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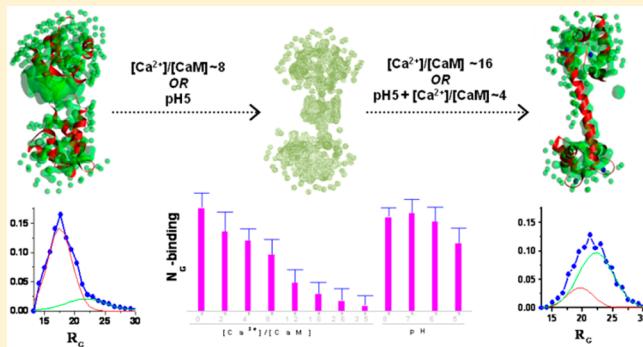
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Low pH Overrides the Need of Calcium Ions for the Shape–Function Relationship of Calmodulin: Resolving Prevailing Debates

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ABSTRACT: Calmodulin (CaM) regulates numerous cellular functions by sensing Ca^{2+} levels inside cells. Although its structure as a function of the Ca^{2+} -bound state remains hotly debated, no report is available on how pH independently or in interaction with Ca^{2+} ions regulates shape and function of CaM. From SAXS data analysis of CaM at different levels of Ca^{2+} -ion concentration and buffer pH, we found that (1) CaM molecules possess a Gaussian-chain-like shape in solution even in the presence of Ca^{2+} ion or at low pH, (2) the global shape of apo CaM is very similar to its NMR structure rather than the crystal structures, (3) about 16 Ca^{2+} ions or more are required per CaM molecule in solution to achieve the four- Ca^{2+} -bound crystal structure, (4) low pH alone can impart shape changes in CaM similar to Ca^{2+} ions, and (5) at different $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio or pH values, the predominant shape of CaM is essentially a weighted average of its apo and fully activated shape. Results were further substantiated by analysis of sedimentation coefficient values from analytical ultracentrifugation and peptide binding assays using two peptides, each known to preferentially bind the apo or the Ca^{2+} -activated state.



INTRODUCTION

Calmodulin (CaM) is a small, ubiquitously found, Ca^{2+} -binding protein. It is known to sense free Ca^{2+} levels to regulate a wide variety of cellular activities ranging from gene expression, signal transmission, ion transport, learning, and memory formation to neuronal plasticity.¹ To relay the changes in the Ca^{2+} levels, apo as well as partially and fully activated CaM have been reported to bind numerous peptides/proteins to affect specific signaling pathways.² In the literature, CaM has been implicated in having a seemingly promiscuous behavior, but one can suggest that the absence of complete understanding of how its global shape varies as a function of free Ca^{2+} ions or other factors in its environment limits our understanding or creates contradictory interpretations.^{2b} Prime support for the dexterous nature of its structure in Ca^{2+} -free state comes from the variance among the 25 models solved within the constraints obtained from NMR spectroscopy (PDB ID: 1CFC)³ and the presence of up to eight structures in the same unit cell (PDB ID: 1QX5).⁴ Importantly, in both structures, the linker connecting the two N- and C-terminal of Ca^{2+} -binding domains was found to be unstructured. Apart from this similarity, the structures of the apo-CaM differ significantly because the one solved within NMR constraints showed a more bilobal shape with D_{\max} (maximum linear dimension) close to 68 Å, whereas the structures seen in crystalline state adopted a collapsed globular shape with D_{\max} about 54 Å. For Ca^{2+} -activated CaM, only the structure of fully activated CaM bound to four Ca^{2+} ions has been resolved from X-ray crystallography (PDB IDs: 1CLL,

3CLN).⁵ Apart from minor differences among the 4 Ca^{2+} -CaM structures, all the structures have a common dumbbell-like shape, where the Ca^{2+} -binding domains are widely spaced as the central linker adopted a rigid α -helical structure. Questioning the structure perceived from crystallography, circular dichroism (CD) experiments interpreted a far less helical content in the Ca^{2+} -activated structure.⁶ It was argued that the long continuous helix (across residues 66–92) is not a feature in the activated structure in solution, rather an artifact of crystallization conditions, because helix promoting fluorinated solvents were used in the crystallization buffer. This notion was also upheld when the helical content was substantially increased in the CD data when trifluoroethanol was added in the buffer.⁷ The ambiguity about the linker's structure in Ca^{2+} -bound CaM got further compounded from time-resolved tyrosine fluorescence anisotropy studies which inferred that isotropic rotational motion which reflects inherent flexibility in the CaM molecules does not change significantly upon Ca^{2+} binding.⁶

Alongside NMR and X-ray crystallography high-resolution information, small-angle X-ray scattering (SAXS) data were also used to interpret the global shape of CaM in buffer devoid of and containing Ca^{2+} ions.⁸ In a study using bovine brain CaM and its mutants, SAXS data analysis suggested that the R_G

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(radius of gyration) and D_{\max} (maximum linear dimension) of molecules increased from 20.6 to 21.5 Å and 58 to 62 Å, respectively, upon increasing the $[Ca^{2+}]/[CaM]$ ratio from 2.3 to 10.3.^{8a} Similarly, dynamic light scattering (DLS) experiments deduced the hydrodynamic radius (R_h) of 25 and 30 Å for the apo and Ca^{2+} -activated compounds ($[Ca^{2+}]/[CaM]$ ratio ~10), respectively.⁹ Though the light and low angle X-ray scattering values agreed well for the activated form, there was a discrepancy between the results obtained for the apo-form which was attributed to the limitation of the light scattering technique. Another study involving SAXS data analysis of some deletion mutants of CaM affirmed that the R_g and D_{\max} changed from 19.4 to 20 Å and 58 to 62 Å, respectively.^{8b} Interestingly, this paper concluded that the central helical linker remains stable or rigid enough despite the deletions in that portion, yet it is flexible enough to permit interaction in the terminal lobes to affect collapse in structure upon peptide binding. In close agreement, SAXS and SANS studies on bovine brain CaM concluded that the R_g and D_{\max} of this protein was about 19.6 and 59 Å and 21.3 and 63 Å, respectively, under Ca^{2+} -free and Ca^{2+} -containing buffers ($[Ca^{2+}]/[CaM]$ ~17–28).^{8c} But all these experimental studies were limited to analyzing mainly the apo and the fully activated forms, which provided little (or no) information on the intermediate structures. In physiology, it would be the intermediate shapes/structures that regulate the biology of this protein and an incomplete understanding has concluded a promiscuous nature of CaM toward its ligands. In the absence of any structural information about partially activated CaM, it is difficult to rule out that CaM molecules actually possess Ca^{2+} -concentration-dependent unique global structure(s) in solution which in turn could be ligand specific.

Although Ca^{2+} ions dominate in influencing the global shape of CaM and other calcium-binding proteins, slight changes in the environmental pH have been shown to drastically alter the effect of Ca^{2+} ions (sometimes even overriding their need).¹⁰ Though there are several studies related to Ca^{2+} activation or Ca^{2+} -induced structural changes of CaM, no direct structural information is available for low pH activation of CaM. An NMR-based analysis of binding of Ca^{2+} ions to CaM at low pH suggested that H^+ ions can compete with Ca^{2+} ions for the low affinity sites in CaM, supporting the notion that buffer pH can modulate activities of CaM.¹¹ We feel that the ubiquitous presence of CaM and its role in apoptosis and ion-based signaling supports that variation in pH should modulate the functional behavior of CaM. To understand global shapes of CaM under varying Ca^{2+} and pH conditions, we carried out different biophysical and biochemical experiments. Briefly, we tried to address (1) if the global shape of apo-CaM is a collapsed globular one like its crystal structure or an extended bilobal similar to its NMR structure, (2) whether the extended dumbbell shape of fully activated CaM seen from crystallography can be actually achieved in solution, as denied earlier, (3) whether the large degree of conformational changes associated with Ca^{2+} activation of CaM occur via partially activated unique intermediates, and (4) whether pH alone can bring about changes similar to Ca^{2+} ions or these two factors act in synchronization?

MATERIALS AND METHODS

Protein Expression and Purification. CaM gene inserted in the pET303 vector was amplified in DH5α and overexpressed in BL21-DE3 by 1 mM IPTG induction (LB Medium;

Merck). The cells containing overexpressed protein were harvested by centrifugation at 6000 rpm for 15 min at 4 °C. The harvested cells were subjected to sonication in 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.2 mM PMSF, and 1 mM DTT conditions followed by centrifugation at 8000 rpm at 4 °C for 45 min. The supernatant obtained after sonication was loaded (with a final $CaCl_2$ concentration of 5 mM) onto activated phenyl Sepharose beads (GE Life Sciences).¹² The column was washed with two column volumes of a buffer composed of 50 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, and 1 mM DTT, followed by another column volume of the same buffer supplemented with 500 mM NaCl. Finally, CaM was eluted with 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 1 mM DTT buffer. Size-exclusion chromatography using Sephadex 200 column attached to AKTA Explorer FPLC system was used for final purification (GE Life Sciences). Presence and purity of the samples were checked by SDS-PAGE (15% resolving gel) and concentration of the pure samples was determined by UV-vis spectrophotometer using $A_{280(1\text{ mg/mL})} = 0.174$. Observed mass of 16.7 kDa for the purified fraction of CaM confirmed its identity (Voyager 4402, Applied Biosystems). Whenever required, purified CaM was concentrated by filtration through a membrane with a 10 kDa molecular mass cutoff (Amicon-Millipore).

Electrophoretic Light Scattering. All experiments were carried out using Beckman Coulter DelsaNanoC instrument having a ζ potential cell. The cell was filled with 10 mg/mL of CaM protein in buffer containing varying free Ca^{2+} ions and pH. For each sample/experiment, diffusion coefficient values were calculated at nine different points in cell to compute the curvature of movement of molecules as a function of voltage applied. All data were analyzed using DelsaNano software to compute the ζ potential value of the protein.

Circular Dichroism Experiments. CD spectra of CaM in buffer varying in free Ca^{2+} -ion concentration and having different pH values were acquired using JASCO CD spectrophotometer (J-815) and quartz cuvette of path length 2 mm. Data were recorded three times, with a scan rate of 10 nm/min, in the range 195–250 nm while varying the sample temperature from 25 to 90 °C. For CD experiments, 10 mM Tris buffer pH 7.5 was used for Ca^{2+} titrations. For pH 8 experiments, we used 10 mM Tris, for pH 7 and 6, 10 mM sodium phosphate was used, and 10 mM sodium acetate was used for pH 5. All the buffers used contained 1 mM EGTA. Baseline spectra were subtracted from sample spectra and the resulting values were averaged for each data set. Mean residue ellipticity (MRE), θ , estimated from spectra as a function of wavelength was plotted using standard software with JASCO instrument.

SWAXS Data Acquisition. Starting with a stock solution of 25 mg/mL, final concentrations of proteins used during SWAXS experiments were 10 mg/mL. For Ca^{2+} -titration experiments, varying amounts of $CaCl_2$ were added from a 50 mM stock solution. The free Ca^{2+} levels were estimated by using web-based software WEBMAXCLITE v1.15. For pH-based experiments, dialysis was done against buffers of pH 8 (50 mM Tris, 1 mM EGTA), 7 (50 mM Sodium Phosphate, 1 mM EGTA), 6 (50 mM Sodium Phosphate, 1 mM EGTA), and 5 (50 mM Sodium Acetate, 1 mM EGTA) at 4 °C. All samples of CaM containing incremental amounts of $CaCl_2$, different pH values and their matching buffers were prepared about 45 min before scattering experiments. Additionally, for estimation of beam intensity at zero angles, a dilution series of

Hen Egg White Lysozyme dissolved in and dialyzed against 40 mM sodium acetate buffer (pH 3.8) having 150 mM NaCl, purified by FPLC and concentrated using membrane concentrators was used. X-ray scattering data was collected at beamline X9 at National Synchrotron Light Source (Brookhaven National Laboratory). Systematically positioned two charge coupled detectors (CCDs) collected scattering data simultaneously at smaller (SAXS) and wider (WAXS) angles. The images recorded on two CCDs from protein solutions were circularly averaged and merged to obtain a composite SWAXS profile. Using programs written by Dr. Lin Yang (Brookhaven National Lab), the SAXS and WAXS data were merged over a Q range of $0.08\text{--}0.12 \text{ \AA}^{-1}$. About $70 \mu\text{L}$ of samples and their matched buffer were exposed for 120 s in quartz flow cell at 15°C with a flow rate of $30 \mu\text{L}/\text{min}$. All the SWAXS experiments were carried out in duplicate. For each sample, the contribution from buffer was subtracted to obtain the scattering intensity (I) from the protein sample as a function of momentum transfer vector, Q ($Q = [4\pi \sin \theta]/\lambda$), where λ is the beam wavelength and θ is the scattering angle. SAXS data on lysozyme concentration series were also collected under identical conditions. Similarity in the migration pattern in SDS-PAGE of CaM samples collected after exposure to X-rays and protein that was never exposed to radiation confirmed that CaM did not suffer radiation induced damage or during travel.

SWAXS Data Analysis. Kratky plots [$I(Q) \times Q^2$ vs Q] of each data set were prepared to examine the globular or Gaussian-chain-like nature of the protein in solution.¹³ The Guinier approximation was employed to estimate the R_G of the scattering particle. As per this approximation, for a monodisperse sample of globular protein, a plot of $\ln(I(Q))$ versus Q^2 , where $Q \times R_G \leq 1.3$, should be linear and fit into the following equation (1):

$$\ln[I(Q)] = \ln[I_0] - (R_G^2/3) \times Q^2 \quad (1)$$

I_0 , defined as the intensity of scattering at zero angles, is directly proportional to the product of molar concentration and molecular mass of the scattering sample, and can be approximated by extrapolating SAXS data to $Q \sim 0$. R_G is defined as the root-mean-square of all elemental volumes from the center-of-mass of the particle, weighted by their scattering densities, and is characteristic of the overall shape of the molecule. For this study, Guinier analysis was performed using the primus software package.¹⁴ Using GNOM45 software,¹⁵ indirect Fourier transformation of the scattering data over the measured Q range computed a pairwise distribution function of interatomic vectors, $P(r)$ (eq 2).

$$P(r) = (1/2\pi) \int I(Q) Q \times r \sin(Q \times r) dQ \quad (2)$$

$P(r)$ is a histogram of the frequency of vector lengths connecting small volume elements within the entire volume of the scattering particle. During indirect Fourier transformation, $P(r)$ was considered to be zero for vector lengths equal to 0 and D_{\max} . The analysis also provided R_G and I_0 from the second moment and the start of $P(r)$, respectively. CRYSTAL26 program was used to compare our experimental SWAXS profiles with the theoretical scattering profiles computed using structures resolved by NMR and X-ray diffraction.¹⁶

Structure Reconstruction with SWAXS Profiles. To visualize the Ca^{2+} -binding and pH-induced changes in the

global structure of the CaM, low resolution models of CaM were reconstructed within the shape constraints computed during $P(r)$ analysis using GASBOR22IQ software.¹⁷ Structures were restored for each data set considering no shape and symmetry bias using 150 dummy residues. A Fibonacci grid order of 13 (378 water molecules) was used to model the hydration layer around the chain ensemble. Using SUPCOMB20 software, the inertial axes of the resultant low resolution shapes for CaM and known structures from NMR and X-ray diffraction were superimposed.¹⁸

Ensemble Optimization Method (EOM). To probe the variation in conformation with increasing amounts of free Ca^{2+} ions as well as different pHs, we performed EOM¹⁹ using SAXS data as a reference. For ensemble optimization, the scattering object was considered as an ensemble of N conformations and the scattering, $I(Q)$, for the ensemble was computed by averaging the contribution from each conformation within the ensemble, where $I_n(Q)$ is the scattering intensity profile from nth conformer of the subset and n varies from 1 to N (eq 3).

$$I(Q) = (1/N) \sum I_n(Q) \quad (3)$$

To find a possible ensemble, representative pool of a large number of random conformers covering entire conformational space was generated. A genetic algorithm was further employed to select the subset of conformations which best fits the experimental data. Weighted estimation of conformations within a shape helped in filtering an ensemble of conformations possible for the scattering species. For our analysis, we generated a random pool of 5000 structures for each SAXS $I(Q)$ data set.

Analytical Ultracentrifuge (AUC). All AUC experiments were carried out in a Beckman-Coulter ProteomeLab XL-I protein characterization system using an An-50Ti rotor (Beckman-Coulter) at a speed of 40 000 rpm. Briefly, $420 \mu\text{L}$ of buffer and $400 \mu\text{L}$ of sample were placed in dual-sectored quartz cells with charcoal-filled Epon centerpieces for data collection. All the data sets were collected in absorbance mode at 280 nm using a step size of 0.003 cm without averaging. For data collection, the cells were radially scanned every 2 min. For data analysis and estimation of sedimentation coefficient ($s_{20,w}$; sedimentation coefficient corrected with reference to water at 20°C), 2-dimensional spectrum (2DSA), genetic algorithm (GA), and Monte Carlo (MC) analyses incorporated in Ultrascan III software (www.ultrascan.uthscsa.edu) was employed.²⁰ During data analysis, the time invariant as well as radial invariant noise correction was done in 2DSA and the corrected data was further used for Monte Carlo analysis which provided population distribution of the species in solution. Time derivative or dc/dt ²¹ and van Holde–Weischet analyses were done using Ultrascan software package to calculate sedimentation coefficient distributions, and the values were corrected using values for water at 20°C . The processed data was further corrected to remove contribution coming from diffusion to boundary shape using enhanced van Holde–Weischet analysis.²²

Peptide Synthesis, Purification, and Labeling. Peptides (M_p and N_G) known to interact with CaM in the presence and absence of Ca^{2+} ions, respectively were synthesized employing 9-fluorenylmethoxycarbonyl (F-moc)-chemistry-based solid phase peptide synthesis.²³ The peptides were labeled with Texas red dye then purified using reversed phase high performance liquid

chromatography (RP-HPLC; Waters HPLC system attached with C18 column) and lyophilized for further use. The purity (>97%) and mass was verified by matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis (Voyager 4402).

Peptide-Based Assay. For peptide-based assay, 1.5 μ g of CaM (under EGTA as well as at different $[Ca^{2+}]/[CaM]$ conditions) was incubated overnight at 4 °C onto ELISA plates (Nunc F96 Microwell Maxisorp). For coating 0.1 M NaHCO₃ buffer, pH 8.8 was used and to obtain different Ca²⁺ concentrations required amounts of CaCl₂ was added. Coating of CaM at different buffers was done using 0.1 M sodium bicarbonate (pH 8), 0.1 M sodium phosphate (pH 7 and 6), and 0.1 M sodium acetate (pH 5). After coating, all the wells were washed three times with 1X PBS followed by addition of protein free blocking buffer (Pierce) for 1 h at room temperature. Subsequently, the wells were washed twice with PBST (1X PBS + 0.05% Tween-20) and then three times with 1X PBS. Texas red labeled peptides M_p (in 100 mM Tris-HCl; pH 7.6, 100 mM NaCl) and N_G (in 20 mM HEPES; pH 8, 100 mM NaCl) were added (50 times excess to CaM) to respective wells and incubated at room temperature for 1 h. M_p was incubated in 1.5 mM CaCl₂ whereas N_G was incubated in EGTA. After incubation, the wells were washed twice with 1X PBS and fluorescence was measured on automated 96-well infinite M200 Pro plate reader (Tecan, Crailsheim, Germany) using excitation and emission parameters of 596 and 622 nm, respectively.

CaM Concentrations Used for Different Experiments. We used the following CaM concentrations: SDS-PAGE, 1.6 mg/mL; ELS, 10 mg/mL; CD experiments (wavelength and temperature scan), 0.17 mg/mL (10 μ M). Final concentration of CaM: all SAXS experiments, 10 mg/mL; AUC, 4.4 mg/mL; peptide-binding assay, 0.012 mg/mL.

RESULTS AND DISCUSSION

Ca²⁺ Ions or Low pH Alters Surface Charge Distribution of CaM. It is known that migration of CaM protein under Laemmli conditions or in SDS-PAGE conditions is dependent on Ca²⁺ levels in the running buffer.^{11,24} In the absence of Ca²⁺ ions, CaM has been shown to migrate to apparent high molecular mass and this anomalous behavior is indicative of changes in the shape and/or surface properties of CaM when it binds to Ca²⁺ ions. We also found that CaM migration was increased with increase in $[Ca^{2+}]/[CaM]$ ratios (Figure 1A). Interestingly, lowering the pH to 6 and 5 also induced a similar change in CaM's migration pattern suggesting that low pH can also induce changes in CaM analogous to Ca²⁺ ions. To check whether this effect also happens in solution (and not just under SDS-PAGE conditions), we carried out ELS experiments and computed the ζ potential of the protein under varying free Ca²⁺ ions and pH of buffer (Table 1). Experiments revealed that the ζ potential of CaM molecules or charge distribution of CaM molecules changes when Ca²⁺ ions are added or when the pH levels of the solutions are lowered (Figure 1B). A less negative ζ potential value with an increase in free Ca²⁺ ions or an increase in H₃O⁺ ions (decrease in pH) indicated that the surface charge of CaM becomes less negative with binding to Ca²⁺ ions or partial neutralization of negatively charged residues. Whereas an increase in $[Ca^{2+}]/[CaM]$ from 0 to 16 increased the ζ potential from -19.5 to -16.3, a decrease of buffer pH under Ca²⁺ ions free conditions from 8 to 5 increased the ζ potential from -19.5 to -13.9. Results from

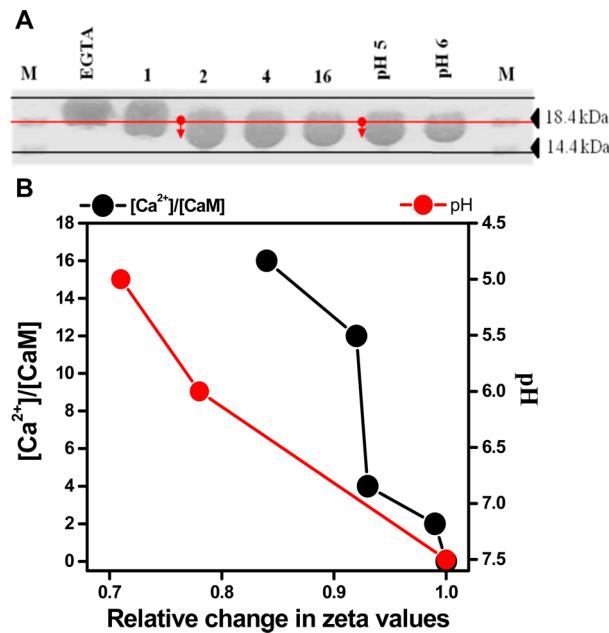


Figure 1. (A) Changes in the migration profile of purified CaM as a function of $[Ca^{2+}]/[CaM]$ ratios and buffer pH. The migration of molecular mass standards of 18.4 kDa is shown with a red line. The small red arrows highlight the shift in migration of CaM molecules to lower mass. (B) Changes in the ζ potential values of purified CaM molecules as a function of Ca²⁺ ions and buffer pH are plotted here.

Table 1. ζ Potential Analysis for CaM as a Function of Varying Ca²⁺ Ion Levels and Buffer pH^a

sample	ζ potential (mV)	mobility (cm ² /(V s))	Z_{eff}	rel change in Z_{eff}
$[Ca^{2+}]/[CaM]$ pH 7.4				
EGTA	-32.43	-2.38×10^{-4}	-19.46	reference
2:1	-30.28	-2.21×10^{-4}	-18.17	0.93
4:1	-30.11	-2.18×10^{-4}	-18.07	0.93
12:1	-29.71	-2.06×10^{-4}	-17.83	0.92
16:1	-27.18	-2.00×10^{-4}	-16.31	0.84
EGTA-CaM				
pH6	-25.32	-1.93×10^{-4}	-15.19	0.78
pH5	-23.13	-1.70×10^{-4}	-13.88	0.71

^aFinal concentration of CaM in all electrophoretic light scattering (ELS) experiments was 10 mg/mL.

our SDS-PAGE and ELS experiments provided first conclusive cue that low pH can also bring about changes in surface properties of CaM similar to those induced by Ca²⁺ ions.

CD Studies: Variation in Secondary Structural Content as a Function of Ca²⁺ and pH. To understand the changes in the secondary structural content in CaM molecules as a function of Ca²⁺ ions and buffer pH, we performed far-UV CD experiments including temperature variation. In correlation to previous reports, we also observed two negative peaks for apo as well as Ca²⁺-CaM.²⁵ Earlier CD studies carried out at $[Ca^{2+}]/[CaM]$ ratios of 0 and 40 concluded that the decrease in MRE value was due to either an increase in α -helical content by about 20% or reorientation of the existing ones, in comparison with NMR and X-ray diffraction data.^{25a,26} Actually, an NMR data interpretation upheld the possibility of reorientation, where the apo protein showed similar overall helical content as seen in the crystal structure of the Ca²⁺-bound CaM (PDB ID: 1CLL).²⁶ Studying a more broader

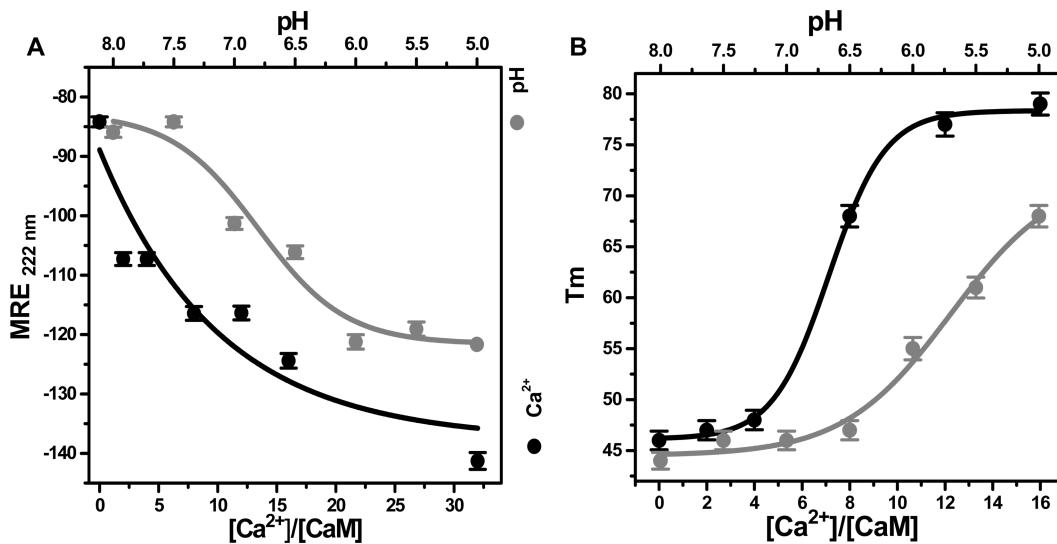


Figure 2. (A) Decrease in the mean residual ellipticity (MRE) of CaM molecules as a function of increase in Ca^{2+} ion concentration (black circle) and lowering of buffer pH (gray circle). (B) Increase in the T_m values of CaM values as a function of increasing amounts of Ca^{2+} (black circle) and lowering of pH (gray circle).

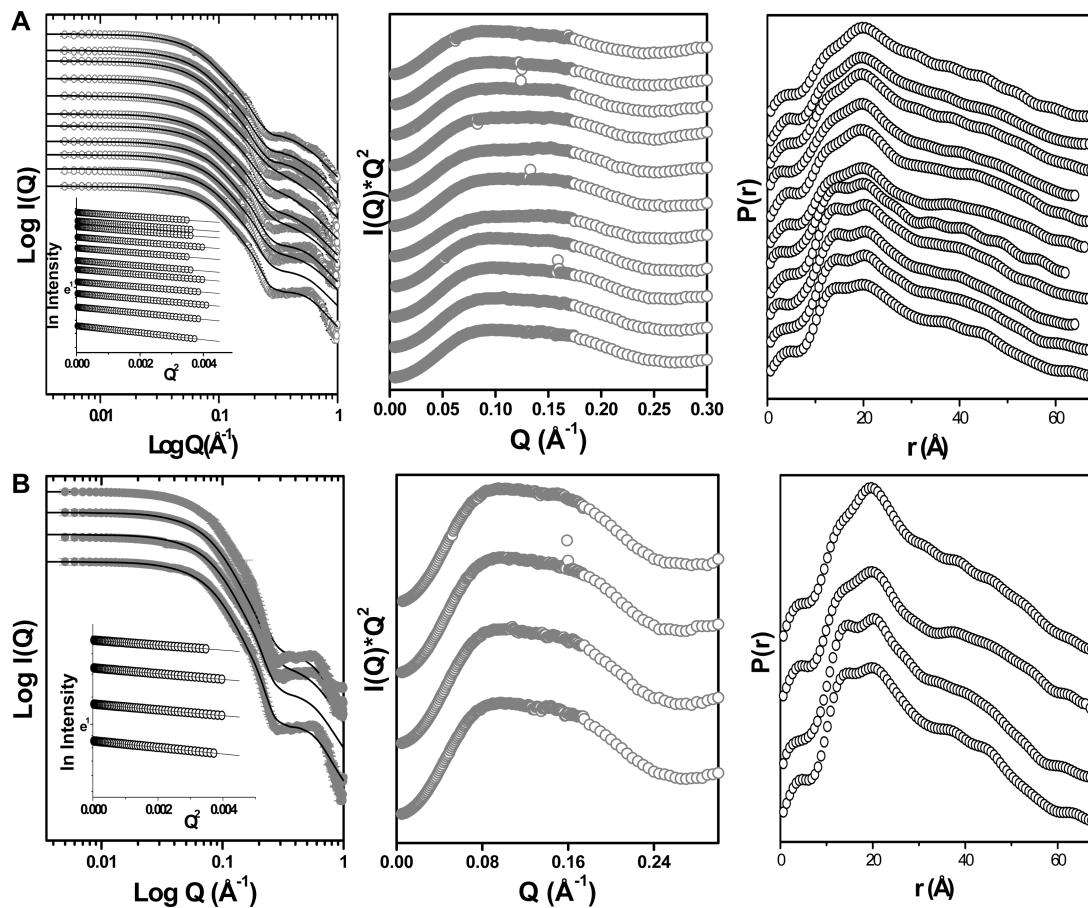


Figure 3. SWAXS data and analysis of scattering intensity profiles acquired from CaM solutions having varying levels of Ca^{2+} ions (A) and pH (B). The left panel shows SWAXS $I(Q)$ profiles acquired from samples of CaM. The regularized scattering profiles (shown as black lines) are superimposed over the acquired experimental data. The inset shows the Guinier regions of the data sets. Middle panels show the Kratky plots, which highlight the Gaussian-chain-like profile of CaM molecules in solution. The right panels show respective $P(r)$ curves computed for the CaM molecules in different samples. For all the three stacked figures, the $[\text{Ca}^{2+}]/[\text{CaM}]$ values are 0, 0.0008, 0.19, 1.1, 2.9, 5.2, 7.6, 12.2, 16.8, 26.1, and 35.4 from bottom to top and pH values are 8, 7, 6, and 5 under EGTA conditions from bottom to top.

range of Ca^{2+} ion concentration in buffer, we repeated the CD studies with CaM while varying the $[\text{Ca}^{2+}]/[\text{CaM}]$ from 0 to

32 and plotted the systematic decrease in the MRE values observed at 222 nm (Figure 2A). Progressive decrease in MRE

Table 2. Computed Structural Parameters and Extrapolated I_0 Values by Performing Indirect Fourier Transformation of the SWAXS Data Collected from Samples of CaM Containing Increasing Amounts of Free Ca^{2+} Ions and at Low pH

	$[\text{Ca}^{2+}]/[\text{CaM}]$	D_{\max} (Å)	R_G (Å)	I_0	I_0/c
pCa					
EGTA	0	67	21.2 ± 0.07	143.8 ± 0.7	13.6
6.3	8×10^{-4}	64	21.1 ± 0.07	147.1 ± 0.7	13.9
3.9	1.9×10^{-1}	67	20.4 ± 0.05	139.4 ± 0.5	13.2
3.1	1.1	62	21.1 ± 0.07	145.7 ± 0.7	13.8
2.7	2.9	66	20.7 ± 0.04	134.2 ± 0.4	12.7
2.4	5.2	67	21.8 ± 0.06	139.2 ± 0.6	13.2
2.3	7.6	64	21.9 ± 0.06	143.9 ± 0.6	13.6
2.1	12.2	64	20.8 ± 0.04	134.6 ± 0.4	12.7
1.9	16.8	68	21.7 ± 0.07	147.1 ± 0.8	13.9
1.8	26.1	66	21.8 ± 0.05	140.3 ± 0.5	13.3
1.6	35.4	68	21.9 ± 0.12	141.5 ± 0.1	13.4
pH					
pH 8	0	68	21.1 ± 0.05	142.2 ± 0.3	12.9
pH 7	0	67	20.8 ± 0.06	140.6 ± 0.1	13.1
pH 6	0	67	21.3 ± 0.03	141.6 ± 0.5	13.2
pH 5	0	69	21.4 ± 0.02	139.9 ± 0.7	13.8

value supports a gain in the α -helical content by about 24% in the CaM molecules with increase in Ca^{2+} ions. This increase is definitely not a mere redistribution as concluded from the NMR study of the apo CaM, rather a clear gain of secondary structural content. It is important to highlight here that the increase in α -helical content beyond a $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio of 4 indicated additional shape changes were induced by excess Ca^{2+} ions in buffer than the four that are picked up by Ca^{2+} -binding sites in CaM molecule. Interestingly, no previous study has reported a variation in secondary structural content in CaM as a function of buffer pH. As seen with increasing Ca^{2+} ions, we observed a sequential decrement in MRE values at 222 nm on lowering down of pH in EGTA buffer or Ca^{2+} -free conditions (Figure 2B). There was a clear gain in the α -helical content by about 15% from pH 8–5, which implied that pH can also modulate the structural rearrangements in CaM.

We also evaluated change in the melting temperature (T_m) of CaM by carrying out CD experiments at different temperatures

with differential amounts of Ca^{2+} ions in buffer and lowering the buffer pH. Results revealed that T_m of CaM increases from 45 to 80 °C as the $[\text{Ca}^{2+}]/[\text{CaM}]$ increased from 0 to 16 at pH 7.5 clearly supporting that Ca^{2+} binding and the presence of excess Ca^{2+} ions increase overall stability of the protein. This observation has been reported earlier that Ca^{2+} ions impart stability to CaM by increasing T_m from 50 to 90 °C.^{25b} The nominal differences in our results are probably due to use of higher concentration of protein and/or consideration of three-state unfolding model by the previous authors vs employment of two-state model of unfolding and lower concentration of CaM in our CD experiments. Somewhat unexpectedly, we observed an increase in the T_m value of CaM from 45 to 68 °C as the buffer pH was decreased from 8 to 5 in the absence of Ca^{2+} ions (Figure 2B). This increase in T_m value corroborated with a partial increase in α -helical content or an increase in structural stability in the CaM molecules upon sensing low pH their environment. Results from SDS-PAGE, ELS, and CD experiments concluded that low pH can induce changes in surface charge distribution and secondary structural content of CaM molecules analogous to those induced by Ca^{2+} ions. These observations questioned if the low pH induces global shape rearrangements like Ca^{2+} ions at pH 8 or if the rearrangements represent an alternative pathway for structural transition?

SWAXS Data from CaM Molecules in the Presence of Varying Ca^{2+} and pH. Measured SAXS $I(Q)$ profiles from samples of CaM containing incremental amounts of free Ca^{2+} ions and at different pH values are presented in Figure 3A,B, left panels. Linearity of the intensity data as a function of Q^2 in the Guinier region confirmed the lack of aggregation or interparticulate effect in our samples under EGTA conditions, in the presence of Ca^{2+} and at low pH (Figure 3A,B, insets in left panels).²⁷ Aware of earlier published results that showed that CaM can aggregate under synchrotron conditions,^{8d} we acquired data using flow-cell where protein molecules interacted with passing wavefront of X-rays only once. The slope of the Guinier approximation considering globular nature of scattering particles suggested that R_G of the molecules was around 21.2 Å under EGTA conditions at pH 8 and it modestly increased to 21.9 Å in samples containing almost 35-fold molar excess of free Ca^{2+} ions at pH 8, whereas it was 21.4 Å at pH 5 with no free Ca^{2+} ions in buffer. Interestingly, Kratky analyses of the data sets revealed that CaM molecules adopt a Gaussian-

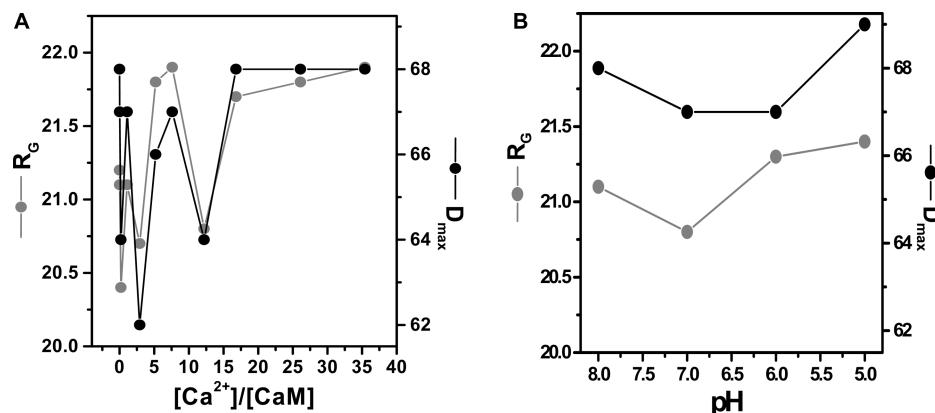


Figure 4. Fluctuation in the R_G and D_{\max} values as computed from the indirect Fourier transformation of the SAXS data for the predominant shape adopted by the CaM molecules as a function of varying levels of Ca^{2+} ions in buffer (A) and in buffers with lower pH (B). Gray and black circles denote the R_G and D_{\max} values with units in Å.

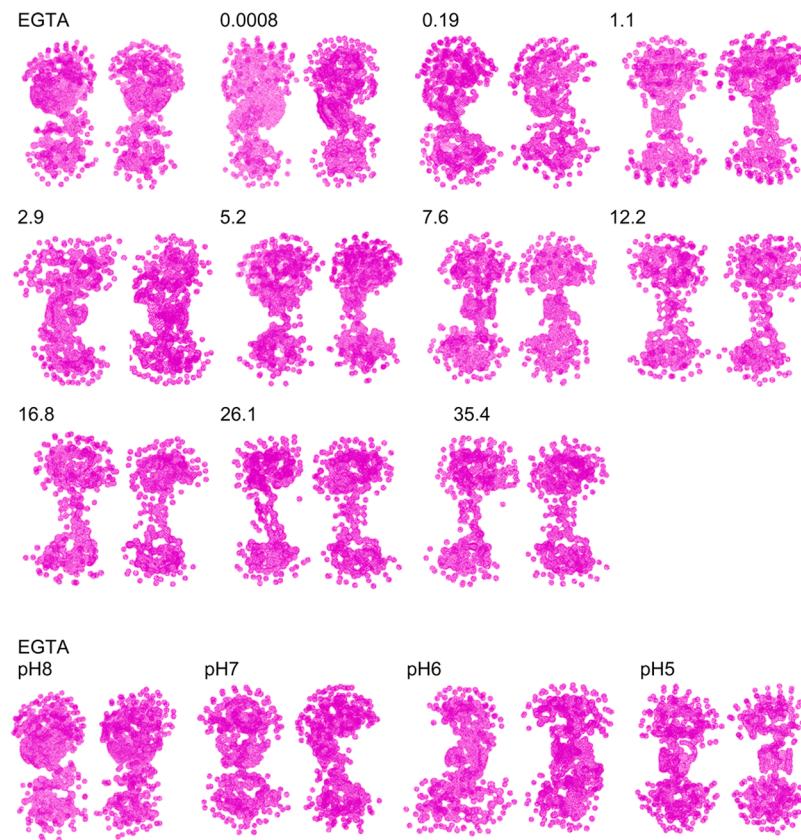


Figure 5. Ab initio models in space filled mode generated using the chain-ensemble modeling approach to visualize the predominant scattering shape of CaM in buffers containing varying levels of Ca^{2+} ions and at different pH. Two views of each model rotated along the long axis are shown here. The estimated value of $[\text{Ca}^{2+}]/[\text{CaM}]$ and pH are mentioned on the top left side of each set.

chain-like profile in solution both under Ca^{2+} -free conditions and in buffer containing increasing levels of free Ca^{2+} ions and under low pH conditions (Figure 3A,B, middle panels). Hyperbolic nature of the $I(Q) \times Q^2$ vs Q plots confirmed that there exists a high order of inherent disorder in the CaM molecules. In other words, a range of conformations similar in global structural features like D_{\max} and R_G values, but lacking similarities at smaller dimensions like secondary structural content or positioning of loops, are adapted by such proteins in solution. Importantly, the observed flexible feature was present in the molecules not only under Ca^{2+} -free conditions but also in samples containing Ca^{2+} ions much above the chelating capacity of the protein, as well as in the pH range of 8–5. Interestingly, results from our Kratky plots explain why so much disagreement exists between interpretation about structure of CaM from X-ray crystallography, NMR, CD, time-resolved fluorescence, and prediction studies,^{6,7,24b,28} and a wide array of activities performed by this protein in apo as well as Ca^{2+} -bound form have been implicated in the presence of a high order of inherent disorder encoded in this protein.^{2b}

Considering monodisperse nature, we performed indirect Fourier transformation of the scattering data sets over a wider Q range (0.005 – 1.0 \AA^{-1}) to obtain the histogram of interatomic vectors constituting the predominant scattering shape of CaM under different conditions. The modeled/regularized scattering intensity during indirect Fourier transformations were superimposed over the measured SWAXS data for comparison (Figure 3A,B, left panels). Good solutions were obtained for data sets with D_{\max} in the range 62 – 68 \AA for Ca^{2+} titrations and 67 – 69 \AA for pH data sets (Figure 3A,B, right

panels). Interestingly, upon using SWAXS data for $P(r)$ curve analysis, two peak profile of the computed $P(r)$ curves for samples having $[\text{Ca}^{2+}]/[\text{CaM}] < 4$ and, at pH 8 to 6 supported that prior to complete activation, a bilobal shape is preferred by CaM molecules in solution. The calculated molecular D_{\max} , R_G , and I_0 values from $P(r)$ analyses have been compiled in Table 2 and presented in Figure 4. Interestingly, in our experiments, calculated R_G and D_{\max} values remained close to 21.2 and 67 \AA , respectively, under Ca^{2+} -free conditions at pH 8, and these values then “wobbled up and down” until the $[\text{Ca}^{2+}]/[\text{CaM}]$ ratios were past 16 , suggesting that the ensemble of conformations accessible to CaM in solution was continually changing until it shifted in favor of a shape which is characterized by a D_{\max} and R_G of 68 and $21.5 \pm 0.4 \text{ \AA}$, respectively. It must be noted that ours is the first SAXS tracking of shape of CaM molecules as Ca^{2+} ions were increasing systematically. Additionally, we found that pH also affected the global shape of CaM by slightly changing D_{\max} and R_G values from 67 to 69 \AA and 20.8 to 21.4 \AA , respectively. Overall, in correlation with the results seen from CD studies, similarities in the trend by which the global shape of the CaM molecules changed as a function of varying Ca^{2+} ions and pH implied that low pH can induce shape changes in calcium binding protein, CaM similar to Ca^{2+} ions.

Our observations clearly place a disagreement with previous SAXS/SANS observations that reported a clear increase in R_G and D_{\max} of CaM as a function of Ca^{2+} ions: (1) from 20.6 to 21.5 \AA and 58 to 62 \AA , respectively,^{8a} (2) from 19.4 to 20 \AA and 58 to 62 \AA , respectively,^{8b} and (3) from 19.6 to 21.3 \AA and 59 to 63 \AA .^{8c} Of course, the $[\text{Ca}^{2+}]/[\text{CaM}]$ values differed in the

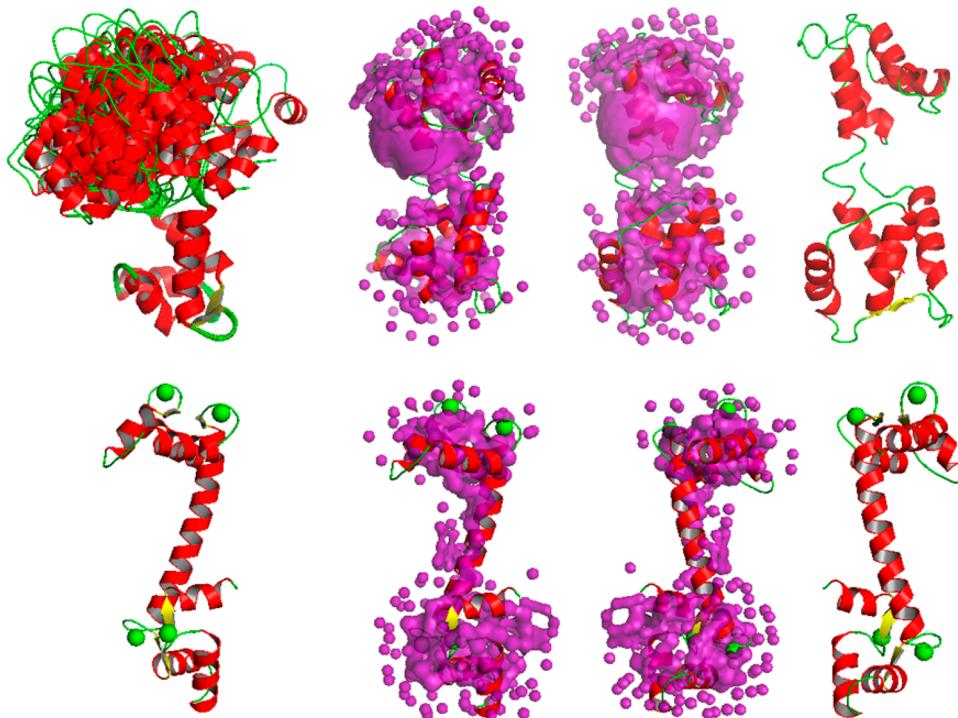


Figure 6. Comparison between our SAXS data models and the structures known for this protein using NMR (top) and X-ray diffraction (bottom). (Top) left to right: 25 models in the NMR data PDB submission 1CFC depicted as ribbons and secondary structure coloring (red, helix; green, loops), one overlay of our SAXS data model in magenta transparent space-filled mode with 1CFD, a rotated view of the same overlap, and finally a ribbon mode model of 1CFD. (Bottom) left to right: ribbon model of PDB submission 1CLL with four Ca^{2+} ions depicted in green cpk mode, an overlay of our SAXS model in magenta transparent space-filled mode with 1CLL, a rotated view of the same overlay, and another view of 1CLL.

previous papers (and have been mentioned in Introduction). At same time, our conclusions actually agree with similar structural parameters of apo-CaM as seen from NMR (PDB IDs: 1CFC and 1CFD) and Ca^{2+} -bound structures from crystallography (PDB IDs: 1CLL and 3CLN). The NMR-based structural information on apo-CaM, where R_g and D_{\max} were computed to be 20.0 and 68 Å (PDB ID: 1CFD), and the same parameters of Ca^{2+} -bound CaM are 21.0 and 69 Å, respectively (PDB ID: 1CLL) and 21.1 and 70 Å, respectively (PDB ID: 3CLN). It is of importance that we never observed any collapsed shape of CaM as seen in the crystal structure of the protein under Ca^{2+} -ion-free condition (PDB ID: 1QX5). Our SWAXS data analysis of Ca^{2+} -ion and pH variation concludes that the global shape of CaM molecules remain comparable during Ca^{2+} and pH-activation process, something which has been seen earlier from structural studies mentioned above and debated earlier due to lack of difference in interdomain energy transfer time-resolved fluorescence experiments with CaM in buffers containing increasing amounts of Ca^{2+} ions.⁶

Visualizing the Shape Changes in CaM as a Function of Ca^{2+} Ions and Low pH. To visualize how the changes in the shape parameters of CaM relate with changes in the global shape of the protein, we performed ab initio structure reconstruction using dummy residues. Results presented here are best solutions from the chain-ensemble modeling routines, as uniform density modeling followed by averaging provided no distinguishable information (Figure 5). In correlation with calculated $P(r)$ curves, shape restoration showed that the structure of this protein remains bilobal from its apo to fully- Ca^{2+} -bound state. The effect of increasing amounts of Ca^{2+} ions in initiating local structural rearrangement in the Ca^{2+} -binding domains could be seen in the models solved for samples having

EGTA to about 1.1 molar excess of free Ca^{2+} ions relative to CaM concentration. Interestingly, the model solved for a data set having $[\text{Ca}^{2+}]/[\text{CaM}] \sim 2.9$ showed an enlarged volume for both N- and C-terminal lobes (Figure 5). Further increments in Ca^{2+} values led to shrinkage in terminal lobes to volumes as seen in earlier data sets indicating some kind of quenching in molecular motion. Models solved at $[\text{Ca}^{2+}]/[\text{CaM}]$ ratios higher than 7.6 indicated a clear formation of two domains separated by a linker. To gain insight into the low pH induced shape rearrangements in CaM, we restored predominant scattering shape of the protein molecules in buffers containing lower pH. Comparison of measured SAXS $I(Q)$ [Q range 0.01–0.5 Å⁻¹] profiles with each other revealed that some changes do occur in the global shape of CaM molecules as pH was lowered from 8 to 5. Comparison of the measured SAXS $I(Q)$ profile and the model restored from the sample at pH 7 revealed some shape rearrangement which was comparable to CaM in buffer having $[\text{Ca}^{2+}]/[\text{CaM}]$ of 0.2. Interestingly, the model solved for CaM at pH 6 was significantly open in both ends and similar to the $[\text{Ca}^{2+}]/[\text{CaM}]$ about 3, and at pH 5 the shape of CaM became bilobal similar to the protein in $[\text{Ca}^{2+}]/[\text{CaM}]$ close to 8. These observations were also supported by χ^2 value of 1.2–1.4 when the SAXS $I(Q)$ profiles of these pH and Ca^{2+} sets were compared in the Q range 0.01–0.5 Å⁻¹. Importantly, SAXS results supported our earlier observations that low pH can induce Ca^{2+} -binding-like shape changes in CaM.

To compare our modeling results with known structures of CaM from other structural methods, one structure of apo-CaM solved within NMR constraints (PDB ID: 1CFC), another structure from X-ray crystallography (PDB ID: 1QX5) and couple of structures of 4 Ca^{2+} -bound CaM or the fully activated

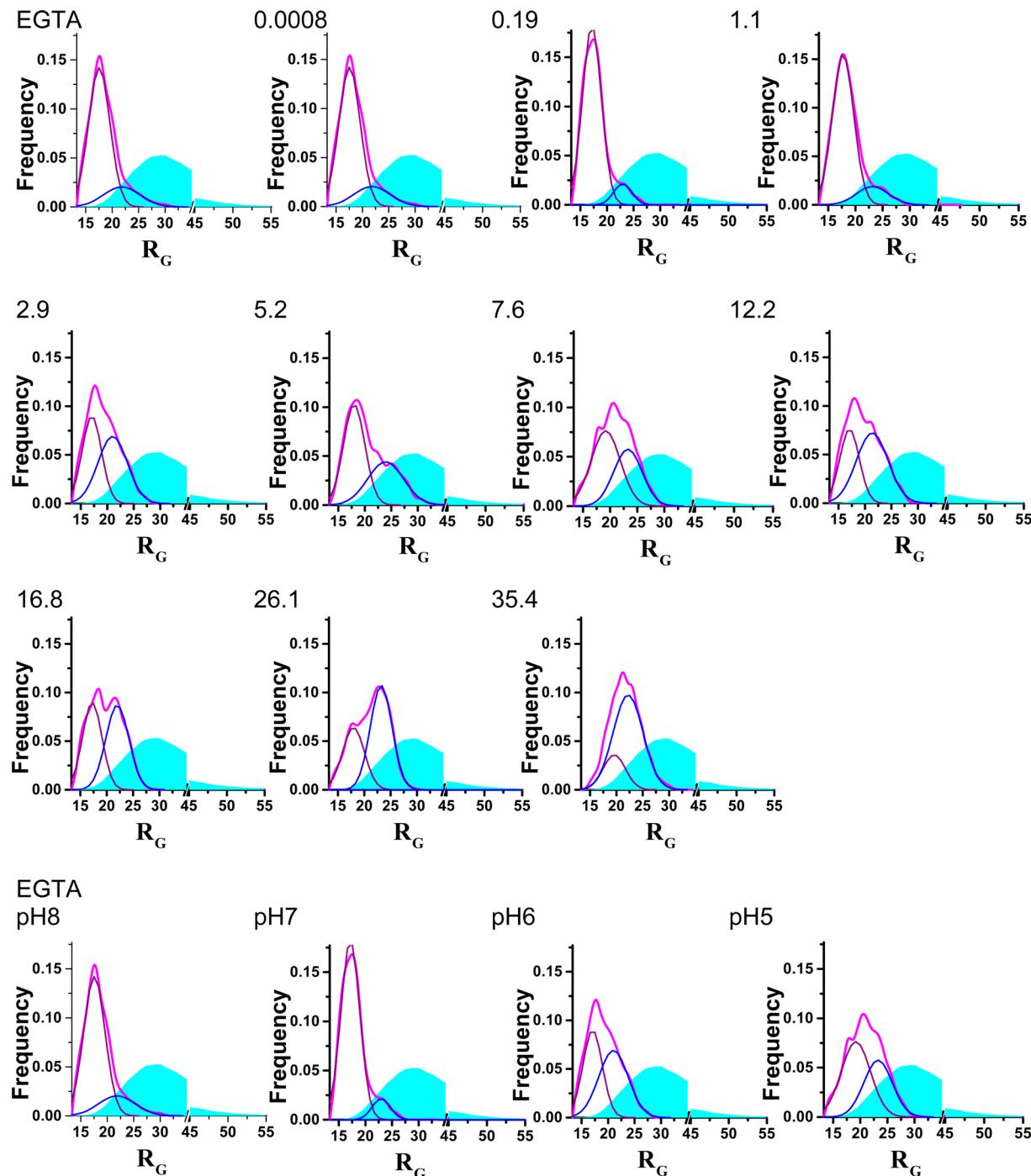


Figure 7. Results of the EOM analyses of the SAXS data sets acquired from solutions of CaM in the presence of varying levels of Ca^{2+} ions and pH. The cyan-colored area in the graph represents the random pool, and the magenta line represents the selected pool of R_G values selected against the measured data. The blue lines show deconvolution of the populations represented by magenta line into two populations.

molecule (PDB ID: 3CLN, 1CLL) are available. Any similarity between our results of predominant solution shape of apo-CaM and crystal structure (PDB ID: 1QXS) was easily ruled out because the D_{\max} of the eight structures in the unit cell were around 54 Å, about 13 Å smaller than the D_{\max} estimated from our analysis. Because dimensions of our model were comparable to the structures solved within constraints offered by NMR experiments, we compared our model of apo-CaM only with the NMR structure of this protein. Interestingly, the 25 structures solved for apo-CaM using NMR suggested that if one domain of the molecule is overlapped, then the other

domain can adopt different positions in space (Figure 6, top left). For clarity, we superimposed our dummy atom model of apoprotein on the coordinates of apo-CaM from 1CFD, where the latter was obtained by energy minimizing 25 structures from NMR (Figure 6, top right). The overlay shown in the middle panels of Figure 6 (top) were generated by superimposing inertial axes of our SAXS model and those of 1CFD. In the two rotated views presented, one can clearly see that the shape features match very well in the lower half of the molecule, except there is an additional volume in our structure which resides in the upper half. The presence of extra volume in our

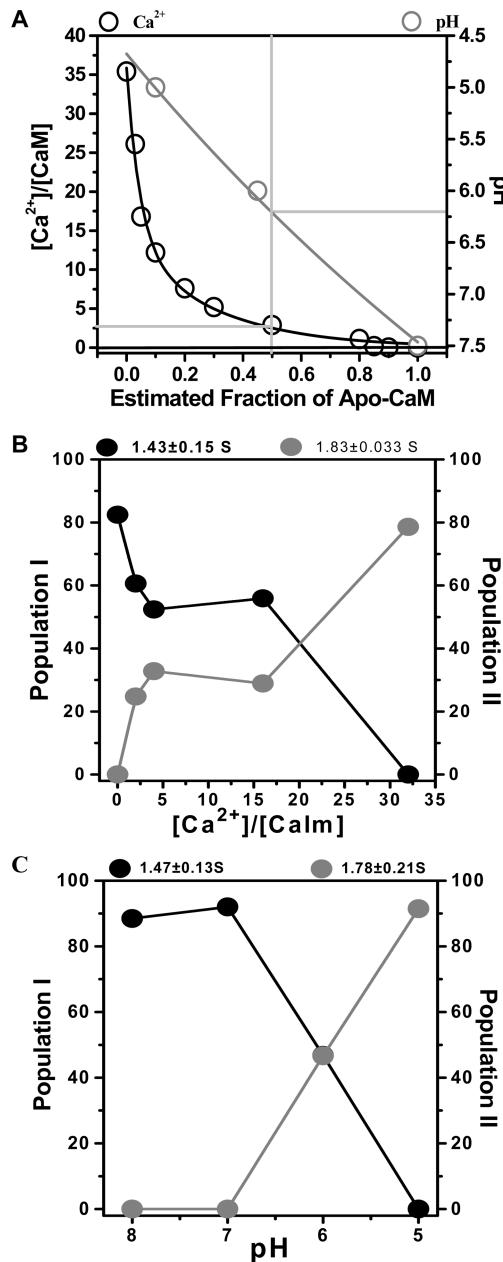


Figure 8. (A) Estimation of the change in the fraction of apo-form in the total ensemble of populations accessible to CaM molecules as a function of varying $[Ca^{2+}]/[CaM]$ ratios and at different pH values. Fitting a first-order exponential decay pattern to the estimated values suggested that the half-change of the trend occurs close to $[Ca^{2+}]/[CaM]$ value of ~ 3 and for pH the half change was seen at ~ 6.2 . For pH data plotting we have considered pH 7.5, 6, and 5. (B) Shift in the fraction of two populations with different sedimentation coefficient values plotted as a function of Ca^{2+} ions in buffer. (C) Variation in the populations as a function of buffer pH. The shift in abundance of molecules observed by SAXS and AUC suggested that the shifts in the ensemble of conformations accessible to CaM molecules are similar at higher Ca^{2+} concentration and at lower pH values.

models suggests that the scattering data model is capable of reflecting local disorder or flexibility in molecules seen from complementary techniques.

For the Ca^{2+} -activated structure, it was clear that the extended dumbbell shape seen from crystallography, and doubted to exist in solution, is adopted at a much larger

$[Ca^{2+}]/[CaM]$ ratio than the chelating ability of the protein. To assess the ability of our SAXS data model in interpreting shape of activated CaM, we superimposed our model containing almost 17-fold excess Ca^{2+} ions over the crystal structure of four- Ca^{2+} -bound CaM (PDB ID: 1CLL). The two rotated views in the middle of the lower part of Figure 6 highlight the remarkable similarities between the low resolution SAXS data model and crystal structure, particularly the relative spatial positioning of the Ca^{2+} -bound terminal domains, their volumes and the length of the interdomain linker. Importantly, we provide conclusive evidence that the shape seen in crystal structure can be achieved in solution, but it requires higher amounts of Ca^{2+} ions to shift the range of conformations accessible to this protein in solution toward that direction. There also lies a possibility that the excess Ca^{2+} ions may be eliciting some kind of chaotropic effect in stabilizing the tertiary structure of the Ca^{2+} -activated CaM. Such an observation was seen earlier for another Ca^{2+} -binding protein, gelsolin, whose solution structure equilibrated in a shape seen from crystal structures of its fragments only when the $[Ca^{2+}]/[CaM]$ ratio was about 40.²⁹ This somewhat explains the discrepancy in the results from CD and fluorescence because Ca^{2+} ions in buffer were never this high in those experiments. Earlier, using the collapsed structure of CaM seen in the crystal structure resolved under Ca^{2+} -free conditions (PDB ID: 1QX5) and the fully activated structure (4 Ca^{2+} ions bound per protein molecule) of CaM from crystallography (PDB ID: 3CLN), an approximation was made to understand shape of partially activated structures of CaM.³⁰ Our results clearly show that this approximation was wrong and the apo-form of CaM actually adopts a more extended bilobal shape rather than a collapsed globular shape. Unfortunately, no atomic resolution structures of intermediate stages are available to compare with our SAXS data models.

Uniqueness of Predominant Shape of CaM under Varying Ca^{2+} Ion and pH Conditions. Being aware that CaM remains a Gaussian-chain-like protein in solution under conditions varying in free Ca^{2+} -ion concentration and pH of buffer, we wanted to explore if the predominant solution shape at any given stage can be considered unique or weighted average of unique solutions, possibly apo and fully Ca^{2+} -activated shape. First, we used the SAXS $I(Q)$ data and employed ensemble optimization method (EOM) to understand the conformational polydispersity exhibited by CaM under different conditions. Against a random pool of conformations varying in their R_G values, searches were performed to find the shapes which fall within the shape variation encoded in the SAXS data (the random and selected pool are represented by cyan and magenta curves, Figure 7). The selected population of shapes indicated that under EGTA or Ca^{2+} -free conditions to a $[Ca^{2+}]/[CaM]$ ratio of about 1, the distribution curve of shapes accessible to CaM under solution conditions adopted a single peak and a minor shoulder profile. The profiles could be solved for two populations—one corresponding to 17–18 Å in R_G and another population with R_G around 21–22 Å. Interestingly, similar peak-and-shoulder profile was seen for CaM molecules at pH 8 and 7. The EOM calculations revealed that $[Ca^{2+}]/[CaM]$ values higher than 3 increased the relative population of the larger sized population. The trend prevailed in the data sets having $[Ca^{2+}]/[CaM]$ 3 to 26, and in buffer containing pH 6 and 5, except at higher relative Ca^{2+} value of 35, the population of shapes shifted mainly toward the higher sized one. These

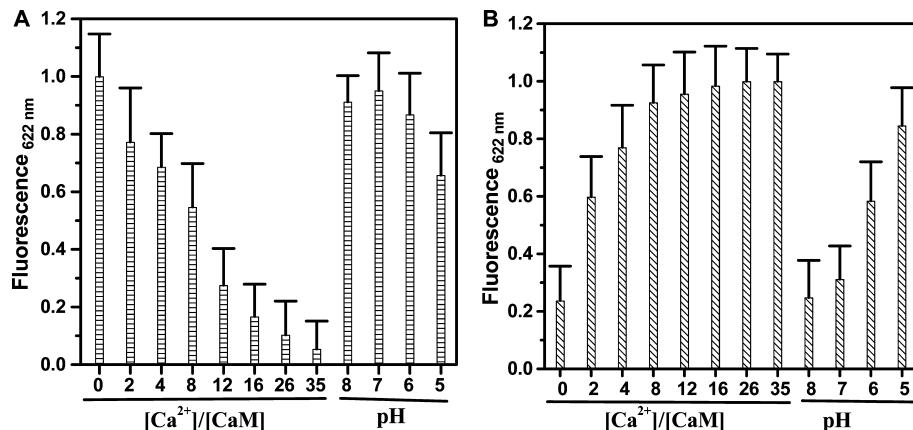


Figure 9. Comparative binding of peptide N_G and M_P with CaM under different Ca²⁺-ion concentration and at different pH values. (A) Binding of N_G to CaM with increasing Ca²⁺ concentrations ([Ca²⁺]/[CaM] varying from 0 to 16) and at pH 8–5. (B) Binding of M_P to CaM with increasing Ca²⁺ concentrations ([Ca²⁺]/[CaM] varying from 0 to 35) and at pH 8–5.

calculations indicated that CaM adopts unique shapes under EGTA and high [Ca²⁺]/[CaM] conditions, and at intermediate Ca²⁺-ion values, CaM molecules essentially adopt a mixture of two populations: apo and fully Ca²⁺-activated shape. In summary, EOM analysis also concluded that low pH can also influence the ensemble of shapes accessible to CaM molecules similar to those induced by Ca²⁺ ions.

Supported by EOM analysis, we used SAXS I(Q) profiles from CaM molecules under different Ca²⁺-ion concentration and pH values to compute the relative population of the two shapes accessible to CaM molecules in solution under those conditions. This was done by comparing the R² value of the SAXS data profile of intermediate data sets (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 Ca²⁺ ions relative to CaM. Our estimation indicated that SAXS data profile of the samples containing just 0.0008, 0.19, 1.1, and 2.9 free Ca²⁺ 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 (Figure 8A). Even in the samples containing 5.2–16.8 molar excess of Ca²⁺ ions, we estimated presence of 30 to 5% of apo structures. Fitting a first-order exponential decay pattern to the estimated values suggested that the half-change of the trend occurs close to [Ca²⁺]/[CaM] value of ~3 (Figure 8A). This analysis implies that unless all Ca²⁺-binding sites are not supersaturated, at any intermediate state, there lies an equilibrium between fully activated as well as some inactive structures. This interpretation can help understand why, on the basis of CD and time-resolved fluorescence studies, previous researchers found little difference in the total α -helical content and cross-talk between the two terminal lobes in samples lacking and containing Ca²⁺ ions, which led to questions about the very existence of orderliness across the central linker.^{28c} Similarly, when we estimated the fraction of apo-CaM at pH 8, 7, 6, and 5 we found that pH 6 contains ~50% of apo-structure whereas pH 5 contains 10% apo-CaM (Figure 8A). The fraction at pH 8 and 7 were very close to pH 7.5 (~100% apo-CaM), and the fraction values of the two populations at pH 6 and 5 resembled the [Ca²⁺]/[CaM] values of ~3 and ~12, respectively.

Assessment of Population Distribution Using Analytical Ultracentrifugation. Along with using the SAXS data to estimate the populations accessible to CaM in solution, we performed analytical ultracentrifugation (AUC) experiments

and deconvoluted the sedimentation velocities to estimate number of major populations. Experiments were carried out with CaM in buffer containing [Ca²⁺]/[CaM] ratio from 0 to 32 and having buffer pH 8 to 5. Importantly, the sedimentation value of 1.43 ± 0.15S obtained for CaM molecules in EGTA or Ca²⁺-ion-free conditions and that of 1.83 ± 0.03S for CaM protein in buffer having [Ca²⁺]/[CaM] ratio of 32 agreed with values published earlier.³¹ At the same time, data analysis at varying ratios of free Ca²⁺ ions in solution or buffer pH showed that the models solved with higher confidence with two species. In correlation with results seen from EOM analysis of SAXS data sets, addition of Ca²⁺ ions resulted in two populations having sedimentation values of 1.43 ± 0.15S and 1.83 ± 0.03S. The relative abundance of the two populations with different sedimentation values as a function of [Ca²⁺]/[CaM] is presented in Figure 8B. For CaM molecules at pH 8, the value of sedimentation was found to be 1.47 ± 0.02S, whereas the value for molecules at pH 5 was computed to be 1.78 ± 0.02S, and the variation in the relative abundance of the two populations as a function of buffer pH is plotted in Figure 8C. Overall, the deconvolution of the SAXS intensity profiles and AUC sedimentation profiles supported the fact that, although the shapes accessible to CaM molecules under Ca²⁺-ion-free and supersaturated state are distinct, a mixture of these two shapes exist at intermediate [Ca²⁺]/[CaM] levels. Moreover, a similar pattern was seen for the populations of global shape in low pH conditions. One query still remained if low pH induced shape changes are on same pathway as those done by Ca²⁺ binding?

Peptide-Based Assay To Assess Ca²⁺ vs pH Effect on CaM. The prime idea for this assay was that if the apo-form exists at different [Ca²⁺]/[CaM] ratios and slowly decreases with increase in Ca²⁺-ion concentrations, the peptide specific to bind the apo-form of CaM would exhibit a decrease in binding ability to CaM molecules. Similarly, our SAXS and AUC data suggested that lower pH should also decrease binding of peptide which primarily binds apo form of CaM. Additionally, increase in Ca²⁺-ion concentration or lowering of pH should increase binding efficacy of peptide which primarily binds Ca²⁺-activated shape of CaM. To carry out these experiments, peptides specific for apo form and Ca²⁺-bound form, neurogranin (N_G)³² and mastoparan (M_P)³³, respectively, labeled with Texas red dye were synthesized, purified, and characterized. Purified peptides were incubated with CaM at different

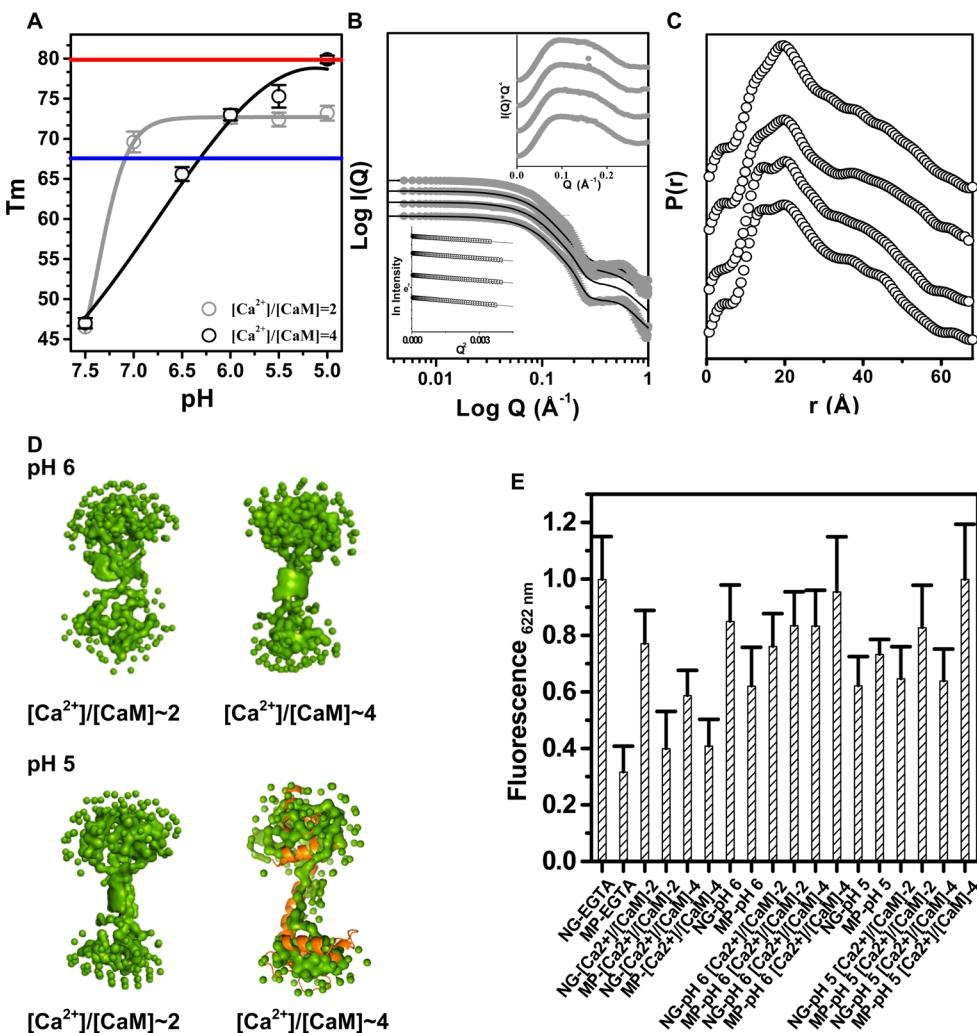


Figure 10. (A) Variation in T_m values of CaM protein as a function of pH. The red line represents the T_m value for the $[Ca^{2+}]/[CaM]$ ratio of 16 and the blue line indicate the T_m for pH 5 CaM. (B) SAXS intensity profiles for CaM molecules in buffers having pH 5 and 6, and $[Ca^{2+}]/[CaM]$ ratio of 2 and 4 at both pH values. The left bottom inset shows the Guinier plots, whereas the right top inset shows the Kratky profile for all the data sets. (C) $P(r)$ curves computed from the SAXS data sets shown in panel (B). In graphs B and C, top to bottom plots represent samples at pH 6 with $[Ca^{2+}]/[CaM] \sim 2$, pH 6 with $[Ca^{2+}]/[CaM] \sim 4$, pH 5 with $[Ca^{2+}]/[CaM] \sim 2$, and pH 5 with $[Ca^{2+}]/[CaM] \sim 4$. (D) Chain-ensemble modeling shapes restored for CaM molecules from measured SAXS data for CaM molecules at pH 6 and 5, and $[Ca^{2+}]/[CaM]$ ratios 2 and 4. The dummy atom models are shown in green space-filled mode. The model solved for pH 5 and $[Ca^{2+}]/[CaM] \sim 4$ has been overlaid on the crystal structure of CaM bound to four Ca^{2+} ions (PDB ID: 1CLL, orange ribbon). (E) Comparative binding of Texas red labeled N_G and M_P to CaM molecules at low pH and low Ca^{2+} ions.

$[Ca^{2+}]/[CaM]$ ratios (0–35) and at different pH buffers (8–5) (see Materials and Methods). Figure 9 summarizes the results from the peptide binding experiments, where the fluorescence values at 622 nm were monitored under different conditions to estimate the amount of bound peptide. Against our expectation, we found that both N_G and M_P could bind to CaM at different levels of Ca^{2+} -ion concentration, albeit the binding potency of the N_G decreased and M_P increased progressively with increase in Ca^{2+} ions. This indicated that either the peptides are not specific for the Ca^{2+} -bound shape of CaM or a portion of apo or fully Ca^{2+} -bound shape always exists among the pool of CaM molecules in solution. The latter possibility correlates with results from our biophysical experiments that two distinct populations always exist and the resultant predominant shape parameters seen at different values of $[Ca^{2+}]/[CaM]$ ratios are a mixture of these two shapes, i.e., the apo form seen from NMR and the four Ca^{2+} -bound crystal structure. Interestingly, the peptide binding trend persisted in the absence of Ca^{2+} ions

but with lower pH (Figure 9). This supported that the shape changes induced by pH are actually the same as those induced by Ca^{2+} ions. In other words, low pH can modulate the structure and hence the function of CaM similar to Ca^{2+} ions. Intrigued with our results that low pH can modulate structure of CaM, we pondered if low pH can override the need for Ca^{2+} ions.

Low pH Can Override the Need for Ca^{2+} Ions. Being ubiquitously present in physiology, there lies a strong possibility that CaM molecules can encounter low pH as well as Ca^{2+} ions simultaneously, and we postulate that these two factors can together affect the predominant solution shape and thus function of CaM. To test this, we first carried out thermal melting CD experiments with CaM at lower pH and lower levels of Ca^{2+} ions in buffer (Figure 10A). Interestingly, we observed that even with $[Ca^{2+}]/[CaM]$ of 2 and 4, the stability of the CaM molecules represented by their T_m values increased from 46 to 70 and 80 °C, respectively. The increase was more

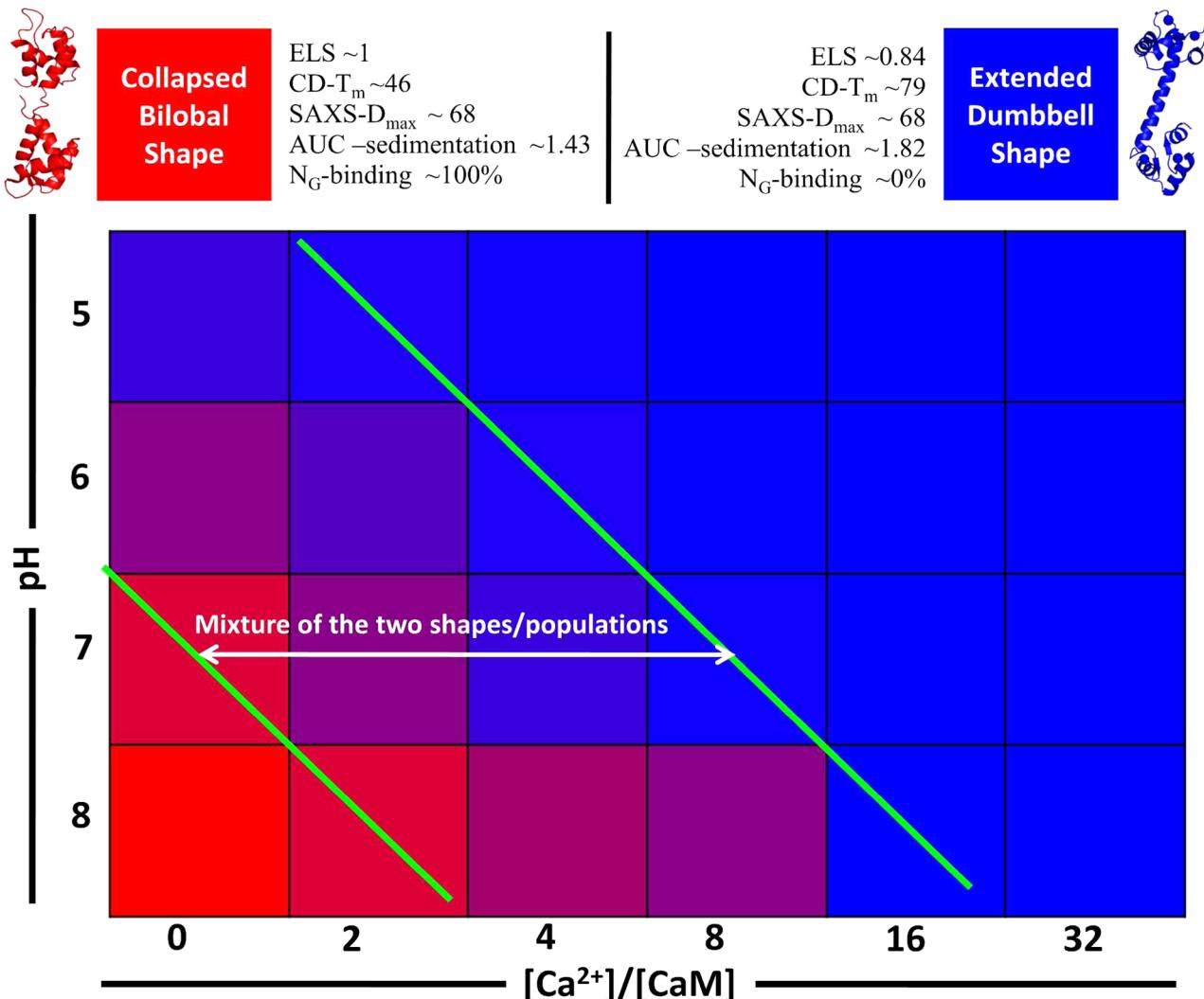


Figure 11. Schematic phase diagram summarizing our biophysical and biochemical results that low pH overrides the need of higher number of Ca²⁺ ions per CaM molecule in transforming the collapsed bilobal shape of CaM into an extended dumbbell shape with central linker exposed.

than that of low pH alone and was comparable to what high levels of Ca²⁺ can induce at pH 8 (>10 Ca²⁺ ions per CaM molecule) (Figure 2B). These results clearly supported that the two factors pH and Ca²⁺ can simultaneously affect the global shape of CaM molecules, and a pH close to 5 compensates for Ca²⁺ ions as high as 16 or more. To gain visual insight into the shape properties of the pH/Ca²⁺-activated CaM, we acquired SAXS data from CaM molecules in buffer containing pH 6 and 5, and free [Ca²⁺]/[CaM] values close to 2 and 4 (Figure 10B–D). Under these conditions, the SAXS $I(Q)$ profiles supported no change in monodisperse Gaussian-chain-like scattering nature of the CaM molecules. Indirect Fourier transformation of the data sets showed that the D_{\max} values observed for CaM at pH 6 and [Ca²⁺]/[CaM] ratios 2 and 4 were 67 and 68 Å, respectively, whereas for samples in buffer having pH 5 and [Ca²⁺]/[CaM] ratios of 2 and 4 were 68 and 68 Å, respectively. The same analysis also provided R_G values of CaM to be 21.4 and 21.6 Å for pH 6 with [Ca²⁺]/[CaM] ~2 and 4, respectively, and at pH 5 the values were 21.6 and 21.8 Å for [Ca²⁺]/[CaM] ~2 and 4, respectively. Using the shape profile encoded in the SAXS profiles, we restored the predominant scattering shape of the CaM molecules under these conditions (Figure 10D). Models showed that at pH 6 and [Ca²⁺]/[CaM] ~2, the

protein shape appeared bilobal and closer to the shape seen for apo CaM at pH 8, but a slight increase in the [Ca²⁺]/[CaM] ratio to 4 transformed the shape into a clearly bilobal one, similar to the partially activated CaM shape seen at the [Ca²⁺]/[CaM] ratio of 1.1 at pH 8. Interestingly, at pH 5, a [Ca²⁺]/[CaM] ratio of 2 appeared, similar to the shape seen for CaM at [Ca²⁺]/[CaM] of 4 at pH 6, and the shape of CaM at [Ca²⁺]/[CaM] ratio of 4 at pH 5 surprisingly appeared very similar to the fully activated CaM shape at a [Ca²⁺]/[CaM] ratio of >16. These results concluded that a slight shift in buffer pH can drastically lower the level of Ca²⁺ ions required to achieve a shape similar to the four-Ca²⁺-ion-bound crystal structure of CaM (PDB ID: 1CLL) (Figure 10D). The tracking of the changes in the global shape of the CaM molecules as a simultaneous effect of Ca²⁺-ion concentration and low pH of buffer were compared with the ability of CaM molecules to bind N_G and M_P peptides, as described earlier (Figure 10E). Our results showed that N_G peptide, which preferentially binds to apo CaM, progressively lost its efficacy of binding to CaM molecules as the pH was reduced from 8 to 5 under Ca²⁺-ion-free conditions. As the [Ca²⁺]/[CaM] ratios were increased to 2 and 6, reduced binding ability of N_G and increased binding ability of M_P peptides substantiated CD- and SAXS-based

conclusions that low pH can induce shape changes in CaM which resemble Ca^{2+} -induced ones.

Overall, we provide the first report that the shape-function relationship of calcium binding protein CaM can be modulated by low pH. The findings reported for CaM in this study are similar, but more holistic to another study where we showed how low pH can activate calcium binding protein, gelsolin in the absence of Ca^{2+} ions.¹⁰ Addressing the early debates, about the shape profile of the apo CaM and its resemblance with NMR or crystal structures, we clearly show that apo CaM adopts a shape that is best represented by the NMR-based structure. Revelation that CaM adopts a Gaussian-chain-like shape in solution, somewhat addressed the promiscuous nature attributed to CaM, but then we report that this behavior does not alter the inherent disorder in the CaM molecules. This indicates that presumed nonspecificity of CaM toward its various ligands is very likely due to our limited understanding of how the different shapes accessible to CaM are modulated by buffer pH too, besides the Ca^{2+} ions. Actually, both of these biochemical factors or secondary messengers modulate functioning of CaM by affecting the ensemble of shapes this protein can adopt in solution. Another interesting fact is that the crystal structures of CaM bound to Ca^{2+} ions at 2.2 and 1.0 Å resolution were obtained from drops set in buffers having pH 5. This brings forth another thought that the crystal structures of CaM are actually influenced by pH as well, not just Ca^{2+} ions. This was the probable reason researchers following biophysical methods failed to capture the overtly helical bilobal shape of CaM with an extended linker, as seen in crystal structures of Ca^{2+} -ion-bound CaM. We show here that very high amounts of free Ca^{2+} ions can induce CaM to adopt a predominant solution conformation very similar to the crystal structure, possibly by affecting chaotropic effects above the chelating limit of the CaM molecules. At same time, we showed here that the need for Ca^{2+} ions is drastically reduced by changing the pH of buffer from 8 to 5.

Results from our biophysical and biochemical experiments showing that low pH can effectively modulate the need for Ca^{2+} ions by CaM molecules to transform their global shape from a collapsed bilobal shape to a dumbbell shape with extended central linker have been summarized in form of a phase diagram (Figure 11). In our schematic, the red and blue color zone represent population representing the apo and activated form of CaM, and intermediate zones are represented by an experimental data-based weighted mixture of the two colors. While this manuscript was being compiled, two crystal structures of CaM were reported, which were obtained by soaking Ca^{2+} -bound CaM crystals in (1) EDTA and (2) strontium ions at low pH.³⁴ Two CaM monomers were found in a single unit cell in both crystals; one with a straight continuous helix and the other with a bent helix. These structures helped in visualizing initial steps of how the central helical linker collapses or bends at atomic resolution. It was intriguing to note that the pH of the crystallization conditions here too were in the range 4–5, making it difficult to dissect the effect of divalent (Ca^{2+} or Sr^{2+}) ions and low pH. Concerted regulation of the shape-function relationship of CaM molecules by pH and Ca^{2+} -ion levels suggests that these two factors interplay to regulate the role of CaM during apoptosis,³⁵ pathogenesis,³⁶ and transport³⁷ where the cell encounters low pH (at one or the other stage). Apart from playing an important role in several signaling processes, CaM is also involved in exocytosis as well as endocytosis events.³⁸ It is very

likely that these two factors lead to a particular balance of the shapes accessible to this protein, which in turn affects a specific function out of the myriad of functions this protein can execute. Overall, our work reveals a new aspect of this ubiquitous protein, and our results will allow researchers to understand this protein better.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CaM	calmodulin
SAXS	small angle X-ray scattering
CD	circular dichroism
AUC	analytical centrifugation

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