Ca²⁺ and Mg²⁺ binding induce conformational stability of Calfumirin-1 from *Dictyostelium discoideum*

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Abstract. The apo-Calfumirin-1 (CAF-1) binds to Ca²⁺ with high affinity and also to Mg²⁺ with high positive cooperativity. The thermal unfolding curves of wtCAF-1 monitored at neutral pH by CD spectroscopy are reversible and show different thermal stabilities in the absence or presence of Ca²⁺ and Mg²⁺ ions. Metal-free wtCAF-1 shows greater thermal stability than EF-IV mutant protein. We observed that GdnHCl-induced unfolding of apo-wtCAF-1 monitored by CD and fluorescence spectroscopies increases co-operative folding with approximately same C_m values. Binding of Ca²⁺ and Mg²⁺ ions to CAF-1 dramatically altered the fluorescence and CD spectra, indicating metal ion-induced conformational changes both in the wild-type and mutant proteins. The hydrophobic probe, ANS is used to observe alteration in surface hydrophobicity of the protein in different ligation states. In apo-wtCAF-1, the exposed hydrophobic surfaces are able to bind ANS which is in contrast to the unfolded or the metal ions ligated conformations. Isothermal titration calorimetry (ITC) results show two possible independent binding sites of comparable affinity for the metal ions. However, their binding to the EF-IV E helix-loop-F helix mutant apo-protein happens with different affinities. The present study demonstrates that Ca²⁺ or Mg²⁺ binding plays a possible role in the conformational stability of the protein.

Keywords. Ca²⁺-binding proteins; EF-hand motifs; protein unfolding; protein conformational stability.

1. Introduction

Calcium-binding proteins (CaBPs) have highly conserved helix-loop-helix structure called as EF-hand motifs within the protein backbone.¹ These EF-hand motif proteins represent the superfamily of CaBPs in cell, and regulate many essential physiological processes.^{2–4} Many EF-hand proteins belong to one of the two categories: (i) signalling proteins or (ii) buffering proteins (e.g. parvalbumin, calbindinD_{9k} and calmodulin are some of the representative buffering and signalling CaBPs).^{5,6} A relatively large number of Ca²⁺-binding proteins CaBPs have been precisely characterized structurally and functionally *in vitro* in order to relate their function *in vivo*. Ca²⁺-binding proteins that contain four EF-hand motifs form two globular domains as N-terminal domain (two EF-hands) and C-terminal domain (two EF-hands), respectively. These domains have either structurally dependent or independent Ca²⁺-binding properties.^{7,8} Binding of Ca²⁺ to these domains

induces conformational changes of variable amplitude, depending on the nature of activity that the protein controls.

Ca²⁺ is known to be widely distributed in all biological systems. Its major concentration in the cytosol is 10^{−7} M, which is four orders lower in magnitude than Mg²⁺. Change in the cytosolic Ca²⁺ concentration initiates a wide range of cellular responses, such as Ca²⁺-regulated cellular activities increases on increasing Ca²⁺ concentration from 10^{−7} M in a resting cell to 10^{−6}–10^{−5} M in the activated cell.^{9,10} Unlike Ca²⁺, Mg²⁺ is another major constituent of cell in high concentrations (0.5–2 mM), and participates in many enzymatic reactions.¹¹ Most of the essential processes of CaBPs in living cells are carried out in presence of high cytosolic concentration of Mg²⁺, about 100–10,000-fold excess than Ca²⁺. The calcium-binding EF-hand motifs of CaBPs are also able to bind Mg²⁺ in the absence of Ca²⁺, either with same or different affinity. Such Ca²⁺/Mg²⁺-binding EF-hand motifs of CaBPs are called mixed sites or structural sites. On the other hand, some of these EF-hand motifs are specific to Ca²⁺

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binding only, and called regulatory sites.¹² The binding specificity of CaBPs to Ca^{2+} and Mg^{2+} or both is dependent on its function.¹³ The CaBPs, such as calmodulin,¹⁴ troponin C,¹⁵ and EhCaBP1¹⁶ are classified as either structural or regulatory proteins based on their binding specificity towards metal ions. In aqueous solution, the protein forms hexagonal coordination in an octahedral rearrangement with Mg^{2+} ion.^{17,18} Mg^{2+} binding induces conformational changes in the EF-hand loops of CaBPs to accommodate the hexagonally coordinated metal ion.^{19–24} The different ionic radii of the two ions ($\text{Ca}^{2+} = 1.14 \text{ \AA}$ and $\text{Mg}^{2+} = 0.86 \text{ \AA}$) enable specificity of CaBPs towards Ca^{2+} over Mg^{2+} binding, with distinguished affinity towards CaBPs.²³ As there is a high concentration of Mg^{2+} in the cell, the binding of Ca^{2+} to CaBPs in the cell can be a process of Ca^{2+} binding to Mg^{2+} -bound protein rather than the apo-protein.

In *Dictyostelium discoideum*, Ca^{2+} -binding proteins play important roles in a wide variety of cellular processes such as signal transduction, cell differentiation and regulation of intracellular Ca^{2+} levels. Previous reports have shown that Calfumirin-1 (CAF-1), a Ca^{2+} -binding protein preferentially expressed at the initial step of *D. discoideum* cell differentiation. Further, overexpression of CAF-1 is found to be possibly promoting cell differentiation through induction of cAMP receptor 1 (CAR1) gene expression.²⁵ Like other CaBPs, CAF-1 possesses characterized four calcium-binding regions called EF-hand (helix-loop-helix) motifs. It is slightly acidic ($\text{pI} = 4.62$) and highly hydrophilic in nature.²⁶ The amino acid sequence of CAF-1 shows significant homology to CaM (26% identity),²⁷ CaBP1 (30% identity),²⁸ CaBP2 (33% identity),²⁹ and CaBP3 (29% identity)³⁰ from *D. discoideum*. These proteins have molecular mass comparable to that of CAF-1 ($\sim 19.5 \text{ kDa}$) and highly conserved EF-hand motifs involved in Ca^{2+} binding.²⁶ Unlike CaM and CaBP3 that behave as a Ca^{2+} sensor protein, CaBP1 and CaBP2 have buffering properties.³⁰ However, functions of CAF-1 in Ca^{2+} -bound and in Ca^{2+} -free forms are still not clear to comment on its role in *D. discoideum* cell differentiation. Here, we have elucidated the conformational and structural stability of Ca^{2+} -free and bound forms of wtCAF-1 and EF-IV mutant. We created a double mutant at C-terminus domain of CAF-1 to remove the Ca^{2+} -binding EF-IV motif and compare the preferential Ca^{2+} -binding and its effect on conformational and structural stability. Thus, we studied the Ca^{2+} and Mg^{2+} induced conformational stability and the energetics of the metal ions binding to the wild-type and mutant CAF-1 to provide the possible biological importance in *D. discoideum*.

2. Experimental

2.1 Plasmid construct, generation of EF4 mutant, protein expression and purification

The coding region of *cafA* gene was amplified by PCR with forward primer (5'-CATATGGCAAGCACCCAA AATATTGTTGAAG-3') and reverse primer (5'-GGA TCCTTATTGATCTGGATTAAATAATTTTG-3'), respectively. To facilitate the cloning procedure, *NdeI* and *BamHI* sites are engineered into the upstream and downstream primers, respectively. The PCR product recovered from 0.7% agarose gel is cloned into pGEM-T Easy vector (Promega) and the ligated mixture is transferred to *Escherichia coli* strain DH5 α . The identity of each construct is confirmed by DNA sequencing and digested with the same restriction enzymes to be inserted to an expression vector pET-15b (Novagen) containing a His₆-tag proceeded by a thrombin digestion site. The construct is named as pET-CAF-1.

The Ca^{2+} -free CAF-1 is mutated in the EF-IV motif using the Quick Change site-directed mutagenesis kit (Stratagene). The double mutant D142 N/E153Q in the EF-IV is created through sequential mutagenesis by changing G to A and G to C, respectively. The PCR product of DNA containing mutant is transferred into *E. coli* DH5 α , and sequenced to confirm the correct amplification. The correct amplified product is ligated to expression vector pET-15b (Novagen) for expression and purification of the mutant protein.

For large-scale purification of the fusion proteins, the plasmid constructs (pET-CAF-1) encoding wild-type CAF-1 and mutant are transferred into the *E. coli* BL21 pLys(DE3) strain. Expression of the fusion proteins harbouring in Luria-Bertani (LB) medium containing ampicillin (50 $\mu\text{g/mL}$) and chloramphenicol (50 $\mu\text{g/mL}$) is induced with 1 mM IPTG (Promega) at an appropriate absorbance of 0.4–0.6 O.D. at 600 nm, 37°C, and further grown overnight at 22°C. The cells are harvested and resuspended into an 1X binding buffer (20 mM MTris-HCl, pH 8.0, 500 mM NaCl and 10 mM Imidazole), sonicated on ice and centrifuged at 17,000 g for 15 min. The fusion proteins are then purified by Ni-affinity chromatography. The fractions containing fusion proteins are concentrated, and the His₆-tag is removed by overnight thrombin treatment at 22°C. Lastly, the soluble protein is further purified by a fast protein liquid chromatography (FPLC) system (AKTA Frac-950, GE-Pharmacia) using a Sephadex G-75 column operated at a flow rate of 1.0 mL/min. Protein samples in 20 mM Tris-HCl, pH 7.2 and 50 mM NaCl are applied to the gel permeation column (120 mL bed volume) equilibrated in the same buffer. Purity

of the recombinant protein is checked by 12% SDS-PAGE and concluded that it represents the full-length pure protein of 169 amino acids. Average yield of the purified protein is 6–14 mg/L of bacterial culture. Metal-free wild-type and double mutant CAF-1 are prepared by incubating the proteins with 1 mM EDTA for 1 h, followed by protein extraction from bacterial lysis which includes extensive dialysis against 20 mM Tris-HCl buffer, pH 7.2 with several changes. The tag-free proteins are concentrated and further dialysed against 2 mM EGTA followed by Chelex-100 resin treatment (presuspended in 20 mM Tris-HCl (pH 7.2) buffer) to remove unbound ions. All buffer solutions used for Ca^{2+} binding studies are passed through Chelex-100 resin and stored in plastic bottle.

2.2 Intrinsic fluorescence

Intrinsic fluorescence spectra are recorded with a Varian Carry Eclipse spectrofluorometer at room temperature using quartz cuvette of 1 cm path length. The protein samples are prepared in 20 mM Tris-HCl buffer (pH 7.2), and fluorescence measurements are performed at a single protein concentration of 7 μM to maintain uniformity in the data output. The tyrosine fluorescence emission spectra are recorded in the range of 280 to 350 nm with the excitation wavelength set at 260 nm. The protein samples are incubated either in the presence or absence of Ca^{2+} and Mg^{2+} or both ions at room temperature for 30 min before each measurement. For hydrophobic probe, ANS-binding experiments, samples containing 200 μM ANS are excited at 370 nm and the emission spectra are recorded between 400 and 650 nm.

2.3 Circular dichroism

Circular dichroism (CD) spectra are recorded with a Jasco J-815 spectropolarimeter equipped with a Jasco ETC-505T temperature controller using a properly sealed quartz cuvette from Hellma, Germany. All solutions are prepared in 20 mM Tris-HCl buffer, pH 7.2 and incubated for 30 min at same temperature before each experiment. The far-UV (190–250 nm) and near-UV (250–350 nm) CD spectra of CAF-1 are recorded either in the absence or presence of Ca^{2+} (0.5 mM) and Mg^{2+} ions (1.0 mM) or both with a resolution or spectral bandwidth of 1 nm, at a scan speed of 50 nm/min with response time of 1 s. For each experiment, five spectra are accumulated and averaged to express the result in molar ellipticity $[\theta]$ ($\text{deg cm}^2 \cdot \text{dmol}^{-1}$). The heat-induced unfolding of apo-, Ca^{2+} - and Mg^{2+} -bound CAF-1 are monitored at fixed wavelength of 222 nm

and the data are collected at $1^\circ\text{C}/\text{min}$ increments from 10 to 90°C . After each unfolding scan, sample is cooled and rescanned to measure the reversibility, which is $>95\%$ for all the thermal scans.^{21,31,32} The background reference spectra obtained at identical conditions are subtracted from the protein spectra. The obtained data are fitted to a two-state model of unfolding transitions from a native (N) to unfolded (U) conformation, and the fraction of unfolding (f_U) is calculated as previously described.^{33,34} Values of ΔH_m at T_m are determined based on the equilibrium unfolding constant for the population of fractional unfolded (f_U) and fractionally natively folded (f_N). Thereafter, a modified Gibbs-Helmholtz equation is used to calculate the change in free energy of unfolding at given temperature (25°C).³⁴

Guanidine hydrochloride (GdnHCl)-induced unfolding of the apo-wtCAF-1 is monitored by measuring the change in molar ellipticity at 222 nm as a function of GdnHCl concentration. Each time a fresh GdnHCl stock solution is prepared for unfolding experiments. The purified apo-wtCAF-1 (10 μM) is added to different GdnHCl concentrations progressively from 0–6 M, and incubated at room temperature before each experiment. The unfolding curves are analysed assuming a two-state unfolding mechanism to determine the unfolding free energy change, ΔG , as a function of GdnHCl concentrations. The ΔG values obtained vary linearly with GdnHCl concentrations, thus the data are analysed by linear extrapolation method to determine the free energy of unfolding in the absence of denaturant, $\Delta G_{(H_2O)}$, which represents the intrinsically conformational stability of the protein.^{35,36} The C_m ($\Delta G_{H_2O}/m$) values obtained explain molar gain in the conformational stability of tertiary and secondary structures.

2.4 Isothermal titration calorimetry

All ITC experiments are performed using a MicroCal VP-ITC instrument at 25°C and the data are processed with Origin Lab (version 7) software supplied by MicroCal. Protein and ligand samples are prepared in Chelex-100 treated 20 mM Tris-HCl buffer (pH 7.2). For wild-type and EF4 mutant proteins, independent Ca^{2+} and Mg^{2+} ion titrations are performed using 50 μM and 100 μM purified apo-proteins, respectively. A series of 7 μL aliquot of either 2 mM CaCl_2 or 2 mM MgCl_2 are injected into the protein sample containing 1.6 mL of 50 μM apo-wtCAF-1, and the corresponding heat signals are monitored calorimetrically. The heat change due to the injections and dilution effects are corrected by subtracting heat change obtained on 2 mM

Ca^{2+} or Mg^{2+} ion titration against the reaction buffer. Two sets of binding sites fitting models are used to fit the ITC data using a nonlinear least-squares minimizations method to calculate the dissociation constant (K_d) and enthalpy changes for each binding experiment.³⁷

3. Results and discussion

The purified recombinant protein CAF-1 in presence of 1 mM CaCl_2 shows insignificant mobility shift but in presence of 2 mM EGTA, a slow migration and distinguishable mobility shift was observed as shown in figure 1. This is similar to the previous report on CaBPs, where different mobility shifts were shown in SDS-PAGE depending on the presence of Ca^{2+} or EGTA in reaction mixture.³⁰ The mobility shift results clearly indicate that recombinant protein CAF-1 retains its conformation in the Ca^{2+} -bound state.

In figure 2, thermal unfolding of apo-, Ca^{2+} - and Mg^{2+} - bound wtCAF-1 followed by change in ellipticity at 222 nm as a function of temperature indicates single cooperative unfolding transition. Thermal stability index, T_m of the protein shows a strong dependence on presence of the metal ions with the highest T_m value in presence of 0.25 mM Ca^{2+} ions followed by the value in presence of 0.5 mM Mg^{2+} ions. From table 1, the melting temperature, T_m values for Mg^{2+} - and Ca^{2+} -bound forms shows significant enhancement in thermal

Figure 2. Comparative thermal unfolding curves of wtCAF-1 monitored by observing the change in ellipticity $[\theta]$ at 222 nm at a scan rate of $1^\circ\text{C}/\text{min}$. Curves represent melting profile of apo- (■), Mg^{2+} -bound (●), and Ca^{2+} -bound (★) wtCAF-1, respectively.

stability than the apo-wtCAF-1. However, lower amplitude in thermal stability was observed for Mg^{2+} -bound form than the Ca^{2+} -bound wtCAF-1. Similar patterns in thermal stability change have been reported for CaM and TnC, CaBPs.^{38,39} Based on the equilibrium constant of unfolding, values of enthalpy change, ΔH_m at T_m for apo-, Mg^{2+} - and Ca^{2+} -bound wtCAF-1 show

Figure 1. SDS-PAGE profile of purified wtCAF-1 ($M_w \sim 19.5$ kDa) expressed in *E. coli* BL21 (DE3) pLyS cells (A). Effect of Ca^{2+} binding on the electrophoretic mobility of CAF-1 (B). Lane 1 (wtCAF-1), lane 2 (1 mM CaCl_2), and lane 3 (2 mM EGTA), respectively.

Table 1. Thermodynamic parameters of thermal unfolding of wtCAF-1 in the presence and absence of Ca^{2+} - and Mg^{2+} -ions at pH 7.2.

Conditions	$T_m(^{\circ}\text{C})$	$\Delta H_m(\text{kcal mol}^{-1})$	$\Delta G(\text{kcal mol}^{-1})$
wtCAF-1	47	30.0 ± 0.8	-1.6
wtCAF-1+ Mg^{2+}	52	35.9 ± 0.4	-2.3
wtCAF-1+ Ca^{2+}	60	52.4 ± 0.8	-3.9

significant difference that correlates with the observed difference in the conformational stability of the protein in presence of the metal ions. Relatively small values of enthalpy variation, ΔH_m at T_m for wtCAF-1 in absence and presence of metal ions are comparable to the values reported for high affinity calcium-binding sites of TnC and CaM.⁴⁰ The value of ΔC_p was roughly estimated using model compound data by multiplying the number of residues with 12 cal/mol/degree.⁴¹ With the estimated ΔC_p value, the calculated free energy change (table 1), $\Delta G(T)$ at 25°C for apo-, Mg^{2+} -, and Ca^{2+} -bound protein are small and in good agreement with the low values of T_m . Evidently from the ΔH_m and $\Delta G(T)$ values, Ca^{2+} binding is more favourable towards wtCAF-1 stability than the Mg^{2+} binding. Thus, Ca^{2+} -bound protein is relatively more stable and active than the Mg^{2+} -bound and apo-wtCAF-1.

Figure 3 represents the GdnHCl-induced equilibrium unfolding of apo-wtCAF-1, monitored by change in fluorescence intensity at 305 nm and ellipticity at 222 nm. The data points obtained fit satisfactorily to a two-state model,^{30,31} and midpoint molar concentration, C_m of GdnHCl obtained from the CD and fluorescence spectroscopy data are 1.1 and 1.0 M, respectively. The C_m values from both, CD and fluorescence spectroscopies indicate co-operative unfolding of apo-wtCAF-1 with

Figure 3. GdnHCl-induced unfolding curves of apo-wtCAF-1 monitored by change in molar ellipticity at 222 nm (★) and change in intrinsic fluorescence at 305 nm (●) with protein concentrations of 10 and 7 μM , respectively.

increasing GdnHCl concentrations from 0–6 M. The resulting Gibb's free energies at zero GdnHCl concentration ΔG_{H_2O} are 2.6 ± 0.3 kcal/mol and 1.8 ± 0.2 kcal/mol from CD and fluorescence spectroscopies, respectively. These values are distinctly smaller than the reported values of metal-free Ca^{2+} -binding protein S100A2.⁴² The denaturant sensitivity factor, m values obtained from CD and fluorescence spectroscopy measurements are comparable, which is an indication of increase in co-operative folding in presence of the ions. Since value of m is an indication of the exposure of hydrophobic surfaces on unfolding, observed m value explains increased conformational stability of ion bound wtCAF-1 against GdnHCl.

The far-UVCD spectra of apo-wtCAF-1 (figure 4A) shows an intense positive peak at 195 nm and two negative peaks around 208 and 222 nm suggesting that the protein is predominantly an α -helical conformation protein. Presence of Ca^{2+} and Mg^{2+} ions increases the respective peak intensities, which indicates that the binding of Ca^{2+} and Mg^{2+} ions impart more α -helical conformation, thus enhancing the degree of folding. Further, binding of Mg^{2+} ion to the Ca^{2+} -bound protein increases the respective negative peaks, while binding of Ca^{2+} ion to the Mg^{2+} -bound protein decreases the respective negative peak intensities. This indicates that both the Ca^{2+} and Mg^{2+} ions have different binding affinities and both ions influence secondary structure of the protein differently.

WtCAF-1 contains seven tyrosine, seven phenylalanine and is devoid of tryptophan residues. In figure 4B, near-UV-CD spectrum of wtCAF-1 shows distinct bands at 276 nm and 255 nm, which are tyrosine and phenylalanine signals, respectively. These bands indicate that wtCAF-1 has a significant amount of tertiary structure in absence of the metal ions. Additionally, the shape of near-UV-CD spectrum of apo-wtCAF-1, especially at 276 nm, significantly increased by addition of Ca^{2+} and Mg^{2+} ions. Since near-UV-CD spectrum is sensitive to structural changes in the vicinity of aromatic amino acid side chains or to changes in the mobility of aromatic side chains, this result suggests that tertiary structure of wtCAF-1 is rearranged to more stable conformation upon metal ion binding. These

Figure 4. Conformational change in the wild-type protein upon Ca^{2+} and Mg^{2+} binding, monitored by CD spectroscopy. (A) Far-UV-CD spectra of apo protein (■); 0.5 mM Ca^{2+} -bound (●) and 1 mM Mg^{2+} -bound (▲); 1 mM Mg^{2+} binding to 0.5 mM Ca^{2+} -bound (▼) and 0.5 mM Ca^{2+} binding to 1 mM Mg^{2+} -bound wtCAF-1 (★). (B) Near-UV CD spectra of apo-wtCAF-1 (■); 0.5 mM Ca^{2+} -bound (●) and 1 mM Mg^{2+} -bound CAF-1 (★) at 25°C. Protein concentration was 50 μM for both the near and far UV-CD experiments.

significant changes both in secondary as well as in tertiary structure of wtCAF-1 rationalizes that the apo-wtCAF-1 has relatively loose and dynamic conformational assemblies that upon binding to metal ions attain more compact and rigid conformation.

Fluorescence emission spectrum of the apo-protein is dominated by the contribution of tyrosine residues with an emission maximum around 305 nm. In

Figure 5. Conformational changes of wtCAF-1 upon Ca^{2+} - and Mg^{2+} -binding monitored by fluorescence spectroscopy. Fluorescence emission spectra of apo-wtCAF-1 (■); 0.5 mM Ca^{2+} -bound (●); 1 mM Mg^{2+} -bound (▲); 1 mM Mg^{2+} binding to 0.5 mM Ca^{2+} -bound CAF-1 (▼) and 0.5 mM Ca^{2+} binding to 1 mM Mg^{2+} -bound CAF-1 (★).

figure 5, binding of Ca^{2+} ion to the apo-wtCAF-1 results in slight decrease of fluorescence intensity without any change in the emission maxima. This is in contrast to many CaBPs that upon binding to Ca^{2+} ion exposes hydrophobic patches, thereby tyrosine fluorescence intensity increases.⁴³ However, in presence of Mg^{2+} ion, fluorescence intensity increases slightly compared to the apo-protein. This could be due to the rearrangement of hydrophobic residues in the protein molecule. A trend of decrease in fluorescence intensity both for Ca^{2+} -binding to Mg^{2+} -bound and Mg^{2+} -binding to Ca^{2+} -bound form of proteins is observed. This change in tyrosine fluorescence reflects two possibilities, either a global structural change or a localized residue rearrangement around the metal-binding sites without changing the amino acid residues involved in the binding.

Extrinsic hydrophobic probe, 8-anilidonaphthalene-1-sulphonic acid (ANS) has been used to monitor the fluctuation in protein structures such as various unfolding events or functional and structural changes.⁴⁴ ANS fluorescence depends on polarity of the local environment; it exhibits lower fluorescence intensity in a polar environment than in the non-polar environment or upon binding to non-polar binding sites or cavities within the protein molecule. Fluorescence intensity of ANS excited at 370 nm yields a low intensity emission maximum around 520 nm (figure 6). Fluorescence intensity of ANS in presence of apo-wtCAF-1 is increased five-fold with peak maxima at ~ 505 nm,

Figure 6. Fluorescence emission spectra of hydrophobic probe ANS alone or in the presence of apo-, Ca^{2+} -bound and Mg^{2+} -bound wtCAF-1. Also shown are emission spectra of ANS in 6 M GdnHCl denatured apo-wtCAF-1. Protein and probe concentrations are $7 \mu\text{M}$ and $200 \mu\text{M}$, respectively, at an excitation wavelength of 370 nm and bandwidths of 5 nm from a range of 420 to 650 nm .

whereas ANS fluorescence intensity in presence of Ca^{2+} -bound wtCAF-1 protein increases only four-fold with a blue shift of 14 nm . Furthermore, the ANS fluorescence emission intensity in presence of Mg^{2+} -bound wtCAF-1 protein is less than the Ca^{2+} -bound protein, which in turn is less than the apo-protein, but higher than the ANS alone. ANS fluorescence intensity in presence of GdnHCl-induced unfolded apo-protein is significantly suppressed, demonstrating that binding of the hydrophobic probe requires a conformational rearrangement to provide a hydrophobic patch for ANS binding. Hydrophobic surface in apo-protein is more exposed to interact with ANS than the Ca^{2+} - and Mg^{2+} -bound protein. Interestingly, we observed that increase in ANS fluorescence is ligand-specific; Mg^{2+} -ion-bound protein provides lesser hydrophobic surfaces than the Ca^{2+} ion-bound protein. Thus, ANS binding data further support the spectroscopic data for specific binding of Ca^{2+} and Mg^{2+} ions to different extent, and strengthen the indication of conformational changes. The apo-wtCAF-1 has a more open conformational arrangement of hydrophobic surfaces than that of Ca^{2+} -bound form. Addition of Mg^{2+} induces further reduction in the exposure of hydrophobic surfaces, and induces relatively compact conformation. Thus, the binding affinity of Ca^{2+} and Mg^{2+} ions causes different conformational rearrangement in the protein.

ITC is used here to analyse the energetics of Ca^{2+} and Mg^{2+} ions binding to the wild-type and mutant proteins.

To evaluate the binding affinities of Ca^{2+} and Mg^{2+} ions to the apo-protein, a titration of either 2 mM CaCl_2 or 2 mM MgCl_2 against $50 \mu\text{M}$ apo-protein is performed and the data are fitted according to a model allowing for maximum co-operative binding (figure 7). Stoichiometry of Ca^{2+} binding is obtained by fitting the data points to the possible binding site models. We observed that the data could not be fitted with one, three or four binding sites, but is well fitted with two classes of binding sites with different affinities. From table 2, it is clear that both the binding sites of Ca^{2+} -ion to the protein are enthalpy- and entropy-driven processes. From the ITC data, binding of Ca^{2+} to apo-protein shows the association constants, K_A of $2.5 \times 10^4 \text{ M}^{-1}$ ($K_d = 40 \mu\text{M}$) and $1.8 \times 10^5 \text{ M}^{-1}$ ($K_d = 6 \mu\text{M}$) for first and second binding sites, respectively. Overall binding affinity of the wild-type protein molecule for Ca^{2+} binding, K_d is $15 \mu\text{M}$, which is comparable to the affinity reported for many Ca^{2+} -binding proteins. The Mg^{2+} ion titration isotherm that fitted to two classes of binding site models yields apparent K_d values of $20 \mu\text{M}$ for site 1 ($K_A = 5.1 \times 10^4 \text{ M}^{-1}$) and $9 \mu\text{M}$ for site 2 ($K_A = 1.5 \times 10^5 \text{ M}^{-1}$). Overall dissociation constant of Mg^{2+} ion binding to apo-protein is $12 \mu\text{M}$. As seen from table 2, specificity for Ca^{2+} ion binding over Mg^{2+} ion is reflected in the ratio of the overall binding affinity ($K_{d,\text{Ca}^{2+}}/K_{d,\text{Mg}^{2+}}$), which is greater than one-fold for the wild-type protein. The results given here suggest that the four EF-hand motifs of apo-wtCAF-1 bind both Ca^{2+} and Mg^{2+} ions with high affinity but to different magnitudes. However, it is required to distinguish the binding specificity of the four EF-hand motifs in the wtCAF-1 towards Ca^{2+} and Mg^{2+} ion binding.

Thermal unfolding of apo-wtCAF-1 and apo-EF-IV mutant is monitored by CD spectroscopy (figure 8). Change in molar ellipticity at 222 nm as a function of temperature is plotted to obtain the midpoint of the protein melting curves. The T_m values of 39°C obtained for apo-EF-IV mutant shows a decrease in thermal stability by 8°C compared to the apo-wtCAF-1 ($T_m = 47^\circ\text{C}$). Such decrease in thermal stability was also accompanied by decrease in enthalpy change, ΔH_m to $25.6 \pm 0.3 \text{ kcal/mol}$ from $30.0 \pm 0.8 \text{ kcal/mol}$, i.e., for apo-wtCAF-1. However, $\Delta G(T)$ values at 25°C for both apo-wtCAF-1 and mutant proteins are approximately the same. This suggests that the wild-type and mutant proteins fold co-operatively as seen from the melting profile, but with different extent of stability. The apo-wtCAF-1 is comparatively more stable than the EF-IV-mutant.

In figure 9, the far-UV-CD spectra of EF-IV mutant show increase in the α -helical content in the Ca^{2+} - and Mg^{2+} -bound mutant proteins. Binding of Ca^{2+} ion to

Figure 7. Effect of Ca^{2+} and Mg^{2+} binding to wtCAF-1 measured by ITC. (A) Titration of Ca^{2+} binding to apo-wtCAF-1, carried out with 2 mM CaCl_2 and protein concentration of 50 μM . Data were fitted to a two sets of site binding models. (B) Mg^{2+} titration to apo-protein and the data fitted to two sets of binding site models. Upper panel shows the raw data for heat released by the binding of Ca^{2+} and Mg^{2+} binding to wtCAF-1 expressed in microcalories per second versus time in minutes, while the lower panel shows peak integration for each injection expressed in kcal/mol of wtCAF-1 versus molar ratio of Ca^{2+} and Mg^{2+} binding to wtCAF-1 in solution.

Mg^{2+} -bound mutant protein shows a distinct increase in the molar ellipticity compared to the binding of Mg^{2+} ion to Ca^{2+} -bound mutant protein. Such comparable changes in the values of molar ellipticity demonstrate that the bound Mg^{2+} ion influences the intense binding of Ca^{2+} ion to the protein. Intrinsic fluorescence intensity decreases for the Ca^{2+} -ion-bound EF-IV mutant protein with respect to the EF-IV mutant apo-

protein. Mg^{2+} ion-bound protein has higher intrinsic fluorescence intensity than the Ca^{2+} -bound protein, but lesser than the mutant apo-protein. This could be due to the rearrangement of hydrophobic residues around the binding sites or the overall conformational rearrangement. Mg^{2+} binding to the Ca^{2+} -bound and Ca^{2+} binding to the Mg^{2+} -bound forms of EF-IV mutant protein experiment indicate that the metal-bound forms are

Table 2. Thermodynamic parameters of Ca^{2+} - and Mg^{2+} -binding to apo-wtCAF-1 determined by ITC.

Titration	Models	Sites	$K_A(\text{M}^{-1})$	$K_d^a(\mu\text{M})$	ΔH (kcal mol $^{-1}$)	$T\Delta S$ (kcal mol $^{-1}$)	ΔG^0 (kcal mol $^{-1}$)
Apo- Ca^{2+}	2 sets of sites	Site 1	$2.5 \times 10^4 \pm 1.3 \times 10^4$	15	-5.2 ± 0.1	-0.5	-4.7
		Site 2	$1.8 \times 10^5 \pm 0.3 \times 10^5$		-12.4 ± 1.1	-5.3	-7.2
Apo- Mg^{2+}	2 sets of sites	Site 1	$5.1 \times 10^4 \pm 1.2 \times 10^6$	12	-4.3 ± 0.4	-2.1	-6.4
		Site 2	$1.5 \times 10^5 \pm 1.3 \times 10^6$		-3.3 ± 1.0	-3.8	-7.0

^a $K_d = 1/\sqrt{K_1 K_2}$, where K_d represents the overall binding affinity. Mean values of K and ΔH (standard deviations shown in parenthesis) were determined from a nonlinear least-square fit of the heat exchanged per injection as a function of total injections using MicroCal Origin software.

Figure 8. Heat-induced unfolding curves of wild-type and EF-IV mutant proteins. Curves represent the melting profile of apo-wtCAF-1 (●) and apo-EF4 mutant protein (★) as a function of temperature.

already rearranged conformations, which do not allow further change in fluorescence intensity in presence of another metal ion.

Independent Ca^{2+} and Mg^{2+} ions binding to EF-IV mutant protein are performed using ITC as shown in figure 10. The ITC titration for Ca^{2+} binding in absence

Figure 9. Conformational changes induced upon Ca^{2+} and Mg^{2+} binding to EF-IV mutant protein. (A) Change in the far-UV-CD spectra of EF4 mutant protein upon Ca^{2+} and Mg^{2+} binding, protein only (■); Ca^{2+} -bound (●); Mg^{2+} -bound (▲); Ca^{2+} binding to Mg^{2+} -bound form (▼); and Mg^{2+} binding to Ca^{2+} -bound form (★). (B) Change in intrinsic fluorescence spectra of EF4 mutant protein upon Ca^{2+} and Mg^{2+} binding, apo-EF-IV mutant protein (■); 0.5 mM Ca^{2+} bound (●); 1 mM Mg^{2+} bound (▲); 1 mM Mg^{2+} binding to 0.5 mM Ca^{2+} -bound protein (▼) and 0.5 mM Ca^{2+} binding to 1 mM Mg^{2+} -bound protein (★). Protein concentrations of 10 and 7 μM were used for CD and fluorescence experiments, respectively.

Figure 10. Ca^{2+} and Mg^{2+} ions binding to the EF-IV mutant apo-CAF-1 by ITC. (A) Ca^{2+} ion titration to EF-IV mutant apo-protein. (B) Mg^{2+} ion titration to metal-free CAF-1 mutant. Protein concentration for both Ca^{2+} and Mg^{2+} ions titration is $100 \mu\text{M}$ and ligand concentrations are 2 mM CaCl_2 and 2 mM MgCl_2 for Ca^{2+} and Mg^{2+} ion titrations.

of Mg^{2+} ion is fitted to a one-site binding model, and binding parameters such as dissociation constant K_d of $1.3 \mu\text{M}$ ($K_A = 7.9 \pm 10^5 \text{ M}^{-1}$), corresponding binding enthalpy and free energy change obtained are listed in table 3. Mg^{2+} ion titration in the absence of Ca^{2+} ion to the mutant apo-protein exhibits a one-site binding isotherm with dissociation constant K_d of $7.7 \mu\text{M}$ ($K_A = 1.3 \pm 10^5 \text{ M}^{-1}$). Comparison of the dissociation constants shows that the protein has higher binding affinities for Ca^{2+} than for Mg^{2+} ion. Thus, the EF-IV mutant protein binds both Ca^{2+} and Mg^{2+} ion to different affinities with the EF-I, EF-II and EF-III hand motifs. Further investigation of the presence of specific binding sites for Ca^{2+} and Mg^{2+} ions in the protein is required.

4. Conclusion

The present study shows that apo-, Ca^{2+} - and Mg^{2+} -bound wtCAF-1 have substantially different protein conformations. Binding of Ca^{2+} and Mg^{2+} ion to the apo-wtCAF-1 stabilizes the protein structure, and shows different binding affinities with comparable dissociation constants. Furthermore, we observe that the presence of Mg^{2+} ion enhances the binding affinity for Ca^{2+} ion towards protein stability in the wild-type protein. The apo-wtCAF-1 and EF-IV mutant protein are distinct in their binding affinities and structural energies. Both the proteins show a similar cooperative unfolding mechanism. Since Ca^{2+} is a well-known secondary messenger in eukaryotic cells

Table 3. Ca^{2+} - and Mg^{2+} -binding to EF-hand IV disabled mutant of CAF-1 calculated by ITC.

Titration	Models	Sites	$K_A (\text{M}^{-1})$	$K_d^a (\mu\text{M})$	$\Delta H (\text{kcal mol}^{-1})$	$T\Delta S (\text{kcal mol}^{-1})$	$\Delta G^0 (\text{kcal mol}^{-1})$
Ca^{2+} vs. apo	1 set of sites	Site 1	$7.9 \times 10^5 \pm 0.2 \times 10^5$	1.3	-21.8 ± 2.1	-13.7	-8.1
Mg^{2+} vs. apo	1 set of sites	Site 1	$1.3 \times 10^5 \pm 0.5 \times 10^5$	7.7	-3.8 ± 0.4	2.8	-6.7

^a $K_d = 1/K_A$

that help in cellular development, the differential Ca^{2+} and Mg^{2+} ions binding to wtCAF-1 correlate with possible changes in *D. discoideum* development as the local metal ions environment changes around the wtCAF-1 protein.

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