CHAPTER-IV
Crystal structure of calcium binding residues
mutant N-terminal domain of calcium binding
protein-1 from Entamoeba histolytica
5.1 Abstract

*E. histolytica* calcium binding protein-1 (EhCaBP1) is one of the calcium sensing proteins which has been characterized structurally as well as functionally. EhCaBP1 is a 14.7 kDa protein, which shares low (29%) sequence identity with the well-studied eukaryotic EF-hand containing protein, calmodulin (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. Transition from 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 of EhCaBP1 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 role of EF-hand motif in 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 Nt-EhCaBP1 EF-2 mutant was overexpressed and purified to its maximum homogeneity by using Ni-NTA chromatography. The buffer of Ni-NTA purified protein was exchanged by dialysis before the crystallization trials. The purified protein was subjected to crystallization and it was crystallized in MPD 58%- 63% sodium acetate buffer pH 5.0-5.5 with 5mM CaCl\(_2\). Diffraction data was collected at 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\(_1\)2\(_1\)2\(_1\), with unit cell parameters a= 44.6, b= 101.3, c= 107.4 Å. The Matthews coefficient V\(_M\) is 2.90 Å\(^3\)Da\(^{-1}\), 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 (14) using the native structure of EhCaBP1 (2NXQ) as the search model. The structure represents the six molecules in the asymmetric unit which forms hexamer unlike to native structure which forms trimer. The hexamer forms due to 2.2 degree bend in the helix-III. Owing to this structural change one trimer interacts to other trimer and forms hexamer. This 2.2 degree bend occur 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 coordination sphere indicates that the residues which are mutated changes the intrinsic capacity of
coordination sphere which are expected to bind calcium with more affinity than the native protein.
5.2 Introduction

EhCaBP1, a 14.7 kDa (134 amino acid residues) protein from *E. histolytica*, 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). The three-dimensional solution structure of EhCaBP1 in calcium bound state was determined by multidimensional nuclear magnetic resonance (NMR) spectroscopy (Sahu et al., 1999, Atreya et al., 2001) and preliminary X-ray crystallography data was reported (Gopal et al., 1998). Interestingly, NMR studies showed that EhCaBP1 has structural similarity to CaM and TroponinC (TnC), with two globular domains connected by a flexible linker region spanning eight amino acid residues. Each domain consists of a pair of canonical calcium binding EF hand motifs. EhCaBP1 binds four calcium ions with different affinity for calcium (Gopal et al., 1998). However, differences exist which make EhCaBP1 distinct from CaM. The inability of EhCaBP1 to activate c-AMP phosphodiesterase differentiates it from all known CaMs (Yadava et al., 1997). Structural studies indicated a more open C-terminal domain for EhCaBP1 with larger hydrophobic surface area exposed as compared with CaM and TnC. Moreover, the central linker of EhCaBP1 contains two glycine residues (G63 and G67) making it more flexible compared with CaM (Jain et al., 2008).

The crystal structure of the *E. histolytica* calcium binding protein-1 (EhCaBP1) has been determined at 2.4 Å resolution (Kumar et al., 2007). Unlike CaM, the first two EF hand motifs in EhCaBP1 are connected by a long helix and form a dumbbell shaped structure. Owing to domain swapping oligomerization three EhCaBP1 molecules interact in a head to tail manner to form a triangular trimer and the C-terminal half could not be traced owing to missing electron density. This arrangement allows EF-hand motif of one molecule to interact with that of an adjacent molecule to form a two EF-hand domain similar to that seen in the N-terminal domain of the NMR structure of CaBP1, calmodulin and troponin C (Kumar et al., 2007). The oligomeric state of EhCaBP1 results in reduced flexibility between domains and may be responsible for the more limited set of targets recognized by EhCaBP1 (Kumar et al., 2007). Independent domain (N-terminal and C-terminal) of EhCaBP1 were characterized with respect to their structure, as well as specific functional features, such as ability to activate kinase and actin binding. The domains were also expressed in *E. histolytica* cells along with green fluorescent protein. The results suggest that the N-terminal domain retains
some of the properties as native protein, such as localization in phagocytic cups and activation of kinase. Crystal structure of EhCaBP1 with Phenylalanine revealed that the assembled domains, which are similar to the calmodulin N-terminal domain, bind to Phenylalanine revealing the binding mode to the target proteins. The C-terminal domain alone did not show any of the activities tested (Jain et al., 2009). However, over-expression in amoebic cells led to a dominant negative phenotype. This result shows that the two domains of EhCaBP1 are functionally and structurally different from each other (Jain et al., 2009). Dynamic nature of EhCaBP1 and to validate the trimerization of N-terminal domain at physiological conditions, the crystal structure of N-terminal domain was determined. The final structure consists of EF-1 and EF-2 motifs separated by a long straight helix as seen in the full-length protein. The spectroscopic and stability studies provided clear evidence for a conversion from trimeric state to monomeric state. As the pH was lowered from the physiological pH, a dynamic trimer to monomer transition was observed (Kumar et al., 2010). Later it was found that EhC2PK a C2-domain-containing protein kinase initiate erythrophagocytosis and EhC2PK binds phosphatidylserine in the presence of calcium and thereby recruits EhCaBP1 and actin to the membrane (Somlata et al., 2011). Recently EhCaBP1 was functionally manipulated by converting Y81F to bring in Phe-Phe interaction. The Y81F mutation in EhCaBP1 resulted in a more compact structure for the C-terminal domain of the mutant as in the case of calmodulin and troponin C. The compact structure is favoured by the presence of π-π interaction between F81 and F129 along with several hydrophobic interactions of F81, which are not seen in the wild type protein. Further, the biological assays reveal preferential membrane localization of the mutant, loss of its colocalization with actin in the phagocytic cups while retaining its ability to bind G- and F-actin (Rout et al., 2013).

Calcium binding ability of CaBPs is mostly controlled by the canonical calcium-binding loops with very high sequence homology. The loop consists of a contiguous stretch of twelve amino acid residues and bind to calcium in pentagonal bipyramidal geometry (Biekofsky et al., 1998). The residues in the loop at positions +1, +3, +5, +7, +9 and +12 coordinate with calcium. In addition, calcium binding affinity is controlled by other factors such as intrinsic binding affinity of each binding loop, conformational cost upon calcium binding (Atreya et al., 2003, Mustafi et al., 2004), EF-β-scaffold etc. Thus, despite the presence of highly conserved residues in the primary sequence of calcium binding loops, a wide range of calcium binding affinities were observed in different EF of these CaBPs (Nelson et al., 1998).
To design and understand the role of calcium affinity in context to protein structure and function, we have generated an EF-hand motif mutant of N-terminal domain of EhCaBP1 in which second EF-hand motif is mutated. We have explained the high affinity binding on the basis of change in coordination difference of mutant EF-hand motif to native EF-hand motif structure. To understand the high calcium binding affinity in context to its structure, we have crystallized the Nt-EhCaBP1 EF-2 mutant and structure was solved at 1.9Å resolution in calcium bound state.
Calcium Binding Protein-1 (EhCaBP1)

i. Calcium Binding Protein-1 (EhCaBP1) belongs to the class of EF hand containing proteins in *E. histolytica* and it has four EF-hand motifs.

ii. EhCaBP1 gene encodes a 134 amino acid and bioinformatics software predicted EhCaBP5 contains two EF-hand motifs (Bhattacharya et al., 2006).

iii. The crystal structure of EhCaBP1 represents the arrangement of trimer (Figure 1, chapter I), in which only half of the molecules residue 2 to 66 traced. Schematic representation of EhCaBP1 is shown below.

iv. The C-terminal half (residue number 67 to 134) is disorder and could not be traced in electron density. Structure of N-terminal half represents the same trimeric arrangement (kumar et al., 2010) as full length EhCaBP1, therefore only N-terminal half of the protein was taken to study the effect of EF-hand motif mutation on its structure and calcium binding.

v. To predict and design high calcium binding affinity are the following residues which have been mutated A47K, N50D, E52F, Q55F, and N56E.

vi. Expected molecular weight and isoelectric points (pI) of NtEhCaBP1EF-2 mutant is 7.0 KDa, 5.3 respectively, calculated by ExPasy server [1].

For the structure determination of EF-hand motif mutant EhCaBP1, mutations were created by site directed mutagenesis in the EF-2 of N-terminal EhCaBP1, and it was overexpressed, purified, crystallized and structure was determined successfully.
4.4.1 Cloning of Nt EhCaBP1EF-2 mutant

The gene fragments corresponding to the N-terminal domains of EhCaBP1 protein were cloned by using existing N-terminal clone of EhCaBP1 as a template in the bacterial expression vector, pET 28(b). The mutations were created (in EhCaBP1 EF-II) at site 141, 150, 156, 165, 168 by site directed mutagenesis. The following primers were used for the mutation.

CaBP1 Mut K2 Fp 5’-CAAATCTATTGATAAAGATGGAAATGG-3’
CaBP1 Mut K2 Rp5’-CCATTTCCATCTTTATCAATAGATTTG-3’
CaBP1Mut D5, F7 Fp 5’-CTATTGATAAAGATGGAGATGGATTTATTGATCAAATGAATTTGC-3’
CaBP1 Mut D5, F7 Rp GCAAATTCATTITTTGATCAATAAAATCCATCTCCATCTTTATCAATAG-3’
CaBP1Mut F10 Fp 5’-ATTITTATGGATTITTAATGAATTTGC-3’
CaBP1Mut F10 Rp 5’-GCAAATTCATTAAATCTAAATAAAT-3’
CaBP1Mut E11 Fp 5’-ATTITATGGATTITTTGAAGAATTITGC-3’
CaBP1Mut E11 Rp 5’-GCAAATTCTTCAAATAATTAAAT-3’
CaBP1 Fp-5’-CATGCCATGGCAATGGCTGAAGCACTTTTTAAAG-3’
CaBP1 Rp-5’-CGGCTCGAGGAGTGAAAACTCAAGGAATTCTTC-3’

After mutating all five residues, finally the insert was cloned in pET-28b vector. Mutations were confirmed by sequencing.

5.4.2 Overexpression and Purification

The NtEhCaBP1 EF-2 mutant construct was transformed into E.coli strain BL21 (DE3) for expression. Cells were grown in LB medium supplemented with 50 mg ml^{-1} kanamycin at 310 K. The culture was induced with 0.8 mM IPTG when the OD reached 0.7 at 600 nM. It was then incubated at the same temperature for 3 h for further growth. Cells were harvested by centrifugation at 7000 rev min^{-1} for 10 min. The cell pellet was suspended in suspension buffer (50 mM Tris pH 8.0, 50 mM NaCl, 5 mM CaCl_2). The cells were then lysed by freeze–thaw followed by sonication. Clear supernatant was obtained by centrifugation at 12000 rev min^{-1} for 30 min. The protein supernatant was passed through Ni–NTA column pre-equilibrated with ten bed volumes of suspension buffer for affinity purification. It was then washed with 30–40 ml wash buffer (50 mM Tris, 70 mM NaCl,) to remove nonspecific binding. Finally, the protein was eluted with elution buffer (50 mM Tris, 50 mM NaCl, 5 mM CaCl_2, 50 mM imidazole). The purity of the protein was checked using 15% SDS–PAGE.
The Ni-NTA purified protein was pure enough to use for the crystallization. Before crystallization trials Ni-NTA purified protein buffer was exchanged by dialysis against the buffer (50 mM Tris pH 8.0, 5 mM CaCl$_2$). The purified and dialyzed protein was concentrated to 15 mg ml$^{-1}$ using a 3 kDa cutoff centicon prior to crystallization.

### 5.4.3. Crystallization of NtEhCaBP1 EF-2 Mutant

Initial crystallization trials were carried out by the hanging-drop vapour-diffusion method in 24 well plates using 2 µl of protein solution was mixed with an equal volume of precipitant solution and equilibrated against 500 ml reservoir solution (precipitant). Initially the same crystallization condition (18% PEG 500 MME, 100 mM acetate buffer pH 3.6–3.8, 15–18% PEG 400, 5 mM CaCl$_2$, 10% Isopropanol, 50 mM NaCl and 100 mM acetate buffer pH 3.6–4.2, 100 mM of KCl solution and a mixture of 1 mM each of glycine, proline, glucose, and sucrose as an additive) was used in which native NtEhCaBP1 (N-terminal EhCaBP1) was crystallized (Kumar et al., 2010). The crystallization plates were incubated at 289 K. We could not get crystals in native NtEhCaBP1 crystallization condition rather the condition was more close to native EhCaBP1 crystallization condition (Kumar et al., 2007). The NtEhCaBP1 EF-2 Mutant was crystallized in MPD 58%- 63% sodium acetate buffer pH 5.0-5.5 with 5mM CaCl$_2$.

### 5.4.4 X-ray diffraction, Data Collection, processing and structure solution

Crystals were soaked in cryoprotectant solution consisting of 65% MPD, 100 mM sodium acetate pH 5.3, 5 mM CaCl$_2$. Single crystals were picked up in cryoloops and flash-cooled in liquid nitrogen. The initial crystal diffraction was done in house rotating anode generator (MICROSTAR, Bruker AXS) at AIRF and crystals diffracted up to 2.7Å resolution. Higher resolution data were collected at 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$_1$2$_1$2$_1$, with unit cell parameters a=44.6, b= 101.3, c= 107.4 Å. The Matthews coefficient $V_M$ is 2.90 Å$^3$Da$^{-1}$, 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 (Storoni et al., 2004) using the native structure of EhCaBP1 (2NXQ) as the search model and assembled trimer was used for molecular replacement, keeping six molecule in asymmetric unit. Twelve calcium atoms, (two calcium in each chain) were identified in the electron density and
included in the refinement. The structure was refined by iterative model building using COOT graphics package (Emsley et al., 2004) combined with Translation, Liberation and Screw-rotation (TLS) displacement parameters were determined and TLS -restrained refinement was performed (Winn et al., 2001). For the final model, the $R_{\text{work}}$ is 22.1 % and $R_{\text{free}}$ is 26.6%. The structure has good stereochemistry as indicated by program PROCHECK (Laskowski et al., 1993) with 97.6% of residues lying in the most favoured regions of the Ramachandran plot. The data collection and final refinement statistics are shown in Table 1 and 2.
Table 1:
Crystallographic data-collection statistics of NtEhCaBP1 EF-2mutant

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<td>X-ray source</td>
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<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Space Group</td>
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<tr>
<td>Cell parameters (Å, °)</td>
<td>a = 44.69, b = 101.35, c = 44.5</td>
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<tr>
<td></td>
<td>α = 90, β = 90, γ = 90</td>
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<tr>
<td>No. of molecules in an asymmetric unit</td>
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</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>B-factor Wilson plot (Å²)</td>
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<td>Mosaicity range (°)</td>
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<tr>
<td>Redundancy</td>
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<tr>
<td>Mean I/σ (I)</td>
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<tr>
<td>Rmerge(%)</td>
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Table 2:
Refinement and Ramachandran plot statistics NtEhCaBP1 EF-2mutant

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<tr>
<td>$R_{\text{work}}$</td>
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<tr>
<td>$R_{\text{free}}$ (%)</td>
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<tr>
<td>r.m.s.d. bond length (Å)</td>
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<tr>
<td>r.m.s.d. bond angles (°)</td>
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<tr>
<td>Favoured region (%)</td>
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<tr>
<td>Additional allowed region (%)</td>
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<tr>
<td>Generously allowed regions (%)</td>
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5.5 Result

5.5.1 Expression, purification and crystallization of Nt-EhCaBP1 EF-2 mutant
The NtEhCaBP1 EF-2 mutant protein was overexpressed in the presence of 0.8 mM IPTG at
37° C temperature. The protein was purified in the single step using Ni-NTA chromatography
and the purity was checked on 15% SDS PAGE (Figure 1). Before crystallization trials buffer
were exchanged by dialysis to remove unwanted salt and exchanged buffer which was used
50 mM Tris pH 8.0 and 5 mM CaCl\(_2\). We could not get crystal in native NtEhCaBP1
crystallization condition. The NtEhCaBP1 EF-2 mutant protein was crystallized in MPD
58%-63% sodium acetate buffer pH 5.0-5.5 with 5mM CaCl\(_2\) crystal appeared after 5-6
days of equilibration (Figure 2).
Figure 1: Purification NtEhCaBP1 EF-2 mutant: 15% SDS PAGE image showing the purification profile of the NtEhCaBP1 EF-2 mutant protein. Lane M represents, molecular-mass marker (labelled in kDa); lanes 1 and 2, different fractions of Ni-NTA chromatography.

Figure 2: Image showing crystals of Nt EhCaBP1 EF-II Mutant
5.5.2 Overall structure of NtEhCaBP1 EF-2 Mutant

The NtEhCaBP1 EF-2 Mutant structure contains six molecules in asymmetric unit, which forms double trimer, unlike native N-terminal EhCaBP1 structure which forms trimer. The model is refined with good electron density (Figure 3). Two EF-hand motifs of NtEhCaBP1 EF-2 mutant were separated by a long helix, similar to that crystal structure of N-terminal domain of NtEhCaBP1 (Kumar et al., 2010) with one calcium bound at each EF-hand motifs (Figure 4).

![Figure 3: Electron density map](image)

**Figure 3: Electron density map:** Image showing the electron density map of few residues at 1.5σ cutoff, in which Phe6, Lys7, Glu8, Ile9, Asp10, Val11, Asn12, Gly13, Asp14 and Gly15 are represented in the electron density.
Figure 4: NtEhCaBP1 EF-2 mutant Monomer: Image showing the monomer of NtEhCaBP1 EF-2 mutant, in which EF-1 and EF-2 adopts extended conformation and both EF-hand motifs are separated by long central helix.
The calcium coordinates with seven of its ligands, including one water molecule, in a pentagonal bipyramidal manner. The coordination geometry of both the calcium binding loops is shown (Figure 5A & B). All the five residues A47K, N50D, E52F, Q55F, and N56E which were mutated in EF-2 of NtEhCaBP1 were traced in crystal structure with good electron density. The extended conformation of NtEhCaBP1 EF-2 mutant formed a domain-swapped trimer exactly similar to native N-terminal domain structure, where three symmetry-related molecules interacted in a head-to-tail manner which leads to trimerization of N-terminal domain. EF-hand 1 of one molecule interacted with EF-hand 2 of the symmetry-related molecule to form a trimer, exactly similar to native N-terminal EhCaBP1 structure (Kumar et al., 2010).

Figure 5A & 5B Image showing the Calcium co-ordination A) Calcium co-ordination in EF-2 of native N-terminal EhCaBP1. B) Image showing the calcium co-ordination in N-terminal EhCaBP1 EF-2 mutant, almost all the co-ordination distance is shrink by ~0.15Å.
5.5.3 Comparison with native NtEhCaBP1 structure

The NtEhCaBP1 EF-II mutant structure is almost similar to native NtEhCaBP1 structure. Monomer of NtEhCaBP1 structure shows extended conformation and the two EF-hand motifs of EhCaBP1 is separated by long helix as in case of native structure. Both the structure superimposed well with r.m.s.d. is 0.69Å. The EF-2 of mutant structure shows small deviation to native structure and as the calcium coordinating distance of all coordinating residues decrease by an average distance of ~0.15Å (Figure 5A, 5B). Due to this distance shrink in the calcium binding coordination sphere helix third bend towards helix second (central helix) with the difference of 2.2 degree (Figure 6). Due to the bend in the helix-III of the mutant structure, one trimer gets close to the other trimer and forms hexamer (Figure 7). The two assembled domains of one trimer accommodated one assembled domains of other trimer and forms star like arrangement of two trimers. The critical residues which are involved in interaction are Gln36 of chain A to Ser64 and Ile65 of chain D. And Gln36 of chain D interact to Ser64 and Ile65 to chain A. In the same manner chain B interact to chain F and chain C interact to chain E, all the interacting residues are same as they interact in case of chain A and D. These interacting residues forms hydrogen bonds and weak interactions are also observed within the interacting residues.
Figure 6: Aligned image of NtEhCaBP1 native (yellow) with NtEhCaBP1 EF-2 mutant (blue): Both the structures aligned with R.M.S.D. value of 0.69 Å. All the three helices of NtEhCaBP1 EF-2 mutant aligned well with the native structure with little deviation that helix-III bend by 2.2° degree from the original position, suggest the structural change after mutation in EF-2 of NtEhCaBP1 mutant structure.
Figure 7: Overall structure of *Entamoeba histolytica* Nt-EhCaBP1EF-2 mutant at 1.9Å resolution: Image shows the formation of hexamer, in which two trimer of Nt-EhCaBP1 comes in contact to each other and leads to the formation of hexamer.
5.6 Discussion

In the pursuit of designing a higher calcium binding affinity EF-hand motif, mutations were created successfully in the second EF hand motif of N-terminal EhCaBP1. It was overexpressed in E. Coli BL21 (DE3) and the expressed protein was purified by using Ni-NTA chromatography followed by gel filtration chromatography. The gel filtration profile indicated the hexameric nature of the protein with a molecular mass of about 42 kDa, and SDS-PAGE indicated the expected molecular mass of 7.0 kDa. The mutant protein did not get crystallized in the same condition which is used for the crystallization of native NtEhCaBP1. The N-terminal EhCaBP1 mutant protein crystallized in MPD 58%- 63% sodium acetate buffer pH 5.0-5.5 with 5mM CaCl₂. The different crystallization condition indicates the structural difference with native NtEhCaBP1. Crystals of NtEhCaBP1 EF-2 mutant diffracted up to 1.9 Å resolution at ESRF DBT-BM14 France. Diffraction data were processed, indexed and scaled using HKL2000 (Otwinowski & Minor, 1997). The crystals belonged to space group P2₁2₁2₁, with unit cell parameters a= 44.6, b= 101.3, c= 107.4 Å. The Matthews volume (V_M) is 2.90 Å³Da⁻¹, indicates 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 (14) by using the native structure of EhCaBP1 (Kumar et al., 2007, PDB 2NXQ) as the search model. The structure was refined by iterative model building using COOT graphics package (15) combined Translation, Liberation and Screw-rotation (TLS) displacement parameters were determined and TLS-restrained refinement was performed (Winn et al., 2001). The final model is refined up to Rwork 22.1 % and Rfree is 26.6%. Two EF-hand motifs of NtEhCaBP1 EF-2 mutant were separated by a long helix. The extended conformation of NtEhCaBP1 EF-II mutant formed a domain swapped trimer exactly similar to native N-terminal domain structure, where three symmetry-related molecules interacted in a head-to-tail manner which leads to trimerization of N-terminal domain.

Unlike native N-terminal EhCaBP1 structure which forms trimer, mutant structure forms hexamer. The main reason behind formation of hexamer structure could be that in the NtEhCaBP1 EF-2 mutant structure the second EF-hand motif bend by 2.2 degree. This bend in the helix-III is 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 suggest that the mutant protein binding calcium with higher affinity than the native protein. This structural change in the intering helix of the mutant structure brings other trimer close and forms
hexamer. The critical residues which are involved in interaction are Gln36 of chain A to Ser64 and Ile65 of chain D. And Gln36 of chain D interact to Ser64 and Ile65 to chain A. In the same manner chain B interact to chain F and chain C interact to chain E, all the interacting residues are same as they interact in case of chain A and D. Overall structure is quite different from native structure as the native NtEhCaBP1 forms trimer and NtEhCaBP1 EF-2 mutant forms 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 proteins.