Long-Range Effects on Calcium Binding and Conformational Change in the N-Domain of Calmodulin†

Abdessamad Ababou, Ryan A. Shenvi, and John R. Desjarlais*

Department of Chemistry, 408 Chandlee Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT: Proteins within the EF-hand protein family exhibit different conformational responses to Ca^{2+} binding. Calmodulin and other members of the EF-hand protein family undergo major changes in conformation upon binding Ca^{2+}. However, some EF-hand proteins, such as calbindin D9k (Clb), bind Ca^{2+} without a significant change in conformation. Here, we investigate the effects of replacement of a leucine at position 39 of the N-terminal domain of calmodulin (N-Cam) with a phenylalanine derived from Clb. This variant is studied alone and in the context of other mutations that affect the conformational properties of N-Cam. Strikingly, the introduction of Phe39, which is distant from the calcium binding sites, leads to a significant enhancement of Ca^{2+} binding affinity, even in the context of other mutations which trap the protein in the closed form. The results yield novel insights into the evolution of EF-hand proteins as calcium sensors versus calcium buffers.

The extraordinary ability of proteins to change conformation is an essential property of the function of a variety of proteins. An ability to understand and predict the presence and extent of conformational change in proteins is key to generating insight into the normal or pathological mechanisms of a wide variety of biological processes.

EF-hand proteins such as calmodulin are known to change conformation upon calcium (Ca^{2+}) binding and often are termed Ca^{2+}-sensor proteins (Figure 1). However, EF-hand proteins that appear to be Ca^{2+} buffering or transporter proteins, such as Clb,[1] exhibit only subtle changes in conformation (*1*−*3*). Despite the wealth of information about the structural differences in this large family (*1*−*13*), the fundamental reasons for (i) the disparate abilities of EF-hand proteins to change conformation and (ii) the striking differences of their Ca^{2+} affinities have yet to be fully elucidated.

We have recently demonstrated that the burial of polar groups in the closed form of N-Cam[1] serves to modulate the energetics of conformational change (*14*). Mutation of two key polar groups Gln41 and Lys75 in N-Cam to the corresponding nonpolar groups Leu and Ile from Clb resulted in the stabilization of the closed form of N-Cam such that it no longer changes conformation to the open form when saturated with Ca^{2+}. This result has shown clearly that the balance of solvation energetics between closed and open states is a fundamental determinant of conformational activity. However, trapping the protein in the closed state results in a significant decrease of its Ca^{2+} binding affinity (*14*, *15*). In the present work we focus on the effect of the amino acid at position 39, which is close in space to the side chains at positions 41 and 75, suggesting that it might also contribute to the energetics of conformational change. A structural alignment (*16*) between N-Cam (1cfd) (*17*) and Clb (1clb) (*7*) shows that the position of the Leu side chain found at position 39 in N-Cam is most closely represented by Phe36 in the Clb structure. An alignment of calmodulin-like domains reveals that Phe is in fact rare at this position. In addition, the calculated change in the nonpolar solvent-accessible surface area using an open form model of Clb (*14*) suggested that Phe at this position may affect the conformational and/or Ca^{2+} binding properties of a domain. In this work we show that the mutation from Leu to Phe at position 39 in N-Cam and a set of N-Cam variants significantly increases Ca^{2+} binding affinity regardless of the conformational activity of the domain. Furthermore, the L39F substitution perturbs the conformational equilibrium between calcium-saturated open and closed states.

MATERIALS AND METHODS

Protein Cloning and Purification. The isolated N-terminal domain of the recombinant human calmodulin used in this work (N-Cam), residues 1−78, was created by gene synthesis using standard techniques. Residues Ala1, Phe19, and Asp78 were changed to Met1 (for purposes of expression), Tyr19 (see below), and Tyr78, respectively, to yield the protein N-CamY, used as a wild-type reference for this study. The original Tyr78 mutation was introduced to facilitate protein quantitation by UV absorbance spectroscopy. An important mutation from Phe to Tyr was introduced at position 19 as described (*14*). This mutant is more spectroscopically active than N-Cam (fluorescence and near-UV CD) and has calcium binding and apo-state folding properties similar to those of N-Cam. Additional mutations L39F, Q41L, and K75I were made independently and in various combinations by cassette mutagenesis. The N-CamY protein and its variants were

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* Corresponding author. Phone: 814-865-4304. Fax: 814-863-8403. E-mail: jrd@chem.psu.edu.

[1] Abbreviations: N-Cam, N-terminal domain of calmodulin; N-CamY, Tyr19 mutant of N-Cam; Clb, calbindin D9k; 5NBAPTA, 5-nitro-1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ANS, 8-anilino-1-naphthalenesulfonic acid; GdmCl, guanidinium chloride.

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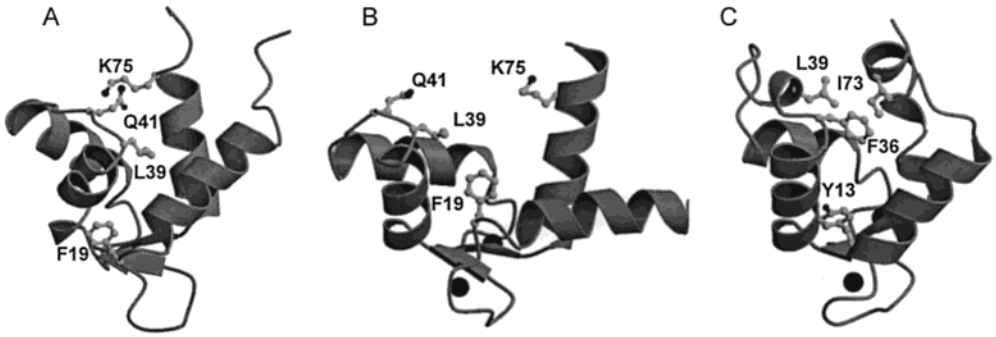

FIGURE 1: Ca^{2+}-induced conformational changes in the N-terminal domain of calmodulin. Ribbon diagrams show the differences in the conformations of (A) Ca^{2+}-free N-Cam (*17*), (B) Ca^{2+}-saturated N-Cam (*31*), and (C) Ca^{2+}-saturated Clb (*32*). Residues Leu39, Gln41, and Lys75 in N-Cam examined in this study, and their structural analogues Phe36, Leu38, and Ile73 in Clb, are displayed. Phe19 from N-Cam, which is replaced by Tyr in all variants studied here, is also highlighted. Black spheres represent the Ca^{2+} ions. Figures were made with MOLSCRIPT (*33*) and Raster 3d (*34*).

overexpressed in *Escherichia coli* Bl21(DE3) cells. The integrity of all of the clones was confirmed by DNA sequencing. The proteins are purified in two stages. The first purification consists of an anion-exchange column, following a published protocol (*18*). The second purification was achieved using reversed-phase HPLC, followed by extensive dialysis to reduce the residual Ca^{2+}. Mass spectrometric analysis of the proteins confirmed the purity and integrity of the protein samples. The purified proteins have also been checked by SDS gel electrophoresis. The concentrations of the proteins were determined in 6 M GdmCl using absorbance spectroscopy and a molar extinction coefficient of Tyr at 280 nm of 1420 M^{-1} cm^{-1}.

Chemical Materials. The chromophoric Ca^{2+} chelator 5-nitro-BAPTA (5NBAPTA) was purchased from Molecular Probes (Eugene, OR). The fluorometric hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS), GdmCl, and MOPS were purchased from Sigma (St. Louis, MO). Solvents for HPLC and other routine laboratory chemicals were of the highest grade commercially available.

Absorbance and Fluorescence Measurements. The Ca^{2+} binding assays using the binding competition between the chelator 5NBAPTA and the proteins (*19*) were carried out using the absorbance signal of the chelator at 430 nm. The absorbance measurements were conducted on a BioSpec-1601 (Shimadzu Scientific Instruments Inc., Columbia, MD). All measurements were performed using 37 μM protein and 25 μM 5NBAPTA, 100 mM KCl, and 20 mM MOPS, pH 7.2.

Fluorescence measurements were conducted on a Fluorolog-3 spectrofluorometer (Jobin Yvon−Spex, Edison, NJ), and the spectra were uncorrected from the photomultiplier response function. The excitation wavelength for ANS was 370 nm. The excitation and emission bandwidths were set at 4 nm, and all of the measurements were conducted at the temperature of 25 °C, using a Neslab water bath circulator (Neslab Instruments, Inc., Portsmouth, NH). All of the measurements were done using 25 μM protein and 5 μM ANS, 100 mM KCl, and 20 mM MOPS, pH 7.2. For Ca^{2+} free and saturated measurements, 0.1 mM EDTA and 1 mM $CaCl_2$ were used, respectively.

Circular Dichroism Measurements. CD spectra were collected on an Aviv Model 62DS CD spectrophotometer equipped with a thermoelectric cell holder and with a Hamilton microlab 500 series automatic titrator. Far-UV CD spectra were collected from 200 to 260 nm using a 1 mm cell path length and a 3 s averaging time.

Thermal unfolding data were collected at 222 nm in the temperature range of 2−96 °C. Samples were placed in a 2 mm cuvette and heated in increments of 2 °C, using an equilibration time of 2.5 min and a 30 s averaging time. The protein concentration was held constant at 25 μM.

Chemical unfolding at 25 °C with GdmCl was conducted by titrating an initial 2 mL of the folded protein placed in 1 × 1 cm cuvette containing a stir bar. CD spectra between 260 and 218 nm were collected with a 3 s averaging time at each denaturant concentration. The titration was performed by removing an aliquot of the folded protein solution and replacing the same volume from an unfolded protein stock prepared in buffer containing ~7.1 M GdmCl. The unfolded protein stock was loaded into the titrator. After each automated increase in the denaturant concentration, the sample was allowed to equilibrate for 2 min in the baseline regions and 3.5 min in the transition regions. The system was shown to be at equilibrium by obtaining identical results after doubling the equilibration time. All CD measurements were corrected by subtracting the buffer spectra. The GdmCl or urea stock solutions were prepared fresh daily, and the concentrations were calculated by their refractive index (*20*) using a Reichert-Jung ABBE MARK II digital refractometer. All experiments were carried out in 20 mM MOPS, pH 7.2, and 100 mM KCl.

Data Analysis. The macroscopic binding constants, K_1 and K_2, of the Ca^{2+} binding of the proteins were determined using the methodology described by Linse et al. (*19*). Data were fitted to single-site and two-site Adair functions describing the competition between 5NBAPTA and a two-site protein as described. Analysis was performed using a Fortran 90 program developed in this laboratory.

We determined the K_D of 5NBAPTA using its absorbance signal at 430 nm in 20 mM MOPS and 100 mM KCl, pH 7.2, buffer. The K_D obtained was 23.0 μM, in close agreement with previously reported values (*21*).

Errors estimates for log K_1, log K_2, and log K_1K_2 were determined by two methods. The first estimate was obtained directly as the standard deviation of fitted values derived from several independent titrations. The second estimate was based on a determination of a confidence interval for each parameter according to a single data set. In all cases, the variability of parameters determined from independent ti-

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        10        20        30        40        50        60        70
         •         •-        -   •       - •-        •         •         •
LTEEQIAEFKEAFSLFD-KDGDGT-ITTKELGTVMRS-LG-QNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMK  N-Cam
  MKSPEELKGIFEKYAAKEGDPNQLSKEELKLLLQTEFPSLLKGPSTLDELFEELDKNGDGEVSFEEFQVLVKKISQ  Clb
      EIREAFRVFD-KDGNGY-ISAAELRHVMTN-LG-EKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTA      C-cam
LSEEMIAEFKAAFDMFD-ADGGGD-ISTKELGTVMRM-LG-QNPTKEELDAIIEEVDEDGSGTIDFEEFLVMMVRQMK  N-TnC
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FIGURE 2: Alignment of EF-hand domain sequences: the N- and C-terminal domains of calmodulin (N-Cam, C-Cam), the regulatory domain of skeletal troponin C (N-tnC), and calbindin D9k (Clb). The numbering scheme is based on the full-length calmodulin sequence. The positions that are the focus of this work are highlighted in black.

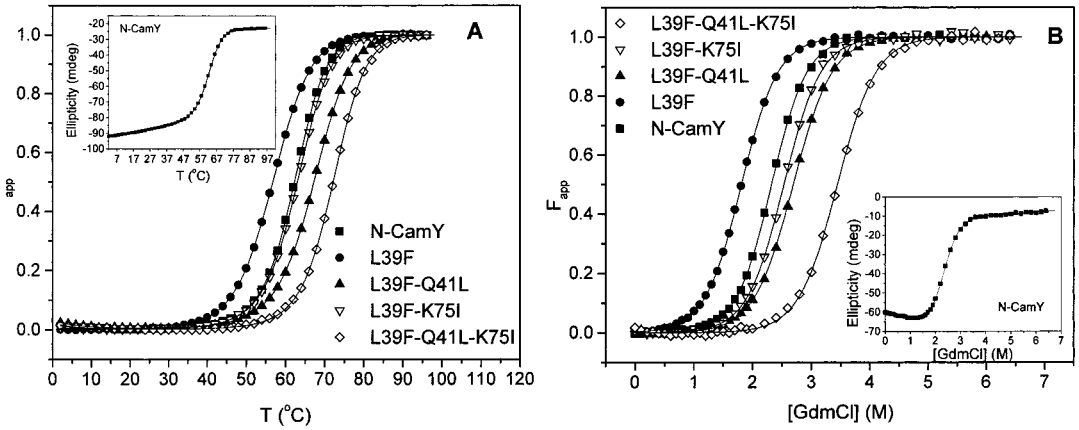

FIGURE 3: Fraction of unfolded protein, F_{app}, for apo-N-Cam, N-CamY, and the mutants as function of (A) temperature and (B) GdmCl concentration. The unfolding was monitored by far-UV CD. The mutants are identified by symbols in the figure insert.

trations exceeded that estimated by the confidence interval calculation. The first (larger) error estimate is thus reported in Table 2.

The unfolding experiments were fitted to a two-state model. Hence, the CD unfolding data were fitted to

$$S = S_N + (S_U - S_N)/[1 + \exp(+\Delta G_{NU}/RT)]$$

where S is the observed signal and S_N and S_U are the CD signals for the native and the unfolded protein, respectively. ΔG_{NU} is the free energy difference of the unfolding reaction. Both the S_N and S_U were assumed to depend linearly on the temperature and the denaturant concentration and to retain this linearity in the transition region.

For the thermal unfolding we assume that the heat capacity, ΔC_p^0, is temperature independent in the range of measurement (*22*). Hence, the free energy of unfolding is

$$\Delta G_{NU}(T) = \Delta H_m + \Delta C_p^0(T - T_m) - T[\Delta H_m/T_m + \Delta C_p^0 \ln(T/T_m)]$$

where T_m is the melting temperature and ΔH_m is the enthalpy difference at T_m.

For the chemical unfolding, using the linear extrapolation model (*23*), the free energy of unfolding is

$$\Delta G_{NU} = \Delta G_{NU}^\circ - m_D[\text{denaturant}]$$

where ΔG_{NU}° is the free energy difference in the absence of denaturant and m_D reflects the sensitivity of ΔG_{NU} to the denaturant concentration. The denaturant concentration at which the protein is 50% unfolded ($\Delta G_{NU} = 0$) is given by C_m.

Denaturation data were fit using the program ORIGIN (Microcal Software, Inc., 1997; Microcal Software, Inc., Northampton, MA). The errors are calculated as the confidence intervals at 68.3%.

RESULTS

Identification of Important Residues. Figure 2 shows a structure-based alignment of N-Cam and Clb. Positions 41 and 75 of the N-Cam protein contain the polar amino acids Gln and Lys while Clb contains the nonpolar residues Leu and Ile at structurally analogous positions. While many EF-hand domains that change conformation tend to contain a Leu at position 39, Clb, which does not change conformation, contains a Phe (Figure 2). Although the residue at position 39 is nonpolar in both proteins, the larger size of Phe versus Leu and its spatial proximity to other positions that influence conformational activity encouraged an investigation of its effects on the properties of N-Cam.

To investigate the importance of the side chain at position 39 on the properties of N-Cam, a variant of N-CamY (*14*) containing the L39F mutation was produced. In addition, the L39F mutation was also made in the context of mutations Q41L, K75I, and Q41L-K75I, substitutions that dramatically alter the conformational equilibrium of the calcium-saturated state.

Stability of Calcium-Free Proteins. Folding stabilities of the apo state of N-CamY and its L39F variants were monitored by thermal and chemical denaturation (GdmCl) using circular dichroism (CD) to monitor the extent of unfolding. The results from the two experiments reveal the same trends in stability, as seen in Figure 3. The L39F mutation destabilizes the apo state of N-CamY. The combination of L39F with the single mutants, Q41L and K75I,

Table 1: Thermodynamic Parameters for Unfolding Experiments of the Apoproteins

	thermal unfolding			chemical unfolding			
protein	ΔH_m (kcal/mol)[a]	T_m (°C)[a]	ΔC_p^0 [kcal/(mol K)][b]	m_{GdmCl} [kcal/(mol M)][a]	C_m (M)	$\Delta G°_{NU}$ (kcal/mol)[a]	$\Delta\Delta G°_{NU}$ (kcal/mol)
N-CamY	53.9 ± 3.4	62.3 ± 0.4	1.3	2.0 ± 0.1	2.3	4.6 ± 0.3	
L39F	44.4 ± 2.1	56.8 ± 0.3	0.7	1.9 ± 0.1	1.8	3.5 ± 0.2	−1.1
L39F-Q41L	47.9 ± 4.4	67.3 ± 0.9	1.1	1.8 ± 0.1	2.7	4.9 ± 0.3	0.3
L39F-K75I	51.5 ± 3.2	63.1 ± 0.4	1.1	1.9 ± 0.2	2.5	4.8 ± 0.5	0.2
L39F-Q41L-K75I	55.9 ± 5.9	72.2 ± 1.1	1.2	1.8 ± 0.2	3.4	6.2 ± 0.9	1.6

[a] Data were well fitted to a two-state model, and the errors are given as ±confidence intervals at the 68.3% level. [b] These values are poorly determined and are listed for completeness only.

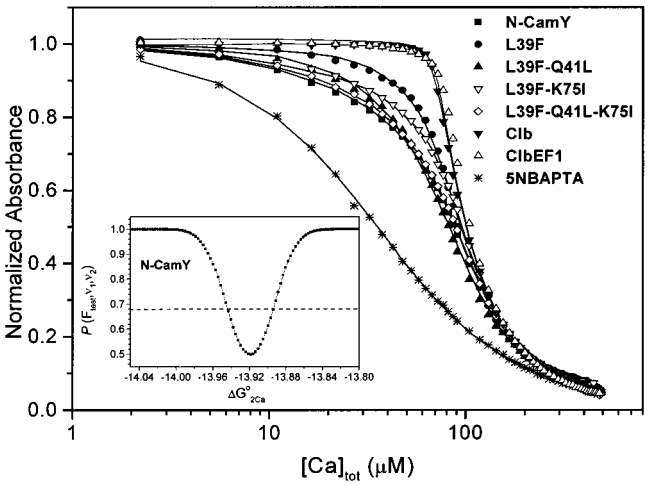

FIGURE 4: Calcium binding assays. The normalized Ca^{2+} competition curves of N-CamY and its mutants, as well as Clb and ClbEF1, are shown. Calcium affinity is measured by competition between the protein and 5NBAPTA (5-nitro-BAPTA), monitored by the absorbance of 5NBAPTA at 430 nm. The symbols are the experimental data and the curves through the points are the least-squares fits. Binding parameters determined from the fits are listed in Table 2. The inset shows the calculated confidence interval (*35*) for $\Delta G°_{2Ca}$ from a representative titration of N-CamY.

and the double mutant, Q41L-K75I, keep nearly the same trend in the apo-state stability as was shown previously (*14*). Indeed, the highest stability is found for the triple mutant L39F-Q41L-K75I, which has an increase of T_m of about 10 °C relative to N-CamY and an increase in C_m of 1.1 M. Smaller increases in stability are also observed for the mutants L39F-Q41L and L39F-K75I, relative to N-CamY.

Chemical denaturation data using GdmCl fit well with a two-state model as shown in Figure 3B (see Materials and Methods). The free energies of unfolding in the absence of denaturant, $\Delta G°_{NU}$, the transition midpoint, C_m, and the change in free energy, $\Delta\Delta G°_{NU}$, relative to N-CamY, are summarized in Table 1. The triple mutant L39F-Q41L-K75I is the most significantly stabilized variant with an increase in unfolding free energy of 1.6 kcal/mol relative to N-CamY. It is worth noting that the mutants show a systematic decrease in their apo-state stability with introduction of the L39F mutation. The effect of the L39F mutation is approximately additive.

Calcium Binding Affinities. The titrations of N-CamY and all mutants with Ca^{2+} in the presence of the chromophoric chelator 5-nitro-BAPTA (5NBAPTA) are shown in Figure 4. A clear competition for Ca^{2+} binding between the protein and the 5NBAPTA is observed in all cases, indicating that all of the variants retain significant affinity for Ca^{2+}. The L39F has a higher affinity for Ca^{2+} than N-CamY, which is manifested by the stronger competition for Ca^{2+} binding with the 5NBAPTA than N-CamY. The macroscopic Ca^{2+} binding constants for all of the mutants are summarized in Table 2. We have shown that the substitution of buried polar groups (Q41, K75) with nonpolar groups (L41, I75) leads to a decrease in Ca^{2+} binding affinity, consistent with the stabilization of the closed state relative to the open state, and that the double mutant Q41L-K75I has the lowest Ca^{2+} affinity, with a Ca^{2+} binding free energy of −12.6 kcal/mol (*14*). The introduction of L39F in several variants results in increases of at least 10-fold in Ca^{2+} binding affinity (only 2-fold in the case of Q41L). Indeed, the introduction of L39F in the Q41L-K75I mutant has decreased its Ca^{2+} binding free energy by −1.5 kcal/mol.

Characterization of Conformational Changes. A useful probe of conformational change in calmodulin-like proteins is the Ca^{2+}-dependent binding of the hydrophobic fluorophore ANS (*24*). The fluorescence emission of ANS in the presence of the apoproteins was compared to that in the presence of the holoproteins. Table 2 summarizes the observed changes in fluorescence intensity and maximum wavelength of emission (λ_{max}). In the case of N-CamY the ANS fluorescence shows a large increase in intensity and a significant decrease of λ_{max} in the presence of Ca^{2+} (Figure 5). The L39F and L39F-Q41L variants, however, show a smaller increase in intensity. The effects of the Q41L and L39F mutations on ANS binding are not additive: the ANS fluorescence is more intense for the Q41L-L39F double mutant than for either mutation alone. Finally, the L39F-K75I and L39F-Q41L-K75I mutants show very little change in the ANS fluorescence intensity with addition of Ca^{2+}. Although ANS binding is a qualitative criterion, the data suggest that these variants no longer adopt an open state structure.

The near-UV CD spectrum of N-CamY and its variants is an alternative indicator of the open/closed equilibrium of the protein (*14*). Consistent with expectations of a closed form, all apoproteins have identical spectral shapes and, within the limits of experimental error, identical extremum values (Figure 6A). Two minima at 263 and 270 nm characteristic of phenylalanine spectra are prominent. For a subset of the mutants, the addition of saturating concentrations of Ca^{2+} results in the rise of a broad minimum between 275 and 290 nm that is characteristic of tyrosines (Figure 6B). Control experiments with the original N-Cam construct (data not shown), which lacks Tyr19, indicate that the calcium-dependent change in the near-UV CD signal is primarily caused by the change in environment of Tyr19 (Figure 1A,B). Such a change is expected from the experi-

Table 2: Calcium Binding Parameters and Fluorescence Spectral Changes of ANS in the Presence of Apo- and Holoproteins

protein	log K_1[a]	log K_2[a]	log K_1K_2	$\Delta G°_{2Ca}$ (kcal/mol)[a]	ESS[b]	$\Delta\lambda_{max}$[c]	$I_{max}(+Ca^{2+})/I_{max}(-Ca^{2+})$
N-CamY	4.97	5.17	10.14	−13.9	$1.27\ 10^{-5}$	27	4.5
L39F	5.94	5.30	11.25	−15.4	$1.52\ 10^{-4}$	23	4.0
L39F-Q41L	5.55	4.64	10.19	−14.0	$9.66\ 10^{-5}$	19	4.1
L39F-K75I	4.99	5.64	10.63	−14.6	$6.20\ 10^{-5}$	5	1.4
L39F-Q41L-K75I	5.08	5.15	10.23	−14.1	$1.49\ 10^{-5}$	2	1.4
Clb	7.28 ± 0.17	6.51	13.79	−18.9 ± 0.3	$8.10\ 10^{-5}$	2	1.0
ClbEF1	6.79 ± 0.18	6.65	13.44	−18.4 ± 0.3	$1.26\ 10^{-4}$	1	1.1

[a] K_1 and K_2 are in molar concentration units. [b] Error squared sum of the fit (see Materials and Methods). [c] Difference of λ_{max} of ANS spectra in the presence of apo- and holoproteins. The estimated uncertainties (see Materials and Methods) are ±0.10−0.15 in log K_1 and log K_2 and ±0.15−0.20 in $\Delta G°_{2Ca}$, unless otherwise stated. Clb is calbindin D9k and ClbEF1 is Clb with a canonical EF-hand instead of the pseudo-EF-hand.

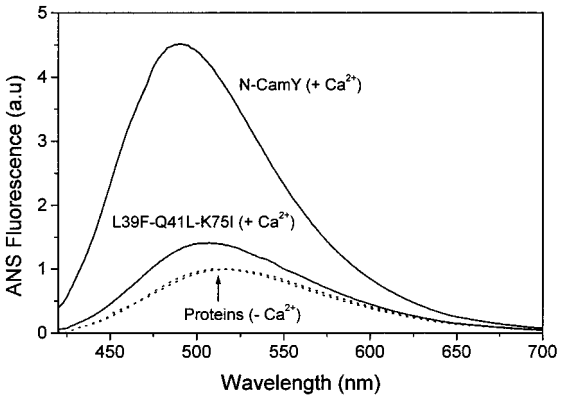

FIGURE 5: Conformational changes measured by ANS binding. Fluorescence emission spectra of ANS in the presence of protein as illustrated in the case of N-CamY and L39F-Q41L-K75I, in the absence of Ca^{2+} (dashed line), normalized to unity, and its relative fluorescence enhancement in the presence of Ca^{2+} (solid line). Table 2 lists the relative ANS intensity for all of the variants in the absence or presence of Ca^{2+}.

mentally determined structure of Ca^{2+}-saturated calmodulin (*25*) and NMR measurements (*14*). The effect is strongest in the case of N-CamY, which is assumed to exist predominantly in the open form when saturated with calcium. The L39F and L39F-Q41L variants have a significantly reduced spectral change, suggesting that their calcium-saturated forms populate the closed and open states in roughly equal proportions. The effects of the Q41L and L39F are again seen to be nonadditive: while L39F alone perturbs the calcium-saturated equilibrium toward a closed form, it appears to have the opposite effect in the context of the Q41L mutation. Finally, the spectral change is seen only weakly in the case of L39F-K75I and is completely absent in the case of the triple mutant L39F-Q41L-K75I. These spectra suggest clearly that these last variants are trapped in a closed structure.

Importantly, the results from the ANS fluorescence and near-UV CD directly parallel each other. The triple mutant L39F-Q41L-K75I loses ANS binding capacity and has no near-UV spectral change, strongly suggesting that it no longer adopts an open state structure. However, as we have shown for the variant Q41L-K75I (*14*), the triple mutant clearly binds Ca^{2+} (Figure 4) and, furthermore, with slightly higher affinity than N-CamY.

DISCUSSION

Our long-term objective is to understand the determinants of conformational change in calcium binding proteins. Several hypotheses exist regarding these determinants. First, it is expected that a significantly higher affinity of calcium for the open state is the driving force for conformational change. The driving force must be significant as the exposure of a large nonpolar surface upon opening the structure is unfavorable. Consistent with these ideas, mutations that trap the N-terminal domain of calmodulin in the closed form significantly reduce calcium binding affinity (*15*). However, these hypotheses raise additional questions regarding the mechanism by which Clb, which always exists in a closed conformation, maintains a high affinity for calcium. One possibility is that Clb accomplishes this by utilizing a pseudo-EF-hand to bind calcium, since the pseudo-EF-hand appears to create a preformed calcium site in the apo state (*1*).

We recently demonstrated that a precise balance of solvation energetics is also important for conformational change (*14*). Perturbations of this balance led to deficiencies in the ability to change conformation and a reduction in Ca^{2+} binding affinity. Indeed, for many variants, a correlation is observed between the Ca^{2+} binding and unfolding free energies. However, as observed for the double mutant Q41L-K75I, if the protein does not change conformation, the Ca^{2+} binding free energy no longer increases with increased folding stability, because the intrinsic Ca^{2+} affinity for the closed state has been reached.

In this work, biophysical analysis of the same variants together with the L39F mutation indicates that the introduction of Phe at position 39 decreases their apo-state stability relative to the unfolded state in an additive way, with the exception of the Q41L context. All of the variants with the L39F mutation also showed an increase of their Ca^{2+} affinity relative to the variants without the L39F mutation, in most cases leading to affinity increases greater than 10-fold. The experimental results indicate a trend in which the decrease in unfolding free energy is similar in magnitude to the decrease in calcium binding free energy (Figure 7). The L39F and Q41L mutations do not have additive effects on these properties or others. This nonadditivity is not surprising, given the spatial proximity of these side chains in the structure of N-Cam.

Effects of the L39F mutation on the open/closed equilibrium of the calcium-saturated proteins are also potentially additive, again with the exception of the Q41L variant. In the case of N-CamY itself, the L39F mutation leads to an experimentally observable shift in the equilibrium toward the closed form. Such an effect implies that the free energy of the calcium-saturated open form has increased relative to the closed form. Consistent with this, the L39F mutation leads to no observable change in the behavior of the K75I

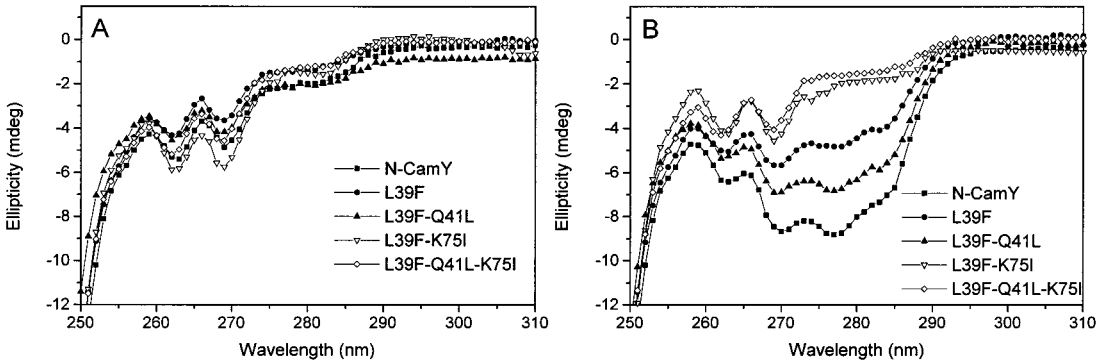

FIGURE 6: Conformational changes measured by near-UV CD spectra. Spectra of all of the variants were collected with (A) 0.1 mM EDTA or (B) 1 mM $CaCl_2$.

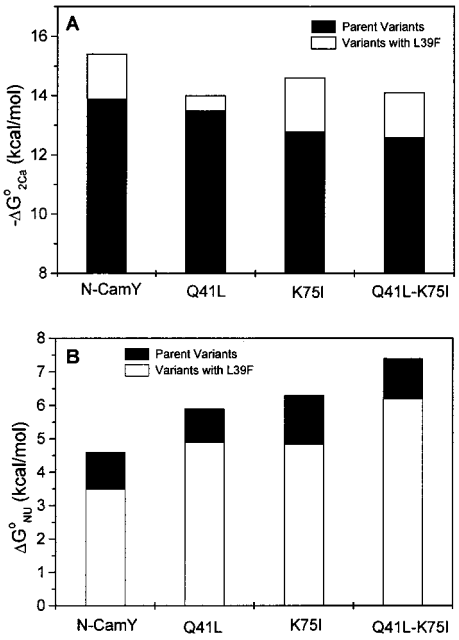

FIGURE 7: Additive effects of the L39F mutation. (A) Changes in calcium binding free energy due to the L39F mutation in the context of several variants of N-CamY. (B) Changes in unfolding free energy due to the L39F mutation.

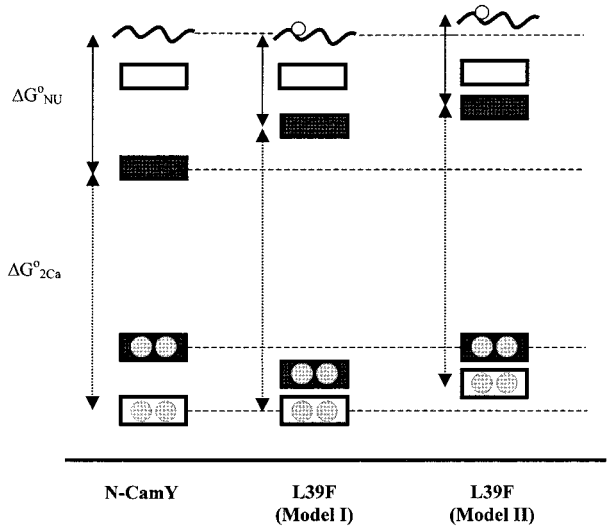

FIGURE 8: Possible models for free energy changes due to L39F mutations. Both models satisfy the condition that changes in calcium binding and unfolding free energies are opposite in direction but approximately equal in magnitude, and a shift in the equilibrium of the calcium-saturated domain toward the closed form. Filled symbols represent closed forms of the domain, and open symbols represent open forms. The unfolded state is represented by a squiggly line. Filled circles represent bound calcium ions. The circle in the unfolded state cartoon indicates the mutation L39F. Intermediate states in which a single calcium ion is bound are not shown.

or Q41L-K75I variants. Since these variants already favor a closed form, a relative increase in the free energy of the open state will serve to shift the equilibrium further toward the closed form. Because the experimental observables (ANS binding and near-UV CD) of a closed form are close to saturation for the parent variants, further perturbation of the equilibrium by L39F is assumed but experimentally not detectable.

In summary, the overall trend of the L39F mutations is an increase in calcium binding affinity, a decrease in the free energy required to unfold the apoprotein, and an apparent shift in the calcium-saturated equilibrium toward the closed form. Two simple free energy models are consistent with these observations, as shown in Figure 8. The first is an increase in the free energy of the apo/closed form and a decrease in the free energy of the calcium-saturated closed form. The second is an increase in the free energy of the apo/closed form combined with an increase in the free energy of the calcium-saturated open form. However, more complex models cannot be excluded.

Several observations from our studies are conspicuous. First, the L39F mutation alone increases the calcium binding affinity by roughly 13-fold relative to N-CamY. This effect correlates with a destabilization of the apo state relative to the unfolded state. Notably, the L39F mutation has a similar effect on calcium affinity in the context of the Q41L-K75I variant, despite the fact that the L39F-Q41L-K75I triple mutant remains in the closed form when saturated with calcium. This result, combined with the overall additivity of L39F effects, strongly suggests that the increased calcium affinity in these proteins stems directly from an increase in calcium affinity of the closed form of the domain, as in model I of Figure 8. Remarkably, the triple mutant has calcium affinity similar to that of N-CamY, demonstrating that the closed form is not inherently incompatible with high-affinity calcium binding. These observations are consistent with the idea that conformationally active EF-hand domains such as N-Cam have evolved to specifically decrease the calcium binding affinity of the closed form.

A comparison of the calcium binding free energies with unfolding free energies determined for the calcium-free state of each variant provides further insight into the behavior of

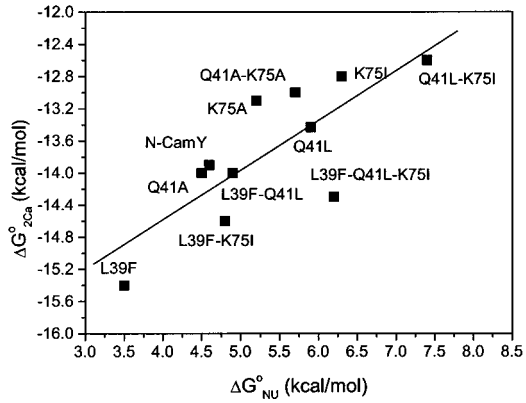

FIGURE 9: Relationship between calcium binding and unfolding free energies.

these proteins. The L39F variant has a roughly additive effect on the unfolding free energies, as shown in Figure 7B. Interestingly, there is a reasonable overall correlation between the unfolding free energy of each protein and its calcium binding free energy (Figure 9). As discussed previously (*14*), a correlation makes sense for mutants that have related effects on the free energies of the open and unfolded states and for which a conformational change still exists. Such mutants include many of those examined in our previous study, which perturb the extent of nonpolar surface exposure in the open and unfolded states. For other variants, such as those including the L39F mutation, a correlation is also expected when perturbations of calcium binding affinity are due to an alteration of the free energy of the apo/closed state of the protein, as in Figure 8. The offset seen in Figure 9 between the L39F variants and the other variants is consistent with a different mechanism for the relationship between calcium binding and unfolding free energies.

A model that explains the increased affinity of L39F variants for calcium is difficult to construct. The site of mutation is at least 15 Å from the calcium binding sites, so the effect is long range. The simplest explanation is that the larger side chain cannot readily be accommodated in the standard closed form structure, and an ensuing perturbation of the structure exacerbates the unfavorable apposition of the calcium ligands. Alternatively, the mutation somehow changes the dynamics of the overall system so that calcium binding is more favorable. An effect of EF-hand dynamics has been speculated to be important to the high affinity of Clb and a canonical EF-hand variant of Clb (*26*), suggesting that a preordered calcium binding loop is a key determinant of calcium affinity, regardless of the nature of the loop.

Long-range effects on calcium binding affinity have been reported in previous studies. The Coulombic influence of charged side chains distant from the calcium binding sites was demonstrated in an early study on the calcium binding properties of Clb (*27*). The importance of proper packing of core side chains, at some distance from the calcium binding sites, has been demonstrated with a series of volume reduction mutations in the core of Clb. The mutations generally lead to a decrease in calcium affinity (*28*). The 10-fold (or greater) increase in calcium affinity observed for L39F mutants described here is significant and more rare. However, other examples of mutations that increase calcium affinity have been reported. The most dramatic example is a F66W mutation in Clb that results in a 25-fold increase in calcium affinity (*10*, *28*). As is the case here, a rationalization of the increase is not obvious. Such mutations serve to remind us that the calcium affinities of natural proteins are precisely tuned by evolution for proper functioning of the protein under appropriate conditions, suggesting perhaps that the ease with which affinity-perturbing mutations are discovered should not be surprising.

Our results raise some interesting questions regarding the mechanism of conformational change in EF-hand proteins. The L39F mutation appears to increase calcium affinity for the closed form of the N-terminal domain of calmodulin, at the same time perturbing the equilibrium of calcium-saturated conformations. Clb is another EF-hand protein that remains in the closed form yet has high affinity for calcium. However, Clb contains an N-terminal pseudo-EF-hand, potentially explaining its higher calcium affinity. A detailed structural analysis of apo and holo forms of Clb has been reported and revealed that the pseudo-EF-hand is preformed for binding Ca^{2+} in comparison with a canonical EF-hand (*1*). To investigate the importance of the pseudo-EF-hand in conferring high-affinity calcium binding to the closed form, we replaced the pseudo-EF-hand of Clb with the entire canonical EF-hand from N-Cam to obtain a Clb with two canonical EF-hands (ClbEF1). Strikingly, the Ca^{2+} affinities of ClbEF1 and Clb are found to be similar, and ClbEF1 shows no conformational change upon Ca^{2+} binding (Table 2). This is in good agreement with an earlier report on the deletion of two amino acids from the pseudo-EF-hand of Clb to generate a canonical EF-hand (*29*, *30*). Hence, the combination of a closed form domain and a canonical EF-hand is not inherently incompatible with high-affinity calcium binding.

CONCLUSION

Our results indicate that calcium affinity and conformational equilibrium are complex traits that can be dramatically influenced by residues distant from the binding sites. The mechanism for the increased affinity is unclear. However, our data suggest that, for the variants studied herein, the increase involves the closed form of the domain.

The determinants of conformational change in EF-hand proteins are presumably numerous. Our results on a set of L39F variants, together with a canonical EF-hand variant of Clb, suggest that conformationally active EF-hand proteins must constrain the domain so as to reduce calcium binding affinity of the closed form while retaining increased affinity for the open form. Future studies might benefit from a focus on which elements of N-Cam and other domains serve to prevent a closed domain from having high affinity for calcium.

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