is the solution structure of \(\alpha\)-syntrophin. I chose \(\alpha\)-syntrophin to study as it acts as an adaptor protein plays fundamental roles in eukaryotes.

\(\alpha\)-syntrophin is a cytoplasmic adapter protein that associates with dystrophin glycoprotein complex and has putative signaling and structural roles at neuromuscular junctions. The solution structure of N-terminal PH-PDZ tandem domain of \(\alpha\)-syntrophin was determined by multidimensional NMR spectroscopy. The PH domain of \(\alpha\)-syntrophin adopts a \(\beta\)-sandwich structure capped by a long \(\alpha\)-helix on one edge. The PDZ domain of \(\alpha\)-syntrophin is composed of a partially
opened barrel capped by two α-helices on each open side. The link region of the two domains is mainly random coil except for the carboxyl terminal extension of the PDZ domain. Although the PH domain has a PDZ sequence insertion in its primary structure, its tertiary structure is not affected by the PDZ domain. In the PH-PDZ tandem of α-syntrophin, each domain is well folded, but the orientation between the two domains is not defined due to the lack of inter domain contacts.

We further found that the PH-PDZ tandem of α-syntrophin displays a significantly stronger binding towards phospholipids when compared to the isolated PH and PDZ domains. We conclude that, when covalently connected, the PH and PDZ domains function synergistically in binding to their common partners. The structure of the PH-PDZ tandem derived from this study can provide a mechanistic explanation to the above experimental observation. The data provided in this study reinforce an emerging concept that covalently connected protein-protein interaction domains often function as supra-modules in regulating signal transductions.
CHAPTER 1
INTRODUCTION

1.1 \(\alpha\)-Syntrophin

1.1.1 Dystrophin Glycoprotein Complex (DGC)

The muscular dystrophies are a heterogeneous group of degenerative disorders that arise from defects or deficiencies of proteins such as extra-cellular matrix protein (laminin-2), membrane associated proteins (sarcoglycan) and cytoplasmic protease (calpain-3). One example of such muscular dystrophies is Duchenne muscular dystrophy (DMD), caused by defect of dystrophin gene (Koenig et al., 1988; Sicinski et al., 1989). Dystrophin protein family consists of dystrophin, utrophin and dystrobrevin. They are mainly expressed in skeletal muscle, cardiac muscle and brain (Tokarz et al., 1998). As a scaffold protein, dystrophin associates with many proteins and forms dystrophin glycoprotein complex (DGC).

The dystrophin glycoprotein complex (DGC) is a multimeric transmembrane protein complex first isolated from skeletal muscle membranes (Ervasti and Campbell, 1993; Rando, 2001) (Fig1.1). In addition to skeletal muscle, the complex is also strongly expressed in heart and smooth muscle, as well as many non-muscle tissues including brain and peripheral nerves (Michele and Campbell, 2003). The complex components diverge from extra-cellular protein (laminin-2) and trans-membrane proteins (dystroglycan, sarcoglycan), to intra-cellular binding
proteins (F-actin, filament–2) and signaling proteins (nNos, camodulin). The primary function of dystrophin glycoprotein complex is to form a structural link between the actin cytoskeleton and the extracellular matrix by binding directly to actin and dystroglycan complex. It also localizes signaling proteins to the membrane (Albrecht and Froehner, 2002). Dystrophin deficiency likely causes DMD (Sicinski and al., 1989), mental retardation (Hodgson et al., 1992), abnormal electroretinograms (Pillers et al., 1993), and gastrointestinal disturbances (Barohn et al., 1988), respectively. Extensively genetic studies also show that deficiencies of many other members of DGC cause similar muscular dystrophies and neuronal abnormality. Therefore, understanding the function of individual component is of paramount importance for study of many pathogenetic processes related to DGC.

Syntrophin, one of dystrophin associated proteins firstly found in 1995 (Adams et al., 1995; Ahn et al., 1996), is believed to be an essential component for DGC functions. The syntrophin knockout mice showed obvious neuromuscular junction aberrance.

1.1.2 Syntrophin family

The syntrophins are a family of modular adapter proteins composed of at least one pleckstrin homology domain (PH), one PSD-95/DLG /ZO-1(PDZ) domain and one carboxyl terminal syntrophin unique region (Fig1.2). There are currently five known syntrophin genes, and they are α1, β1, β2, γ1, γ2 (Ahn et al., 1996; Piluso et al., 2000). Although syntrophins were found from mammal,
Fig. 1.1: Dystrophin Glycoprotein Complex (DGC). This diagrammatic representation includes components of the complex. SYN represents syntrophin, nNOS represents neuronal NO synthase, P is the phosphorylation sites of dystrophin and dystrobrevin. C and N represent carboxyl terminus and amino terminus of dystrophin, respectively.
its homologs were found in *C. elegans* (*stn–1*) and *Drosophila* (*SYN1* and *SYN2*) (Dekkers et al., 2004; Grisoni et al., 2003). α-syntrophin and β-syntrophin are acidic and basic proteins, respectively. α-syntrophin binds directly to dystrophin family proteins (dystrophin, utrophin and dystrobrevin) by the second PH domain and SU domain (Ahn and Kunkel, 1995; Dwyer and Froehner, 1995; Kachinsky et al., 1999; Yang et al., 1995). This leaves the PDZ domain free to associate with other signaling and channel proteins. The PDZ domain of α-syntrophin is known to bind to a variety of signaling molecules, including sodium channels (Gee et al., 1998; Schultz et al., 1998; Thomas et al., 2003), neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996; Hashida-Okumura et al., 1999; Hillier et al., 1999) and stress-activated protein kinase-3 (SAPK3) (Hasegawa et al., 1999; Lumeng et al., 1999). It is proposed that α-syntrophin functions as an adaptor protein which recruits signaling and channel proteins to dystrophin glycoprotein complex (DGC).

Although similar in their primary sequence, variant isoforms of syntrophin have very different cellular distributions (Kramarcy and Sealock, 2000; Peters et al., 1997). α-syntrophin is most abundant in sarcolemma of skeletal muscle and neuromuscular junctions (NMJ), whereas β1 and β2 syntrophin are ubiquitously expressed (Ahn et al., 1996). γ-syntrophin is a newly found member which is mainly expressed in central nervous system (CNS) (Piluso et al., 2000). The syntrophins also differ in developmental regulation, with α-syntrophin being the first to appear on the postsynaptic membrane (Kramarcy and Sealock, 2000). This
complex distribution and differential developmental expression pattern suggest that each syntrophin isoform plays unique functions.

![Diagram of syntrophin family](image)

α, β1, β2  
\[ \text{N} \quad \text{PDZ} \quad \text{PH1a} \quad \text{PH1b} \quad \text{PH2} \quad \text{SU} \quad \text{C} \]

γ1, γ2  
\[ \text{N} \quad \text{PDZ} \quad \text{PH} \quad \text{SU} \quad \text{C} \]

Fig. 1.2: Domain composition of the syntrophin family. N and C represent amino terminus and carboxyl terminus, respectively; PDZ is PSD-95/ DLG/ ZO-1 domain. PH1a and PH1b are sub-domains of the first Pleckstrin Homology domain of syntrophin, which is split by PDZ domain. PH2 is the second Pleckstrin Homology domain. SU refers to the Syntrophin Unique domain.

α-syntrophin knockout mice showed no evidence of myopathy but totally lost neuronal NO synthase (nNOS) from sarcolemma and leads to structurally aberrant neuromuscular junctions (Adams et al., 2000; Kameya et al., 1999). Both β1 and β2 syntrophin knockout mice showed no phenotype in NMJ formation, whereas α/β syntrophin null mice have more severe structural aberrant NMJs (Adams et al., 2004; Shiao et al., 2004). So α-syntrophin is suggested to play an important role in synapse formation and utrophin organization in neuromuscular synapse formation (Adams et al., 2000).
1.1.3 PDZ and the embedded PH domains of α-syntrophin

PDZ (PSD-95/DLG/ZO-1) domain is a protein–protein interaction module of approximately 90 amino acids. As one of the most abundant protein domains found in genomes of metazoans, PDZ domain plays an important role in targeting signaling proteins to specific plasma membranes, as well as assembling proteins into supra-molecular signaling complexes. It achieves these functions by recognizing specific carboxyl terminal amino acid sequence motifs. Recent studies have revealed other modes of PDZ – mediated protein interactions, one such example is that PDZ domains can interact with internal peptide sequences that adopt a β-hairpin structure (Tochio et al., 2000; Tochio et al., 1999). Although PDZ may be the sole protein interaction domain within a cytoplasmic protein, they are often found in combination with other protein interaction domains (for instance, SH3, PTB) participating in complexes that facilitate signaling or determine the localization of receptors (Fan and Zhang, 2002; Nourry et al., 2003; Zhang and Wang, 2003). A study by Zimmermann et al. even raised the provocative idea that PDZ domains can function as lipid binding modules (Zimmermann et al., 2002).

Biochemical and genetic studies have uncovered a number of fundamental roles of α-syntrophin PDZ domain. For example, the syntrophin PDZ domain for correct localization of nNOS to DGC is required (Brenman et al., 1996; Hillier et al., 1999). The recruitment of aquaporin-4 to plasma membrane relies on the PDZ domain of syntrophin (Adams et al., 2001; Neely et al., 2001). The structure of α-syntrophin PDZ domain has been determined by both NMR and X-ray
crystallographic techniques (Hillier et al., 1999; Schultz et al., 1998)

Pleckstrin homology (PH) domain is a membrane target domain and also has been implicated as a protein-protein and protein-lipid interaction domain (Lemmon and Ferguson, 2000). The core of PH domain is a twisted β-sandwich composed of 7 anti-parallel β strands and an α-helix. Three variable loops, β1-β2 (VL1), β3-β4 (VL2), β5-β6 (VL3) are most diverse both in length and amino acid sequences. Most, if not all, PH domains bind to phosphoinositides present in cell membranes (Harlan et al., 1994; Lemmon and Ferguson, 2000), although they do so with quite different affinities and specificity. In a few cases, the phosphoinositides binding to PH domain is strong and specific. One example is the Bruton’s tyrosine kinase (Btk) PH domain which binds to Phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P3) specifically with high affinity (K_D=40 nM) (Salim et al., 1996). The lipid binding pocket is formed by the variable loops VL1 and VL2 (Hyvonen and Saraste, 1997; Komander et al., 2004; Milburn et al., 2003). Studies using green fluorescent proteins fusions showed that such binding is sufficient for the signal regulated targeting of the proteins to the membrane (Gray et al., 1999; Varnai et al., 1999). The majority of PH domain show only weak and quite non-specific binding to phosphoinositides. Although the physiological significance of such weak lipid binding of most PH domains is unknown, some of those PH domains either participate in membrane association through co-operation with other domains in the same protein (Falasca et al., 1998; Stam et al., 1997) or requires oligomerization of its host protein in order to increase membrane binding avidity (Klein et al., 1998).
Earlier biochemical study suggested that the second PH domain is required for the α-syntrophin to be localized to DGC (Kachinsky et al., 1999), and the first PH domain showed ability to bind to phospholipids (Chockalingam et al., 1999). The unique domain organization (Fig 1.2) also attracts research on the first PH domain. Unlike canonical head to tail tandem arrangement, the PDZ domain locates in the middle of the first PH domain sequence, and splits the PH domain into two segments. This interesting feature can also been seen in other cases such as phospholipase Cγ (Fig 1.3), of which the second PH domain and the catalytic domain are split by two SH2 domain and a SH3 domain.

Fig. 1.3: Domain composition of PLCγ. PLC is phospholipase C. PH1 refers to the first PH domain of PLCγ; Domain X and Domain Y is the catalytic domain which is split by 4 other domains; PH2a and PH2b are subdomains of the second PH domain embedded by 3 domains; SH2 represents Src Homology 2 domain; SH3 is Src Homology 3 domain; N and C represent amino terminus and carboxyl terminus.
1.2 Protein structure determined by NMR

Nuclear magnetic resonance (NMR) spectroscopy is one of the principal experimental techniques in structural biology. Due to the tremendous progresses in the past two decades, NMR spectroscopy becomes to be a major experimental method for determining the three dimensional structures of large biological molecules.

Fig 1.4 shows a simplified flowchart diagram of NMR–based structure determination. The whole process can be roughly divided into 6 steps:

The first step is sample preparation. Purified $^{13}$C and $^{15}$N labeled samples are dissolved into suitable buffer at sufficient high concentration (usually >1 mM). Several conditions must be considered before data acquisition: buffer, pH, ionic strength and sample temperature. The high concentration sample should be stable under certain conditions before the acquisition. Normally $^{1}$H–$^{15}$N HSQC (Kay, 1992) spectra of is used to $^{15}$N – labeled proteins applied to check the protein quality and optimize the experimental condition.

The second step is NMR spectra recording and processing. For small proteins, 2D $^{1}$H–$^{1}$H TOCSY and NOESY with different mixing time and DQF-COSY with unlabelled samples are used to determine protein structure (Wuthrich, 1986). To large proteins, due to spectra overlap and linewidth broadening, a series of 3D experiments using $^{15}$N–labeled and $^{15}$N, $^{13}$C–labeled samples in H$_2$O or D$_2$O are applied instead. Normally used experiments can be divided into 3 catalogues according to their goals: HNCO (Muhandriam, 1994),
HNCACB (Witterkind, 1993), CBCA(CO)NH (Grzesiek, 1992), HCCH-TOCSY (Bax, 1993) for sequential resonance assignment, \(^{15}\text{N}\)-separated NOESY (Zhang, 1994) and \(^{13}\text{C}\)-separated NOESY (Lee, 1994) and 2D \(^1\text{H}-{\text{H}}\) NOESY for NOEs data, and HNHA (Vuister, 1993), \(^{15}\text{N}-{\text{H}}\) HMQC–J (Kay, 1990a) for J_{\text{HN–Ha}} data. The acquired data is multinuclear and multidimensional data and needs to be processed by computer before analysis. The process includes baseline correction, solvent suppression, resolution enhancement and Fourier-transform. The spectra are processed and graphically displayed by nmrPipe and nmrDraw (Delaglio et al., 1995), two most-commonly used software-packages.

Sequential resonance assignment is the third step of NMR-based structural determination. In this step, as many as possible of NMR resonance should be assigned to a specific nuclei in each residue. For small proteins less than seventy amino acids, homonuclear spectroscopy (2D TOCSY and NOESY) are applied to achieve this goal as described by Wüthrich (Wüthrich, 1986). Different assignment strategies are employed to larger proteins because of the complexity of "finger-print region" (containing amide proton and \(\alpha\)-proton cross peaks). In this case, normally used method is based on triple resonance experiments which link one residue with its adjacent neighbors by heteronuclear couplings by recording spectra of \(^{15}\text{N}, \(^{13}\text{C}\)-labeled samples. By this method, chemical shift of \(\text{C}_\alpha\) and \(\text{C}_\beta\) of each amino acid residues and the previous one can be obtained by HNCACB and CBCA(CO)NH. Sequential assignment can be determined by matching intra-and inter-residue \(\text{C}_\alpha\), \(\text{C}_\beta\) chemical shift and amino acids sequence. Side-chain
assignment is obtained by analyzing HCCH-TOCSY spectrum recorded on $^{15}$N, $^{13}$C–labeled samples in D$_2$O.

The fourth step is structural restraints collection. There are two types of restraints can be obtained from NMR: NOEs and coupling constants, reflect inter–proton distance and torsion angles ranges. Based on assignments of the previous steps, NOEs can be extracted from $^{15}$N–separated NOESY and $^{13}$C–separated NOESY experiments. Then the NOEs are divided into three classes according to peak intensity. They are short-, medium-, and long-range NOEs, defined as distances up to 2.9 Å, 3.5Å and 5 Å, respectively. NOE assignment need particular attention because some NOEs are ambiguous and mis-assignment can lead to a wrong calculated structure. Ambiguous NOEs are often assigned based on calculated structures derived from unambiguous restraints obtained in earlier iteration cycle. Although high quality structures are determined primarily by distance constrains, scalar coupling constants is also needed. Particular interest is put on $H^N$-$H^\alpha$ coupling constants, which directly relates to polypeptide backbone dihedral angle $\phi$. The experiments used to measure coupling constants are HNHA and $^{15}$N-$^1$H HMQC–J. The relationship between coupling constants and dihedral angle $\phi$ is:

$$^3J_{HN-H\alpha}=A\cos^2(\phi - 60^\circ)+B\cos(\phi - 60^\circ)+C$$

A, B and C are constants depend on particular nuclei involved in the covalent bonds.

The fifth step is structural calculation. Structural calculation is carried out
in two stages. The first stage is to identify secondary structure elements and
topology by measurable NMR parameters associated with backbone assignment
(chemical shift index), sequential short- medium-, and long-range NOEs, and
HN-Hα coupling constants. In the second stage, computational methods, such as
distance geometry and molecular dynamic simulated annealing, are used. One of the
most useful computer programs is XPLOR (Brunger, 1992), and its derivative, CNS
(Brunger et al., 1998). At this stage, non-sequential long-range NOEs and
stereo-specific assignments of diastereotopic protons are used. This step needs to be
iterated coupled with the analysis of structural restraints, and the violation of
restraints is analyzed during the iteration. It is performed until majority of
experimental restraints are in agreement with an ensemble of protein conformation
representing the solution structure.

The final step is structural refinement. In the final step, the structure
obtained is refined by using Ramachandran plot, which reflect our knowledge about
conformational preference of proteins. The quality of calculated structures is
assessed by software the PROCHECK-NMR (Laskowski et al., 1996).
Fig. 1.4: Simplified flow-chart of general strategies of NMR structure determination.
CHAPTER 2
MATERIALS AND METHODS

In this thesis, the N-terminal PH-PDZ tandem of α-syntrophin was cloned, expressed and purified. To compare with this protein sample, the isolated forms of the PDZ domain and the PH domain of α-syntrophin with the PDZ domain deleted region are also purified.

2.1 Cloning, expression and purification of α-syntrophin N-terminus

The mouse α-syntrophin gene was PCR amplified by using the following two primers: 5' - CAGGATCCGCGTCAGGCAGGCCGCGC-3' (5' primer) and 5' - GACTCGAGTTATGTGCCGATCTGCCTTGG-3' (3' primer). The PCR-amplified fragment was inserted into the plasmid pET-M. The pET-M plasmid was then transformed into E-Coli BL21-CodonPlus (DE3) host cells. To express the target proteins, the host cells containing the syntrophin expressing plasmids were growing in LB medium at 37°C until the $A_{600}$ reached 0.6-1.0. The expression of the protein was induced by the addition of isopropyl-D-thiogalactopyranoside (IPTG) to a final concentration of 0.25 mM. The culture was incubated at 16 °C for 16 hours after the addition of IPTG before harvesting by centrifugation.

Uniformly $^{15}$N-labelled α-syntrophin was prepared by growing the bacteria in M9 minimal medium using $^{15}$NH$_4$Cl (1g/liter) as the sole nitrogen source. Uniformly $^{13}$C $^{15}$N-labelled α-syntrophin was prepared by growing the bacteria in
M9 minimal medium using $^{15}$NH$_4$Cl (1g/liter) and $^{13}$C$_6$-glucose (1g/liter) as the sole nitrogen and carbon sources, respectively.

Recombinant syntrophin proteins were purified by a Ni$^{2+}$-NTA affinity column and other chromatographic techniques. Cell pellets from 1-liter culture were resuspended in 30 ml of Ni$^{2+}$-NTA column binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) containing 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml of antipain. Cells were lysed by three passes through a French press, and the viscosity of the suspension was reduced by sonication. And the lysate was centrifuged at 18000 rpm for 30 min. Soluble syntrophin proteins in the supernatant was purified on the Ni$^{2+}$-NTA column following the instructions of the manufacturer (Novagen). The His6-tagged syntrophin proteins eluted from the Ni$^{2+}$-NTA column was directly applied to a Sephacryl-100 gel filtration column (Amersham Pharmacia Biotech) to remove small amounts of contaminating proteins. The purified syntrophin proteins were extensively dialyzed against 10 mM NH$_4$HCO$_3$ buffer, freeze dried, and stored at -80 °C.

2.2 NMR sample preparation

The PH-PDZ tandem of $\alpha$-syntrophin is dissolved in 100 mM potassium phosphate buffer, pH 6.5 containing 10 mM DTT in 90% H$_2$O/10%D$_2$O or 99.99% D$_2$O. The concentration of NMR samples was approximately 1.2 mM. Total 4 NMR samples were prepared for structure determination: (unlabelled $\alpha$-syntrophin in D$_2$O, $^{15}$N-labelled $\alpha$-syntrophin in H$_2$O, $^{13}$C $^{15}$N-labelled $\alpha$-syntrophin in D$_2$O, 10%
fractionally $^{13}$C-labelled $\alpha$-syntrophin). To help determination of the PH-PDZ tandem structure, isolated PDZ domain and PH domain is also prepared. Isolated PDZ domain is dissolved into the same buffer as used for the the PH-PDZ tandem domain. Isolated PH domain is dissolved into 100 mM Tris buffer, pH 7.0 containing 10 mM DTT in 90% H$_2$O/10%D$_2$O.

2.3 NMR experiment

All NMR spectra were recorded on Varian UNITY Inova 500 MHz and 750 MHz NMR spectrometers each equipped with a pulse field gradient driver and a z-axis actively shielded $^1$H($^{13}$C, $^{15}$N)-triple resonance probe head. Sequential backbone resonance assignments were achieved by through-bond heteronuclear correlation experiments including HNCACB (Witterkind, 1993), CBCA(CO)NH (Grzesiek, 1992), HNCO (Muhandriam, 1994), HNCA (Kay, 1990b), HNCOCA, and the non-aromatic side-chain assignment were obtained by HCCH-TOCSY (Bax, 1993) experiment. The aromatic rings were assigned using homonuclear 2D $^1$H-$^1$H NOESY and TOCSY spectra. Stereospecific assignment assignment of methyl groups of Valine and Leucine residues were obtained using a fractionally $^{13}$C-labeled $\alpha$I-syntrophin (Neri et al., 1989). All NMR spectra were processed with the NMRPipe (Delaglio et al., 1995) software package and analysed with PIPP (Garrett, 1991).
2.4 Structural calculation

Approximate interproton distances were obtained from 3D $^{15}$N-separated (mixing time 100 ms), $^{13}$C-separated (mixing time 100 ms), and homonuclear $^1$H 2D (mixing time 50 ms and 100 ms) NOESY spectra. The distance restraints were divided into three classes for NOEs unrelated to NH protons, 1.8-2.7, 1.8-3.3, and 1.8-5.0 Å, corresponding to strong, medium and weak NOEs, respectively. The NOEs involving NH protons were grouped into three groups as well, 1.8-2.9, 1.8-3.5, and 1.8-5.0 Å, corresponding to strong, medium and weak NOEs, respectively. Hydrogen bond restraints were obtained by analyzing standard backbone NOE patterns characteristic of α-helices and β-strands. Two distance restraints, $r_{\text{NH-O}}$(1.8-2.2Å) and $r_{\text{N-O}}$(2.2-3.3Å), were used for each hydrogen bond. Phi and Psi dihedral angle restraints were obtained by chemical shift indices and NOE patterns, values of $-60^\circ\pm30^\circ$ and $-40^\circ\pm30^\circ$ were used for phi and psi dihedral angle of α-helices regions respectively, and $-120^\circ\pm50^\circ$ and $120^\circ\pm50^\circ$ for β-strands. Structures were calculated by standard simulated annealing protocol using the program CNS (Brunger et al., 1998). The final structures were refined by the torsion angle molecular dynamics protocol. Typically, twenty structures with the lowest energies from a total of 100 calculated structures were chosen for structure analysis. The quality of calculated structures was assessed with the program PROCHECK-NMR (Laskowski et al., 1996).
2.5 Illustrations

The graphics presentation of α-synctrophin, including ribbon diagrams and structural ensemble superposition, were prepared using the programs MOLMOL (Koradi et al., 1996), MOLSCRIPT (Kraulis, 1991), and RASTER3D (Merritt, 1994).

2.6 lipid binding assay

Brain Extracts I from Folch Extract, bovine brain (Sigma-Aldrich, Inc.) was used for lipid binding assay. It contains 50% phosphatidylserine (PS) and 10% phosphatidylinositol. The buffer solution contains 20mM HEPES, 150 mM NaCl and 1mM DTT, pH equals 7.4. The brain extract was dissolved by two cycles of ultrasonication on ice with a microprobe for 15s with 45s cooling intervals. The final lipid concentration was 2mg/mL. Prepared vesicles were used within 24 hours.

The lipid binding assay is a described standard method (Chockalingam et al., 1999; Wang and Spudich, 1984). The protein to be analyzed was dialyzed in buffer solution(20 mM HEPES, 150mM NaCl, pH 7.4) overnight at 4°C and centrifuged at a speed of 65,000 rpm for 15 minutes at 4°C in polycarbonate tubes. The supernatant protein solution was used for lipid binding assay.

Lipid vesicles of 30μL were added to the tubes containing 10μL protein solution sample. The final lipid concentration was 1.5 mg/mL and the protein concentration was 12.5mM in the assays. After incubation at room temperature for 10 minutes and on ice for 5 minutes, the tubes were centrifuged at 65,000 rpm for
15 minutes at 4 °C. The supernatant (S) was saved and the pellet was rinsed once by the buffer solution.

The pellet was resuspended in 40μL of Laemmli sample (solution) buffer (Peters et al., 1997). Both the supernatant and pellet samples are loaded to the same gel. The gel was stained with Coomassie brilliant blue.
CHAPTER 3

RESULTS

3.1 $^1$H, $^{13}$C, $^{15}$N resonance assignment

Sequence-specific assignments of the backbone resonances were achieved by triple-resonance experiment. Matching the intra- and inter-residue $^{13}$C$_\alpha$ and $^{13}$C$_\beta$ chemical shifts derived from HNCA, HNCOCA, HNCACB and CBCA(CO)NH spectra for a given residue (associated with a $^1$H-$^{15}$N correlation peak) and the previous residue in the primary sequence, the backbone resonance assignment can be obtained. Fig 3.1 shows an example of this process. Fig 3.2 shows the $^1$H-$^{15}$N HSQC spectrum and backbone assignment for $\alpha$-syntrophin at pH 6.5 and 30°C, which also verifies the overall quality and homogeneity of NMR spectroscopic samples. The peaks connected by strait lines are side chain amide cross-peaks of Asn and Gln residues. Prolines are missing because they have no amide protons.

Side-chain resonances were assigned from analysis of HNCA and HCCH-TOCSY spectra. The ring resonances of the aromatic residues (3 tryptophans, 4 phenylalanines and 4 tyrosines) were assigned by analyzing 2D NOESY and TOCSY of unlabelled $\alpha$-syntrophin samples in D$_2$O.

To help analyzing the PH-PDZ tandem of $\alpha$-syntrophin, I also assigned resonance of the isolated PDZ and PH domains spectra. Fig 3.4 shows the $^1$H-$^{15}$N HSQC spectrum of the isolated PDZ domain of $\alpha$-syntrophin and Fig 3.5 shows the $^1$H-$^{15}$N HSQC spectrum of the isolated PH domain of $\alpha$-syntrophin.
Fig. 3.1: Partial $^{13}C_\alpha$ and $^{13}C_\beta$ obtained from CBCA(CO)NH and HNCA CB. The correlation involving amides of residues from H141 to V145 are illustrated. For each residue, PC$_\alpha$ and PC$_\beta$ represent the cross peak of the backbone HN and the previous residue's C$_\alpha$ and C$_\beta$. For each residue, C$_\alpha$C$_\beta$ chemical shift of intra-residue and previous residue can be obtained from HNCA CB. C$_\alpha$C$_\beta$ chemical shift of previous residue can be obtained from CBCA(CO)NH. Based on primary sequence, the connectivity of residues is acquired.
Fig. 3.2: The $^1$H-$^{15}$N HSQC spectrum of $\alpha$-syntrophin acquired at a 750 MHz spectrometer of $\sim$1.0 mM, pH 6.5, 303 K. All assignments are labeled on the figure. The peaks connected by strait lines are side chain amide cross peaks of Asn and Gln residues. The Prolines were not assigned on this spectrum because they lack backbone HN.
Fig. 3.3: The $^1$H-$^{15}$N HSQC spectra of α-syntrophin PDZ domain acquired at a 500 MHz of ~1.0 mM, pH 6.5, 303 K. All assignments are labeled in the PH-PDZ tandem residue number. The peaks connected by strait lines are side chain amide cross peaks of Asn and Gln residues. The Proline103 is not assigned on this spectrum because it lacks backbone HN.
Fig. 3.4: The $^1$H-$^{15}$N HSQC spectrum of $\alpha$-syntrophin PH domain acquired at a 750 MHz of ~1.0 mM, pH 7.0, 303 K. All assignments are labeled in PH-PDZ the tandem residue number. The peaks connected by straight lines are side chain amide cross peaks of Asn and Gln residues. The Prolines were not assigned on this spectrum because they lack backbone HN.
3.2 Secondary structure of the PH-PDZ tandem of the $\alpha$-syntrophin

Qualitative analysis of the short- and medium-range NOEs and chemical shift index were served to identify the secondary structure elements of the PH-PDZ tandem of $\alpha$-syntrophin (Fig 3.5 and Fig 3.6). Generally, the characteristic NOE patterns of the medium $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha\alpha}(i,i+3)$ NOEs, strong $d_{NN}(i,i+1)$ NOEs indicate the $\alpha$-helix secondary structure which chemical shift index value is -1. Based on these judgements, two $\alpha$-helices can be identified in the PDZ domain and a long $\alpha$-helix was identified in the carboxyl terminus of the PH domain. For $\beta$-strands, three characters are normally identified: the first is the typical NOE pattern of very strong $d_{\alpha\alpha}(i,i+1)$, the second is the chemical shift index value is +1, and the third is the characteristic long-range NOEs between cross-strands ($H^\alpha_i-H^\alpha_j$, $H^\alpha_i-H^N_j$) if some $\beta$-strands form $\beta$-sheet. Seven $\beta$-strands were identified in the PH domain and six $\beta$-strands were identified in the PDZ domain. The boundaries of the secondary elements were also confirmed in the structure calculation.
Fig. 3.5: Summary of the chemical shift index and sequential NOE connectivities observed in the PH domain of the PH-PDZ tandem of α-syntrophin. Bars indicate NOESY cross-peaks observed between residues. The bars heights indicate the strength of the NOE. “.....” shows the loop sequence inserted by the PDZ domain of the PH domain.
Fig. 3.6: Summary of the chemical shift index and sequential NOE connectivities observed in the PDZ domain of the PH-PDZ tandem of α-syntrophin. Bars indicate NOESY cross-peaks observed between residues. The bars heights indicate the strength of the NOE.
3.3 Tertiary structure of α-syntrophin

A total of 3651 experiment restraints derived from NMR spectroscopy were used for structure calculation. For the secondary structure regions of the two domains, root mean square deviations (RMSDs) are 0.54 Å (the PH domain) and 0.57 Å (the PDZ domain) for backbone heavy atoms. The structural statistics are summarized in Table 3.1. A stereo-view showing the best-fit superposition of the backbone atoms of the final 20 structures of α-syntrophin PH domain and PDZ domain are shown in Fig 3.7 and Fig 3.8, respectively. The backbone conformations of the α-syntrophin, with ribbon diagrams of PH domain, PDZ domain and the tandem domain structure are shown in Fig 3.9, Fig 3.10 and Fig 3.11.

The α-syntrophin PH domain is a typical PH domain which consists a β-sandwich fold and a long α helix in one edge of the sandwich (Lemmon et al., 2002). The β-sandwich is composed by two nearly orthogonal β-sheets, one contains 4 antiparallel β strands (β1-β4) and the other contains three (β5-β7). The right-handed twist of the two orthogonally packed β-sheets in the sandwich results in their close contact at two close corners. One of these close corners is spanned by strand β1, the other close corner is completed by a β-turn between strands β4 and β5. One splayed corner is capped by the C-terminal α-helix. The second splayed corner is only partially filled by the side chains of β4. β1-β2 loop (VL1) and β5-β6 loop (VL3) are highly variable and β3-β4 loop is inserted by the PDZ domain.

The α-syntrophin PDZ domain (Hillier et al., 1999) is a canonic PDZ domain: six twisted β-strands forms a partially opened barrel and each opened side
capped by two $\alpha$ helix on each sides. The extended groove formed by $\beta B$ and $\alpha B$ binds directly to nNos PDZ domain and other ligands (Hillier et al., 1999).

Fig 3.9 shows the ribbon diagram presentation of one of potential tandem domain structure because the inter–PH, PDZ domain orientation could not be determined due to the lack of inter-domain NOEs. The link region from PHa motif to PDZ domain is 32–residue long and PDZ to PHb link is composed by 36 residues. These two link regions are both Proline rich region and highly variable.
Table 3.1: Structural statistics for the family of 20 structures of α-syntrophin

<table>
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<th>Distance restraints</th>
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<tbody>
<tr>
<td>Intraresidue (i-j=0)</td>
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<tr>
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<td>88</td>
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<tr>
<td>Total</td>
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Mean r.m.s. deviation from experimental restraints

<table>
<thead>
<tr>
<th>Distance (Å)</th>
<th>0.015 ± 0.0001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihedral angle (°)</td>
<td>0.145 ± 0.04</td>
</tr>
</tbody>
</table>

Mean r.m.s. deviation from idealized covalent geometry

| Bond(Å) | 0.001 ± 0.000 |
| Angle(°) | 0.294 ± 0.004 |
| Improper(°) | 0.136 ± 0.008 |

Mean energy (kcal/mol)

| $E_{\text{NOE}}$ | 50.5 ± 3.49 |
| $E_{\text{dih}}$ | 0.24 ± 0.15 |
| $E_{\text{L-J}}$ | -331 ± 24.48 |

Ramachandran plot (%)

| Most favorable region | 59.5 |
| Additional allowed region | 32.4 |
| Generously allowed region | 8.1 |

Atomic r.m.s. difference (Å)

<table>
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</table>

<table>
<thead>
<tr>
<th>PDZ domain</th>
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</tr>
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<tbody>
<tr>
<td>Backbone atoms(N, C', Cω)</td>
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</tr>
<tr>
<td>All heavy atoms</td>
<td>1.14</td>
</tr>
</tbody>
</table>

1The final values of the square-well NOE and dihedral angle potentials were calculated with force constants of 50 kcal mol$^{-1}$ Å$^{-1}$ and 200 kcal mol$^{-1}$ Å$^{-1}$, respectively. 2The program PROCHECK was used to assess the overall quality of the structures. 3For the statistics of PH domain, the two highly variable loops (residues 18-26, 221-228) and linkage of PH-PDZ (residues 46-77, 164-203) are excluded. The secondary definition is shown in Fig 3.5. 4For the statistics of the PDZ domain, the variable βA-βB loop is excluded.
Fig. 3.7: Stereo view of the best-fit superposition of the backbone atoms (N, Cα, C’) of the final 20 structures of PH domain of the PH-PDZ tandem of α-syntrophin. The structures are superimposed against the average structure using the residues 7-48, 200-263. The N-terminus and C-terminus are labeled respectively. The inserted PDZ domain is shown in grey ribbon diagram.
Fig. 3.8: Stereo view of the best-fit superposition of the backbone atoms (N, Cα, C') of the final 20 structures of PDZ domain of the PH-PDZ tandem of α-syntrophin. The structures are superimposed against the average structure using the residues 76-165. The N-terminus and C-terminus are labeled respectively.
Fig. 3.9: Ribbon diagram representation of $\alpha$-syntrophin PH domain structure. $\alpha$-helix is colored in blue and $\beta$-stands are colored in green. The secondary structure is labeled. N and C represent amino terminus and carboxyl terminus, respectively. The inserted PDZ domain is shown in grey ribbon diagram (B), the same structure as (A) but rotate in 90°.
Fig. 3.10: Ribbon diagram representation of \(\alpha\)-syntrophin PDZ domain structure. \(\alpha\)-helix is colored in blue and \(\beta\)-stands are colored in green. The secondary structure is labeled. N and C represent amino terminus and carboxyl terminus, respectively. (B), the same structure as (A) but rotate in 90°.
Fig. 3.11: Ribbon diagram representation of the PH-PDZ tandem of α-syntrophin structure (one potential orientation). The PH and PDZ domain are colored in blue and red, respectively. N and C represent amino terminus and carboxyl terminus, respectively. The secondary structure is labeled.
3.3 PH domain does not interact directly with the inserted PDZ domain

The structure study reveals that the first PH domain and its inserted PDZ domain fold independently and do not contact directly.

There is no direct interaction between the PH domain and the inserted PDZ domain because no inter-domain NOEs were found. Therefore, the orientation of PH domain and PDZ domain can not be determined in structural calculation. Fig. 3.12 shows the overlay plots of $^1$H-$^{15}$N HSQC spectrum of $\alpha$-syntrophin isolated PH domain and the PH domain in the PH-PDZ tandem of $\alpha$-syntrophin. From the spectrum, each peak of the PH domain overlaps well with it's the PH-PDZ tandem counterpart except those random coil peaks. Since chemical shift is very sensitive to its chemical surroundings, this suggests the backbone conformation of PH domain is not affected by the PDZ insertion.

Fig 3.13 shows the overlay plots of $^1$H-$^{15}$N HSQC spectra of the isolated $\alpha$-syntrophin PDZ domain and the PH-PDZ tandem domain. Unlike the phenomenon of Fig 3.10, PDZ domain shows some chemical shift perturbation if it is isolated from the PH-PDZ tandem domain. Since all peaks are assigned, the perturbed residues can be mapped to the PDZ domain structure (Fig 3.14). The residues with largest chemical shift changes locate at amino terminus and carboxyl terminus of the PDZ domain. It can be easily explained by their difference of neighboring sequences in these two forms. $\beta$B and $\beta$C strands are also obviously perturbed if PDZ domain is isolated from the PH-PDZ tandem. Based on the fact
that PH domain is not perturbed by PDZ sequence insertion, the PDZ C-terminal link region is presumably responsible for this chemical shift perturbation.
Fig. 3.12: Overlapped $^1$H-$^{15}$N HSQC spectrum of isolated PH domain and the PH-PDZ tandem domain of α-syntrophin. The red peaks belong to isolated PH domain and grey peaks are the PH-PDZ tandem of α-syntrophin.
Fig. 3.13: Overlapped $^1$H-$^{15}$N HSQC spectrum of isolated PDZ domain and the PH-PDZ tandem of $\alpha$-syntrophin domains. The red peaks belong to isolated PDZ domain and grey peaks are the PH-PDZ tandem of $\alpha$-syntrophin.
Fig. 3.14: Chemical shift changes of the PDZ domain of the PH-PDZ tandem of α-syntrophin from isolated PDZ domain. The combined $^1$H and $^{15}$N chemical shift changes are defined:

$$\Delta_{\text{ppm}} = (\Delta \delta_{\text{HN}}^2 + (\Delta \delta_{\text{N}} * \alpha_{\text{N}})^2)^{1/2}.$$ 

The scaling factor used to normalize $^1$H and $^{15}$N chemical shift is 0.17. The coloring scheme is presented using a horizontal bar at the top.
3.4 Potential lipid binding of α-syntrophin

PH domain is a phosphoinositide recognition domain (Lemmon and Ferguson, 2000). Previous study suggested that α-syntrophin specifically binds to PI(4,5)P₂ (Chockalingam et al., 1999). Lipid binding sites of most known PH domains is located at variable loop 1 (VL1) and variable loop 2 (VL2) (Cronin et al., 2004; Hyvonen and Saraste, 1997). The consensus sequence motif stretching from β1 to β2 is identified as ΦxKx Gx*K*xx KxR(K)xF(L). In the consensus sequence, ‘Φ’ represents a hydrophobic amino acid, ‘x’ represents any amino acid and ‘*’ represents any number of amino acids. The side chain of the positive residues of the binding pocket form hydrogen bond with phosphoinositides. Structure based sequence alignment analysis of PH domain (Fig 3.15) showed that α-syntrophin PH domain totally lacks the key residues required for lipid binding in the VL1 and VL2 (Lemmon and Ferguson, 2000). This is supported by superimposition of α-syntrophin PH domain with Btk PH domain on its binding pocket (Fig 3.16) (Hyvonen and Saraste, 1997). Structural analysis showed that α-syntrophin PH domain is more like the PH domain of β-spectrin, which binds to PI(4,5)P₂ with residues from VL1 and VL3 (Hyvonen et al., 1995). Fig 3.17 shows the structure superimposition of two PH domains from α-syntrophin and β-spectrin. Several key residues in spectrin phospholipids binding residues do have counterparts in syntrophin PH domain. In a lipid binding assay (Fig 3.18), the PH-PDZ tandem of α-syntrophin showed obvious lipid binding capacity while isolated PH domain binds to phospholipids very weakly. This result is somehow reasonable because
nearly all PH domains bind to phospholipids weakly except those bind in VL1-VL2 pockets.
Fig. 3.15: Structure based sequence alignment of PH domain of α-syntrophin with known PI-binding PH domains. Secondary elements of α-syntrophin are on the top of alignment. The residues reported to be essential for phospholipids binding are in red color.
Fig. 3.16: Superimposition of α-syntrophin PH domain and Btk PH domain on canonic lipid binding site. All essential side-chains in this sort of binding site is absent in α-syntrophin PH domain.
Fig. 3.17: Comparison of α-syntrophin and β-spectrin PH domain on VL1 and VL3. The red one is α-syntrophin and blue one is β spectrin
Fig. 3.18: SDS-PAGE of α-syntrophin phospholipids binding assay.

P represents pellet and S represents supernatant. The left 2 bands used the PH-PDZ tandem of α-syntrophin, the middle two bands used isolated PDZ domain and right 2 bands are isolated PH domain.
CHAPTER 4

DISCUSSION

As a significant component of dystrophin glycoprotein complex, α-syntrophin is a key for understanding the complex physiological function and has drawn a lot of attention. By sequence alignment of all members of syntrophin family (Fig 4.1), although thought to have only one PH domain, γ1 and γ2 syntrophin have a putative split PH domain. And from the alignment, all syntrophins have similar PDZ domain insertion in β3-β4 loop (VL2) except SYN-1, syntrophin homolog in Drosophila, of which the PDZ domain is inserted into the β5-β6 loop. The physiological and structural significance of such conserved sequence arrangement is not totally understood. Another question is how the domains of α-syntrophin cooperate to achieve its biological function. Numerous studies focused on the PDZ domain and proved it to be essential for nNOS localization, utrophin organization and neuromuscular junction formation (Adams et al., 2001; Shiao et al., 2004), and the PH2 domain, the SU motif in syntrophin is required for its localization to DGC (Ahn and Kunkel, 1995), but the function of the N-terminal, split PH domain which is conserved in the syntrophin family is still unknown. These questions need to be uncovered by biological and structural studies.

The structural studies described in this thesis shed lights on these questions. The first PH domain of α-syntrophin and its inserted PDZ domain are
structurally independent and the link region is long and variable. The PH domain structure is not affected by the PDZ domain. This is the first structure of a PH domain inserted by other domains. Lipid binding assay reveals the PH-PDZ tandem binds to phospholipids strongly while the two isolated domains show very weak binding. Although not proven, our biological data suggests some sort of binding specificity of the PH domain. Base on this suggestion, a model exploring the significance of these two tandem domains and their biological function is proposed (Fig 4.2).

In this model, both the PH domain and the PDZ domain are responsible for membrane targeting. The inherent tendency to bind to a specific class of phospholipids of the PH domain provides the α-syntrophin ability to recognize a specific location. Meanwhile, the membrane recruitment increases exponentially because of the appearance of two tandem domains despite the affinity of individual interactions with phospholipids is low. If one domain binds to membrane surface at a binding affinity $K_D$ 1mM with $\Delta G=-4$ kcal/mol, dimmer binding will increase $K_D$ to micromolar range with $\Delta G=-8$ kcal/mol. The specificity provided by the PH domain and increase of lipid binding affinity make α-syntrophin an adaptor for signaling and channel proteins not only can be associate to DGC but also to membrane surface.

Although the first PH domain and the PDZ domain do not interact directly, it can not be interpret that they have no structural relationship. Since our structure is
apo-form which has no PDZ domain ligand, one possibility is the ligand protein binding to the PDZ domain affects the PH-PDZ domain orientation and even their putative lipid binding. Another possibility is the two domains have transient interaction so their orientation can not be solved by traditional NOE-based structure determination. The newly developed NMR technology residual dipolar coupling (RDC) can be used to detect the potential PH-PDZ orientation. This method yields orientation restraints by measuring dipolar coupling in anisotropic solution.

The solution structure of α-syntrophin and the proposed model do not explain the significance of the domain insertion-the PH domain and the PDZ domain are structurally independent. To test whether the link region and the specific domain arrangement is physiological and structurally important, some recombine proteins are going to be expressed and purified. In those proteins, one class is domain recombinant: the PDZ domain is put on the N-terminus or C-terminus of the PH domain; In another class of proteins, the link region is replaced by designed cutting sites. The experiment on these recombinant proteins will help to understand the relationship between the PH domain and the PDZ domain.

It is important to note that although the second PH domain of α-syntrophin binds to dystrophin family, it does not exclude its potential phospholipids binding ability. This may provide its host protein additional lipid binding affinity. So the future study about α-syntrophin and phospholipids binding may use full length protein instead of the PH-PDZ tandem.
Fig. 4.1: Syntrophin family sequence alignment. SNT-1 is syntrophin homolog in *C. elegans* and SYN-1 is homolog in *Drosophila*. PDZ domain insertion appears in β3-β4 loop of all syntrophins except SYN1, which appears in β5-β6 loop. Secondary elements of α-syntrophin are on the top of alignment.
Fig. 4.2: Model for α-syntrophin function in membrane localization.

The PH domain and PDZ domain both contribute to membrane localization. PH domain
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