# SUMMARY

## CHAPTER-I

### Crystal Structure of Calcium Binding protein-5 (EhCaBP5) from *Entamoeba histolytica*

- *E. histolytica* contains 27 calcium binding proteins, the presence of many calcium binding proteins indicate that this organism has extensive and complicated calcium signalling network (Bhattacharya et al., 2005).
- Functional study of *E.histolytica* calcium binding proteins indicates that these calcium binding proteins perform variety of functions in *E. histolytica* (Chakrabarty et al., Sahoo et al., 2004, Jain et al., 2008, Somlata et al., 2011, Saima et al 2012., Grewal et al., 2013).
- Towards structural and functional characterization of calcium binding proteins of *E. histolytica*, we have determined the crystal structure of calcium binding protein-5 (CaBP5) at 1.9 Å resolution.
- For the structure determination of EhCaBP5, gene was cloned, overexpressed and purified to its maximum homogeneity by using Ni-NTA chromatography followed by gel filtration chromatography.
- The purified protein was crystallized by hanging drop vapour diffusion method in 2.6 M-2.8 M sodium acetate trihydrate and 0.1M bis-tris pH 5.5. Crystals were diffracted up to 1.8 Å resolution at ESRF-DBT-BM14 France.
- Diffraction data were processed and scaled using HKL2000 (Otwinowski & Minor., 1997). The crystals belonged to space group C222, with unit cell parameters a= 47.5, b= 133.6, c= 44.5 Å.
- Molecular replacement protocol was applied for the structure solution using Potato Calmodulin (Yun et al., 2004; PDB entry 1RFJ), as a template, which shares 29% sequence identity to EhCaBP5 and is the most similar among known CaM structures. But Molecular replacement failed to give any solution (Kumar et al., 2012).
- Structure was solved with Selenomethionyl derivative protein and crystal was diffracted at 1.9Å resolution. Diffraction data were scaled, indexed and processed in space group C2, crystal parameters were a = 70.55, b = 44.45, c = 47.73, $\alpha$ =90, $\beta$ = 108.9, $\gamma$ = 90
- Structure was solved by single anomalous dispersion (SAD) method. Out of 138 residues 108 were built by auto build programs ARP/WARP (Perrakis et al., 1999), then rest of residues were traced in COOT, refinement was done by Refmac5 software.

- The final structure of EhCaBP5 has been refined to R and $R_{free}$ 18.7 and 22.1. Out of 138 residues, refined structure contains 131 residues except the first six residues of the proteins.
- EhCaBP5 structure shows the presence of only one molecule in the asymmetric unit. The structure is rich in α helices and antiparallel β strands at C-terminal end were also observed. Stretch signature residues indicate the presence of two EF-hand motifs but in the crystal structure only one EF-hand motif was observed with bound calcium.
- Unconventional mode of calcium binding to canonical EF-hand motifs has been observed. Despite of 29% sequence identity to Potato CaM, the overall structure does not shows similarity to Potato CaM.
- EhCaBP5 structure differs to CaM in number of EF-hand motifs and domain composition. EF-hand motif of EhCaBP5 is in closed conformation, whereas CaM EF-hand motifs become open when calcium binds to it. EhCaBP5 structure does not have long central helix as observed in various CaM structure, it is broken in between and connected via a small loop.
- This is the first time an ELC like protein structure has been determined independently in closed conformation. The EhCaBP5 EF-hand motif remains in closed conformation though the calcium is bound to it and calcium coordinating residues are canonical. The closed EF-hand motif is due to presence of more hydrophobic residues as compared to Potato CaM and ELC, so this extensive hydrophobic interaction between these two helices could be the reason for the closed EF-hand motif conformation of EhCaBP5.
- The structural resemblance of EhCaBP5 to ELC indicates that it could interact to myosin or IQ motif.

**CHAPTER-II**

**EhCaBP5 interact with Myosin IB and involved in initiation of phagocytosis**

- Structure based alignment indicates that EhCaBP5 structure is quite similar to essential light chains (ELC) of myosin and it was expected that EhCaBP5 could serve as ELC of *E. histolytica* myosin and it could interact with the myosin of *E. histolytica*.
- Surface plasmon resonance studies (SPR) were performed to check the interaction between EhCaBP5 and IQ motif peptide of Myosin IB and heavy chain myosin.
- Kinetic studies have indicated EhCaBP5 interacts with myosin IB, IQ motif peptide and dissociation constant (Kd) were calculated to be 240 nM.
- It was found that other calcium binding proteins of *E.histolytica* like EhCaBP1, EhCaBP3 and myosin IB are involved in phagocytosis (Jain et al., 2008, Aslam et al., 2012,) which raised the question whether EhCaBP5 is associated in phagocytosis or not as EhCaBP5 was found to be interacting with myosin IB in SPR.
- We checked the cellular co-localization of EhCaBP5 and myosin IB. The subcellular localization of EhCaBP5 was analyzed during RBC uptake by immunostaining with specific anti-EhCaBP5 antibody. Immunostaining result indicates that EhCaBP5 and Myosin IB are co-localized during the process of phagosome formation and once the phagosomes are formed EhCaBP5 leaves the site. Enrichment of actin was also observed in the phagocytic cup as expected and the superimposition of both CaBP5 and actin suggested that both proteins are colocalized in the phagocytic cups.
- In order to further confirm this interaction, we have performed the Co-immunoprecipitation using immobilized anti-CaBP5 antibody and total cell lysate. Anti-EhCaBP5 antibody precipitated myosin IB along with CaBP5 in a calcium independent manner as myosin IB was also observed in the presence of EGTA, suggesting that calcium is not required for the interaction of these two proteins
- Enrichment of actin was also observed in the phagocytic cup as expected and the superimposition of both CaBP5 and actin suggested that both proteins are colocalized in the phagocytic cups. Since fluorescence data clearly shows the colocalization of actin along with EhCaBP5. However we could not get any direct interaction of EhCaBP5 with actin.
- As EhCaBP5 is found to be localized in cytoplasm and involved in erythrophagocytosis, to check whether these proteins (EhCaBP1 and EhCaBP5) are colocalized during process of erythrophagocytosis, we performed colocalization experiment. Our result

suggests that both EhCaBP1 and EhCaBP5 are colocalized at the time of phagocytic cup formation during the process of erythrophagocytosis.

- Though we could not check the direct interaction of both (EhCaBP5 and EhCaBP1) proteins *in vitro* but we can speculate that colocalization of both protein EhCaBP1 and EhCaBP5 have a regulatory function during process of phagocytosis or phagosome formation as EhCaBP1 transiently remains in mature phagocytic cup, whereas EhCaBP5 leaves phagosome once it fully mature. Suggesting EhCaBP5 has some role in phagocytic cup formation along with EhCaBP1.

**CHAPTER-III**

**Modeling of EhCaBP5-IQ motif peptide Complex**

- The surface plasmon resonance (SPR) assay, cell co-localization and pull down assay confirm our hypothesis and EhCaBP5 was found to interact with Myosin IB *in vitro* as well as *in vivo*.
- To know how these two proteins may interact with each other, we modeled EhCaBP5 in complex with IQ-motif peptide.
- The various complex structure of calmodulin-target protein and structures of myosin with its light chain provided basis for the modeling of EhCaBP5-IQ motif complex.
- The EhCaBP5-IQ motif complex model was obtained by using Rosetta FlexPepDock web server (London et al., 2011).
- The EhCaBP5-IQ motif complex model adopts more open C-terminal conformation and the open C-terminal lobe accommodates IQ-motif peptide in the cleft.
- The interaction of EhCaBP5 with IQ motif is mainly governed by hydrophobic residues and nonbonded interaction of complex model.
- Superimposition of native EhCaBP5 structure on EhCaBP5-IQ motif complex model helped to identify structural changes in EhCaBP5 needed to form a complex with IQ motif.
- Peptide binding did not lead to global changes, as binding of peptide to EhCaBP5 did not interfere or alter the structure of N-terminal domain of EhCaBP5 and C-terminal half adopt extended conformation.
- Orientation of the C-terminal part of EhCaBP5 changed as it moved 15 degrees as compared to the native EhCaBP5 structure, to accommodate the peptide resulting in stretching out of loop connecting the two domains.
- To understand local conformational changes at C-terminal upon peptide binding we tried to superimpose the C-terminal of the model upon the crystal structure of EhCaBP5. The RMSD of the alignment was 0.601 suggesting movement of the C-terminal as compared to the N-terminal.
- The overall length of native EhCaBP5 from head to tail is 47.7 Å, whereas length of EhCaBP5-IQ motif complex is 55.1 Å, clearly indicating elongated conformation adopted by EhCaBP5 after peptide binding.
- We expect that the extended conformation of EhCaBP5 with IQ motif may allow to interact with other molecules/partner during the various cellular processes.

## CHAPTER-IV

## Crystal structure of calcium binding residues mutant N-terminal domain of calcium binding protein-1 from *Entamoeba histolytica*

- EhCaBP1 a 14.7 kDa protein, which shares low (29%) sequence identity with the well-studied eukaryotic EF hand-containing protein, calmodulin (CaM) (Prasad et al., 1993). EhCaBP1 was found to be involved in cytoskeleton dynamics and is associated with phagocytic cup formation in calcium- independent manner (Sahoo et al., 2004, Jain et al., 2008).
- Crystal structure of N-terminal domain of EhCaBP1 shows the trimeric organization of EF-hand motif as observed in full length EhCaBP1 structure and trimer to monomer state was observed as the pH was lowered from the physiological pH (Kumar et al., 2007, 2010).
- To understand the role of calcium binding loop (EF-hand motif) and conformational changes upon calcium binding, we mutated the native N-terminal second EF-hand motif with the residues which is expected to bind calcium with higher affinity. We have mutated residues A47K, N50D, E52F, Q55F and N56E.
- To understand the conformational changes upon calcium binding, we have crystallized the Nt-EhCaBP1 EF-2 mutant and structure was solved at 1.9Å resolution in calcium bound state.
- The NtEhCaBP1 EF-2 mutant was overexpressed and purified to its maximum homogeneity by Ni-NTA chromatography.
- The purified protein was subjected to crystallization and it was crystallized in MPD 58%- 63% sodium actetate buffer pH 5.0-5.5 with 5mM $CaCl_2$.
- Diffraction data was collected on ESRF DBT-BM14 France. The crystals diffracted to 1.9 Å resolution. Diffraction data were processed and scaled using HKL2000 (Otwinowski & Minor., 1997). The crystals belonged to space group $P2_12_12_1$, with unit cell parameters a= 44.6, b= 101.3, c= 107.4 Å.
- The Matthews coefficient $V_M$ is 2.90 $A^3Da^{-1}$ indicating the presence of six molecules in the asymmetric unit, with a solvent content of 57.5% (Matthews., 1968).
- The structure was solved by molecular replacement with Phaser program using the native structure of EhCaBP1 (2NXQ) as the search model.
- The structure represents the six molecules in the asymmetric unit which forms a hexamer unlike the native structure which forms a trimer.

- The hexamer forms due the 2.2 degree bend in the helix-III which interacts with the other trimer. This 2.2 degree bend occurs in the structure due to the shrinkage of calcium binding coordination sphere and all the coordination residues shrink at an average of ~0.15Å.
- This shrink in the structure suggests that the mutant protein binding calcium with higher affinity than the native protein.
- The critical residues which are involved in interaction are Gln36 of chain A with Ser64 and Ile65 of chain D. And Gln36 of chain D interact with Ser64 and Ile65 to chain A. In the same manner chain B interacts with chain F and chain C interacts with chain E, all the interacting residues are the same as they interacts in case of chain A and D. Overall structure is quite different from native structure as the native NtEhCaBP1 forms a trimer and NtEhCaBP1 EF-2 mutant forms a hexamer due to the structural change in the helix-III of EF-2.
- This structural change in EF-2 of the NtEhCaBP1 mutant structure leads to the formation of hexamer structure which may affect the function of the protein in context to its physiological property like localization and interaction with other target protein.