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A calbindin D_{9k} mutant containing a novel structural extension: ^1H nuclear magnetic resonance studies

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Abstract

Calbindin D_{9k} is a small, well-studied calcium-binding protein consisting of two helix-loop-helix motifs called EF-hands. The P43MG[2] mutant is one of a series of mutants designed to sequentially lengthen the largely unstructured tether region between the two EF-hands (F36-S44). A lower calcium affinity for P43MG was expected on the basis of simple entropic arguments. However, this is not the case and P43MG (-97 kJ·mol^{-1}) has a stronger calcium affinity than P43M (-93 kJ·mol^{-1}), P43G (-95 kJ·mol^{-1}) and even wild-type protein (-96 kJ·mol^{-1}). An NMR study was initiated to probe the structural basis for these calcium-binding results. The ^1H NMR assignments and $^3J_{HNH\alpha}$ values of the calcium-free and calcium-bound forms of P43MG calbindin D_{9k} mutant are compared with those of P43G. These comparisons reveal that little structure is formed in the tether regions of P43MG(apo), P43G(apo) and P43G(Ca) but a helical turn (S38–K41) appears to stabilize this part of the protein structure for P43MG(Ca). Several characteristic NOEs obtained from 2D and 3D NMR experiments support this novel helix. A similar, short helix exists in the crystal structure of calcium-bound wild-type calbindin D_{9k}—but this is the first observation in solution for wild-type calbindin D_{9k} or any of its mutants.

Keywords: calbindin D_{9k}; calcium-binding constants; calcium-binding protein; induced structure; NMR assignment

Calbindin D_{9k} (Fig. 1) is a small calcium-binding protein belonging to the EF-hand family that includes calmodulin, troponin C, and parvalbumin (Ikura, 1996; Schäfer & Heizmann, 1996). Its small size and readily accessed expression system (Brodin et al., 1986; Linse et al., 1987) provides an excellent model for the study of structure and function in other EF-hand proteins, including the role of intra-cellular calcium and protein stability. The EF-hand family contains helix-loop-helix motifs (the loop generally contains 12 or 14 residues with a calcium-binding site) known as EF-hands (Kretsinger & Nockolds, 1973). Paired EF-hands contain two calcium-binding loops adjacent to each other with a short region of β-sheet between; this structure plays an important role in the cooperativity reported for calcium-binding (Linse et al., 1991). The two EF-hands form a four α-helix module that binds two calcium ions. Several such modules with sites of varying calcium affinity may be present in an EF-hand protein—calmodulin contains two such modules with four high affinity calcium sites connected through a long, predominantly helical, linker (Babu et al., 1988). Recombinant wild-type calbindin D_{9k} consists of 75 amino acid residues in a single four α-helix module (Fig. 1).

In one class of EF-hand proteins, called calcium sensors, calcium-binding results in a large structural change with partial exposure of the hydrophobic core of the four helices (Ikura, 1996). This plays an important role in the regulatory function of proteins such as calmodulin. The second class, called calcium buffers (or transporters) (Ikura, 1996), undergoes much smaller structural changes involving repacking of the hydrophobic core upon calcium-binding. Calbindin D_{9k} is the best characterized EF-hand protein of this second class (Chazin et al., 1989; Kördel et al., 1989; Kördel et al., 1990; Kördel et al., 1992; Kördel et al., 1993; Skelton et al., 1995). The two calcium-binding sites of calbindin D_{9k} act co-operatively,

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Abbreviations: NMR, Nuclear Magnetic Resonance Spectroscopy; NOESY, Nuclear Overhauser Spectroscopy; NOE, Nuclear Overhauser Enhancement; COSY, Correlation Spectroscopy; DQF-COSY, Double-Quantum Correlation Spectroscopy; R-COSY, Relayed Correlation Spectroscopy; PE-COSY, Primitive Exclusive Correlation Spectroscopy; TOCSY, Total Correlation Spectroscopy; ROESY, Rotating-frame Overhauser Spectroscopy; $^3J_{HNH\alpha}$, coupling constant (Hz) between an amide proton and alpha proton; UV, Ultraviolet Spectroscopy; $\Delta H\alpha$ represents the difference in chemical shift for a given Hα proton with its random coil chemical shift; $\Delta\delta$ is the difference in chemical shift between two mutants; ΔG_{TOT} is the total free energy of binding two calcium ions; K_1 and K_2 are calcium binding constants.

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[2]P43 of wild-type calbindin is replaced by G in the P43G mutant and by the two amino acid sequence MG (M43G43a) in P43MG. This nomenclature (for P43MG) is utilized to aid the comparison of the chemically homologous parts of the proteins (residues M0-G42 and S44-Q75). Ca-bound forms of calbindin D_{9k} indicated as (Ca) after the mutant name are defined as complexed with two calcium ions in this paper.

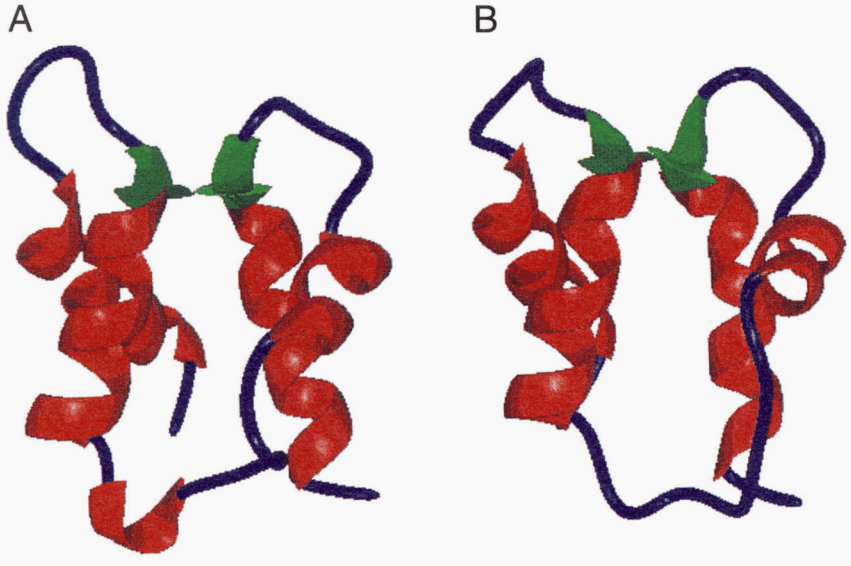

Fig. 1. NMR-derived structures of (**A**) calcium-free (apo) calbindin mutant P43G (PDB file: 1clb.pdb) (Skelton et al., 1995) and (**B**) calcium-bound (Ca) calbindin mutant P43G (PDB file: 2bca.pdb) (Kördel et al., 1993). Helices are colored red and a short stretch of β-sheet between the two calcium-binding loops is shown in green. The tether region is shown at the base of the protein structures and is predominantly blue (random/turn structure). A short helical turn in the tether region of the apo-form structure is defined as irregular in the PDB file. This helical turn is the result of molecular modelling and is not strongly supported by primary NMR data, as discussed in the text.

as do the sites in the majority of EF-hand proteins, and sequential calcium-binding is only observed for mutants that perturb the affinity of one of the calcium-binding loops (Carlström & Chazin, 1993; Wimberly et al., 1995).

Figure 1 illustrates the general structure of calbindin D_{9k} in its calcium-free (apo) (Skelton et al., 1995) and calcium-bound (Ca) forms (Kördel et al., 1993). In the course of our studies with calbindin D_{9k}, we discovered a mutation and amino acid insertion (P43MG) that lead to an increase in calcium affinity. This paper is a preliminary exploration of the structural basis for the higher calcium affinity of this mutant. The tether region between the two EF-hands—some 15 to 20 Å from residues in the calcium-binding loops that are anchored into the hydrophobic core (L23 and V61)—contains the mutation/insertion. The tether is a flexible linker with little or no strong structural characteristics reported to date. Wild-type calbindin D_{9k} provides evidence for this to be the case in solution. A proline residue (P43) in the middle of the tether region undergoes cis-trans isomerization at a rate (0.2 s^{-1}) that is comparable to that found in small, randomly structured peptides (Chazin et al., 1989; Kördel et al., 1990). However, this cis-trans isomerization appears to also occur in the crystal state (Svensson et al., 1992) in which a well-defined helical turn occurs between S38 and K41 (Szebenyi & Moffat, 1986). NMR dynamic studies of the P43G mutant show the tether region to be more mobile than the well-defined helices of the protein with similar dynamic characteristics for both the apo- and calcium-bound forms (Kördel et al., 1992; Akke et al., 1993). By contrast, Kördel et al. (1993) report a hydrogen bond between K41HN and S38O in the NMR structure of P43G(Ca) (21/33 structures); this is consistent with the helical region found in the calcium-bound wild-type crystal structure (Szebenyi & Moffat, 1986). Most calbindin studies by NMR include the mutation P43G or P43M to alleviate the complexity of NMR spectra caused by the two conformations of P43 in native protein.

There has been no rational explanation why a mutation at position 43 leads to such a variation in calcium-binding properties for wild-type P43G and P43M mutants if this is truly a region of little structural importance (Table 1, Fig. 2). The changes in calcium-binding affinity due to the D19N (+4 kJ·mol^{-1}) (Linse et al., 1988) and V61G (+6 kJ·mol^{-1}) (S. Linse, unpubl. work) mutations of the calcium-binding loops (and hydrophobic core for V61G) are of similar magnitude as the change due to the P43M mutation (although many other mutations result in much smaller effects). To summarize, there may be some ill-defined or transitional helical content present in the first few residues of the tether of solvated calbindin D_{9k} and its mutants—similar to that seen in the wild-type crystal structure. However, there is little evidence to suggest a stable structural element in the tether region of wild-type calbindin D_{9k} or any of its mutants studied so far in solution.

We have used NMR spectroscopy to identify and characterize a novel, well-formed structural element found only for P43MG(Ca) in solution. Backbone ^{1}H NMR assignments indicate that the main

Table 1. *Calcium-binding constants of wild-type calbindin D_{9k} and several mutants illustrating the improved calcium affinity of P43MG*

Mutant	ΔG_{TOT}[a,b] (kJ·mol^{-1})	log K_1[b]	log K_2[b]
Wild-type	−96	8.2	8.6
P43G	−95	8.0	8.7
P43M	−93	7.7	8.6
P43MG	−97	8.2	8.8
P43MGG	−93	7.8	8.4
P43M + V61G	−87	6.8	8.5
D19N	−89	7.6	8.0

[a] $\Delta G_{TOT} = -RT \ln K_1 K_2$.
[b] The error limits for ΔG_{TOT} are 0.5 kJ·mol or less, and for log K_1 and log K_2 0.05 to 0.1.

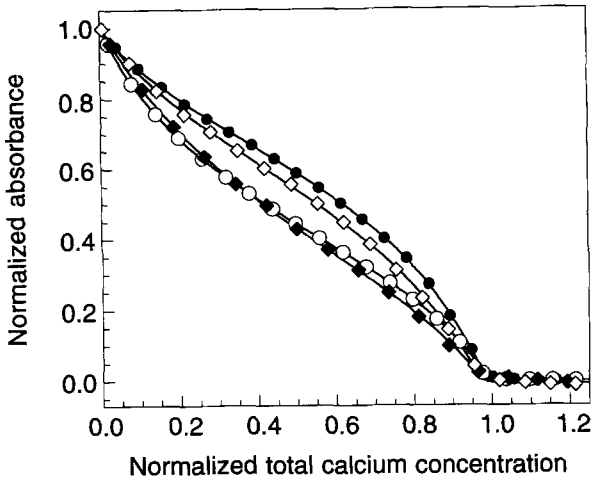

Fig. 2. Determination of calcium binding constants from calcium titrations of 25 μM quin 2 (a chromophoric calcium chelator) in the presence of 25 μM wild-type or mutant calbindin D_{9k}. The UV-absorbance at 263 nm was monitored as a function of total calcium concentration. Experimental data points for wild-type (open diamonds), P43M (open circles), P43MG (filled circles), and P43MGG (filled diamonds). The curves shown with solid lines were obtained by least squares fitting to the data points using two macroscopic calcium-binding constants (K_1 and K_2) for the protein and one pre-determined calcium-binding constant for the chelator (K_Q = 1.92×10^8 M^{-1}). In the graph, the total calcium concentration is normalized so that 1.0 corresponds to one times the chelator concentration plus two times the protein concentration, and the absorbance is normalized so that 1.0 corresponds the absorbance for the solution in complete absence of calcium and 0.0 to the fully calcium-saturated case.

region of structural change is between S38 and K41. Both $\Delta H\alpha$ chemical shifts and $^3J_{HNH\alpha}$ values reveal that residues S38–K41 have helical characteristics for P43MG(Ca)—but not in the absence of calcium. To extract the clearest results from our data, and to show this new structural element to be well-defined, we compare the NMR data for P43MG with the published NMR data of P43G (Kördel et al., 1992; Akke et al., 1993; Kördel et al., 1993; Skelton et al., 1995) that is structurally homologous to wild-type calbindin D_{9k} (Kördel et al., 1993) and to their published structures (Szebenyi & Moffat, 1986; Kördel et al., 1993; Skelton et al., 1995).

Calmodulin contains relatively short tethers between its EF-hand pairs, analogous to calbindin D_{9k}. Three calmodulin-target peptide complexes involving predominantly hydrophobic interactions have been solved to date (Ikura et al. 1992, Meador et al., 1992, 1993). Interactions are observed between calmodulin's tether region and its target peptides (residues 39, 41, 114, and 116; Meador et al., 1993). Residues 112, 114, and 115 may play a role in the binding of neuromodulin "IQ-motif" through their side-chains and the proposed "semi-open" apo calmodulin structure (Swindells & Ikura, 1996). Other EF-hand proteins, such as calbindin D_{28k} (Fullmer & Wasserman, 1987), calretinin (Cheung et al., 1993), recoverin (Flaherty et al., 1993; Tanaka et al., 1995), sarcoplasmic calcium-binding protein (Vijay-Kumar et al., 1992; Cook et al., 1993), and S100 (Isobe & Okuyama, 1978; Drohat et al., 1996), have long putative tether regions between their EF-hands. The structures of most of these proteins are unknown, but recoverin contains a helix between its third and fourth EF-hands (Flaherty et al., 1993; Tanaka et al., 1995). The site of myristoyl binding and potential interaction sites for acidic phospholipid membranes have been reported for recoverin (Tanaka et al., 1995), yet no role has been assigned to the helix between the EF-hands. The present work provides an insight into the possible roles of long tethers and their potential structures.

Results and discussion

General characteristics of P43MG

The smooth preparation and purification of P43MG, one of many calbindin mutants prepared in the lab, suggests similar general characteristics to those of wild-type calbindin and other mutants. The UV spectra of P43MG in its apo- and calcium-bound states (not shown) are typical for calbindin mutants (Brodin et al., 1986). The calcium affinity of P43MG, higher than expected, made the apo-form of the protein difficult to prepare. This difficulty supports the figures given in Table 1 that were measured several times by a competitive UV titration method (Fig. 2) to confirm the additional calcium affinity of P43MG over wild-type calbindin and other similar mutants. The P43MG mutation does not appear to cause any change in the positive cooperativity of calcium binding.

Backbone ^1H NMR assignments

Figure 3 compares the backbone (HN and Hα) assignments of both apo- and calcium- forms of P43G and P43MG. The most striking feature of these plots is the relative amplitude of the $\Delta\delta$ values (ppm) between apo- (Fig. 3A) and calcium-forms (Fig. 3B) for the HN data. The $\Delta\delta$ values for apo-form are small, with only a few residues showing changes greater than 0.2 ppm (and all are less than 0.32 ppm). These small values are typical for a mutation or insertion made in a structurally insensitive region of the protein, i.e., as in a region of no structure. Much of the scatter for the apo-forms arise from their structural or chemical instability under the conditions of study—some resonances drift by up to 0.05 ppm over a two-week period at NMR conditions. The $\Delta\delta$ values for the calcium-bound forms are more dramatic (Fig. 3B). Only moderate $\Delta\delta$ values are observed for the residues flanking the mutation but large $\Delta\delta$ values of about 1 ppm are seen close to the mutation site (L40 and K41, Fig. 3B), suggesting that the insertion is made in a conformationally stable/structured region of the protein. The calcium-bound form of calbindin D_{9k} is chemically stable and there is a good agreement in the amide chemical shifts of the two mutants with little variation for the first 30 residues. Small scatter is seen for the C-terminal end of helix II (residues 24–35, particularly Q33), helix III (residues 46–54) and the C-terminal end of helix IV (residues 62–74). These residues form the base of the hydrophobic core, structurally close to the mutation site. In contrast, the P43G and trans-P43 wild-type amide assignments agree well with much less scatter and maximum differences of about 0.3 ppm in a plot similar to Figure 3B (Kördel et al., 1990). Little structure is reported for the tether of P43G(Ca) (Kördel et al., 1993) and we conclude that P43MG(Ca) contains a "new" structural element, close to the glycine insertion.

The comparison of Hα chemical shifts for the apo-forms of P43G and P43MG (Fig. 3C) shows little difference in the tether region of the mutants (L31 is close to an aromatic ring and sensitive to small changes in local conformation that may or may not be important). The upfield shifts observed for the Hα resonances of S38, L40, and K41 of P43MG(Ca) (Fig. 3D) show a more helical nature than the identical residues of P43G(Ca). A more general chemical shift correlation, made with random-coil values

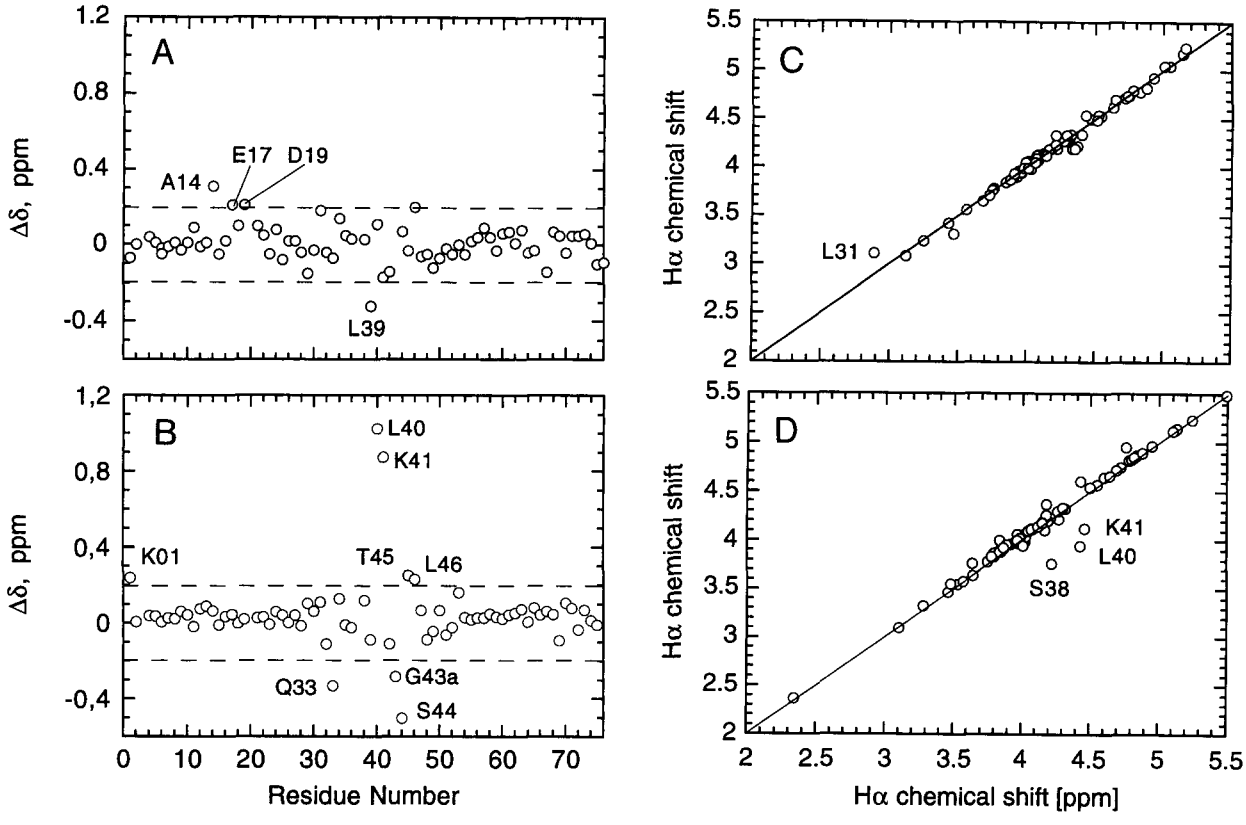

Fig. 3. Comparison of ^{1}H NMR backbone assignments (HN, Hα) for P43G (Kördel et al., 1990; Skelton et al., 1990) and P43MG as a function of residue: (**A**) Difference ($\Delta\delta$, ppm) in HN chemical shifts for apo-forms, (**B**) difference in HN chemical shift ($\Delta\delta$, ppm) for calcium-bound forms. Direct comparison of Hα chemical shifts drawn as P43HG versus PY3G for (**C**) apo-forms and (**D**) calcium bound forms.

of chemical shifts, indicates upfield shifts from random-coil values (ΔHα > 0.38 ppm) as helical (Wishart et al., 1991; Wishart et al., 1995). On this basis, both P43G(apo) and P43MG(apo) contain little helical character; P43G(Ca) has helical character for residue L39 only and P43MG(Ca) has helical character for the region S38–K41.

$^3J_{HNH\alpha}$ assignments

Coupling constants ($^3J_{HNH\alpha}$) characterize secondary structure elements (Wüthrich, 1986). Protein dihedral angles result in small $^3J_{HNH\alpha}$ values (<5.5 Hz) for helices and larger values (>8 Hz) for β-sheet residues. For technical reasons, it is difficult to accurately measure the absolute value of $^3J_{HNH\alpha}$ and we only consider differences >1 Hz between P43G and P43MG to be significant (Fig. 4) (Neuhaus et al., 1985). Only three such differences are observed in the comparison of apo-forms (Fig. 4A). Two of these residues (S62 and E27) reside in the two calcium-binding loops and the insertion at P43MG should not affect them directly. Several residues are observed within the 1 Hz boundaries that belong to different classes (helical, random, and beta-like) for the two mutants. Figure 4A suggests few, if any, large conformational differences between P43G(apo) and P43MG(apo).

More residues fall outside the boundaries or belong to different classes for the comparison of P43G(Ca) with P43MG(Ca) (Fig. 4B). Several helical residues (A14, Q33, D47, and others) retain their class but fall outside the 1 Hz boundaries—this is probably an artefact in measuring small $^3J_{HNH\alpha}$ values. Residues S24 and K29 are outside the boundaries but within the same class. Residues S38, L40, and K41 stand out as the only residues in Figure 4B that both change class and fall far outside the 1 Hz boundaries. The sequence S38–K41 is classed as largely randomly structured for P43G(Ca) while the same residues are helical ($^3J_{HNH\alpha}$ < 5.5 Hz) for P43MG(Ca). Thus, the $^3J_{HNH\alpha}$ data supports the Hα chemical shift data presented above that there is new helical character in the tether region of calcium-bound P34MG calbindin.

Characteristic NOEs for P43MG(Ca)

It is well established that proton pairs of <5 Å distance reveal structural elements through characteristic NOEs (Wüthrich, 1986). Unfortunately, many NOEs characteristic of helical structures that are observed in the 2D NOESY and ROESY spectra are rendered ambiguous either through alternative possible assignments or through being buried under overlapping peaks (assessed using unpublished scripts developed by J. Evenäs). A 3D NOESY-HSQC experiment with ^{15}N-labeled protein was obtained at 600 MHz to solve this problem. This spectrum benefited from the increased sensitivity of our 600 MHz instrument, the use of mild water-suppression techniques that do not affect peak intensities so greatly, and the filtering out of overlapping aromatic resonances in addition to the use of the ^{15}N-filtered third dimension to aid dispersion. Many of the characteristic NOEs shown in Figure 5 were obtained from this spectrum.

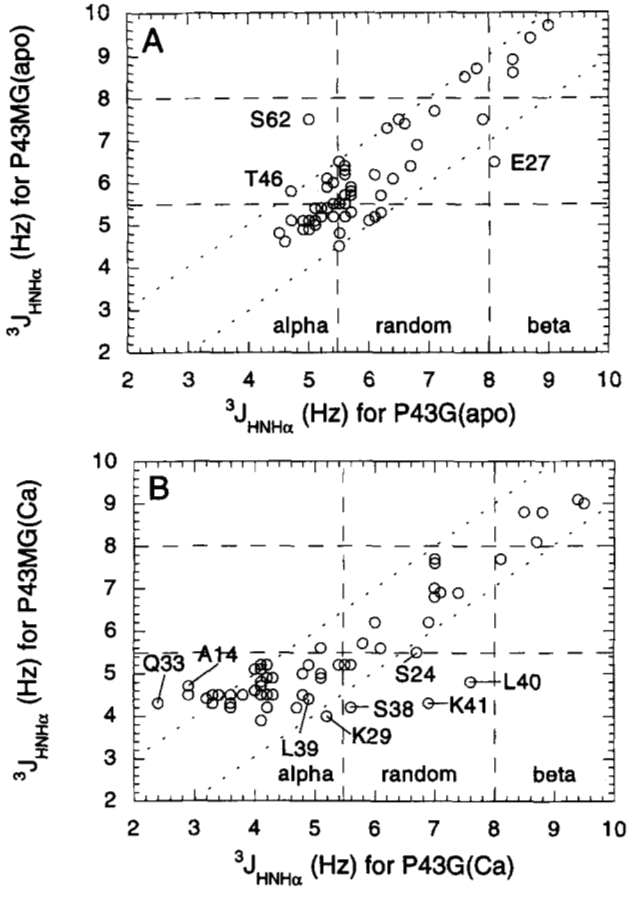

Fig. 4. Comparison of the backbone $^3J_{HNH\alpha}$ coupling constants of P43G and P43MG for (**A**) apo-forms and (**B**) calcium-bound forms. The graph is split into three regions for each axis corresponding to helical region (<5.5 Hz), random region (5.5–8.0 Hz), and β region (>8.0 Hz). Two dashed lines extend diagonally and represent a ±1 Hz error range.

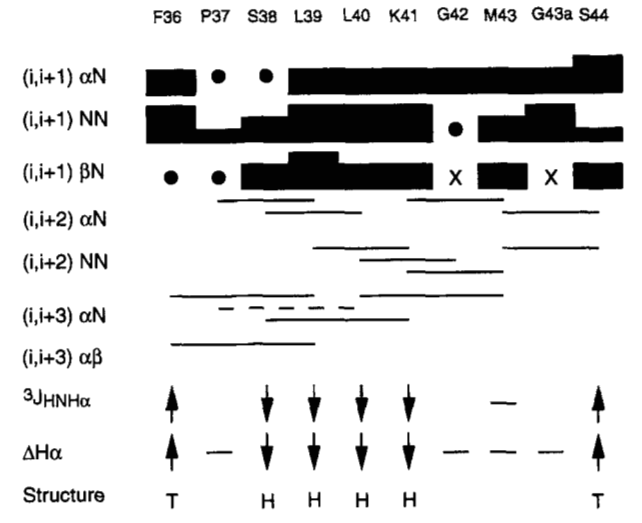

Fig. 5. Summary of unambiguous NOEs involving residues F36–S45 obtained from 2D NOESY, 2D ROESY, and 3D NOESY-HSQC experiments that define the novel helix of calcium-bound P43MG calbindin. The Hδ protons of P37 are used in place of the missing amide proton of this residue (Wüthrich et al., 1984). The height of the bars indicates the strength of the sequential (i, i + 1) NOEs with circles denoting observed but ambiguous crosspeaks. Horizontal lines link residues involved in unambiguous medium range NOEs (i, i + 2 and i, i + 3). No (i, i + 4) αN connectivities were observed in this part of the protein. For $^3J_{HNH\alpha}$ and $\Delta H\alpha$ the "up arrows" denote β-sheet or turn-like structure and the "down arrows" denote helix.

The NOE evidence (Fig. 5) supports the data obtained from values of coupling constants, $^3J_{HNH\alpha}$, and chemical shift differences relative to random coil, $\Delta H\alpha$; the region from S38 to K41 clearly forms a helix with many medium to strong sequential connectivities and several medium-range interactions. For the rest of the tether we observe weak to medium strength sequential NOEs and fewer medium-range NOEs. How long is the new helix? The evidence for the continuation of the helix through residues G42 to G43a is weak. A number of changes in amide chemical shift occur at the C-terminal end of helix II (S24–E35) for P43MG(Ca) but this helix appears to be left intact. The (i, i + 3) connectivities between F36 and L39 with the turn-like attributes of $^3J_{HNH\alpha}$ and $\Delta H\alpha$ for F36 suggest the same kinked relationship of helix II and the new helix as is observed in the wild-type crystal structure (Szebenyi & Moffat, 1986). The S38–K41 region is described as an irregular helix for the crystal structure of wild-type calbindin and as a single turn of 3_{10} helix for the P43G(Ca) NMR structure. It is difficult to differentiate between regular and 3_{10} helix for P43MG(Ca) for such a small sequence due to the few unambiguous, characteristic medium-range NOEs available. However, we can conclude that a helical turn structure is formed.

Several unambiguous NOEs (2D homonuclear NOESY) involving the side chains of L39 and L40 show that these residues are anchored to the base of the hydrophobic core of P43MG(Ca) (Table 2). These NOE assignments would be expected if the helical turn of the crystal structure of wild-type calbindin was maintained in solution. These hydrophobic interactions could play an important driving force for structure formation by providing a framework for the helix and in the differentiation of 3_{10} from regular helix. Interestingly, the residues mentioned in Table 2 are found in the C-terminal end of helix II, Helix III, and the C-terminal end of helix IV—areas that have small $\Delta\delta$ values in Figure 3B (discussed above). This suggests that the small scatter observed in Figure 3B is caused by the same structural reasons as the much larger changes close to the site of mutation. We cannot make a structural comparison of apo- and calcium-bound forms at this time due to the incomplete NOESY assignment of P43MG(apo) and the need to assess peak ambiguity against initial structures. The near-degeneracy of the methyl groups of L39 and L40 for

Table 2. *Long range (i, j > 4) NOE contacts involving the methyl groups of L39 and L40*

NOE from	to
L39Hδ	L32Hδ, F36Hβ M43HN, M43Hγ, G43aHN K72HN, I73Hα
L40Hδ	L32Hβ, Q33HN, F36HN S44HN T45HN, T45Hα, L46HN, L46Hα D47HN, E48HN, L49HN, L49Hβ, F50HN

P43MG(apo) suggests that they are not as well anchored as for P43MG(Ca) and could be part of the predominantly random structured tether that has been previously described (Skelton et al., 1995).

Comparison of other mutants

The general NMR data for P43M(Ca) (T. Drakenberg, unpubl. work) is similar to that for P43G(Ca) with only a few moderate chemical shift differences around the site of mutation. P43M(Ca) shows little helical structure in its tether region on the basis of $^3J_{HNH\alpha}$ and $\Delta H\alpha$ values. Neither P43M(Ca) nor P43G(Ca) appear to contain the helical region found in P43MG(Ca). Glycine is known to be a poor building block for helix formation (O'Neil & DeGrado, 1990) and the data available for P43M(Ca) suggests that this mutant does not contain any more structure than P43G(Ca). This implies that the calcium-induced structure of P43MG is a function of the additional residue (M or G) rather than a function of the type of residue to which P43 is mutated.

The mutant P43MGG has a low calcium-binding affinity similar to P43M (Table 1). One structural explanation for this is that the double extension prevents the formation of helix in the calcium-bound form of the protein. Another is that the double extension now allows the helix formation in both apo and calcium-bound forms, effectively neutralizing the calcium-induced benefit observed with P43MG. A NMR study similar to this one is currently underway with P43MGG to try and answer these questions.

Concluding remarks

This study illustrates the formation of a structured helical element in a region previously described as unstructured in both wild-type calbindin and its mutants. The helical element appears to be anchored to the base of the hydrophobic core, possibly resulting in small structural changes to the protein additional to the small calcium-induced changes in relative helix angles and hydrophobic core packing that are observed for other mutants. No dramatic differences in chemical shift between P43G(Ca) and P43MG(Ca) are observed for residues in the calcium-binding loops. This suggests that the increased calcium-binding affinity of the P43MG mutant arises from an increased protein stability brought about by the calcium induced helix formation. The mutation of P43MG was not expected to result in a higher calcium-binding affinity than native calbindin. Further insights are expected from the comparison of detailed NMR-derived structures of the apo-, the cadmium-bound, and the calcium-bound forms of P43MG, and from NMR dynamic studies to illustrate changes in mobility of the tether upon calcium-binding. These future studies should shed further light on some of the more subtle structural nuances of P43MG(Ca) hinted by the data presented above. In particular, we expect to discover the full relationship between the new structure and the enhanced calcium-binding affinity of P43MG.

The structural changes that occur upon calcium-binding to calbindin D_{9k} are small compared to calcium sensors such as calmodulin. The allosteric effect of calcium-binding to P43MG calbindin induces new structure about 25 Å from the calcium-binding sites. Most EF-hand protein structures solved to date do not include structure in their tether regions. This may be because calbindin D_{9k} and calmodulin contain relatively short tether regions compared to the extended tether of P43MG calbindin and the helix containing tether of recoverin. Calbindin D_{28k} (Fullmer & Wasserman, 1987) calretinin (Cheung et al., 1993) and S-100 (Isobe & Okuyama, 1978) are structurally unsolved EF-hand proteins that contain longer putative tether regions whose role and function are unknown.

The combination of a longer structure containing tether with a calmodulin-like activity could be advantageous: the induced structure could help to stabilize the calcium-bound state and help to define a surface that can be used to bind target peptides/proteins. Calmodulin is a ubiquitous protein known to interact with more than 30 other proteins predominantly through its calcium-induced exposed hydrophobic core. The short, flexible tether regions of calmodulin make hydrophobic contacts with the three target peptides whose complexes have been characterized to date, illustrating that the tether region has an important role in target protein recognition. Most members of the EF-hand family are localized within certain tissue-types and appear to have a more specific role than calmodulin. EF-hand proteins would benefit from a more rigid target protein-binding site afforded by structure (calcium-induced or not) in the tether region to obtain specificity.

Materials and methods

Sample preparation

Previously published methods for the expression and purification of protein (Brodin et al., 1986; Linse et al., 1987) and the preparation of apo-form protein (Skelton et al., 1990) were employed. For this study two oligonucleotides in the synthetic wild-type gene (Brodin et al., 1986) were changed/lengthened to produce the P43MG calbindin D_{9k} mutant. These changes (underlined) are summarized below:

Amino acid sequence	LEU LYS GLY MET GLY SER THR LEU ASP
Oligo 17	5′-GGT CCG ATG TCT ACT CTG GAT G-3′
Oligo 14	3′-AC GAC TTT CCA TAC CCA AGA-5′

The preparation of ^{15}N labeled protein closely followed the preparation of TR_2C, a tryptic fragment of calmodulin (Finn et al., 1995), except for the use of the modified plasmid for calbindin D_{9k}, described above, rather than that of TR_2C.

Calcium-binding constants

The method for the measurement of calcium-binding by competitive UV-spectrophotometry can be found in the references from which several of the calcium-binding results are taken (Linse et al., 1991; Linse et al., 1993). The results presented here and quoted from other studies are the average of several separate determinations. The precision in the total free energy of binding two calcium ions, $\Delta G_{TOT} = -\ln K_1 K_2$, is generally high whereas the division into separate binding steps, K_1 and K_2, is less precise, especially when there is strong positive cooperativity.

2D ^1H NMR spectroscopy of P43MG(apo) and P43MG(Ca)

A 5 mM solution of protein was prepared in 90:10 H_2O:D_2O (or 99.996% D_2O) and adjusted to pH 6 (ignoring isotope effects). NMR experiments were performed at 27 °C and 500.13 MHz on a GE-500 NMR spectrometer fitted with a triple resonance Bruker probe. Several optimised spectral widths were employed of between 6211 and 8000 Hz with 2k or 4k of data collected in t_1 and

solvent suppression used for H_2O samples. In the t_2 dimension, between 400 and 722 fids were collected using the States-TPPI procedure (States et al., 1982). Standard 2D NMR experiments were run and included (mixing times in parenthesis): COSY (Marion & Wüthrich, 1983), DQF-COSY, R-COSY (30 ms) (Wagner, 1983), PE-COSY (35 ms) (Mueller, 1987), TOCSY (10–80 ms) (Braunschweiler & Ernst, 1983), 2Q (30 ms) (Braunschweiler et al., 1983), ROESY (38–150 ms) (Bothner-By et al., 1984), and NOESY (20–200 ms) experiments. All spectra were processed and assigned using FELIX (Molecular Simulations Inc., San Diego). Assignment was assisted by the assignments of the P43M(Ca) (T. Drakenberg, unpubl. work) and P43G (apo and Ca) mutants (Kördel et al., 1990; Skelton et al., 1995). This extensive set of NMR experiments allows us to verify backbone assignments, for example through sequential NOE assignments (Wüthrich, 1986), and will facilitate high resolution NMR structure determinations in the future.

NMR spectroscopy of ^{15}N labelled P43MG(Ca)

A 3.5 mM sample of ^{15}N labelled calbindin P34MG(Ca) was prepared and investigated at pH 6.0 and 27 °C. NMR experiments were run on a Varian Unity-plus 600 at 599.891 MHz (^1H frequency) and 60.793 MHz (^{15}N frequency). A HSQC experiment (Zhang et al., 1994) was rapidly interpreted from the amide chemical shifts determined from 2D spectroscopy and the ^{15}N chemical shifts published for P43G(Ca) (Skelton et al., 1992). A 3D NOESY-HSQC (Zhang et al., 1994) was obtained. The 256 × 128 × 2048 complex data set was collected with 8 scans per fid; the t_1 and t_2 dimensions were linear-predicted to give 256 × 128 real data points and zero-filled to give a final spectrum of 512 × 256 × 512 points. Shifted (60°–75°) squared-sine window functions were applied before transformation and the right hand portion of the t_3 dimension was discarded to reduce memory requirements.

Supplementary material in electronic index

Full ^1H NMR assignments of P43MG(apo) and P43MG(Ca).

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