Calmodulin, a small, acidic Ca^{2+} binding protein is omnipresent in eukaryotic cells. It binds multiple partners in the presence and absence of Ca^{2+} and regulates different cellular activities like cell signalling processes, ion transport, apoptosis to name a few. But despite being extensively studied, to date there is ambiguity regarding the structure of protein in solution. While the NMR structure for apo-CaM was observed to be bilobal, crystal structure has shown four domain swapped dimers in a single unit cell. A similar confusion has persisted with Ca^{2+}-CaM, where one crystal structure has shown the protein to be dumb-bell shaped where two globular domains are separated by a 27 residue long helix linker, other structure was of a compact collapsed CaM with a bent linker. Besides Ca^{2+}, only one or two studies have shown that pH might regulate CaM, but to date, structural evidence is lacking. While individual domains have been shown to function even out of context of the intact protein, the role of the interconnecting linker has remained in doubt. Some studies have shown the linker to act only as a flexible tether for connecting the two domains while others have held it responsible for interdomain association in CaM. Taking all this information into consideration, we have tried to gain visual insight into structural changes in CaM and domain cross-talk with the linker as a function of Ca^{2+} and pH.

The surface charge distribution of CaM was observed to be altered as a function of increasing concentration of Ca^{2+} ions and lowering of buffer pH by SDS-PAGE and ELS experiments. Increase in Ca^{2+} concentration and lowering of buffer pH increased the zeta potential from -19.5 to -16.3 and -19.5 to -13.9, respectively. Circular dichroism experiments with temperature variation revealed that Ca^{2+} and pH also affected the secondary structural content of CaM, where 24% gain was observed in α-helical content as $[Ca^{2+}]/[CaM]$ ratio was increased from 0 to 32. A similar but lesser gain of 15% was observed when the buffer pH was lowered from pH 8 to 5. The increase in Ca^{2+} and lowering of buffer pH increased the stability of CaM as the melting temperature was observed to increase from 45 to 80°C and 45 to 68°C, respectively. In order to visualize whether pH induced changes were analogous to Ca^{2+}, we collected SAXS data. Structural parameters from SAXS data showed the R_g values in the range of 21.2-21.9 Å and 21.2-21.4 Å for Ca^{2+} and pH datasets, respectively. Similarly the D_{max} values were found to vary from 62-68 Å and 67-69 Å as a function of Ca^{2+} and pH, respectively. An interesting observation was the guassian chain-like nature obtained for CaM as a function of varying Ca^{2+} and pH. Comparison of measured SAXS I(Q) profiles and models restored for pH datasets with those of Ca^{2+} revealed shape changes comparable with Ca^{2+}, where the lowest pH 5 was found to be comparable to intermediate Ca^{2+} concentrations of $[Ca^{2+}]/[CaM]$~8. To compare our models under EGTA and $[Ca^{2+}]/[CaM]$~32 with

available high resolution structures, we attempted automatic superimposition of inertial axes of our SAXS based model under EGTA condition with 1CFD and that under $[Ca^{2+}]/[CaM]$~32 with 1CLL. The domain volumes and the length of interconnecting linker match well under Ca^{2+} conditions, however there is presence of extra volume in one domain of our Apo-CaM SAXS model which might reflect the local disorder observed in Apo-CaM from complementary techniques.

The uniqueness of the predominant solution shape observed for CaM was explored by ensemble optimization method where two populations of varying R_g values were obtained – one at 17-18 Å and other at 21-22 Å under varying conditions of Ca^{2+} ions. The trend prevailed for pH datasets as well and the population shape was shifted to single population of larger R_g at $[Ca^{2+}]/[CaM]$~32. Comparison of R^2 value of the SAXS data profile of intermediate datasets (Q range from 0.005 to 0.22 $Å^{-1}$) with the weighted summation of SAXS data profiles for the apo and sample containing 35 molar excess of Ca^{2+} ions relative to CaM indicated that SAXS data profile of the samples containing just 0.0008, 0.19, 1.1 and 2.9 free Ca^{2+} ions per CaM molecule could be modeled as composed of 10%, 15%, 20% and 50% of the SAXS profile of the fully activated structure, respectively . First order exponential decay pattern fitted to the estimated values suggested that the half-change occurs close to $[Ca^{2+}]/[CaM]$ value of ~3. This analysis implied that at any intermediate state, equilibrium existed between fully activated as well as some inactive structures which persisted till all Ca^{2+} binding sites were super-saturated. Results from analytical centrifuge experiments under varying conditions of Ca^{2+} and pH supported this observation where at any given $[Ca^{2+}]/[CaM]$ ratio, two populations were found with sedimentation coefficient values of 1.43 ± 0.15S and 1.83 ± 0.03S. The relative abundance of the two populations shifted to population of higher sedimentation coefficient at $[Ca^{2+}]/[CaM]$~32. A similar trend was observed with lowering of buffer pH from 8 to 5 where two populations were found with sedimentation coefficient values of 1.47 ± 0.02S and 1.78 ± 0.02S. Furthermore, using M_P and N_G peptides having antagonistic binding properties where M_P peptide binds to Ca^{2+}-CaM and N_G to Apo-CaM, it was observed that N_G binding decreased, while M_P binding increased with increase in $[Ca^{2+}]/[CaM]$ ratios. The binding trend observed for M_P and N_G with increasing Ca^{2+} concentrations continued with decrease in pH. These results correlated with our SAXS and AUC results suggesting that a mixture of Apo and $[Ca^{2+}]/[CaM]$~32 shapes existed at intermediate $[Ca^{2+}]/[CaM]$ levels. More importantly, similar pattern was seen for the populations of global shape in low pH conditions. The results also highlighted for the first time that low pH induces changes analogous to Ca^{2+} in CaM. To further demonstrate that low

pH can override the need for Ca^{2+} ions, circular dichroism data was collected for CaM samples at low pH supplemented with low Ca^{2+} ion concentrations. The thermal stability of CaM increased from 45 to 70 and 80 °C, respectively at $[Ca^{2+}]/[CaM]$ ratios of 2 and 4 at low pH 5. Structural insight from SAXS showed that the predominant solution shape of CaM at pH 5 with $[Ca^{2+}]/[CaM]\sim2$ matched that of $[Ca^{2+}]/[CaM]\sim4$ at pH 8, while the shape observed at $[Ca^{2+}]/[CaM]\sim4$ at low pH matched well with fully activated CaM observed at $[Ca^{2+}]/[CaM]\sim32$. The SAXS results were substantiated with peptide binding assays where N_G binding was reduced and M_P binding was increased at low concentration of Ca^{2+} levels following lowering of pH to 5. Overall, our studies have revealed for the first time: 1) low pH can modulate the shape-function relationship of CaM similar to Ca^{2+}, and 2) structural insight into how low pH can override the need for Ca^{2+} ions whereby both these factors act simultaneously and affect the ensemble of shapes available to CaM in solution.

To understand whether the modulation of CaM by varying Ca^{2+} and pH conditions is affected by domain cross-talk with linker, we created CaM mutants where N- and C-domains have been sequentially removed (CaM abstracted mutants) and flexible residues (^{77}KDTDS^{81}) in linker have been deleted (CaM linker mutant). Peptide binding assay with N- and C-domain abstracted mutants using M_P peptide showed that deletion of N-terminal domain inhibited binding of the peptide. While, M_P binding to C domain abstracted CaM increased from EGTA to $[Ca^{2+}]/[CaM]\sim2$, further increase in Ca^{2+} concentrations to $[Ca^{2+}]/[CaM]$ ratio of 32 led to decrease in M_P binding. The trend persisted with lowering of buffer pH from 8 to 5. *Ab initio* modeling of the SAXS data collected for domain abstracted mutants under increasing $[Ca^{2+}]/[CaM]$ ratios showed that C-domain abstracted mutant adopted an elongated shape under EGTA and $[Ca^{2+}]/[CaM]\sim2$ conditions, where a tail-like feature is present which most likely is the interconnecting linker of CaM. With increase in Ca^{2+} concentration, the protein became collapsed with disappearance of the tail-like feature. On the other hand, the N-domain abstracted mutant adopted a collapsed shape under all conditions. The global shape of domain abstracted CaM mutants as observed with increase in Ca^{2+} concentrations prevailed with lowering of pH. Peptide assay data combined with SAXS data highlighted the linker-domain interaction and importance of both domains to attain extended CaM linker conformation required for target peptides activation.

Peptide binding assay with CaM linker mutant showed that N_G binding was reduced from EGTA to $[Ca^{2+}]/[CaM]\sim32$, however the binding was significantly higher when compared to CaM at $[Ca^{2+}]/[CaM]\sim32$. A similar trend was observed when pH was reduced from 8 to 5. The M_P peptide binding, on the other hand, was significantly reduced as

$[Ca^{2+}]/[CaM]$ ratio was increased from 0 to 32, when compared with native CaM. Modeled SAXS data for CaM linker mutant under EGTA, intermediate and saturating Ca^{2+} concentrations and low pH showed bilobal shape for CaM, but conformation achieved by the mutant differed from native CaM which demonstrated the importance of interconnecting CaM linker to achieve the conformation attained by CaM as a function of Ca^{2+} and pH for efficient target binding.

Overall, this thesis has provided a holistic view of shape function relationship of CaM as a function of Ca^{2+} and demonstrated for the first time that low pH modulates shape of CaM and is able to override the need for Ca^{2+} ions. Moreover, we have demonstrated the importance of interdomain connecting linker in CaM for the conformation achieved by CaM in solution and target peptide binding.