Main goals

The topics related to my STRAIN fellowship are reported below:

- The conformational analysis of peptides and proteins and the screening of biomolecules by means of mono- and multi-dimensional NMR (Nuclear Magnetic Resonance) techniques
- The calculation of the tri-dimensional structure of peptides and proteins starting from NMR data
- The design of peptides and peptidomimetic antagonists of protein-protein or protein-ligand interactions by means of molecular docking techniques. I have developed this topic under the tutorship of Dr. Luigi Vitagliano of the IBB-CNR.

I attended 70 hours of theoretical courses related to these topics, and 1430 hours of lab activities during which I focused on two different work packages:

Work package I-NMR conformational studies of a peptide spanning the Mid-Loop region of Ship2-Sam and molecular docking studies with EphA2-Sam.

State of the art

The lipid phosphatase Ship2 is a protein involved in diseases such as diabetes, cancer, neurodegeneration, and atherosclerosis which contains a Sam (Sterile alpha motif) domain. Sam domains are small protein binding modules made up of a 5 helix bundle (Figure 1A). The Sam domain of Ship2 (Ship2-Sam) interacts with the Sam domain of the EphA2 receptor (EphA2-Sam) through a “Mid Loop/End Helix” topology of binding (1). In this complex the central region of Ship2-Sam forms the Mid Loop surface that is needed to bind EphA2-Sam (Figure 1A). This interaction in cancer cells inhibits EphA2 endocytosis and degradation with an increase of receptor pro-oncogenic activity (2). A 22 residue long-peptide encompassing most of the Ship2-Sam Mid Loop interface (called Shiptide), capable of binding EphA2-Sam with a micromolar affinity, (Figure 1B), has been previously identified (3). In this work package the conformational features of the Shiptide were investigated, through solution NMR studies under different conditions (i.e., in phosphate buffer and in presence of trifluoro-ethanol (TFE)). Moreover, molecular docking studies were performed to gain possible interaction models of the EphA2-Sam/Shiptide complex. The gained structural information will contribute to the design of efficient antagonists of Ship2-Sam heterotypic interactions with possible therapeutic applications.

Performed Activities

In order to analyze the conformational features of the Shiptide, 2D NMR experiments were acquired for peptide samples dissolved either in sodium phosphate or in mixtures containing 30% H₂O and 70% TFE (2,2,2-Trifluoroethanol-D3). TFE is a structuring co-solvent that is often used to investigate the inherent conformational preferences of peptides (4). In detail 2D [¹H, ¹H] NMR experiments such as TOCSY (Total Correlation Spectroscopy), NOESY (Nuclear Overhauser Enhancement Spectroscopy), ROESY (Rotating frame Overhauser Enhancement Spectroscopy) and DQFCOSY (Double Quantum-Filtered Correlation Spectroscopy) were recorded. First, proton resonance assignments were determined in aqueous buffer and in presence of TFE. Then, NMR parameters to evaluate the type and content of secondary structure in the Shiptide were measured (i.e., chemical shift deviations from random coil values for Hα protons, analysis of the NOEs pattern, evaluation of temperature coefficients (Δδ/ΔT)). The NMR solution structure of the Shiptide in presence of TFE was calculated as well through distance restraints collected from NOESY spectra. The calculated NMR structure was finally implemented in molecular docking studies to obtain models for a putative EphA2-Sam/Shiptide complex. These studies were conducted with the Haddock web server (5), by using for EphA2-Sam, the NMR structure already present in the Protein Data Bank (PDB id: 2E8N).

Results

NMR experiments conducted in sodium phosphate revealed that the Shiptide was lacking an ordered structure. In fact in the NOESY spectrum a reduced set of correlations was detected (Figure
1C); this trend was confirmed by chemical shifts analysis. In presence of TFE the 2D NOESY spectrum of the Shiptide resulted characterized by the appearance of cross-peaks indicative of an increase of ordered conformations (Figure 1D). The gain of an ordered structure was further confirmed by the analysis of chemical shifts deviations of Hα protons from random coil values, which pointed to the presence of a helical conformation in the C-terminal half of the peptide. The NOE pattern collected in presence of TFE additionally confirmed helical conformations. Temperature coefficients $\Delta \delta / \Delta T$ were also evaluated by following chemical shifts variations of the amide protons as function of the temperature. Low $|\Delta \delta / \Delta T|$ values, generally associated to low solvent exposure and involvement of the $H_N$ amide protons in hydrogen-bonding were measured for 7 out of 22 Shiptide residues.

A complete structure calculation was carried out for the Shiptide in 70% TFE (Figure 1B). The NMR solution structure of the Shiptide resulted composed by an N-terminal segment lacking canonical secondary structure elements, and by a $\alpha$-helix covering the C-terminal region (Figure 1B). The Shiptide 3D structure does not reproduce the structural motives of the ML region of the parent full length domain (=Ship2-Sam), that is instead made up of three helical segments connected by disordered loop regions.

The 3D structure of the Shiptide calculated in TFE was implemented in molecular docking studies to formulate hypotheses about a EphA2-Sam/Shiptide interaction model. Many docking models contain the EphA2-Sam C-terminal $\alpha5$ helix and the Shiptide helical portion facing each other in a fashion which resembles some Sam-Sam associations occurring with the so called "tail-to-tail" model (6). These speculative models suggest novel possible routes for the design of helical Shiptide analogues that could antagonize EphA2-Sam interaction and act as therapeutic agents.

These results have been published in the journal Biopolymers: Mercurio F. A., Scognamiglio P. L., Di Natale C., Marasco D., Pellecchia M., and Leone M. CD and NMR conformational studies of a peptide encompassing the Mid Loop interface of Ship2-Sam. Biopolymers 2014; 101(11): 1088-98 (Cover picture of the online journal)

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**Figure 1:** A model of EphA2-Sam/Ship2-Sam complex (PDB id: 2KSO), with the Ship2-Sam Mid-Loop region represented by the Shiptide (green) (A). One representative NMR conformer of the Shiptide in TFE with its primary sequence indicated on the bottom (B). Shiptide NOESY spectra acquired in phosphate buffer (C), and in $H_2O/TFE$ 30:70 v/v (D).
Workpackage II-NMR conformational studies of disordered phosphopeptides.

**State of the art**

Within this work package NMR conformational studies of disordered phosphopeptides were performed to get further insights into the molecular recognition mechanisms involving Intrinsically Disordered Proteins (IDPs).

In the last few years IDPs have received great attention from the scientific community as they participate in several important biological processes and diseases. These proteins are characterized by conformational plasticity and ability to bind several targets through high specificity/low affinity interactions and enhanced binding kinetics (7). It is assumed that post-translational modifications such as phosphorylation can stimulate a structural rearrangement in IDPs and facilitate their binding to partners (7); to investigate these features three peptides containing a phosphorylated residue either at the C- or N- termini were designed in the group of Prof. F. Rossi of the University "Federico II" of Naples (Table 1).

**Performed Activities**

Within this project three phosphopeptides were analyzed: IDP1, IDP2 and IDP3.

Table 1:IDP peptide sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>IDP1</td>
<td>NH$_2$-AQIREASSPSLQVDNQSDQ$pt$-CONH$_2$</td>
</tr>
<tr>
<td>IDP2</td>
<td>NH$_2$-$pt$AQIREASSPSLQVDNQSDQT-CONH$_2$</td>
</tr>
<tr>
<td>IDP3</td>
<td>NH$_2$-$p$SAQIREASSPSLQVDNQSDQT-CONH$_2$</td>
</tr>
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$pt$ = phosphothreonine  $ps$ = phosphoserine

NMR analysis was performed in H$_2$O and in a mixture H$_2$O/TFE 17/83 v/v for IDP1, and in DMSO (Dimethyl sulfoxide) for IDP2 and IDP3. The following 2D [$^1$H, $^1$H] NMR spectra were acquired: TOCSY, DQFCOSY, NOESY. First complete proton resonance assignments were obtained for all peptides later, chemical shift deviations from random coil values for H$_\alpha$ protons and NOEs pattern were analyzed to get insights into secondary structure elements. Finally, IDP1 structure calculations in H$_2$O/TFE 17/83 v/v were carried out by using distance constraints from NOESY experiments.

**Results**

Initially, the conformational preferences of IDP1 were investigated in H$_2$O/D$_2$O (90/10 v/v). Under these experimental conditions, the almost complete absence of signal in the NOESY experiment indicated that IDP1 was very flexible and disordered (Figure 1A). Next IDP1 NMR experiments were acquired in presence of 83% TFE. In presence of this structuring agent the NOESY spectrum was characterized by the appearance of many cross-peaks, possibly indicating a decrease of flexibility in the peptide (Figure 1B). Chemical shifts deviations of H$_\alpha$ protons from random coil values and analysis of NOEs pattern did not point to any specific secondary structure element. Structural calculations for IDP1 in TFE demonstrated that the peptide was rather flexible and assumed a pseudo-helical turn only in a small region at the C-terminal (Figure 2C).

IDP2 and IDP3 resulted rather insoluble in water and in water/TFE mixtures, thus their structural features were analyzed in DMSO, in which IDP2 and IDP3 showed a very similar conformational behavior. NOESY spectra of both peptides contained many cross-peaks and resembled typical experiments recorded for rigid folded species. Detailed analysis of NOE patterns revealed a clear prevalence of sequential contacts that alone are not sufficient to indicate any specific ordered secondary structure element. Tentative structure calculations were carried out and demonstrated the intrinsic conformational disorder of IDP2 and IDP3 (Figure 2 D,E). As concerning the possible applications of the disordered IDP1, IDP2 and IDP3 peptides, we can certainly envision their use to generate novel peptide amphiphiles (PAs) to be implemented in the field of biomedicine and

![Figure 2](image)

**Figure 2**: IDP1 NOESY spectra acquired in H$_2$O (A), and in H$_2$O/TFE 17:83 v/v (B). The 20 NMR conformers of IDP1 in H$_2$O/TFE 17:83 v/v (backbone atoms superimposed from L11 to Q16), IDP2 in DMSO and IDP3 in DMSO are shown in panels (C) (D) and (E) respectively.

**References:**

I carried out my research activities for the STRAIN fellowship at the Institute of Bionanotechnologies and Bioimaging (IBB) of the National Research Council, in Naples. The aims of IBB are to promote research activities within the fields of syntheses, structural characterization of biomolecular systems such as peptides and proteins and development of new diagnostic and pharmaceutical products. In particular I worked at the NMR laboratory of the Institute, which is equipped with two NMR spectrometers: a Varian Unity 400 MHz and a Varian Unity 600 MHz provided with a cold probe. I performed analysis of NMR spectra, structure calculations and molecular docking on a Linux workstation located in the informatics center of the Host Institution.

My tutor, Dr. Marilisa Leone, is an expert in solution NMR techniques for conformational studies of proteins/peptides and in drug discovery field. She has been a researcher at the IBB since December 2009; she obtained a PhD in chemistry at the University “Federico II” of Naples in 2003 and afterwards she worked for several years at the Sanford - Burnham Medical Research Institute in San Diego (USA).