Solvation energetics and conformational change in EF-hand proteins

ABDESSAMAD ABABOU AND JOHN R. DESJARLAIS

Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802, USA

Abstract

Calmodulin and other members of the EF-hand protein family are known to undergo major changes in conformation upon binding Ca^{2+}. However, some EF-hand proteins, such as calbindin D9k, bind Ca^{2+} without a significant change in conformation. Here, we show the importance of a precise balance of solvation energetics to conformational change, using mutational analysis of partially buried polar groups in the N-terminal domain of calmodulin (N-cam). Several variants were characterized using fluorescence, circular dichroism, and NMR spectroscopy. Strikingly, the replacement of polar side chains glutamine and lysine at positions 41 and 75 with nonpolar side chains leads to dramatic enhancement of the stability of the Ca^{2+}-free state, a corresponding decrease in Ca^{2+}-binding affinity, and an apparent loss of ability to change conformation to the open form. The results suggest a paradigm for conformational change in which energetic strain is accumulated in one state in order to modulate the energetics of change to the alternative state.

Keywords: Conformational change; EF-hand; calmodulin; calbindin; solvation; buried polar; calcium

Conformational changes are intrinsic to the function of a variety of proteins. The changes in structure are typically triggered by a change in environment such as pH, phosphorylation state, or ligand-binding status (Schachman 1987; Ackers et al. 1992; Bullough et al. 1994; Ikura 1996). EF-hand proteins such as calmodulin are known to change conformation upon calcium (Ca^{2+}) binding and often are termed Ca^{2+}-sensor proteins. However, EF-hand proteins that appear to be Ca^{2+}-buffering or -transporter proteins, such as calbindin D9k, exhibit only subtle changes in conformation (Skelton et al. 1994; Chazin 1995; Ikura 1996). A number of three-dimensional structures of EF-hand proteins have been determined in the Ca^{2+}-saturated and -free forms (Herzberg and James 1985; Flaherty et al. 1993; Kordel et al. 1993; Gagne et al. 1995; Kuboniwa et al. 1995; Skelton et al. 1995; Slupsky and Sykes 1995; Drohat et al. 1996, 1998; Sastry et al. 1998). The recent progress of atomic-resolution structural analysis has provided valuable infor-

mation about Ca^{2+}-induced conformational change in EF-hand proteins.

Several groups have made important contributions in this direction through structure determination, mutational and structural analysis, and comparative studies (Skelton et al. 1994, 1995; Chazin 1995; Ikura 1995, 1966; Slupsky and Sykes 1995; Biekofsky et al. 1998; Julenius et al. 1998; Nelson and Chazin 1998a,b). Other studies have focused on individual Ca^{2+}-ligating residues that have key roles in transducing the Ca^{2+}-binding event into structural change (Evenas et al. 1997, 1998; Gagne et al. 1997). Despite this wealth of information about the structural differences in this family, the fundamental reasons for the disparate abilities of EF-hand proteins to change conformation have yet to be fully elucidated.

In calmodulin-like Ca^{2+}-sensor proteins, the change in structure to the activated open-state (Fig. 1) results in the energetically unfavorable exposure of a large nonpolar surface. The increased affinity of Ca^{2+} for the open state provides the essential driving force to overcome this significant difference in energy. However, other factors may also make crucial contributions to the balance of energetics between structural forms. We used structures of the N-terminal domain of calmodulin (N-cam) in its Ca^{2+}-free and -saturated
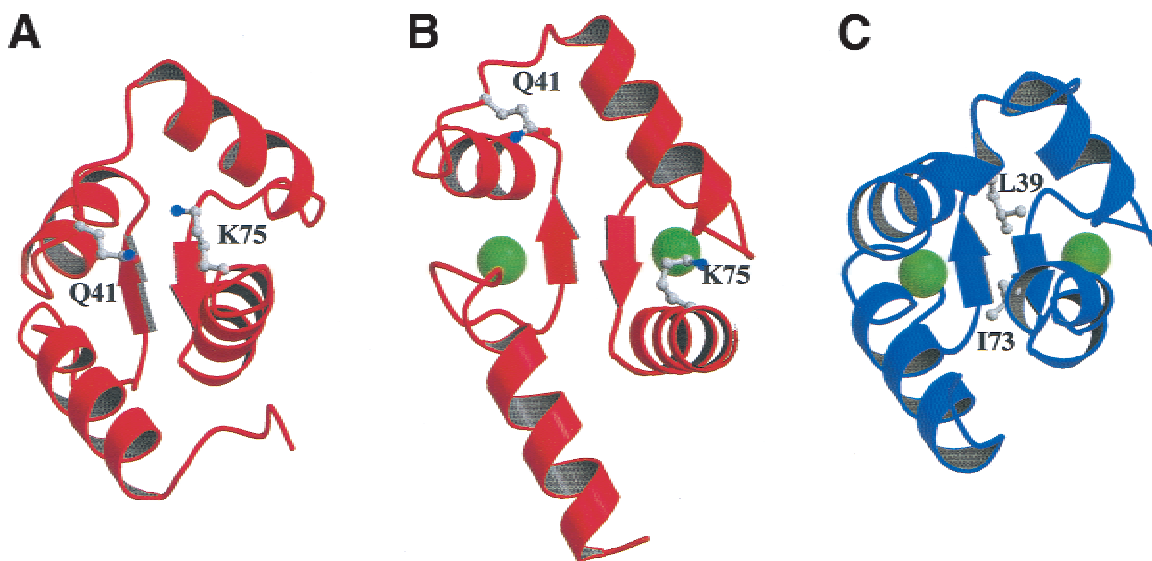
Fig. 1. Ca^{2+}-induced conformational changes in the N-terminal domain of calmodulin. (*A*) Ribbon diagrams show the differences in the conformations of Ca^{2+}-free N-Cam (Kuboniwa et al. 1995); (*B*) Ca^{2+}-saturated N-cam (Chattopadhyaya et al. 1992); and (*C*) Ca^{2+}-saturated calbindin D9k (Svensson et al. 1992). The residues Gln41 and Lys75 in N-Cam examined in this study, as well as Leu38 and Ile73 in calbindin D9k, are displayed. Green spheres represent the Ca^{2+} ions. The figures were made with MOLSCRIPT (Kraulis 1991) and Raster 3D (Merrit and Bacon 1997).

forms, the structure of calbindin in its Ca^{2+}-free form, and a model of calbindin in a hypothetical open-state structure to estimate the contributions of individual amino acids to the differential solvation energetics of conformational change. Based on the analysis, we hypothesized that the burial of polar groups in the closed form of N-cam serves to modulate the energetics of conformational change.

Consistent with this hypothesis, mutation of two key polar groups at positions 41 and 75 in N-cam to the corresponding nonpolar groups from calbindin resulted in the stabilization of the closed form of calmodulin, such that it no longer changes conformation to the open form when saturated with Ca^{2+}.

Results

Partial burial of polar groups in the closed form of calmodulin

Figure 2A shows an alignment of several EF-hand domain sequences, including N-cam. Interestingly, positions 41 and 75 of the N-cam protein contain the polar amino acids Gln and Lys, whereas calbindin contains the nonpolar residues Leu and Ile at structurally equivalent positions. Other EF-hand domains that change conformation also tend to contain polar groups at these positions. Observation of the structural orientation of these residues in N-cam reveals that they are partially buried at the top of the cleft of the domain, as shown in Figure 2B.

The extent of burial of the glutamine and lysine functional groups at positions 41 and 75 in N-cam is unexpectedly high and energetically unfavorable. Surface-area calculations performed on the deposited ensemble of NMR structures (1cfc; Kuboniwa et al. 1995) reveal that the extent of burial varies widely within the ensemble. In some of the structures, the burial is complete, whereas in others it is modest. The expected unfavorable energetics could stem directly from the desolvation cost of the polar moieties or indirectly through the reduction in conformational entropy associated with an inability to solvate a large number of side-chain configurations. In any event, their presence may have an important contribution to the balance of energetics involved in the ability of N-cam to change conformation, particularly because they become fully exposed to solvent in the open-state structure (Fig. 1B).

Our first indication of the presence of partially buried polar groups in N-cam came from analysis of the extent of nonpolar surface area exposed when changing conformation. Changes in nonpolar accessible surface area calculated from structures of N-cam in its Ca^{2+}-free (Kuboniwa et al. 1995) and -saturated states (Babu et al. 1988) revealed a significant increase at several positions.

Comparison of these changes to those expected for a similar conformational change in calbindin (see Materials and Methods) reveals some disparity between the two proteins at a number of positions, as shown in Figure 3. In particular, the change in nonpolar surface exposure at positions 41 and 75 in N-cam is markedly reduced compared to the change at equivalent positions in calbindin. The dif-

A

B
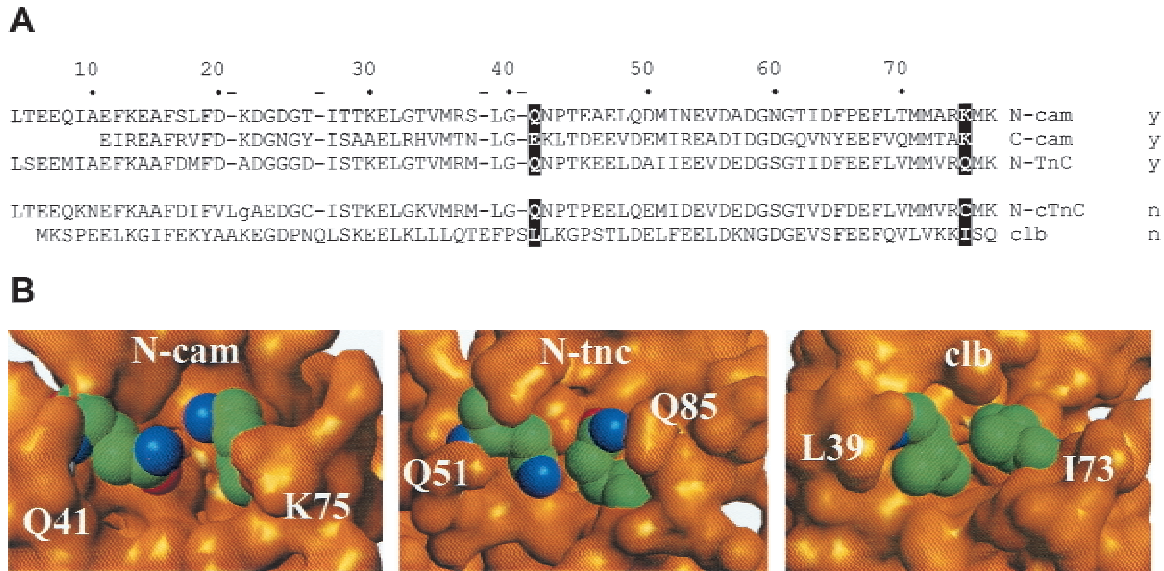
Fig. 2. Partially buried polar groups in EF-hand proteins. (*A*) Alignment of EF-hand domain sequences: the N- and C-terminal domains of calmodulin (N-cam, C-cam), the regulatory domain of skeletal troponin C (N-tnC), the regulatory domain of cardiac troponin C (N-ctnC), and calbindin D9k (clb). The numbering scheme is based on the full-length calmodulin sequence. The positions that are the focus of this work are highlighted in black. The ability to change conformation (y) or not (n) is listed for each domain. (*B*) Close-ups of buried polar groups in Ca^{2+}-free structures of N-cam and the regulatory domain of troponin C (N-tnc). Calbindin (clb), in contrast, contains nonpolar groups at structurally equivalent positions.

ference largely stems from the fact that N-cam contains polar groups at these positions, whereas calbindin contains nonpolar groups, as discussed above. Below, we present a mutational analysis to investigate the importance of the buried polar groups to conformational change.

Model system

To facilitate our study of conformational change in N-cam (calmodulin residues 1–78), we introduced a single mutation from Phe to Tyr at position 19 to yield the protein N-camY. This protein is more spectroscopically active than N-cam and has been determined to have thermal and chemical stability and Ca^{2+}-binding affinity similar to that of N-cam (Tables 1, 2). In addition, preliminary chemical shift assignments of N-camY in the apo and holo states (A. Ababou and J.R. Desjarlais, unpubl.) reveal a precise correlation with the published assignments for N-cam (Ikura et al. 1990, 1991; Kuboniwa et al. 1995; Zhang et al. 1995; Elshorst et al. 1999). The N-camY protein is used throughout this study as a wild-type reference. All variants described are constructed in this background.

Stability of the apo-proteins

If the buried polar groups in the closed state of N-camY are indeed unfavorable, we expect that their replacement with nonpolar groups will result in the stabilization of the folding of the apo state and a decrease in Ca^{2+}-binding affinity. Substitution at the same positions with alanine might be

expected to have an intermediate effect on the stability of the domain, because they will relieve the effect of polar burial but will not contribute a greater amount of hydrophobicity.

Folding stabilities of the apo state of N-camY and its 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 qualitative trends in stability, as seen in Figure 4. Indeed, the melting temperatures (T_m) correlate extremely well with the midpoints of unfolding determined from chemical denaturation (C_m). The stabilities that we determine for N-cam (cam 1–78) are higher than values reported previously using slightly different conditions and protein fragments (cam 1–75; Sorensen and Shea 1998; Masino et al. 2000). It appears to be possible that the few extra amino acids contained in our constructs significantly influence the stability of the domain.

The substitution of the buried polar residues with nonpolar residues had significant effects on the stability of the apo state (Fig. 4; Table 1). The most dramatic effects are observed for the double-mutant Q41L-K75I, in which the polar residues are replaced by similarly sized nonpolar residues. This variant has an increase of T_m of 16°C and an increase in C_m of 1.7 M. Smaller but significant increases in stability are also observed for the single mutations Q41L and K75I. As expected, the alanine variants show an intermediate stability between N-camY and the nonpolar variants.
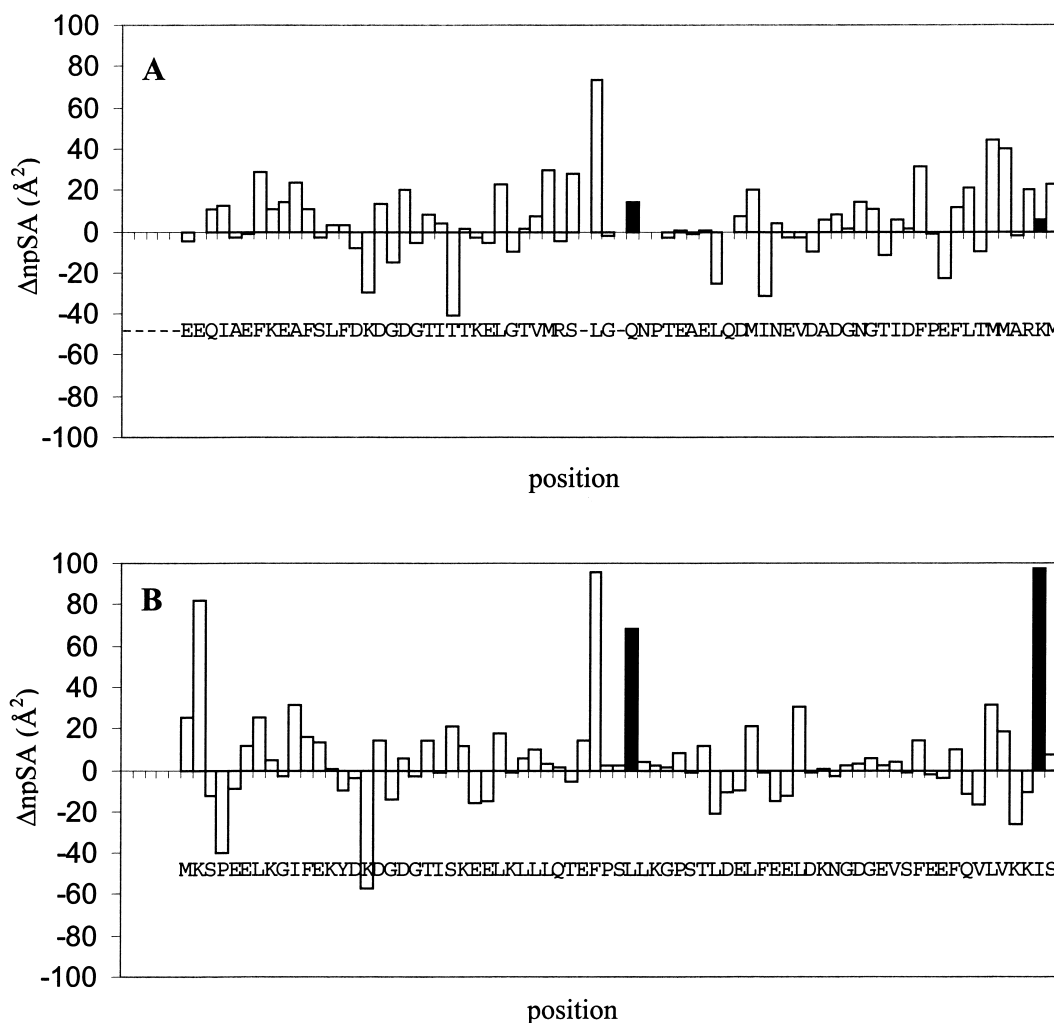
Fig. 3. Position-specific changes in nonpolar surface area (ΔnpSA). (*A*) Change in npSA of N-cam residues, plotted as the difference in accessible surface areas calculated from the Ca^{2+}-saturated and -free structures. (*B*) Predicted change in npSA for a hybrid protein clb-EF1, constructed in order to model a hypothetical open state for calbindin (see Materials and Methods). Positions 41 and 75 from N-camY are highlighted, as are their counterparts in the clb-EF1 sequence.

Chemical denaturation data using GdmCl fit well with a two-state model as shown in Figure 4B (see Materials and Methods). The free energies of unfolding in the absence of denaturant, $\Delta G°_{NU}$(H$_2$O), 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 double-mutant Q41L-K75I is the most significantly stabilized variant with an increase in unfolding free energy of 2.8 kcal/mole relative to N-camY.

Measurement of the folding stabilities of the Ca^{2+}-saturated states of each variant would be extremely informative. However, such experiments are impractical due to the already high stability of N-camY and the dependence of Ca^{2+}-binding affinity on ionic strength (Linse et al. 1991a, b).

Ca^{2+}-binding affinities

An increase in stability of the apo/closed state relative to the unfolded state is likely to coincide with an increase in stability of the apo state relative to the Ca^{2+}-bound open state. Because Ca^{2+} binding in N-camY is coupled to the change in conformation, changes in the relative stability of closed and open states should have a direct effect on Ca^{2+}-binding affinity (in the absence of any more direct effects on Ca^{2+} binding).

The titration of N-CamY and variants with Ca^{2+} in the presence of the chromophoric chelator 5-nitro-BAPTA (5NBAPTA) is shown in Figure 5. A clear competition for Ca^{2+} binding between protein and the 5NBAPTA is observed in all cases, indicating that all of the variants retain

Table 1. *Thermodynamic parameters for unfolding experiments*

Protein	Thermal denaturation			Chemical denaturation			
	ΔH_m (kcal/mol)[a]	T_m (°C)[a]	$\Delta C°_p$ (kcal/mol K)[b]	C_m (M)[c]	m_{GdmCl} (kcal/mol M)[a]	$\Delta G°_{NU}$ (kcal/mol)[a]	$\Delta\Delta G°_{NU}$ (kcal/mol)
N-Cam	53.1 ± 5.1	63.2 ± 0.5	1.3	2.4	1.9 ± 0.2	4.6 ± 0.6	0.0
N-CamY	53.9 ± 3.4	62.3 ± 0.4	1.3	2.3	2.0 ± 0.1	4.6 ± 0.3	—
Q41L	54.8 ± 5.3	71.3 ± 1.1	1.2	3.3	1.8 ± 0.1	5.9 ± 0.3	1.3
K75I	60.9 ± 4.9	67.7 ± 0.5	1.4	3.2	2.0 ± 0.1	6.3 ± 0.5	1.7
Q41L–K75I	57.5 ± 6.5	77.8 ± 2.0	1.2	4.0	1.8 ± 0.2	7.4 ± 0.9	2.8
Q41A	49.8 ± 4.1	63.2 ± 0.7	1.2	2.4	1.9 ± 0.1	4.5 ± 0.2	−0.1
K75A	54.6 ± 5.8	63.7 ± 0.7	1.3	2.7	1.9 ± 0.2	5.2 ± 0.6	0.6
Q41A–K75A	57.2 ± 5.2	67.0 ± 0.6	1.3	2.9	2.0 ± 0.1	5.7 ± 0.5	1.0

[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.

significant affinity for Ca^{2+}. Analysis of the data indicates that N-CamY binds two Ca^{2+} ions with a free energy of $\Delta G°_{2Ca} = -13.9$ kcal/mole, in close agreement with previously published results (Linse et al. 1991a). 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. The double-mutant Q41L-K75I has the lowest Ca^{2+} affinity, with a Ca^{2+}-binding free energy of −12.6 kcal/mole. This corresponds to an increase in Ca^{2+}-binding free energy of 1.3 kcal/mole relative to N-camY. Interestingly, this represents roughly half of the effect of the double substitution on the unfolding free energy. The macroscopic Ca^{2+}-binding constants for all of the variants are summarized in Table 2. Again, as expected, the alanine variants have intermediate effects on Ca^{2+}-binding affinity.

There are three possible explanations for an observed decrease in Ca^{2+}-binding affinity. First, the substitutions could have a direct effect on the structure of the Ca^{2+}-binding loops. We view this possibility as unlikely, because the sites of mutation are far removed from the Ca^{2+}-binding loops. Alternatively, the substitutions could lead to a relative stabilization of the closed form, with the open state still being formed in the presence of Ca^{2+}. In this case, Ca^{2+} binding is coupled to conformational change; thus, the Ca^{2+} affinity should decrease as the stability of the closed state increases. Finally, if the open form is destabilized to the extent that the Ca^{2+}-saturated closed state is predominant, the measured Ca^{2+}-binding affinity will approach the intrinsic affinity of the closed form.

### Characterization of conformational changes

A hallmark of conformational change in calmodulin like proteins is the Ca^{2+}-dependent binding of the hydrophobic fluorophore 8-anilino-1-naphthalene-sulfonic acid (ANS; Bayley et al. 1984), which presumably binds in the large hydrophobic pocket formed by the open state. The fluorescence emission of ANS in the presence of the apo-proteins was compared to that in the presence of the holo-proteins. 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+} (Fig. 6). Similar effects are observed for the Q41L, the Q41A, and modestly for the other alanine variants (Table 2). However, the K75I and the Q41L-K75I variants show little to no change in the ANS fluorescence intensity upon addition of high concentrations of Ca^{2+}. Assuming that ANS-binding capacity is indicative of the pres-

Table 2. *Calcium-binding parameters and fluorescence spectra changes of ANS in the presence of apo- and holo-proteins*

| Protein	Log K_1[a]	Log K_2[a]	Log K_1K_2	$\Delta G°_{2Ca}$ (kcal/mol)	ESS[b]	$\Delta\lambda_{max}$	I_{max}(+ Ca^{2+})/I_{max}(− Ca^{2+})
N-Cam	4.84	5.14	9.98	−13.7	1.04×10^{-5}		
N-CamY	4.97	5.17	10.14	−13.9	1.27×10^{-5}	29	5.1
Q41L	4.87	4.95	9.82	−13.5	3.27×10^{-6}	21	3.9
K75I	4.76	4.55	9.31	−12.8	1.76×10^{-5}	11	1.4
Q41L–K75I	4.45	4.73	9.18	−12.6	1.31×10^{-5}	5	1.2
Q41A	5.05	5.16	10.21	−14.0	1.27×10^{-4}	26	2.6
K75A	4.97	4.55	9.52	−13.1	6.48×10^{-5}	9	1.8
Q41A–K75A	4.69	4.77	9.46	−13.0	1.28×10^{-5}	16	1.7

[a] K_1 and K_2 are in molar concentration units.
[b] Error squared sum of the fit (see Materials and Methods).
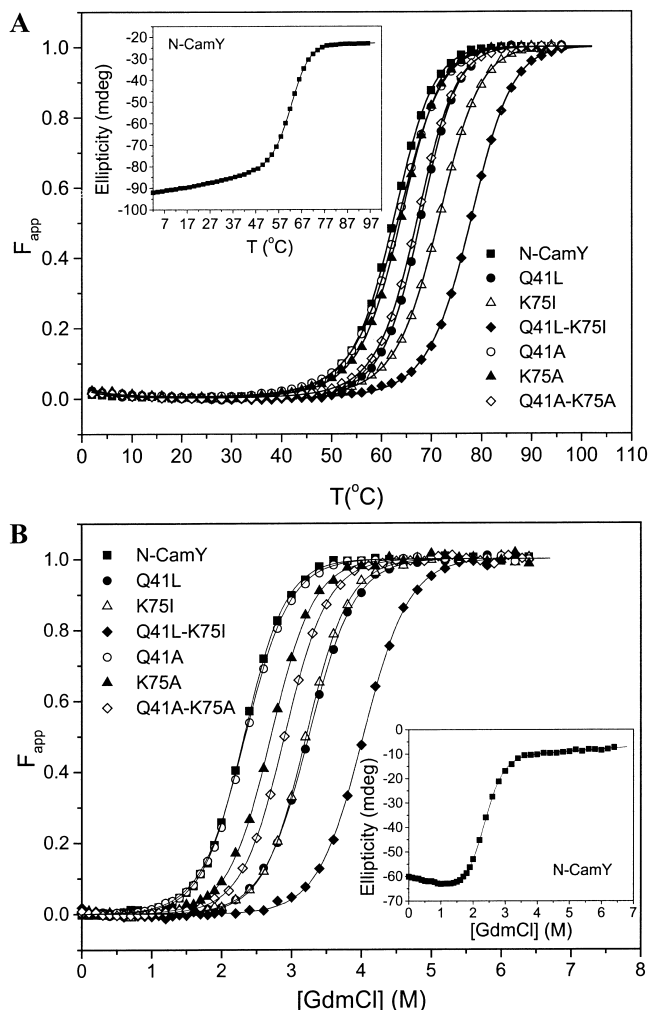
Fig. 4. Stability of Ca^{2+}-free N-camY variants. (*A*) Thermal Denaturation. The apparent fraction of unfolded protein F_{app} for N-CamY and the variants is plotted as a function of temperature. The extent of unfolding was monitored by circular dichroism (CD) at a wavelength of 222 nm. (*B*) Chemical Denaturation. The F_{app} as a function of GdmCl concentration is shown for each variant. The unfolding was monitored by far-UV CD at a wavelength of 232 nm. For each figure, the inset shows the uncorrected denaturation data and initial fits for the N-camY protein.

ence of the open-state structure, the data suggest that these variants no longer adopt an open-state structure. However, we note that ANS binding is a qualitative assay in which a change in fluorescence intensity can reflect a change in conformational equilibrium or a change in ANS-binding affinity for the open-state structure.

Close packing of aromatic groups in native protein cores gives rise to near-UV CD spectra that are dependent on the solvent and the proximity of other functional groups (Tawell and Manning 1994). To investigate the local structure and environment of aromatic residues, we have recorded the near-UV CD spectra of the apo- and holo-proteins, as shown in Figure 7. All apo-proteins have identical
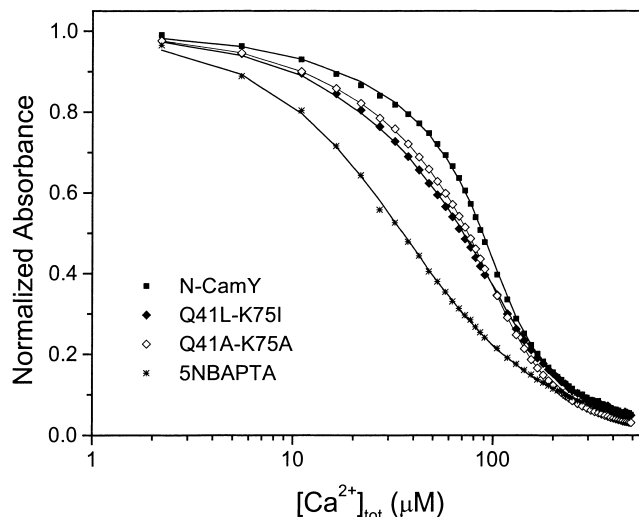

Fig. 5. Ca^{2+}-binding assays for representative N-camY variants. Logarithmic representations of the normalized Ca^{2+} competition curves of N-CamY and the mutants monitored by the absorbance of 5NBAPTA (5-nitro-BAPTA) at 430 nm. The symbols are the experimental data and the curves through the points are the least-squares fits. Binding parameters determined from the fits are listed in Table 2.

spectral shapes and, within the limits of experimental error, identical extremum values. Two minima at 263 nm and 270 nm, characteristic of phenylalanine spectra, are prominent. For a subset of the variants, the addition of saturating concentrations of Ca^{2+} results in the rise of a broad minimum between 275 nm and 290 nm characteristic of tyrosines.
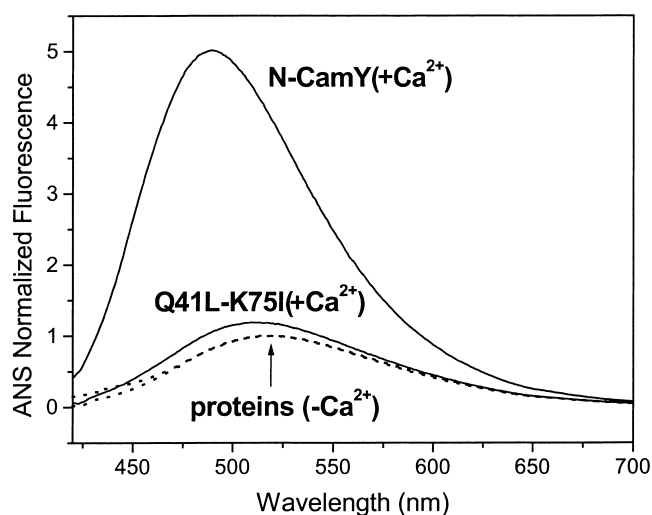
Fig. 6. Conformational changes measured by ANS binding. Fluorescence emission spectra of ANS in the presence of protein as illustrated in the case of N-CamY and Q41L-K75I, in the absence of Ca^{2+} (dashed line), normalized to unity, and its relative fluorescence enhancement in the presence of Ca^{2+} (solid line). Table 2 lists the relative ANS intensity for all of the variants in the presence or absence of Ca^{2+}.
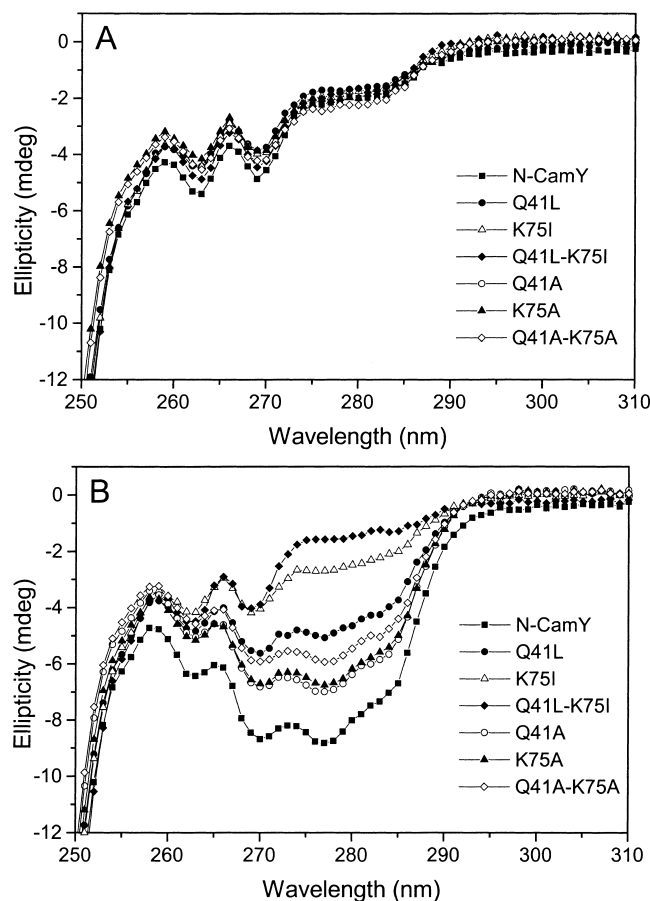
Fig. 7. Conformational changes measured by near-UV CD spectra. Spectra of all of the variants were collected with 0.1 mM EDTA (*A*) or 1 mM CaCl₂(*B*).

This result clearly indicates that the environment of Tyr19 changes in response to Ca²⁺ binding in such a way that it is highly packed in the core of the protein and most probably with the cluster of phenylalanines at positions 12, 65, and 68, as expected from the experimentally determined structure of Ca²⁺-saturated calmodulin (Babu et al. 1988). The effect is strong in the case of N-CamY, Q41L, and the alanine variants. It is seen only weakly in the case of K75I and is completely absent in the case of the double-mutant Q41L-K75I. These spectra appear to be a signature of the difference in conformation between Ca²⁺-saturated N-CamY and Q41L-K75 and suggest clearly that the double mutant is trapped in a closed structure. In contrast to the ANS assay, the near-UV CD spectra are likely to be a more direct measure of the equilibrium between two conformations.

Importantly, the results from the ANS fluorescence and near-UV CD directly parallel each other. The double-mutant 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, the variant

clearly binds Ca²⁺ as seen in Figure 5. Ca²⁺ binding to this variant is further supported by the large chemical shift perturbations observed for protons in the vicinity of both Ca²⁺-binding sites, as listed in Table 3. Hence, for this protein, Ca²⁺ binding is apparently uncoupled from a major conformational change, as is the case for calbindin D9k.

The Ca²⁺-induced change in the near-UV CD spectra of the Q41L and alanine variants is approximately half of that observed for N-camY. In light of this reduced spectral change and the stability and Ca²⁺-binding data, we speculate that these variants exist in equilibrium between closed and open states in the presence of Ca²⁺. Precedence for this phenomenon has been reported for Ca²⁺-binding-loop substitutions (Evenas et al. 1997, 1998).

NMR spectroscopy

NMR spectroscopy has been used to further investigate the conformational changes in these proteins upon Ca²⁺ binding. We have collected NMR data for N-CamY and the double-mutant Q41L-K75, which has exhibited the most dramatic differences from N-CamY and is apparently trapped in a closed form in the presence of Ca²⁺. The spectra collected for the Ca²⁺-free form of Q41L-K75I are extremely similar to those of apo-N-camY (data not shown). In contrast, the spectra of the Ca²⁺-saturated form of Q41L-K75I differ considerably from those of Ca²⁺-saturated N-camY. These differences are prominent in the aromatic regions of the NOESY spectra, as shown in Figure 8. In particular, Tyr19, whose aromatic resonances were assigned using COSY and TOCSY data, has several NOE crosspeaks to other aromatic resonances in the Ca²⁺-saturated form of N-camY. These include the aromatic protons from Phe68, consistent with the structure of Ca²⁺-saturated calmodulin, as well as published assignments (Ikura et al. 1990, 1991; Elshorst et al. 1999). In contrast, only a single weak crosspeak between Tyr19 and another aromatic reso-

Table 3. *Selected chemical shifts in the calcium binding loops*[a]

Residue	N-CamY (− Ca²⁺)		Q41L–K75I (− Ca²⁺)	
	NH	αH	NH	αH
Gly25	10.15	4.23 ; 3.79	10.18	4.23 ; 3.79
Ile27	8.17	ND	8.22	ND
Gly61	9.99	4.10 ; 3.84	9.83	4.07 ; 3.89
Ile63	8.85	4.54	8.87	4.62
	N-CamY (+ Ca²⁺)		Q41L–K75I (+ Ca²⁺)	
Gly25	10.61	4.31 ; 3.63	10.53	4.30 ; 3.62
Ile27	9.77	4.96	9.57	5.02
Gly61	10.57	4.15 ; 3.42	10.52	4.14 ; 3.44
Ile63	8.90	5.14	9.01	5.19

[a] Chemical shifts are listed at 298 K with respect to the water line at 4.70 ppm. ND, not determined.
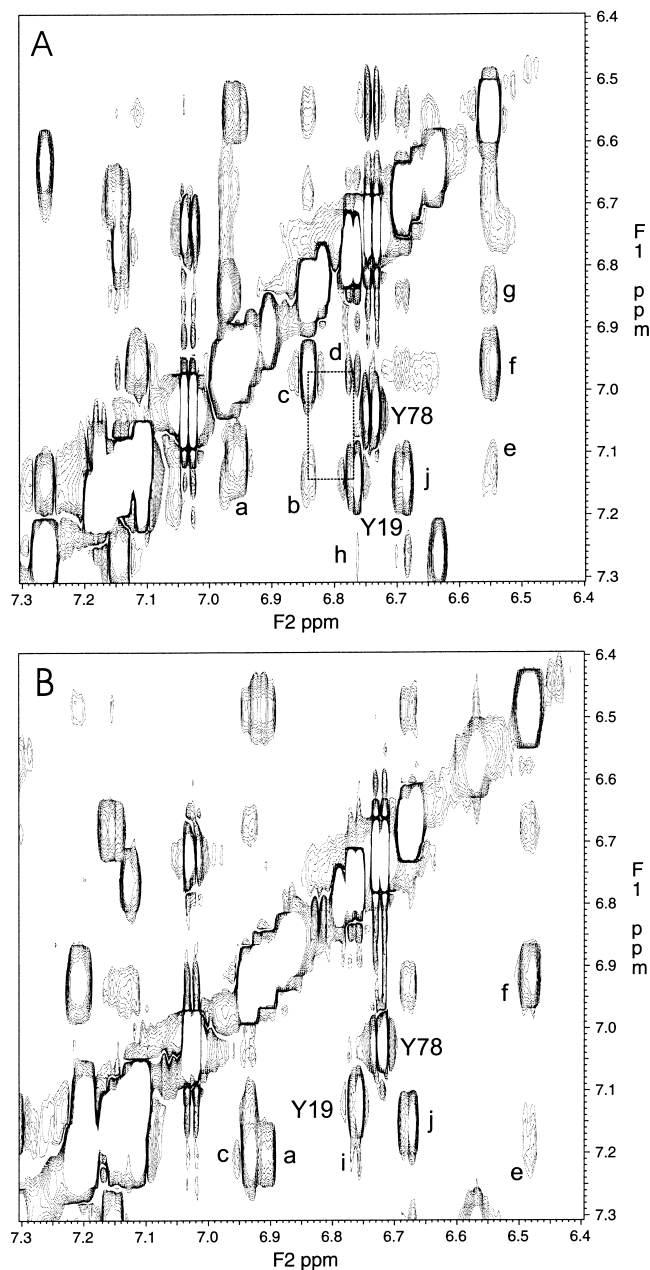
Fig. 8. Aromatic regions of ^{1}H-^{1}H NOESY spectra. Spectra are shown for Ca^{2+}-saturated N-camY (*A*) and Ca^{2+}-saturated Q41L-K75I (*B*). As expected from the open-state structure of N-cam, several cross-peaks between Tyr19 and other aromatics are observed (dashed box). These cross-peaks are not expected for the closed form. Assignments—based on COSY, TOCSY, and NOESY correlations, similarities with published assignments (Ikura et al. 1990, 1991; Elshorst et al. 1999), and consistency with the know structures—are as follows. For (*A*) Y19, Y19 (Hε, 6.77) to (Hδ, 7.14); Y78, Y78 (Hε, 6.74) to (Hδ, 7.05); a, F16 (Hε, 6.96) to (Hζ, 7.11); b, F68 (Hε, 6.84) to Y19 (Hδ, 7.14); c, F68 (Hε, 6.84) to (Hζ, 6.97); d, Y19 (Hε, 6.77) to F68 (Hζ, 6.97); e, F16 (Hδ, 6.55) to (Hζ, 7.11); f, F16 (Hδ, 6.55) to (Hε, 6.96); g, F16 (Hδ, 6.55) to F68 (Hε, 6.84); h, Y19 (Hε, 6.77) to F65* j, F65* (H*, 6.69) to F65* (Hε, 7.15). For (*B*) Y19, Y19 (Hε, 6.76) to (Hδ, 7.13); Y78, Y78 (Hε, 6.72) to (Hδ, 7.02); a, F16 (Hε, 6.92) to (Hζ, 7.21); c, F68 (H*, 6.93) to (H*, 7.21); e, F16 (Hδ, 6.48) to (Hζ, 7.21); f, F16 (Hδ, 6.48) to (Hε, 6.92); i, Y19 (Hε, 6.76) to (*); j, F65* (H*, 6.68) to F65* (Hε, 7.15). *Not determined or uncertainty in the assignment.

nance (unassigned) is observed in the spectrum of the Q41L-K75I variant. Furthermore, several significant chemical shift differences between the two spectra are apparent. These observations, together with the fluorescence and CD data, suggest the Ca^{2+}-saturated Q41L-K75I variant exists in a structural form that is significantly different from the canonical open-state structure expected for a Ca^{2+}-saturated domain.

Discussion

The results described above support a view in which suboptimal stability has evolved into the Ca^{2+}-free, closed form of the N-terminal domain of calmodulin in order to mitigate the unfavorable energetics associated with adopting the functionally activated open-state structure. The results of our mutational analysis are consistent with the concept of a careful balance of solvation energetics between structural forms. Perturbations of this balance lead to deficiencies in the ability to change conformation and/or the ability to bind Ca^{2+} with high affinity.

Mutational analysis indicates that a major requirement for conformational change in N-cam is the absence of large hydrophobic groups at positions 41 and 75. Mutation of Q41 and K75 to Leu and Ile, respectively, leads to a dramatic stabilization of the apo state of N-camY and a reduction of Ca^{2+}-binding affinity. Finally, spectroscopic analysis strongly suggests that for this variant, the equilibrium between Ca^{2+}-saturated closed and open forms is perturbed to an extent such that the Ca^{2+}-saturated closed form dominates.

Comparison of the results of alanine mutations at positions 41 and 75 versus the substitutions with larger nonpolar groups indicates that the burial of polar groups is important but not essential for conformational change. However, the presence of the polar groups of Gln and Lys does appear to be a significant factor for retaining appropriate levels of Ca^{2+}-binding affinity and a more complete conversion of the protein population to the open-state structure. Interestingly, several other N-cam-related domains, including the C-terminal domain of calmodulin and the regulatory N-terminal domain of troponin C, also contain polar groups at these positions, as shown in Figure 2. Inspection of a high-resolution structure of the N-terminal domain of troponin C (Gagne et al. 1995) reveals that the polar moieties from two glutamine residues are indeed partially buried in its Ca^{2+}-free form (Fig. 2B). We speculate that the inclusion of buried polar groups is an important energetic factor in many other EF-hand proteins of the Ca^{2+} sensor class.

As shown in Figure 9, data from the set of variants reveal a correlation between the Ca^{2+}-binding and -unfolding free energies. For many of the variants, the correlation is roughly linear because Ca^{2+} binding is coupled to conformational change. However, as observed for the double-mutant Q41L-
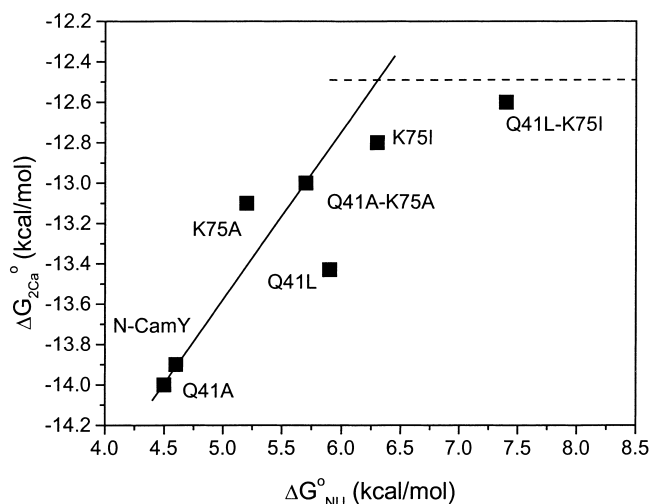
Fig. 9. Relationship between calcium-binding and -unfolding free energies. For proteins that change conformation, a linear correlation might be expected, denoted by the solid line. However, as the closed state becomes stabilized to the extent that calcium binding does not induce a conformational change, the free energies will become uncoupled. At this point, the calcium-binding free energy will approach the intrinsic affinity of the closed state, as denoted by the dashed line.

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. Any further increases in the closed-state stability should have little effect on Ca^{2+} affinity.

Buried polar groups have been shown to have significant effects on the stability of protein structure. Substitution of buried nonpolar residues with polar residues has been shown to lead to large decreases in protein stability (Dao-pin et al. 1991; Stites et al. 1991; Blaber et al. 1993). Alternatively, replacement of native buried polars with appropriately selected nonpolar residues has been shown to stabilize a protein significantly (Waldburger et al. 1995; Hendsch et al. 1996). Other studies have highlighted the role of buried polar groups in providing oligomeric state specificity to leucine zipper motifs (Lumb and Kim 1995; Gonzalez et al. 1996; Zeng et al. 1997). According to our results, the effect is also likely to be a major factor in the ability of some classes of proteins to undergo unimolecular changes in conformation.

This study was initiated by a comparison between the properties of calbindin and N-cam. A complete understanding of the differential ability of these domains to change conformation will only be shown by mutational conversion of the conformationally inert protein calbindin D9k into one that changes conformation in response to Ca^{2+} binding. Although a variety of substitutions will likely be necessary in this endeavor, our results indicate that close attention must be paid to the balance of solvation energetics in the two

structural forms of the protein and that the introduction of polar groups at key positions in calbindin will make an important contribution.

A general requirement for conformational change is the presence of structural ambivalence, which can occur in many ways. In an elegant demonstration of this principle, Young et al. (1999) showed that structural ambiguity in the form of secondary-structure propensity can be used with marked success to predict regions of proteins that undergo changes in conformation. In the case of the N-terminal domain of calmodulin and, presumably, similar domains, structural ambivalence depends, at least in part, on the presence of partially buried or restricted polar groups. Their presence foreshadows the existence of an alternative form in which the polar groups can become fully solvated. Perhaps the identification of suboptimal structural regions, often ascribed to the incorrect assignment of structure, might in some cases lead to the identification of conformationally active regions of proteins. In light of the central role of conformational change in a variety of disease states (Carrell and Gooptu 1998; Dobson 1999), this may yield important insights into the mechanism of transformation to pathological structural 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 using the standard cloning techniques. A single mutation from Phe to Tyr was introduced at position 19 to yield the protein N-camY, the protein used throughout the study as a wild-type reference. Mutagenic oligonucleotides were purchased from Integrated DNA Technologies. The residues Ala1, Phe19, and Asp78 were changed to Met1, Tyr19, and Tyr78, respectively. The N-CamY and its variants were overexpressed in *Escherichia coli* Bl21(DE3) cells. The integrity of all the clones was confirmed by DNA sequencing. The yields of purified proteins from this expression system are consistently high, with an average of ~150 mg/L of culture medium. The proteins are purified in two stages. The first purification consists of an anion exchange column, following a published protocol (Meyer et al. 1996). 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 were checked by SDS gel electrophoresis, and all the proteins showed >98% purity. The concentrations of the proteins were determined in 6M GdmCl using absorbance spectroscopy and a molar extinction coefficient of Tyr at 280 nm of 1420 M^{-1} cm^{-1}.

Chemical materials

The fluorimetric Ca^{2+} chelator 5NBAPTA was purchased from Molecular Probes. The fluorimetric hydrophobic probe ANS, GdmCl, and MOPS were purchased from Sigma. 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 (Linse et al. 1991b) was carried out using the absorbance signal of the chelator at 430 nm.

The absorbance measurements were conducted on a BioSpec-1601 (SHIMADZU Scientific Instruments).

All measurements were performed using 37 μM protein and 25 μM 5NBAPTA, 100 mM KCl, and 20 mM MOPS at pH 7.2.

Fluorescence measurements were conducted on a Fluorolog-3 spectrofluorimeter (JOBIN YVON-SPEX), 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 the measurements were conducted at 25°C, using a NESLAB water-bath circulator (NESLAB Instruments). All the measurements were done using 25 μM protein and 5 μM ANS, 100 mM KCl, and 20 mM MOPS at 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 nm to 260 nm using a 1-mm cell path length and a 3 s averaging time. Thermal unfolding data were collected at 222 nm from 2°C to 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 nm 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 (Pace 1986) using a Reichert-Jung ABBE MARK II digital refractometer. All experiments were carried out in 20 mM MOPS at pH 7.2, 100 mM KCl.

A model of calbindin in the open state

The simplest approach to modeling an open-state structure of calbindin is to use that of N-cam as a reference. In order to facilitate this approach, we modeled a calbindin sequence in which the N-terminal Ca^{2+}-binding loop (residues 15–24) was replaced by the loop from calmodulin (residues 20–27) to create the protein clb-EF1. Hence, the structure of N-cam in the Ca^{2+}-saturated state can be used directly as a structural template from which to build an open-state model of calbindin. The backbone model for a projected form of calbindin (clb-EF1) in the open state was assembled by grafting the connecting loop structure (between the EF-hands) from calbindin onto the open-state structure of the N-terminal domain of calmodulin. This model assumes that any conformational change achieved in the clb-EF1 system will resemble that which takes place for calmodulin, consisting primarily of a hinge movement that alters the relative orientation of two of the alpha helices relative to the others. Side chains of the clb-EF1 sequence were built on the hypothetical backbone structure using the program SPA (Raha et al. 2000). Although the models are expected to be imperfect, they are useful for generating hypotheses addressing the inability of calbindin to change conformation.

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. (1991b). Data were fitted to single- 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 our laboratory. We determined the K_D of 5NBAPTA using its absorbance signal at 430 nm in 20 mM MOPS, 100 mM KCl at pH 7.2 buffer. The K_D obtained was 23.0 μM, which was in close agreement with previously reported values (Rand et al. 1997). 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 unfolded and the native 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 is temperature independent in the range of measurement (Privalov 1979). Hence, the free energy of unfolding is

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

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 (Greene and Pace 1974), the free energy of unfolding is

$$\Delta G_{NU} = \Delta G_{NU}(H_2O) - m_D [\text{Denaturant}]$$

where $\Delta G_{NU}(H_2O)$ 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 1997). The calculated errors in the unfolding experiments are given as ± confidence intervals at the 68.3% level.

NMR measurements

All NMR samples were 600 μL in volume, the buffer conditions were 100 mM KCl, 0.15 mM sodium azide in 95% H_2O/5% D_2O at pH 7.2. The protein concentrations were 1 mM with 0.1 mM

EDTA for the Ca^{2+}-free sample and 4 mM $CaCl_2$ for the Ca^{2+}-loaded sample.

All spectra were acquired at a proton frequency of 600 MHz and at 298 K using a Bruker DRX-600 spectrometer. ^1H-^1H DQF-COSY, ^1H-^1H TOCSY, and ^1H-^1H NOESY spectra were acquired in the quadrature mode and with TPPI (Marion and Wuthrich 1983). A total of 64 scans were collected for each of the 512 t_1 values and the t_2 values. Mixing times of 45 ms (TOCSY), 100 ms (NOESY), and a relaxation delay of 1.5 sec were used for a spectral window of 13.4 ppm. The carrier was always set on the water resonance and its suppression was applied using the WATER-GATE technique (Piotto et al. 1992).

Spectral widths were as follows: DQF-COSY, 8013 Hz in both t_2 (4096 complex points) and t_1 (482–512 increments); NOESY and TOCSY, 12,500 Hz in t_2 (4096 complex points) and 8013 Hz in t_1 (512 increments). All the data were processed with NMRPipe program (Delaglio et al. 1995) and shifted squared sine-bell window function and zero filling were applied in both dimensions prior to Fourier transformation. A polynomial baseline correction was applied in both dimensions in the frequency domain. The proton chemical shifts were indirectly referenced to sodium 2,2-dimethyl-2-silapentane-5sulfonate through the water signal (Wishart et al. 1995).

Acknowledgments

References

Ackers, G.K., Doyle, M.L., Myers, D., and Daugherty, M.A. 1992. Molecular code for cooperativity in hemoglobin. *Science* **255:** 54–63.

Babu, Y.S., Bugg, C.E., and Cook, W.J. 1988. Structure of calmodulin refined at 2.2 Å resolution. *J. Mol. Biol.* **204:** 191–204.

Bayley, P., Ahlstrom, P., Martin, S.R., and Forsen, S. 1984. The kinetics of calcium binding to calmodulin: Quin 2 and ANS stopped-flow fluorescence studies. *Biochem. Biophys. Res. Commun.* **120:** 185–91.

Biekofsky, R.R., Martin, S.R., Browne, J.P., Bayley, P.M., and Feeney, J. 1998. Ca^{2+} coordination to backbone carbonyl oxygen atoms in calmodulin and other EF-hand proteins: 15N chemical shifts as probes for monitoring individual-site Ca^{2+} coordination. *Biochemistry* **37:** 7617–29.

Blaber, M., Lindstrom, J.D., Gassner, N., Xu, J., Heinz, D.W., and Matthews, B.W. 1993. Energetic cost and structural consequences of burying a hydroxyl group within the core of a protein determined from Ala–Ser and Val–Thr substitutions in T4 lysozyme. *Biochemistry* **32:** 11363–11373.

Bullough, P.A., Hughson, F.M., Skehel, J.J., and Wiley, D.C. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* **371:** 37–43.

Carrell, R.W. and Gooptu, B. 1998. Conformational changes and disease—Serpins, prions and Alzheimer's. *Curr. Opin. Struct. Biol.* **8:** 799–809.

Chattopadhyaya, R., Meador, W.E., Means, A.R., and Quiocho, F.A. 1992. Calmodulin structure refined at 1.7 Å resolution. *J. Mol. Biol.* **228:** 1177–92.

Chazin, W.J. 1995. Releasing the calcium trigger. *Nat. Struct. Biol.* **2:** 707–10.

Dao-pin, S., Anderson, D.E., Baase, W.A., Dahlquist, F.W., and Matthews, B.W. 1991. Structural and thermodynamic consequences of burying a charged residue within the hydrophobic core of T4 lysozyme. *Biochemistry* **30:** 11521–9.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. 1995. NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6:** 277–93.

Dobson, C.M. 1999. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* **24:** 329–32.

Drohat, A.C., Amburgey, J.C., Abildgaard, F., Starich, M.R., Baldisseri, D., and Weber, D.J. 1996. Solution structure of rat apo-S100B(beta beta) as determined by NMR spectroscopy. *Biochemistry* **35:** 11577–88.

Drohat, A.C., Baldisseri, D.M., Rustandi, R.R., and Weber, D.J. 1998. Solution structure of calcium-bound rat S100B(betabeta) as determined by nuclear magnetic resonance spectroscopy. *Biochemistry* **37:** 2729–40.

Elshorst, B., Hennig, M., Forsterling, H., Diener, A., Maurer, M., Schulte, P., Schwalbe, H., Griesinger, C., Krebs, J., Schmid, H., et al. 1999. NMR solution structure of a complex of calmodulin with a binding peptide of the Ca^{2+} pump. *Biochem-istry* **36:** 3448–57.

Evenas, J., Thulin, E., Malmendal, A., Forsen, S., and Carlstrom, G. 1997. NMR studies of the E140Q mutant of the carboxy-terminal domain of calmodulin reveal global conformational exchange in the Ca^{2+}-saturated state. *Biochem-istry* **36:** 3448–57.

Evenas, J., Malmendal, A., Thulin, E., Carlstrom, G., and Forsen, S. 1998. Ca^{2+} binding and conformational changes in a calmodulin domain. *Biochemistry* **37:** 13744–54.

Flaherty, K.M., Zozulya, S., Stryer, L., and McKay, D.B. 1993. Three-dimensional structure of recoverin, a calcium sensor in vision. *Cell* **75:** 709–16.

Gagne, S.M., Li, M.X., and Sykes, B.D. 1997. Mechanism of direct coupling between binding and induced structural change in regulatory calcium binding proteins. *Biochemistry* **36:** 4386–92.

Gagne, S.M., Tsuda, S., Li, M.X., Smillie, L.B., and Sykes, B.D. 1995. Structures of the troponin C regulatory domains in the apo and calcium-saturated states. *Nat. Struct. Biol.* **2:** 784–789.

Gonzalez Jr., L., Woolfson, D.N., and Alber, T. 1996. Buried polar residues and structural specificity in the GCN4 leucine zipper. *Nat. Struct. Biol.* **3:** 1011–8.

Greene Jr., R.F. and Pace, C.N. 1974. Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, alpha-chymotrypsin, and beta-lactoglobulin. *J. Biol. Chem.* **249:** 5388–93.

Hendsch, Z.S., Jonsson, T., Sauer, R.T., and Tidor, B. 1996. Protein stabilization by removal of unsatisfied polar groups: Computational approaches and experimental tests. *Biochemistry* **35:** 7621–5.

Herzberg, O. and James, M.N. 1985. Structure of the calcium regulatory muscle protein troponin-C at 2.8 A resolution. *Nature* **313:** 653–9.

Ikura, M. 1995. Molecular and structutral basis of target recognition by calmodulin. *Annu. Rev. Biophys. Biomol. Struct.* **24:** 85–116.

Ikura, M. 1996. Calcium binding and conformational response in EF-hand proteins. *Trends Biochem. Sci.* **21:** 14–7.

Ikura, M., Marion, D., Kay, L.E., Shih, H., Krinks, M., Klee, C.B., and Bax, A. 1990. Heteronuclear 3D NMR and isotopic labeling of calmodulin. Towards the complete assignment of the 1H NMR spectrum. *Biochem. Pharmacol.* **40:** 153–60.

Ikura, M., Spera, S., Barbato, G., Kay, L.E., Krinks, M., and Bax, A. 1991. Secondary structure and side-chain 1H and 13C resonance assignments of calmodulin in solution by heteronuclear multidimensional NMR spectroscopy. *Biochemistry* **30:** 9216–28.

Julenius, K., Thulin, E., Linse, S., and Finn, B.E. 1998. Hydrophobic core substitutions in Calbindin D9k: Effects on stability and structure. Biochemistry 37: 8915-8925.

Kordel, J., Skelton, N.J., M., A., and Chazin, W.J. 1993. High-resolution structure of calcium-loaded calbindin D9k. *J. Mol. Biol.* **231:** 711–734.

Kraulis, P.J. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24:** 946–950.

Kuboniwa, H., N., T., Grzesiek, S., Ren, H., Klee, C.B., and Bax, A. 1995. Solution structure of calcium-free Calmodulin. Nature Structural Biology 2: 768-776.

Linse, S., Helmersson, A., and Forsen, S. 1991a. Calcium binding to calmodulin and its globular domains. *J. Biol. Chem.* **266:** 8050–4.

Linse, S., Johansson, C., Brodin, P., Grundstrom, T., Drakenberg, T., and Forsen, S. 1991b. Electrostatic contributions to the binding of Ca^{2+} in calbindin D9k. *Biochemistry* **30:** 154–62.

Lumb, K.J. and Kim, P.S. 1995. A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. *Biochemistry* **34:** 8642–8.

Marion, D. and Wuthrich, K. 1983. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of 1H–1H spin–spin coupling constants in proteins. *Biochem. Biophys. Res. Commun.* **113:** 967–74.

Masino, L., Martin, S.R., and Bayley, P.M. 2000. Ligand binding and thermo-dynamic stability of a multidomain protein, calmodulin. *Prot. Sci.* **9:** 1519–29.

Merrit, E.A. and Bacon, D.J. 1997. Raster3D: Photorealistic Molecular Graphics. *Meth. Enzymol.* **277:** 505–524.

Meyer, D.F., Mabuchi, Y., and Grabarek, Z. 1996. The role of Phe-92 in the Ca(2+)-induced conformational transition in the C-terminal domain of cal-modulin. *J. Biol. Chem.* **271:** 11284–90.

Nelson, M.R. and Chazin, W.J. 1998a. An interaction-based analysis of cal-cium-induced conformational changes in Ca²⁺ sensor proteins. *Prot. Sci.* **7:** 270–82.

Nelson, M.R. and Chazin, W.J. 1998b. Structures of EF-hand Ca(2+)-binding proteins: Diversity in the organization, packing and response to Ca²⁺ bind-ing. *Biometals* **11:** 297–318.

Pace, C.N. 1986. Determination and analysis of urea and guanidine hydrochlo-ride denaturation curves. *Meth. Enzymol.* **131:** 266–80.

Piotto, M., Saudek, V., and Sklenar, V. 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR* **2:** 661–5.

Privalov, P.L. 1979. Stability of proteins: Small globular proteins. *Adv. Prot. Chem.* **33:** 167–241.

Raha, K., Wollacott, A.M., Italia, M.J., and Desjarlais, J.R. 2000. Prediction of amino acid sequence from structure. *Prot. Sci.* **9:** 1106–19.

Rand, M.D., Lindblom, A., Carlson, J., Villoutreix, B.O., and Stenflo, J. 1997. Calcium binding to tandem repeats of EGF-like modules. Expression and characterization of the EGF-like modules of human Notch-1 implicated in receptor-ligand interactions. *Prot. Sci.* **6:** 2059–71.

Sastry, M., Ketchem, R.R., Crescenzi, O., Weber, C., Lubienski, M.J., Hidaka, H., and Chazin, W.J. 1998. The three-dimensional structure of Ca(2+)-bound calcyclin: Implications for Ca(2+)-signal transduction by S100 pro-teins. *Structure* **6:** 223–31.

Schachman, H.K. 1987. From allostery to mutagenesis: 20 years with aspartate transcarbamoylase. *Biochem. Soc. Trans.* **15:** 772–5.

Skelton, N.J., Kordel, J., Akke, M., Forsen, S., and Chazin, W.J. 1994. Signal transduction versus buffering activity in Ca(2+)-binding proteins. *Nat. Struct. Biol.* **1:** 239–45.

Skelton, N.J., Kordel, J., and Chazin, W.J. 1995. Determination of the solution structure of Apo calbindin D9k by NMR spectroscopy. *J. Mol. Biol.* **249:** 441–462.

Slupsky, C.M. and Sykes, B.D. 1995. NMR solution structure of calcium-saturated skeletal muscle troponin C. *Biochemistry* **34:** 15953–64.

Sorensen, B.R. and Shea, M.A. 1998. Interactions between domains of apo calmodulin alter calcium binding and stability. *Biochemistry* **37:** 4244–53.

Stites, W.E., Gittis, A.G., Lattman, E.E., and Shortle, D. 1991. In a staphylo-coccal nuclease mutant the side-chain of a lysine replacing valine 66 is fully buried in the hydrophobic core. *J. Mol. Biol.* **221:** 7–14.

Svensson, L.A., Thulin, E., and Forsen, S. 1992. Proline cis-trans isomers in calbindin D9k observed by X-ray crystallography. *J. Mol. Biol.* **223:** 601–6.

Waldburger, C.D., Schildbach, J.F., and Sauer, R.T. 1995. Are buried salt bridges important for protein stability and conformational specificity? *Nat. Struct. Biol.* **2:** 122–8.

Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. 1995. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **6:** 135–40.

Young, M., Kirshenbaum, K., Dill, K.A., and Highsmith, S. 1999. Predicting conformational switches in proteins. *Prot. Sci.* **8:** 1752–64.

Zeng, X., Herndon, A.M., and Hu, J.C. 1997. Buried asparagines determine the dimerization specificities of leucine zipper mutants. *Proc. Nat. Acad. Sci.* **94:** 3673–8.

Zhang, M., Tanaka, T., and Ikura, M. 1995. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat. Struct. Biol.* **2:** 758–767.