Synopsis
of

Bioinformatic Studies in Modeling of Proteins and Protein Complexes

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The Overview:

In modern Molecular Biology and Biophysics the central issue is to understand the interactions stabilizing the molecular complexes. Knowledge of these aspects is essential for designing simple biomolecule having desired properties which can be used for controlling cellular processes. Although the characterization of the interactions between DNA and the protein and protein-protein is possible through X-ray crystallography, NMR or some other biochemical experiments but the detail characterization of dynamic features in atomics level cannot be obtained from experiments. These can be done by molecular modeling and molecular dynamics study.

Protein-protein Complex:

The interactions between proteins are important for numerous biological functions; signals from the exterior of a cell are mediated to the inside of that cell by protein–protein interactions of the signaling molecules. This process, called signal transduction, plays a fundamental role in many biological processes including those involving diseases (e.g. cancers, multiple sclerosis). Proteins might interact with other proteins for a long time to form a part of a protein complex, a protein may help another protein or a protein may interact briefly with another protein just to modify it (for example, a protein kinase will add a phosphate to a target protein). This modification of proteins can itself change protein–protein interactions. Protein–Protein interactions are thus central to virtually every process in a living cell. Information about these interactions improves our understanding of diseases and can provide the basis for new therapeutic approaches.

Protein–protein interaction prediction is a field combining bioinformatics approach and structural biological studies in an attempt to identify and catalog physical interactions between pairs or groups of proteins. Experimentally, physical interactions between pairs of proteins can be inferred from a variety of experimental techniques, including yeast two-hybrids systems, protein-fragment complementation assays (PCA) etc. Protein-protein structure complex can be studied by X-ray crystallography but is normally a time consuming process and involves lots of experimental difficulties exists.

Protein–protein docking methods can provide an atomistic understanding of 1) nature of interactions between interacting proteins 2) spatial configuration adopted by interacting proteins 3) strength and specificity of the interacting proteins involved. A prerequisite for protein-protein docking to be successful is that the 3D structure for each of the interacting proteins is available. The structure can be experimentally solved or computed using homologous protein structures. Different algorithms/software for automatic protein-protein docking are available. We have studied two protein-protein complexes

involved in signal transduction for which experimental data was available (Bonvin, 2006; Gray, 2006). One is smad4 Hoxa9 complex structure which is a part of SMAD pathway. Second one is Notch-Delta Complex involved in Notch Signaling pathway which is responsible for Cell-Cell communication. The Notch and TGFβ signaling pathways play critical roles in control of cell fate during metazoan development. There is a cross talk between Notch and TGFβ signaling pathways (Blokzijl et al. 2003).

The SMAD proteins are homologs of both the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA. The name is a combination of the two.

Members of the transforming growth factor-β (TGF-β) superfamily bind to two different serine/threonine kinase receptors, i.e. type I and type II receptors. Upon ligand binding, type I receptors specifically activate intracellular Smad proteins. Receptor regulated smads called R-Smads form complexes with Co-Smads and translocate into the nucleus, where they binds to another transcription factors and regulate the transcription of target genes. This pathway is called smad pathway.

The structure of Smad family members is very similar. Smad proteins contain three distinct regions, the Mad-homology domains 1 (MH1) and 2 (MH2) located at the N terminal and C terminal regions respectively and a linker region in between. The sequence of the MH1 domain in I-Smads is very short, while the MH1 domains in R-Smads and Co-Smads are composed of approximately 130 amino acids whose sequences are highly conserved. The crystal structure of the MH1 domain shows it to be a compact globular fold composed of four α-helices, six short β strands and five loops. Additionally, the MH1 domain also has a β- hairpin structure made up of 11 amino acids through which MH1 can directly bind to DNA when activated Smads are translocated into the nucleus. In the static state (Fig1), MH1 can inhibit the biological activity of the MH2 domain due to interactions between these two domains and vice versa [Heta et al 1997] The canonical MH2 domain contains about 200 amino acids and its amino acid sequence is highly conserved. When activated, Smads can compact into homo-oligomeric complexes through interaction with MH2 domains. Each monomer consists of a β-sandwich core flanked by three α-helices in a bundle on one side and several loops and an α-helix on the other side. Homomeric interfaces are formed by extensive contacts between the three-helix bundle of one monomer and the loops on the adjacent monomer [Shi et al 1997]. The linker region between MH1 and MH2 domains is highly variable in length and sequence. This region contributes to the formation of Smad homo-oligomers and Smad Hoxa9 complex.

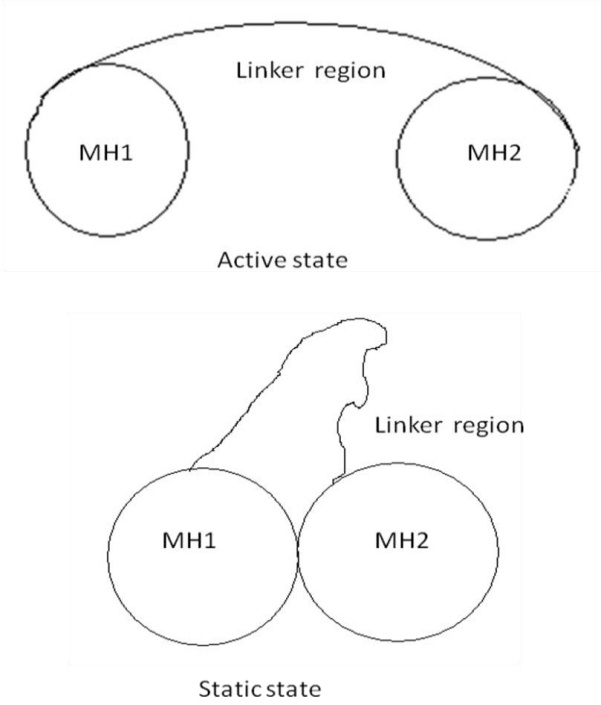


Fig 1: General SMAD structure

Hoxa9 and Hoxc8 act as a repressor for (osteopontin) OPN promoter. SMAD1 and SMAD4 protein after phosphorylation goes into the nucleus and binds to Hoxc8 and Hoxa9 and dislodge it from its DNA binding site. Structural information of the Hoxa9-smad4 complex will help us to get the insight of the pathway and in future structure based drug design would be possible. According to the known experimental information we have proposed two structures of Hoxa9-SMAD4 complex.

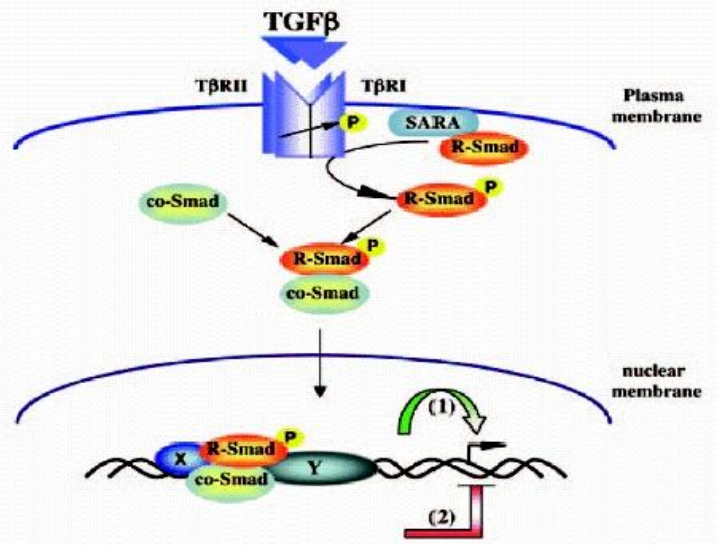


Fig 2: SMAD pathway

The notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms (Artavanis-Tsakonas, S. et al 1999). Once the notch extracellular domain interacts with a

ligand, an ADAM-family metalloprotease called TACE (Tumor Necrosis Factor Alpha Converting Enzyme) cleaves the notch protein just outside the membrane (Brou C et al 2000). This releases the extracellular portion of notch, which continues to interact with the ligand. The ligand plus the notch extracellular domain is then endocytosed by the ligand-expressing cell.

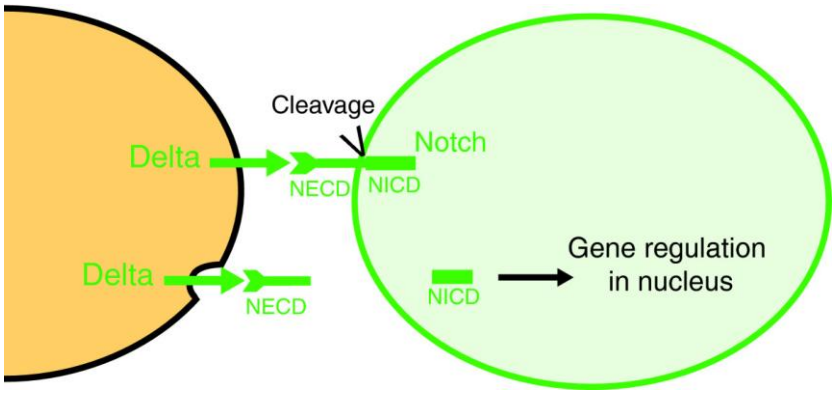


Fig 3:Delta-notch pathway

Notch proteins are conserved cell surface receptors. Both Drosophila Notch and Human Notch1 have extracellular domains containing 36 tandem EGF repeats, as well as other sequences [Bray, 2006]. A ligand binding domain comprising the two EGF repeats 11 and 12 has been defined using a cell adhesion assay. [Rebay et al 1999]. Genetic studies of Notch mutant flies confirm the importance of the EGF repeat 11–12 region in vivo.

Previous experimental result (Xu et al 2005) supports the conclusion that EGF11 and EGF12 are essential for ligand binding, but indicate that other EGF domains also make substantial contributions to ligand binding. Characterization of Notch deletion constructs and O-fucose site mutants further revealed that no single site or region can account for the influence of Fringe on Notch-ligand binding. Additionally, they observed (Xu et al 2005) an influence of Fringe on a Notch fragment including only 4 of its 36 EGF domains (EGF10-13).

Binding of Delta to Notch results in a proteolytic cascade that releases the Notch intracellular domain, allowing it to participate in transcriptional activation in the nucleus. In this molecular modeling study we have taken drosophila Delta protein as a ligand since its interaction with Notch protein is experimentally well established and for Notch1 on the other hand we chose one that belongs to mammalian group with known crystal structure. We have proposed two delta-notch complex structure and analysed the interaction between them.

Protein-DNA complex:

To understand the DNA protein interaction we need to know about general DNA binding domain. A DNA-binding domain (DBD) is an independently folded protein domain which contains at least one motif that recognizes double- or single-stranded DNA. There are different types of DNA binding

domains e.g. Helix turn Helix, Zinc finger, Leucine zipper,Winged helix, Winged helix turn helix and Helix-loop-helix. DNA-binding domains with functions involving DNA structure have biological roles in the replication, repair, storage, and modification of DNA, such as methylation. Many proteins involved in the regulation of gene expression contain DNA-binding domains.

The specificity of DNA-binding proteins can be studied using many biochemical and biophysical techniques, such as gel electrophoresis, analytical ultracentrifugation, calorimetry, DNA mutation, protein structure modification, nuclear magnetic resonance, x-ray crystallography, surface plasmon resonance, electron paramagnetic resonance, cross-linking. For studying the atomic detail molecular dynamics study proves to be a valuable tool.

In eukaryotes, the homeodomain comprises of 3 helices, of which the third recognizes the DNA. They are common in proteins that regulate developmental processes. We have studied a protein-DNA complex (Antennapedia hoemodomain protein DNA complex) with such a HTH motif, a homeodomain protein. Homeodomain protein is an ideal system to study the DNA protein interaction.(Lilley, David, 1995.).

The Problem: The interactions between proteins and protein DNA complexes involved in signaling pathway like SMAD pathway and delta-notch are important for numerous biological functions. The knowledge of their structural features and interactions are essential to gain knowledge about the pathway**.** Although the characterization of the interactions between DNA and the protein and protein-protein is possible through X-ray crystallography, NMR or some other biochemical experiments but the detail characterization of dynamic features in atomics level cannot be obtained from experiments. The problem is to understanding the structural and interaction features of the key protein-protein, protein-DNA complexes (SMAD4-Hoxa9, Delta-Notch and Hoxc8-DNA complex) involved in signaling pathway.

Aim: The aim of the thesis is to standardize a methodology that would lead to a reasonable model which would explain the SMAD4-Hoxa9 and Delta-Notch complex which has a key role in SMAD pathway and Delta-Notch Pathway and also the Molecular Dynamics of the model complex for detail characterization of the dynamical features. The methodology adopted would also help characterize a Protein-DNA complex i.e. Antennapedia-DNA protein complex in a 20ns long molecular dynamics simulation.

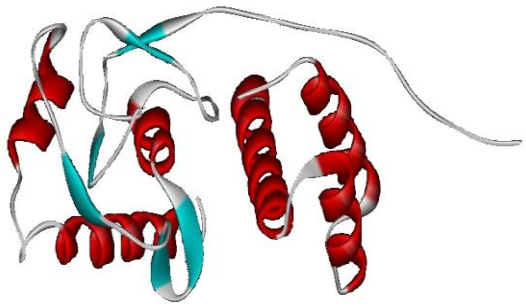
Objectives:

1) Building a reasonable structural model of SMAD4-Hoxa9 complex.
2) Interaction studies between SMAD4 and Hoxa-9 complex.
3) Building a reasonable structural model of Delta-Notch complex.
4) Interaction studies between Delta-Notch complex.
5) Detail characterization of Hoxc8-DNA complex in a 20ns long Molecular Dynamics Simulation.

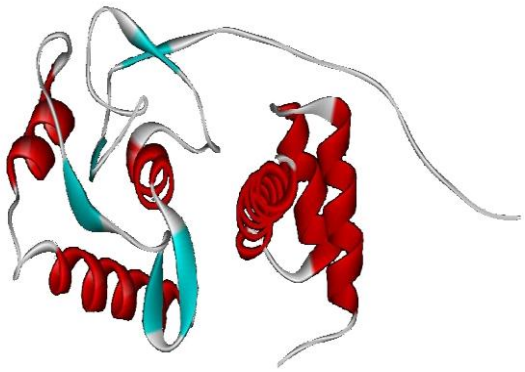
Results:

Building a reasonable structural model of SMAD4-Hoxa9 complex.

The MH1 domain of smad4 was modeled by homology modeling taking 1ozj.pdb as a template. There are three alpha helices in the structure from S32 to E49, from L54 to L63 and from P91 to W99. There are 6 beta strands, T73-Q75, E82 to V84, R87toG89, K110-H111,S125-C127,133Y-134E.
Biochemical experiments suggest that a part of MH1 domain of smad4 and part of the linker interacts with Hoxa9 and dislodge it from DNA. So it is expected that Hoxa9 helix-III which was interacting with DNA, also interacts with SMAD4. A new strategy has been used to build a reasonable structure of long linker region. On the basis of the existing experimental data we have built two models by manual docking so that smad4 residues 101-148 should be interacting with Hoxa9 Helix-III(residue no 49-60). Structural relaxation of the complex was monitored by analyzing the time evolution of the RMSD of the frames with respect to the initial structure. This molecular modeling study leads us to believe that two models developed in this study could be representative of the way Smad4 interacts with Hoxa9.



(a)



(b)

Fig 4 (a) Modeled structure -I is shown as solid ribbon model. (b) Modeled structure -II is shown as solid ribbon model. The structures were viewed and saved as picture file by WebLab Viewer software.

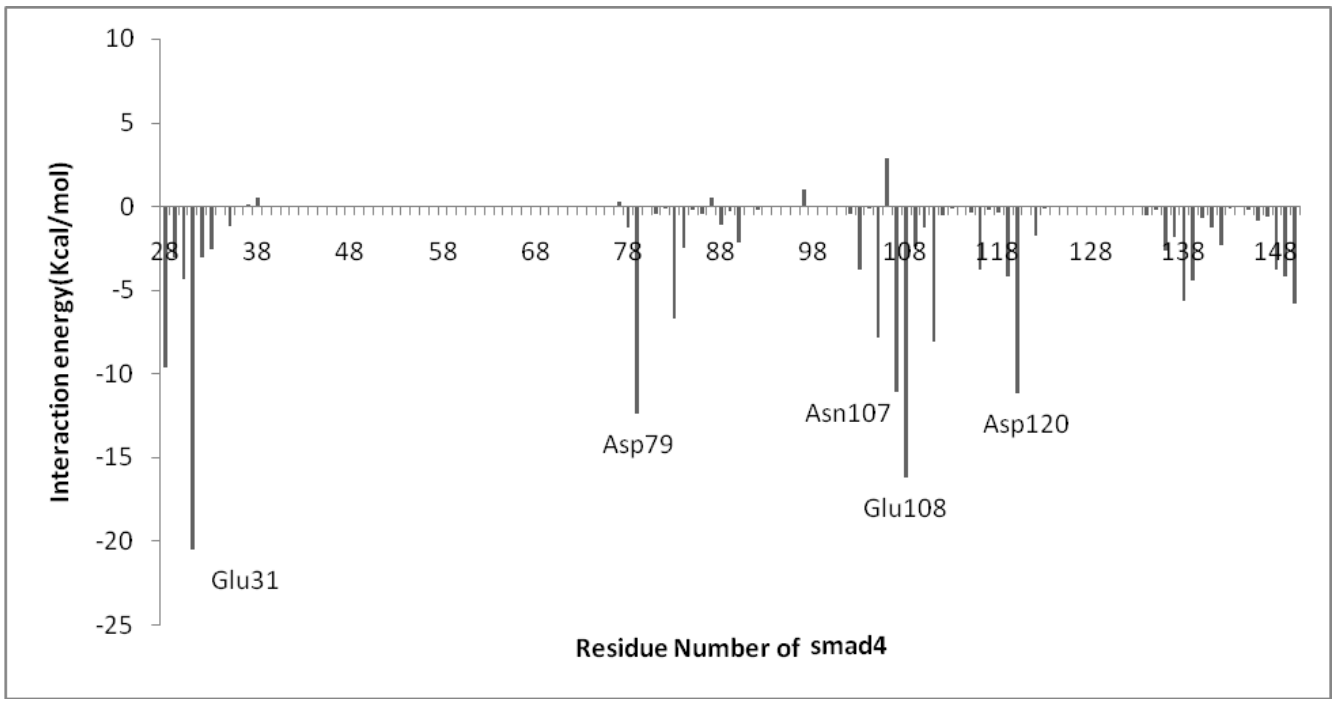
Interaction studies between SMAD4 and Hoxa-9 complex.

The interaction results shows that important residues for complex formation in model-I are Glu31,Asp79,Asn107,Glu108,Asp120 and Asp103,His105,Glu108,Asp120,Gln149 in model-II.

From the interaction studies the important residues have been identified in the models. It has been studied when these important amino acid residues are mutated how the structure interaction is affected.

Total 4 mutations in the 2 models viz at 1) Asp79->Ala79 2) Asn107->Ala107 in model-I and 3)Asp 103->Ala103 4)His105->Ala105 in model-II have been studied.

In four proposed mutation study it is seen that few interactions get reduced where as few other interactions grows so that overall interaction remains same. The interaction pattern is shown in the Fig 5. Thus it is concluded that there are many complementary interactions possible between Hoxa9 and Smad4 MH1 domain if the MH1 domain is open and accessible for interaction.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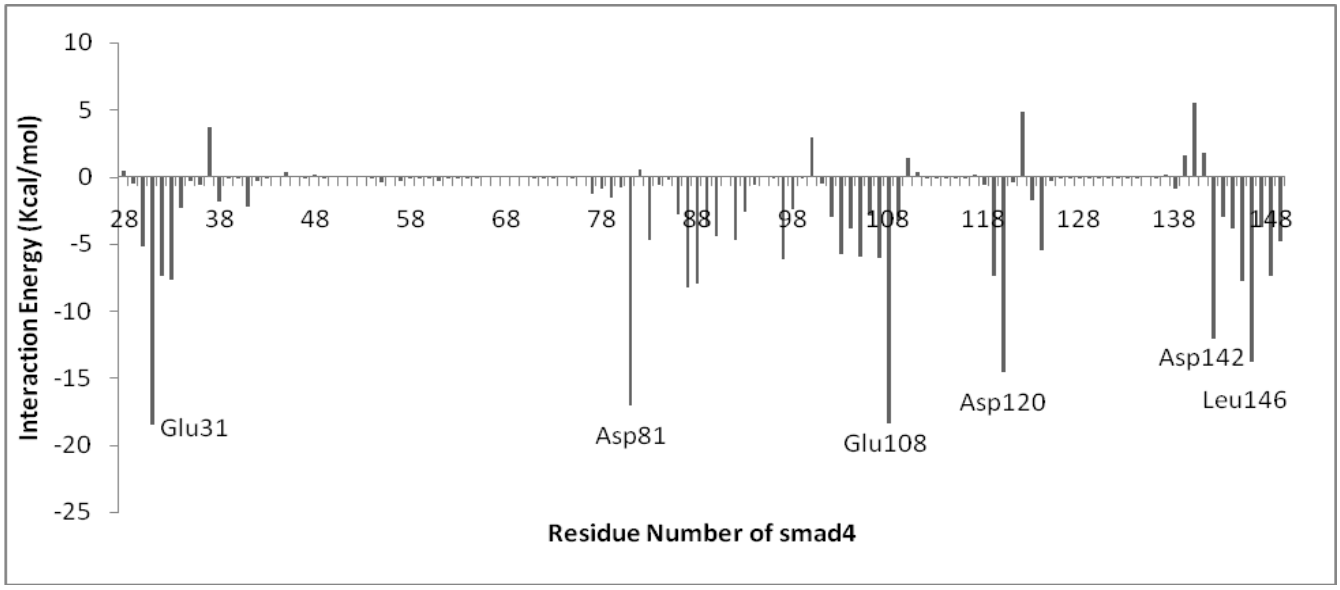
Interaction energy(Kcal/mol)
Asp79
Asn107
Asp120
Glu108
Glu31
Residue Number of smad4

(a)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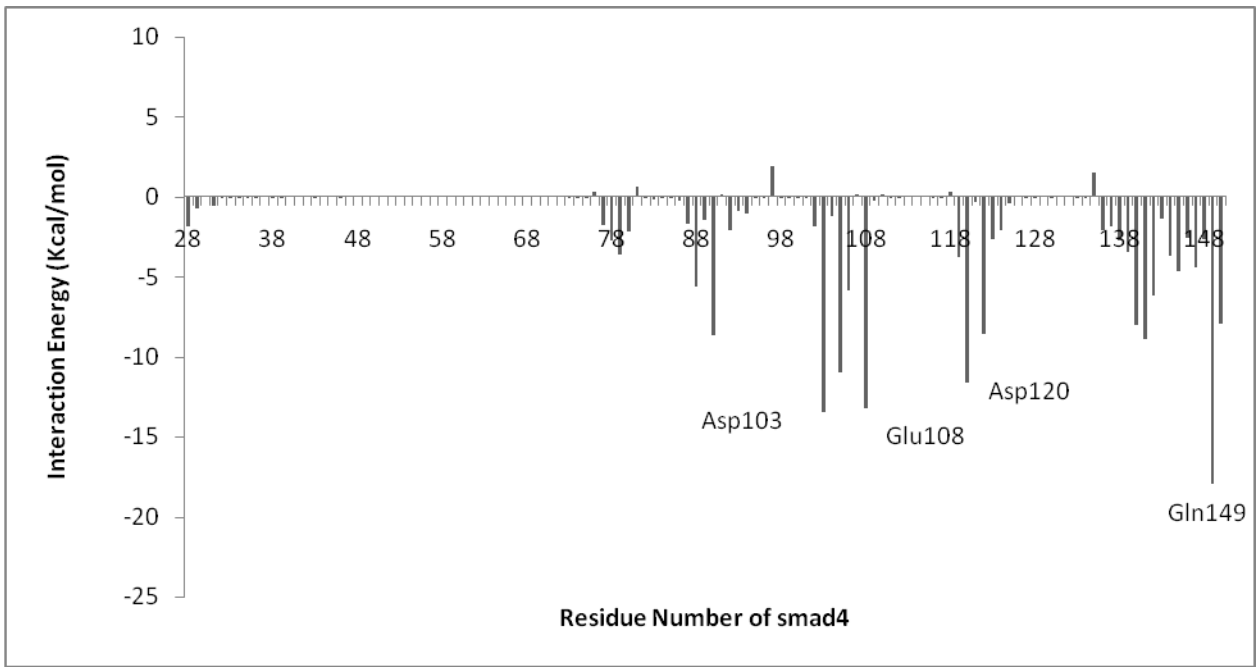
Interaction Energy (Kcal/mol)
Glu31
Asp81
Glu108
Asp120
Asp142
Leu146
Residue Number of smad4

(b)



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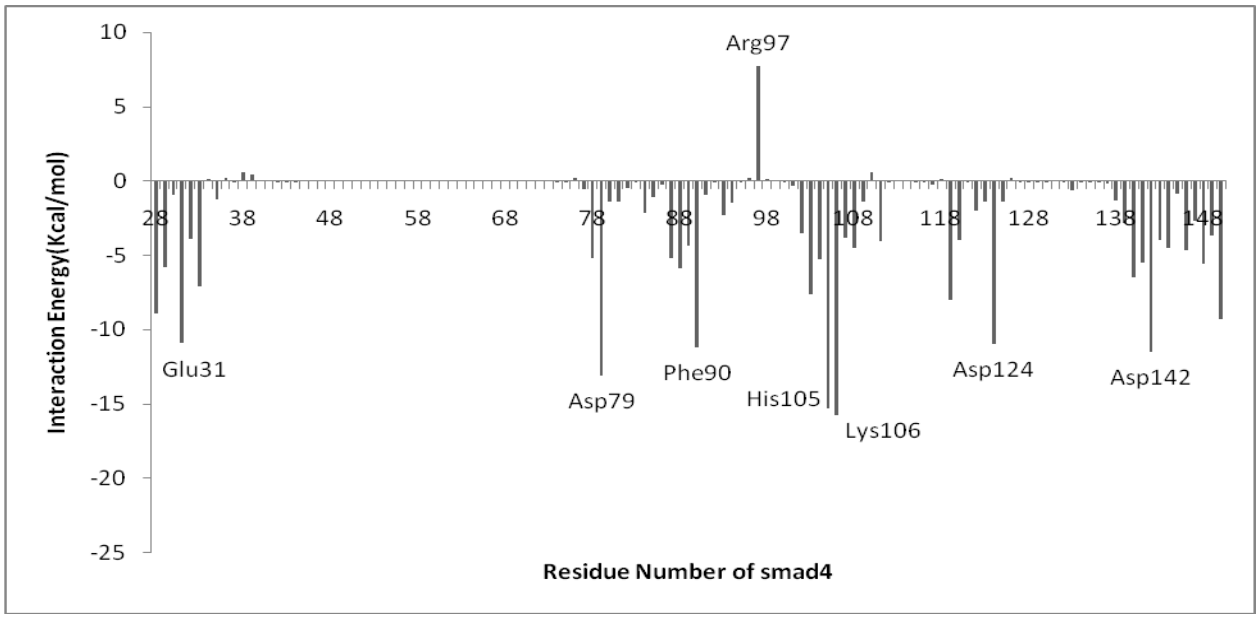
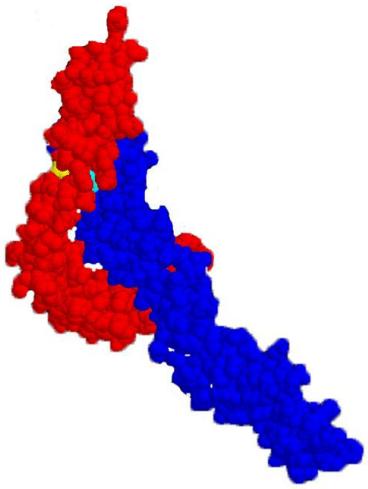


(d)

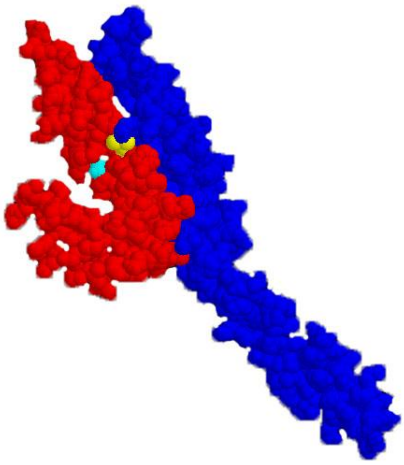
Fig5. Interaction pattern of smad4 with Hoxa9 in (a) wild type Model I, after mutation (b) Asp79 ->Ala79 in Model-I (c) wild type Model-II, after mutation (d) Asp103->Ala103.

Building a reasonable structural model of Delta-Notch complex.

In case of Delta-Notch complex, two probable structures have been proposed which is shown in Fig 6. Experimentally observed important residues of Notch i.e. V453 and G472 are shown in yellow and cyan.



a



b

Fig 6 **:** Space-filled models showing the docked structure of Delta-Notch complex. Model-I (a) and Model-X (b) are shown. Notch ligand binding region and Delta are shown in red and blue respectively. Yellow and cyan residues indicate the positions of V453 and G472 residues of Notch.

Notch1 (henceforth referred to as Notch) ligand-binding domain and part of the Delta were docked by

GRAMM-X. It is known that V453 and G472 of Notch represent the strongest candidate for binding, so these residues were used as the interface residue as constraints for docking. GRAMM-X results give ten possible structures of Delta-Notch complex. Each structure was analyzed for the interaction energy and involved surface area. According to ASA and interaction energy Model-I and Model-X have been selected as most probable structures.

Interaction studies between Delta-Notch complex

Interaction energies were calculated for both the Models. The details are written in the thesis. Here only two interaction patterns are shown in Fig 7.

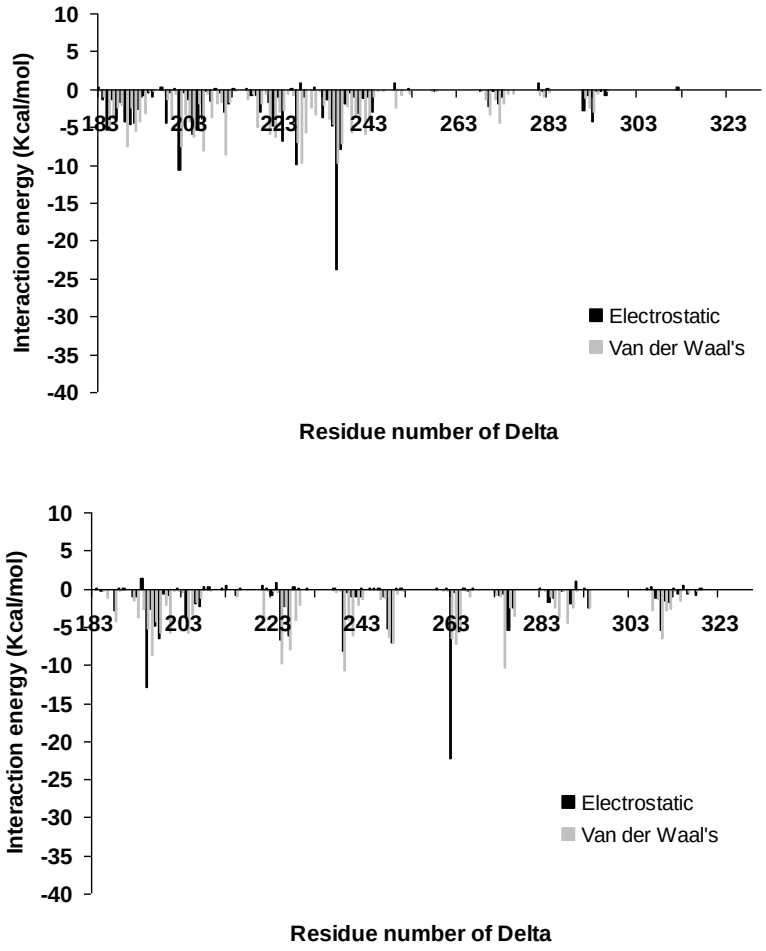


Fig 7. Interaction energy pattern of Delta (a) Model-I and (b) Model-II are shown. The contributions of Van der Waal's and Electrostatic energy are shown by grey and black bars respectively.

The interactions between Delta-Notch in different models have been investigated and suggested that Model-X is most suitable structure. The importance of the conserved residues were also been analyzed.

Detail characterization of Hoxc8-DNA complex in a 20ns long Molecular Dynamics Simulation.

In the analysis of smad pathway, structure functional analysis of Hoxc-8 and DNA complex is important. X-ray crystallographic structure of Hoxc8-DNA structure is not available,so the homology modeling of Hoxc8-DNA structure was done (Roy and Sen, 2005). To understand the detail protein DNA interaction, the crystallography structure is needed as a starting structure.. Antennapedia homeodomain protein has ben chosen as a model system because it has high sequence identity with Hoxc8-DNA complex. The DNA-protein interactions have been studied in 20ns MD simulation of free and bound protein. RMSD vs Time is shown in the Fig 8.

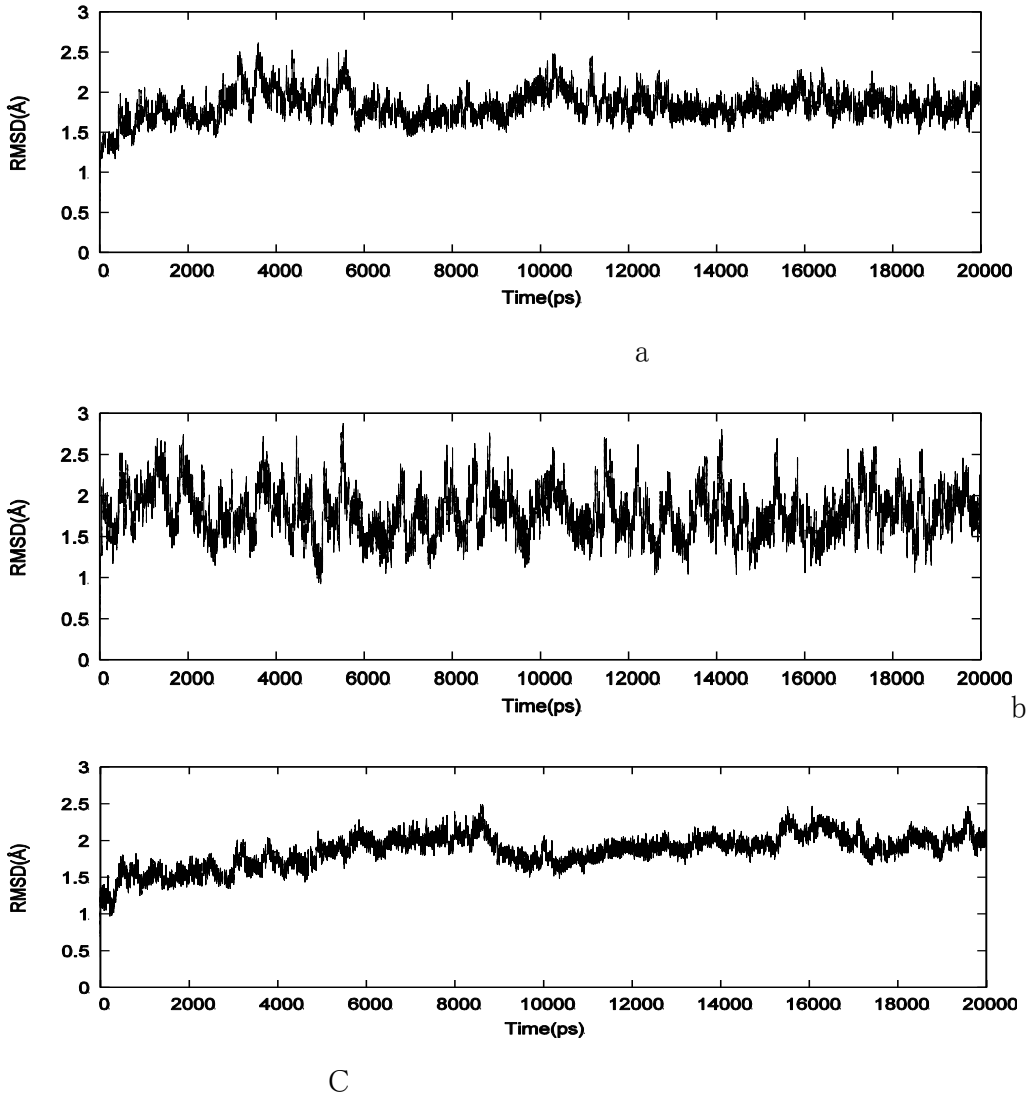
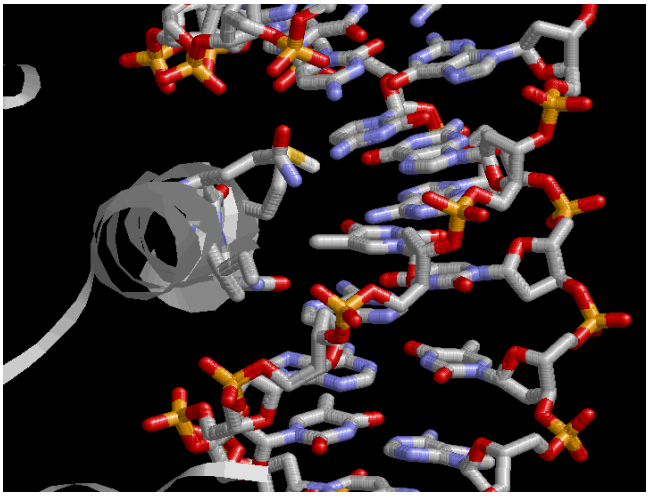
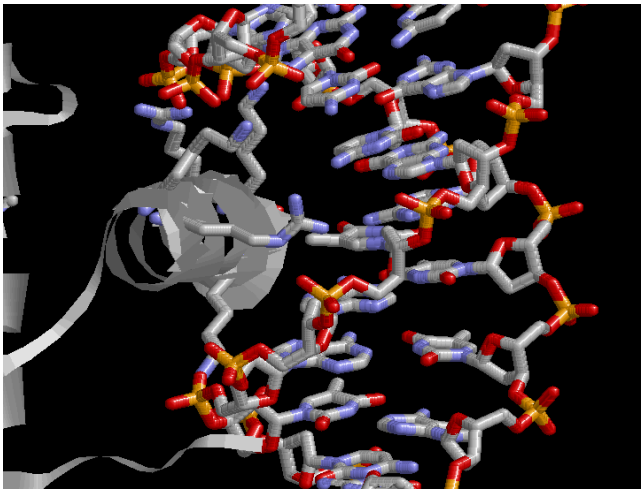


Fig 8. Time evolution of RMSD of the coordinates of the protein-DNA complex with respect to initial structure (excluding end base pairs of DNA and end residues of protein). Time evolution of RMSD of the complex (a). Time evolution of RMSD of the coordinates of the free DNA (b) and free protein (c)



(a)



(b)

Fig 9. The Interaction of the DNA base and the Protein residues are shown in (a). The protein is shown in ribbon model, DNA is shown in stick model and the interactive residues Met54, Asn51, Gln50, and Ile47 are shown in stick model. (a) The Interaction of the DNA back bone is shown in Fig 9(b), the important residues are shown in stick model Arg53, Lys57, Lys46, Arg43, and Lys55.

This study makes use of MD simulation to extend the analysis of the water interactions of the Antenneapedia Homeodomain -DNA which takes into account the role of several water bridges that is important for the DNA-protein recognition. In respect of specific interaction pattern, the dynamical average structure is slightly different from the crystal structure. In this analysis it was found that a typical hydration shell around the protein-DNA interface (Gln 50), with first peak around 2.7 Å and thesecond peak around 5 Å. This hydration shell is absent in the free protein.

It is well accepted now that water play an important role, in homeodomain interaction. Here the role of water along with Gln50 in 20ns long simulation were analyzed followed by an analysis of the trajectory where it was found that the water molecules penetrate into the interface during simulation. Comparison of water bridges with the crystal structure shows crystal structure miss some important water bridge which plays a major role in recognition of the ligand once the molecular dynamics study is undertaken.

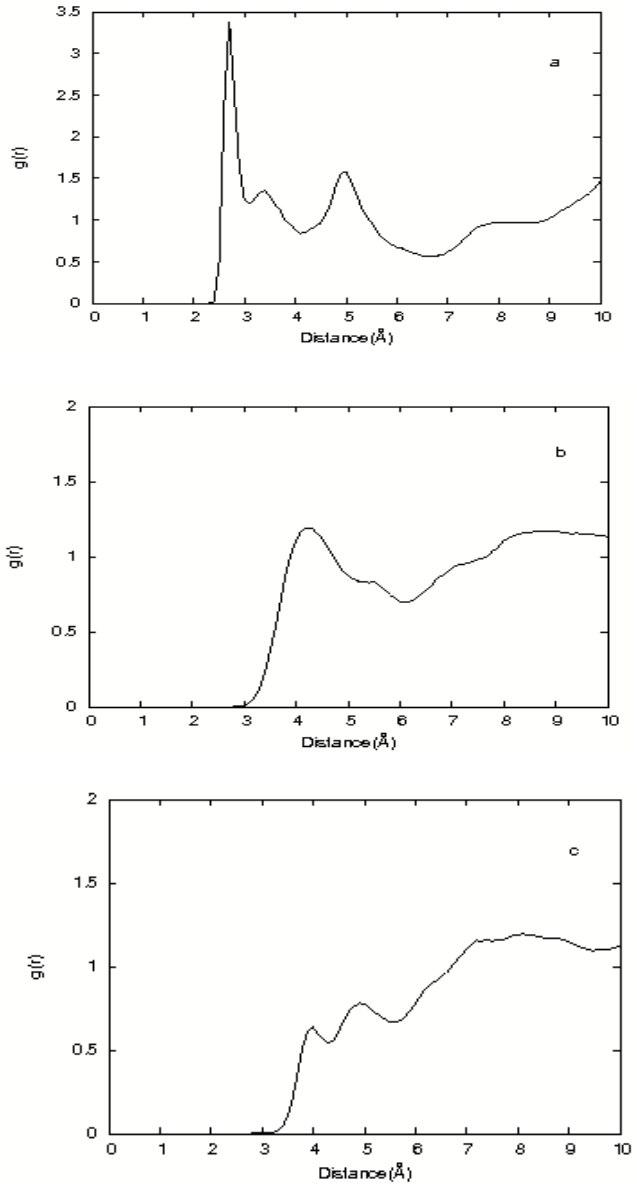


Fig 10. Protein-DNA complex-water radial distribution around a key interacting residue (Gln50) as a function of distance between water oxygen atoms and the nearest protein (Gln-50) atom. The distribution was averaged over last 8ns. (b) The protein water radial distribution around Gln50 in a free protein. (c) The radial distribution of water around DNA-II 6th base (A interaction base in DNA protein complex with Gln50) in the free DNA.

In the DNA deformational table it is easily shows that due to the fluctuations in the major groove at 9^{th} and 10^{th} position (AT/AT) the protein helix-III can enters into the groove. The major groove width at 8^{th} position is highest (18.97 Å) and the fluctuations is less than other positions (in major groove) and thus reflects the stability.

Conclusion:

Here I have proposed 3D structures of important protein-protein complexes like Delta-Notch and Smad4-Hoxa9 complex which are involved in different signaling pathways like SMAD pathway and Delta-Notch pathway and also suggested most probable structure analyzing them according to their interactions, solvent accessible surface area, planarity and salvation energy gain.

I have found the energy basis of the experimentally important residues of Notch protein like V453 and G472 in binding with Delta and analyzed the importance of the conserved residues R196, R198 and R200 of Delta protein in interaction with Notch which facilitates the Notch signaling. Our investigation suggests that Model-X as the most probable structure of Delta-Notch.

On the other hand in case of Smad-Hoxa9 complex both the proposed models are possible. The interaction results shows that important residues for complex formation in model-I are Glu31,Asp79,Asn107,Glu108,Asp120 and Asp 103,His105,Glu108,Asp120,Gln149 in model-II. In four proposed mutation study it is seen that few interactions get reduced where as few other interactions grows so that overall interaction remains same. Thus it is concluded that there are many complementary interactions possible between Hoxa9 and smad4 MH1 domain if the MH1 domain is open and accessible for interaction.

In this thesis I have also discussed about a long dynamics (20ns) simulation of an important protein DNA complex i.e Anteneapedia Homeodomain-DNA complex which is also a part of SMAD pathway. Here a typical hydration shell has been found around the protein-DNA interface (Gln 50), first peak is around 2.7 Å and the second peak is around 5 Å. This hydration shell is absent in the free protein. It is well accepted now that water play an important role, in homeodomain interaction, here I have analyzed the role of water along with Gln50 in 20ns long simulation. I have analyzed the trajectory where it is found that the water molecules penetrate into the interface during simulation. Water Bridges comparison with the crystal structure shows crystal structure miss some important water bridge which comes into play a major role in recognition due to the dynamics. In the DNA deformational table shows

that due to the fluctuations in the major groove at 9^{th} and 10^{th} position (AT/AT) the protein helix-III get enters into the groove. The major groove width at 8^{th} position is highest (18.97 Å) and the fluctuations is less than other positions (in major groove) reflects the stability. In free DNA the major groove was wide at the 6 CC/GG positions which may be shifted to 8^{th} (AT/AT) position due to protein binding, in the protein bound crystal and MD average structure.

This thesis surveys the intricacies of formation and activities of the protein-protein complexes and Protein-DNA complexes in order to establish the mode of functioning of these complexes within the reallife situation using Molecular Dynamics studies. We expect these theoretical studies would help to understand better the exact nature of the structure and the interaction when experimental results are available and to predict the same in other cases.

Significant achievement:

- One of the important partner of signaling pathway (SMAD) is Hoxa9-Smad4 complex. Structural model of Hoxa9-Smad4 has been proposed, can be used for drug design.

- Another important partner in cell-cell communication is Delta-Notch Complex. Structural model of Delta-Notch protein has been build based on biochemical experimental information.

- Detail structural analysis of the Hoxc8-DNA complex, free protein and free DNA during 20ns long simulation suggests how the structures of the proteins and DNA are changed during complex formation. Involvement of the water in the recognition process has been analyzed so that now we know the structural role in transcription regulation process of osteopontin gene.

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