

Metal-ion affinity and specificity in EF-hand proteins: coordination geometry and domain plasticity in parvalbumin

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Background: The EF-hand family is a large set of Ca²⁺-binding proteins that contain characteristic helix-loop-helix binding motifs that are highly conserved in sequence. Members of this family include parvalbumin and many prominent regulatory proteins such as calmodulin and troponin C. EF-hand proteins are involved in a variety of physiological processes including cell-cycle regulation, second messenger production, muscle contraction, microtubule organization and vision.

Results: We have determined the structures of parvalbumin mutants designed to explore the role of the last coordinating residue of the Ca²⁺-binding loop. An E101D substitution has been made in the parvalbumin EF site. The substitution decreases the Ca²⁺-binding affinity 100-fold and increases the Mg²⁺-binding affinity 10-fold. Both the Ca²⁺- and Mg²⁺-bound structures have been determined, and a structural basis has been proposed for the metal-ion-binding properties.

Conclusions: The E101D mutation does not affect the Mg²⁺ coordination geometry of the binding loop, but it does pull the F helix 1.1 Å towards the loop. The E101D–Ca²⁺ structure reveals that this mutant cannot obtain the sevenfold coordination preferred by Ca²⁺, presumably because of strain limits imposed by tertiary structure. Analysis of these results relative to previously reported structural information supports a model wherein the characteristics of the last coordinating residue and the plasticity of the Ca²⁺-binding loop delimit the allowable geometries for the coordinating sphere.

Introduction

Calcium modulates many physiological functions through regulatory Ca²⁺-binding proteins, which must be able to distinguish Ca²⁺ ions in the presence of greater concentrations of other metal cations. A large class of Ca²⁺-binding proteins that display this sort of preferential Ca²⁺-binding is the EF-hand family. EF-hand proteins contain characteristic helix-loop-helix Ca²⁺-binding sites with highly conserved coordinating residues and secondary structure [1–3]. They are involved in many basic physiological processes such as muscle contraction, vision, cell cycle regulation, brain cortex and cerebellum modulation, and microtubule organization [4].

Parvalbumin is a member of the EF-hand family of Ca²⁺-binding proteins. It is a small, 10–12 kDa protein found in type II fast twitch muscle and fast firing nerve cells, particularly in neurons secreting γ -aminobutyric acid (GABA) [5,6]. Parvalbumins exist in two isoforms, an α parvalbumin containing 109 residues, and the β species with 108 residues, which is the species involved in these studies. The two EF-hand binding sites present in wild-type parvalbumin are homologous to the EF-hand binding sites

found in larger, regulatory members of the EF-hand family such as troponin C and calmodulin [1]. However, because of its smaller size and superior crystallization properties, parvalbumin is a more agreeable model for study of Ca²⁺-binding properties in EF-hand proteins.

Within the cell, free Ca²⁺ is maintained at very low concentrations, in the 0.1 μ M range, by Ca²⁺–ATP pumps, which transfer excess Ca²⁺ ions through membranes into the extracellular fluids or into storage organelles such as the reticula. Therefore, the Ca²⁺ concentrations required for interaction with intracellular Ca²⁺-binding proteins generally occur only during a Ca²⁺ influx. This type of influx of free Ca²⁺ occurs as a response to an external stimulus, generally of an electrical, chemical, or hormonal nature, interacting with a transmembrane channel or receptor [1]. Unlike Ca²⁺, Mg²⁺ is found inside the cell at relatively constant concentrations in the millimolar range, readily available for interaction with proteins [3].

That the EF-hand binding site is able to discriminate between the two small, divalent cations Mg²⁺ and Ca²⁺ is remarkable. These two alkaline earth metals, both of which

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have a significant physiological role, are identical in charge and quite close in size. Mg^{2+} has an effective ionic radius usually ranging from 0.57 to 0.72 Å, whereas the Ca^{2+} radius is ordinarily between 1.00 and 1.12 Å [7]. Ca^{2+} -binding proteins must exploit small behavioral differences between these two metal ions to attune the physiological pathways they regulate and affect. Both Mg^{2+} and Ca^{2+} are closed-shell, spherical metal ions, making them relatively 'hard', although Mg^{2+} is more likely to share orbitals with other atoms to form partial covalent bonds. The relevant differences are: first, Mg^{2+} favors coordination by fewer ligands than Ca^{2+} , almost always exhibiting octahedral coordination geometry, with six being the preferred coordination number [7]. Ca^{2+} is most commonly found coordinated by seven or eight ligands and exhibits more variability in coordination number and geometry than Mg^{2+} . The coordination number in spherical metal ions is primarily a function of the size of the ion, because optimal coordination is achieved through closely packing as many ligands as possible around the ion [8]. Second, Ca^{2+} -ligand bond distances are typically 2.3 to 2.6 Å and are more variable than Mg^{2+} -ligand bond lengths, which tend to average about 2.0 to 2.1 Å [7]. Finally, Mg^{2+} has a 10^3 times slower substitution rate than Ca^{2+} ; this is because the free energy of desolvation for ions of like charge increases proportionally to the surface charge density of the ion [8].

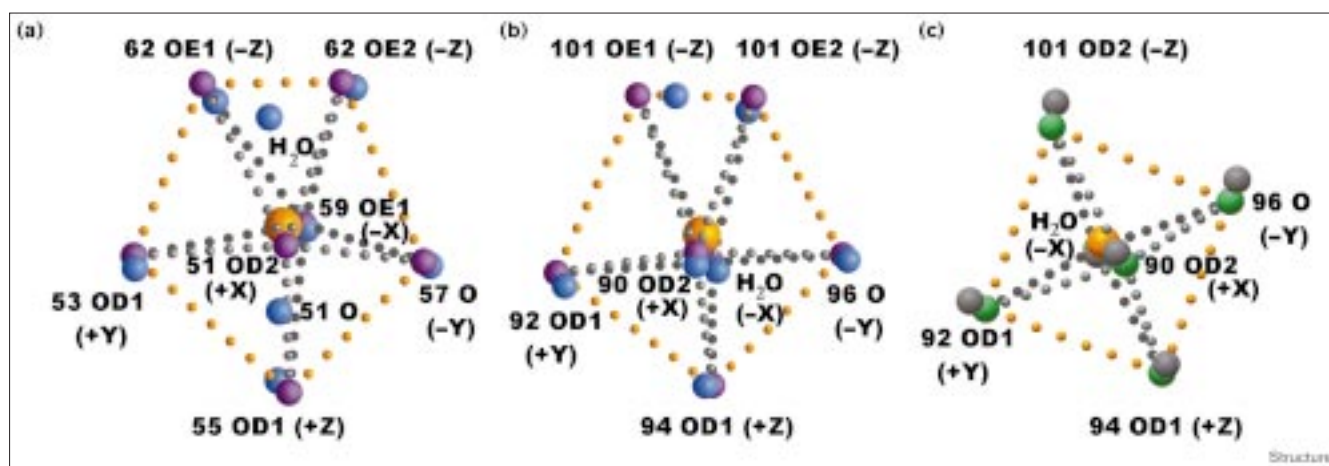
There are two broad classes of Ca^{2+} -binding site within the EF-hand family [3]. The first is Ca^{2+} -specific with a moderate affinity for Ca^{2+} . These sites are regulatory, and as they do not bind Mg^{2+} at physiological concentrations they often remain unoccupied under conditions of normal

resting levels of intracellular Ca^{2+} . All four Ca^{2+} -binding sites in calmodulin and the two N-terminal sites in troponin C are of this type [9–11]. The second class is a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding site that generally has $\sim 10^4$ higher affinity for Ca^{2+} than for Mg^{2+} [3], but commonly contains Mg^{2+} at resting levels of Ca^{2+} because of the greater intracellular concentration of Mg^{2+} . The two C-terminal sites in troponin C and both of the EF-hands in parvalbumin are of this second type [10–12].

The binding loop of the EF-hand helix-loop-helix motif is traditionally defined as the 12 consecutive residues beginning with the first metal-ion-coordinating residue and ending with the last, even though the last three residues are actually part of the second helix. Six of the 12 loop residues participate in both Ca^{2+} and Mg^{2+} binding, although the last coordinating residue (position –Z) is generally a glutamate that changes from a bidentate ligand in the sevenfold ligation of Ca^{2+} to a monodentate ligand in the sixfold coordination of Mg^{2+} [8]. All coordinating ligands are oxygens; however, when the ninth residue of the loop (position –X) does not have a sidechain oxygen to donate, a water molecule is recruited as a ligand. The specific metal-ion-binding residues of the two carp β -parvalbumin binding sites with their X, Y, Z positions and coordination geometry are shown in Figure 1.

Four crystal structures of parvalbumin mutants are reported here, all containing an F102W mutation (using single-letter amino acid notation) for determining metal-ion-binding parameters through monitoring the fluorescence emission

Figure 1



Metal-ion coordination geometry in parvalbumin. (a) The CD loop of the mutant PVEF coordinates calcium with eight ligands (shown here superimposed on wild-type coordination geometry). (b) The characteristic pentagonal bipyramidal geometry of the calcium-bound EF loop's coordinating oxygens in the PVEF mutant is shown

superimposed on the wild-type structure [17]. (c) The EF loop of the PVEF E101D mutant coordinates both calcium and magnesium with the octahedral geometry that is typical of magnesium binding in EF hands. Color coding is as follows: magenta, wild-type carp- Ca^{2+} ; blue, PVEF- Ca^{2+} ; gray, PVEF-E101D- Ca^{2+} ; green, PVEF-E101D- Mg^{2+} .

of the tryptophan. A double mutant, PVEF, was constructed with F102W/D51A substitutions. The D51A mutation replaces the highly conserved first coordinating aspartic acid (position +X) in the N-terminal Ca^{2+} -binding site with an alanine, and thereby inactivates that site at physiological Ca^{2+} levels. Finally, a triple mutant called PVEF-E101D, and containing F102W/D51A/E101D substitutions, has been engineered to explore the role of the glutamic acid at position -Z in the C-terminal Ca^{2+} -binding site. The F102W mutant and the PVEF mutant were crystallized with Ca^{2+} bound in both binding sites. The PVEF-E101D mutant was crystallized with Ca^{2+} in the second site in one case, and with Mg^{2+} in this site in the other case. In both cases, the mutated N-terminal site was unoccupied.

Results and discussion

F102W mutant

Our primary purpose in crystallizing the F102W mutant was to determine whether the Ca^{2+} -binding loop regions were structurally affected by the substitution of tryptophan for phenylalanine at residue 102. It has previously been shown that this substitution allows metal-ion binding to be monitored via an increase in the intensity of the tryptophan fluorescence on Ca^{2+} binding [13–16]. The F102W mutant retains wild-type metal-ion-binding parameters (QL and JDP, unpublished results) and crystallized isomorphously with wild-type parvalbumin [17].

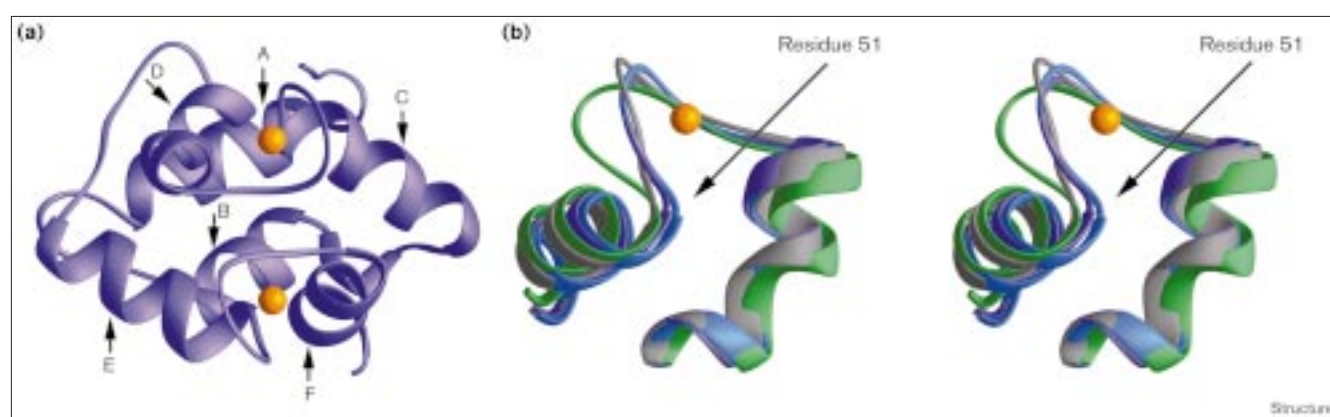
The refined structures of F102W and wild-type carp parvalbumin are also quite similar, with an overall root mean square deviation (rmsd) of 0.67 Å, calculated over all the mainchain atoms. Moreover, calculations comparing fragments representing the binding sites (the last ten

residues from the first helix, the twelve loop residues and the first seven residues of the second helix) of the wild-type versus our mutant displayed even smaller rmsds. Parvalbumin is commonly divided into three domains — AB, CD and EF — each containing a helix-loop-helix motif (Figure 2a). Domain AB contains a deletion of two amino acids in the loop region and consequently does not bind metal ions [18]. Domain CD contains the N-terminal site and EF contains the C-terminal site. The CD sites of wild-type and F102W parvalbumin were overlapped from residues 41–69, and the rmsd between the mainchain atoms of the two regions was 0.13 Å. Similarly, the EF-site regions, containing residues 80–108, were found to have a mainchain rmsd of 0.17 Å. Residue 102 is included in the EF site; it is the first amino acid on the C-terminal side of the binding loop. The very low rmsd observed between the binding-site regions of the mutant and the wild-type structures is consistent with the wild-type metal-ion-binding parameters measured for the F102W mutant. A plot of the inter-residue distance differences between this mutant and the wild type indicates that the N terminus is the primary contributor to the deviations in structure (Figure 3a). This is supported by the fact that the overall rmsd between F102W and the wild type decreases from 0.67 Å to 0.21 Å if you remove the first eight residues from the calculation.

PVEF mutant (F102W/D51A)

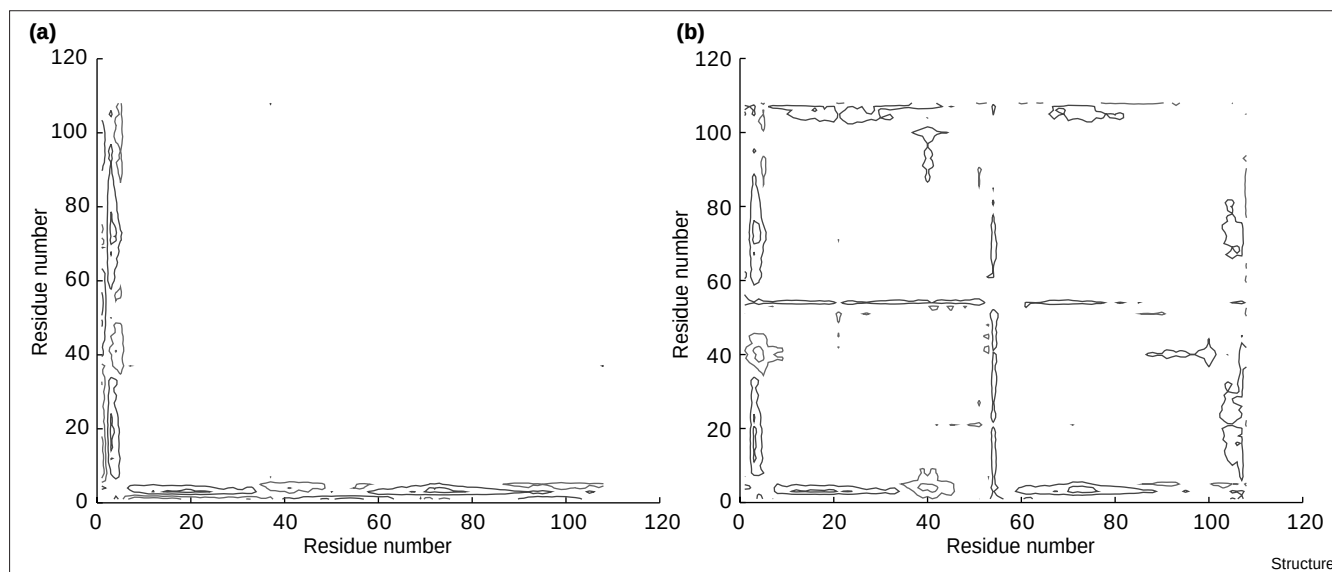
The crystal structure of the PVEF mutant was determined in order to ascertain whether the D51A mutation in the CD loop affects Ca^{2+} coordination in the EF loop. The Ca^{2+} -binding parameters measured in the EF site were of the same order as wild type (QL and JDP, unpublished results). Mainchain rmsd calculations and

Figure 2



Ribbon diagrams of wild-type parvalbumin and the CD loop of mutant parvalbumins. (a) The six helices of wild-type parvalbumin [17] are labeled A–F. (b) Stereoview comparing the CD loop in the parvalbumin mutants with the Ca^{2+} -bound wild type. The D51A mutation is present in all three mutants, but the two E101D mutant structures contain no metal

ion in the CD loop, whereas the PVEF mutant contains Ca^{2+} in an unusual coordination geometry. The mutant PVEF binding site rearranges at residue 51 to allow the carbonyl oxygen of Ala51 to coordinate the Ca^{2+} ion. Color coding is as follows: purple, wild-type carp- Ca^{2+} ; gray, E101D- Ca^{2+} ; blue, PVEF- Ca^{2+} ; green, E101D- Mg^{2+} .

Figure 3

Delta distance plots of parvalbumin mutants versus wild type. (a) F102W–Ca²⁺ minus wild-type carp–Ca²⁺. Contours for Δ of ± 1 Å or greater. The absolute values of the differences in the inter-residue distances between the F102W mutant and wild type are all < 1 Å, except at the termini. The N terminus appears to be especially

dynamic. (b) PVEF–Ca²⁺ minus wild-type carp–Ca²⁺. Contours for Δ of ± 1 Å or greater. The mutation at residue 51 in PVEF affects inter-residue distances primarily in the CD loop (residues 51–62). There also appears to be some variation from wild-type in the C helix (residues 40–50).

inter-residue distance differences (Figure 3b) showed high structural homology between the PVEF and wild-type conformations for the EF site. The rmsd from the wild-type structure for residues 80–108 of PVEF is 0.5 Å. Consequently, it is evident that the double mutation has a greater effect on EF-site structure than the F102W mutation alone. However, when only the Ca²⁺-binding-loop region is considered (residues 90–101), the PVEF to wild-type rmsd decreases to 0.3 Å. The mean Ca²⁺–oxygen distance was within the range expected for wild-type parvalbumin (Table 1). As illustrated in Figure 1b, the sevenfold Ca²⁺ coordination in this site forms the pentagonal bipyramid commonly found in

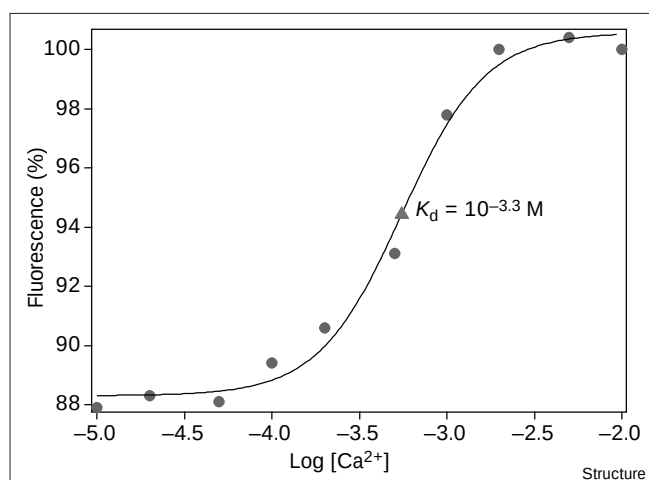
EF-hand proteins. The wild-type and PVEF ligating oxygens superimposed almost identically, with the exception of an approximately 36° tilt in the Glu101 sidechain of the PVEF mutant relative to the wild type.

The original data indicating that the D51A mutation in the CD binding loop inactivated that loop were collected at physiological ratios of Ca²⁺ to mutant protein. Under these conditions, the measured parameters suggested that Ca²⁺ binding was occurring only in the EF loop. However, in our crystals, Ca²⁺ is bound in the CD loop in a nontraditional coordination (Figure 1a). Eight oxygens, instead of the typical seven, are recruited to ligate the Ca²⁺. The

Table 1**Metal ion–oxygen distances in the EF loop.**

Result no.	WT–Ca ²⁺ *	Pike–Ca ²⁺ †	PVEF–Ca ²⁺	E101D–Ca ²⁺	E101D–Mg ²⁺ ‡	E101D–Mg ²⁺ §	Pike–Mg ²⁺ †
101	2.51	2.7	2.44	–	–	–	–
101	2.49	2.49	2.38	2.26 (.04)	1.98 (.07)	2.00 (.06)	2.04
96	2.29	2.33	2.43	2.30 (.04)	1.99 (.08)	1.91 (.06)	2.14
94	2.44	2.3	2.44	2.18 (.04)	2.11 (.09)	2.22 (.08)	2.18
92	2.42	2.26	2.39	2.23 (.05)	2.06 (.10)	2.16 (.07)	2.09
90	2.25	2.19	2.34	2.30 (.04)	1.87 (.07)	1.88 (.06)	2.07
H ₂ O	2.51	2.15	2.29	2.38 (.05)	2.32 (.12)	1.99 (.17)	2.15
<mean>	2.42 ± .01	2.35 ± .04	2.39 ± .003	2.28 ± .005	2.05 ± .03	2.03 ± .02	2.11 ± .003

Distances are in Å. WT, wild-type carp parvalbumin; Pike, wild-type pike parvalbumin. *Distances from [17]. †Distances from [22]. ‡E101D–Mg²⁺: first molecule in asymmetric unit. §E101D–Mg²⁺: second molecule in asymmetric unit. Numbers in parentheses are estimated standard deviations as computed by SHELX-97 [40].

Figure 4

Graph of the PVEF CD loop Ca^{2+} titration performed while monitoring changes in the fluorescence emission of Trp102 at 324 nm (excitation at 290 nm). The EF loop has a K_d of around 10^{-8} M (QL and JDP, unpublished results), and plateaus around 10^{-5} M $[\text{Ca}^{2+}]$. The CD loop does not bind calcium until the concentration is well above the physiological range of intracellular Ca^{2+} levels, as shown, with a K_d of $10^{-3.3}$ M.

residues involved in Ca^{2+} binding in the mutant CD loop are positionally identical to those of the wild-type CD loop, but Ala51 donates the carbonyl oxygen and a water molecule comprises the eighth coordinating oxygen.

In order to clarify these results, Ca^{2+} titrations were performed on the PVEF mutant to determine the point at

which the mutant CD loop begins to bind Ca^{2+} . The data confirmed that the CD loop ($K_d = \sim 10^{-3.3}$) does not bind Ca^{2+} at intracellular concentration levels (Figure 4). The Ca^{2+} concentration in the crystallization drops was very close to the cusp for achieving Ca^{2+} binding in the CD loop in both the PVEF- Ca^{2+} and E101D- Ca^{2+} crystals. However, the PVEF mutant crystallized in a very different space group from the E101D mutants (Table 2), and the crystal-packing conditions in PVEF favored Ca^{2+} binding in the mutant CD loop, whereas in the E101D- Ca^{2+} crystal they did not.

There are at least two existing structures where an alanine residue donates a carbonyl oxygen as the first coordinating residue of a Ca^{2+} -binding loop. The crystal structures of bovine intestinal calbindin D [19] and of domain VI of the small subunit of calpain [20,21] also contain Ca^{2+} -binding loops where this occurs. In these structures the Ca^{2+} -binding loops also exhibit non-conformity when compared to the canonical EF hand, although they do not exhibit the octa-coordination found in PVEF.

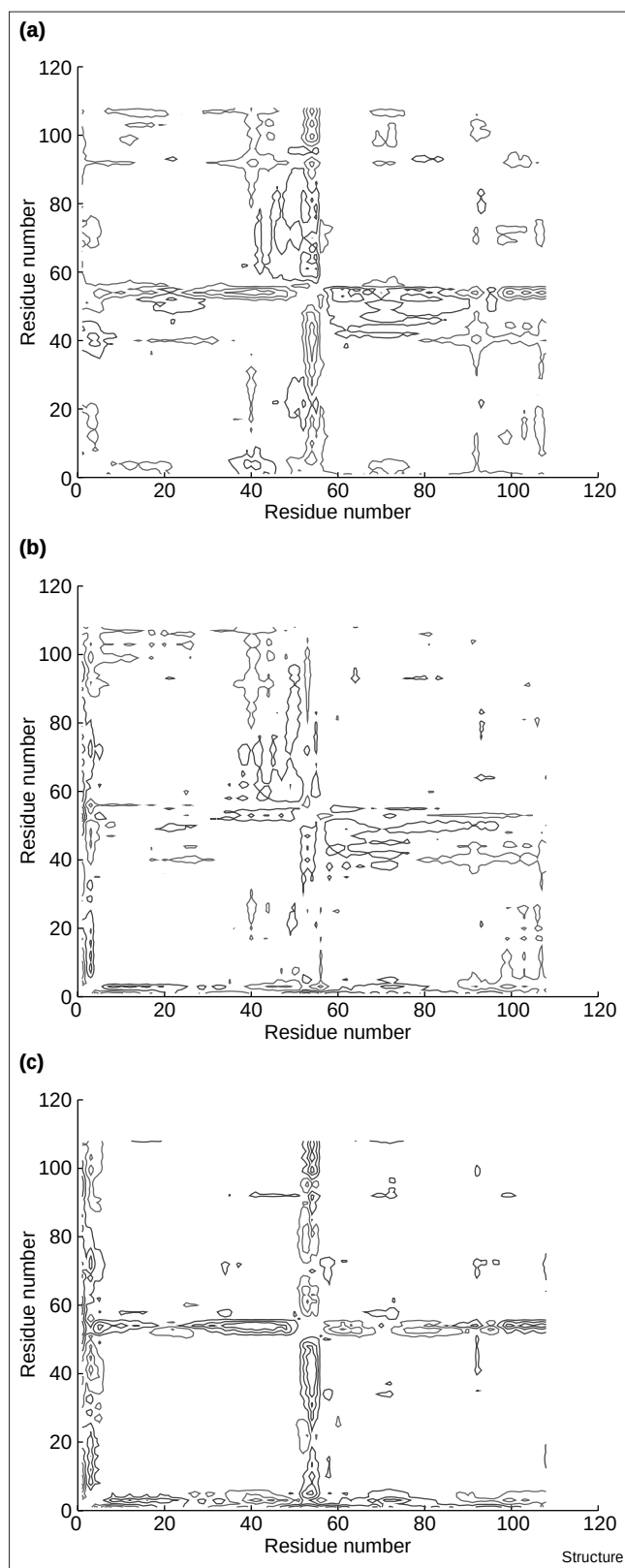
The 0.68 Å mainchain rmsd between the CD sites of wild-type and PVEF parvalbumin is much smaller than the deviations seen between PVEF-E101D and wild-type CD sites where the mutant binding site does not contain a metal ion and the loop structure seems to be substantially relaxed. Nevertheless, the Δ distance plot of Figure 3b shows that rearrangements are required in the C helix of PVEF, and in the residues adjacent to the D51A mutation, presumably to allow the carbonyl oxygen of Ala51 to interact with the Ca^{2+} in the loop

Table 2

Crystallization, processing and refinement statistics for parvalbumin mutants.

Mutant	F102W*	PVEF	PVEF-E101D-Mg ²⁺	PVEF-E101D-Ca ²⁺
Space group	C2	P6 ₅ 22	P2 ₁	P2 ₁
Molecules per asymmetric unit	1	1	2	1
Unit cell				
a (Å)	28.85	33.42	35.23	34.51
b	60.59	33.42	50.21	37.30
c	54.48	298.19	55.37	37.07
α (°)	90.00	90.00	90.00	90.00
β	94.97	90.00	99.22	113.12
γ	90.00	120.00	90.00	90.00
Completeness (%)	95	73	96	94
R _{merge} (%)	7.0	6.9	8.5	6.5
Resolution (Å)	1.9	2.0	2.0	1.7
R (%)				
f _{obs} > 4σ			19.17	16.41
All reflections	17.68	20.96	19.62	16.72
R _{free} (%)				
f _{obs} > 4σ			29.12	22.15
All reflections	27.00	29.02	30.01	22.71

*These are essentially the same unit-cell parameters as wild-type carp parvalbumin: spacegroup C2, a = 28.7, b = 61.1, c = 54.5, β = 94.37 [18].



(Figure 2b). Mean Ca^{2+} -oxygen distances for the PVEF CD loop (Table 3) imply a larger coordination sphere than in wild-type parvalbumin, as would be expected

Figure 5

Delta distance plots of the PVEF-E101D mutants and PVEF. (a) PVEF-E101D- Mg^{2+} minus PVEF- Ca^{2+} . Contours for Δ of ± 1 Å or greater. This plot of the absolute value of the differences in the inter-residue distances between the E101D- Mg^{2+} mutant and PVEF- Ca^{2+} shows considerable rearrangement in the first part of the CD loop (the loop is defined as residues 51–62). The contours in the EF loop region (residues 90–101) represent the difference between the sizes of coordination spheres when Ca^{2+} is in the loop compared with Mg^{2+} . (b) PVEF-E101D- Ca^{2+} minus PVEF- Ca^{2+} . Contours for Δ of ± 1 Å or greater. The E101D- Ca^{2+} mutant also shows differences in the first part of the CD loop when compared to PVEF- Ca^{2+} . The substitution at residue 101 makes very little impact on the backbone conformation. (c) PVEF-E101D- Ca^{2+} minus PVEF-E101D- Mg^{2+} . Contours for Δ of ± 1 Å or greater. Comparison of the two unoccupied CD loops in the Ca^{2+} -bound and Mg^{2+} -bound E101D structures displays different conformations for the two mutants.

from the increase in coordination number, but the difference is within the margin of error for the model.

PVEF-E101D mutant (F102W/D51A/E101D)

The CD binding site is unoccupied in both the E101D- Mg^{2+} and the E101D- Ca^{2+} structures. The CD binding loop encompasses residues 51–62. The CD loop has collapsed in both the mutant structures from residue 51 through 56 in comparison to the wild-type loop (Figures 2b,5). Residue 56 is a glycine that is 96% conserved at the sixth position of the binding loop [8] and the loop resumes wild-type structure from this residue on. The three CD loop residues immediately following the glycine are known to hydrogen bond in an antiparallel fashion with the same three residues of the EF loop. It is likely that these interactions contribute to the positional stability of the region containing the last seven loop residues.

Comparison of the two unoccupied CD loops in the Ca^{2+} -bound and Mg^{2+} -bound E101D structures displays a difference in conformation between the two mutants for residues 51–56 (Figures 2b,5c). In addition, E101D- Ca^{2+}

Table 3

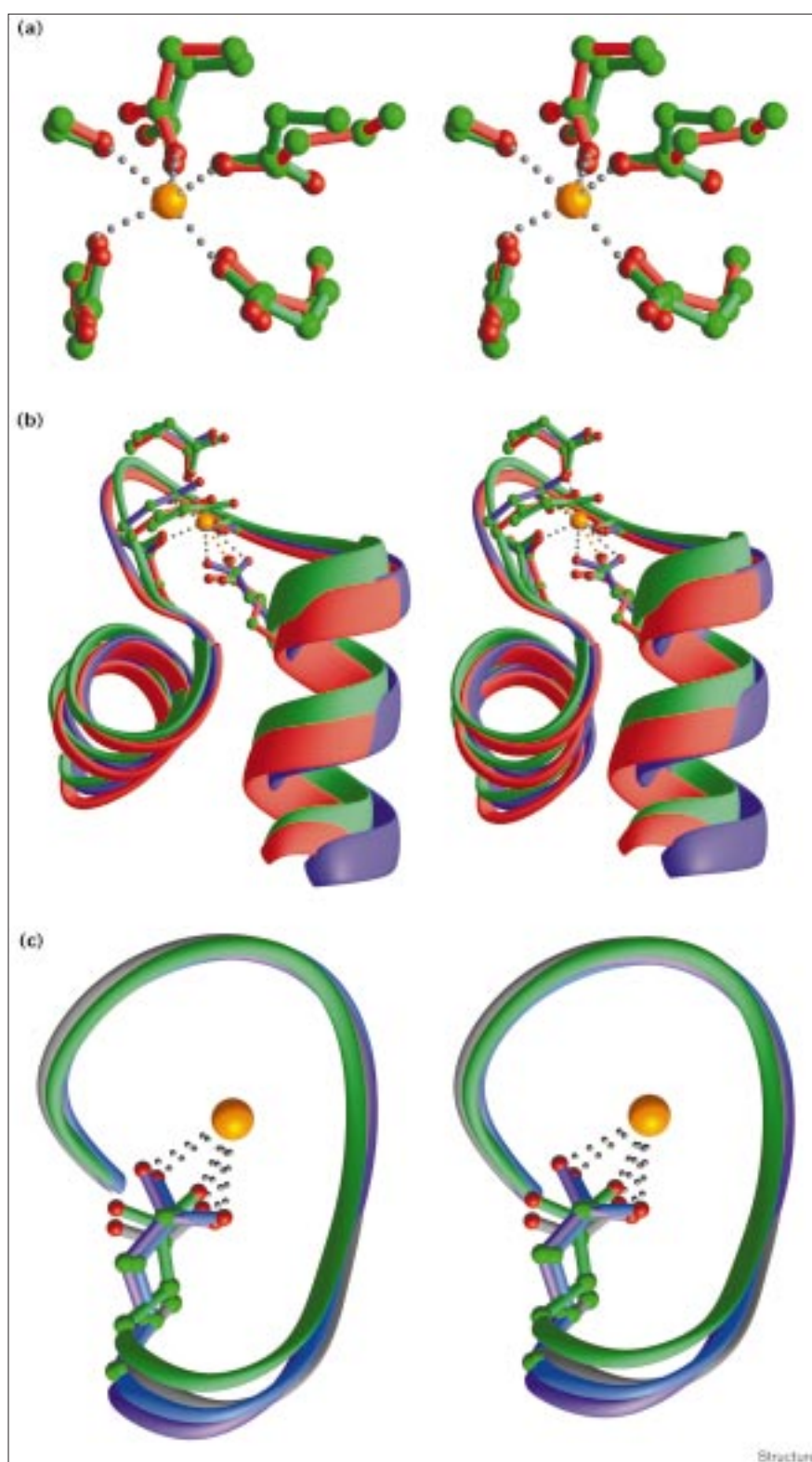
Calcium-oxygen distances in the CD loop.

Result no.	WT- Ca^{2+} *	Pike- Ca^{2+} †	PVEF- Ca^{2+}
62	2.55	2.57	2.51
62	2.43	2.46	2.42
59	2.48	2.48	2.48
57	2.27	2.31	2.36
55	2.6	2.38	2.59
53	2.36	2.47	2.43
51	2.23	2.17	—
51(C-O)	—	—	2.51
H ₂ O	—	—	2.52
<mean>	2.42 $\pm$.02	2.41 $\pm$.02	2.48 $\pm$.01

Distances are in Å. WT, wild-type carp parvalbumin; Pike, wild-type pike parvalbumin. *Distances from [17]. †Distances from [22].

Figure 6

EF loop of parvalbumin. (a) Stereoview superposition of the EF-loop Mg^{2+} -binding residues in the E101D mutant and the pike parvalbumin structure reported by Declercq *et al.* [22]. (b) Stereo diagram of the EF site of the E101D- Mg^{2+} mutant superimposed on the coordinates for the wild-type Ca^{2+} -bound carp parvalbumin structure [17] and the wild-type pike parvalbumin structure with Mg^{2+} bound [22]. The F helix of the E101D mutant is pulled about 1.1 Å into the loop relative to wild type. The fact that the F helix of the Mg^{2+} -bound pike structure is not displaced relative to wild-type carp parvalbumin indicates that this is an effect of the E101D substitution, and not a characteristic of Mg^{2+} binding. (c) Stereoview superposition of residue 101 in the mutant EF loops with respect to the wild-type structure [17]. The distance from the noncoordinating sidechain oxygen to the ion in E101D- Ca^{2+} is 3.85 Å, in E101D- Mg^{2+} it is 3.61 Å. Color coding: gray, E101D- Ca^{2+} ; blue, PVEF- Ca^{2+} ; green, E101D- Mg^{2+} ; purple, carp- Ca^{2+} ; red, pike- Mg^{2+} .



has an rmsd computed over the CD binding site region (residues 41–69) of 1.05 Å relative to wild type, whereas

E101D- Mg^{2+} has one of 2.07 Å. Finally, the B factors increase for residues 51–56 in the E101D mutants,

whereas they do not for the F102W and PVEF mutants where the CD loop contains a Ca^{2+} ion. These results imply that the unoccupied mutant CD loop is flexible.

The E101D mutant structure was determined in order to explore the role of the glutamic acid at the last coordinating position ($-Z$) of the Ca^{2+} -binding loop. This residue supplies two of the seven coordinating oxygens in the conventional Ca^{2+} -bound EF hand. The substitution of aspartic acid for glutamic acid altered the kinetics of the EF loop such that Ca^{2+} affinity decreased 100-fold and Mg^{2+} affinity increased 10-fold, making the likelihood of binding either of the two metal ions in this mutant loop almost equivalent. In addition, the Ca^{2+} off-rate increased drastically from a wild-type k_{off} of the order of about 1 s^{-1} [11] to the mutant k_{off} of 500 s^{-1} (QL and JDP, unpublished results).

The Mg^{2+} -bound mutant displays hexacoordinate octahedral binding geometry (Figure 1c) typical of that found in other Mg^{2+} -EF-hand structures [22,23]. The Mg^{2+} -oxygen distances are comparable to those reported for the Mg^{2+} -bound EF hand from pike parvalbumin (Table 1) and the spatial positions of the coordinating oxygens superimpose well on the wild-type positions, as illustrated in Figure 6a. The substitution of the shorter aspartate residue causes the F helix to be pulled in towards the binding site, as seen in Figure 6b. For the aspartic acid to maintain the appropriate position for the bidentate ligation of Ca^{2+} , the F helix would probably have to undergo even more drastic rearrangement. This presumably contributes to the unexpected result that the Ca^{2+} -bound E101D structure also displayed octahedral coordination (Figure 1c), implying that the shorter aspartic acid residue is unable to coordinate the Ca^{2+} in a bidentate fashion. The wild-type Glu101 has both greater flexibility, conferred by the additional χ_3 torsion angle, and increased reach. It seems probable that a combination of these factors allows the glutamic acid to adopt a close, bidentate conformation in this loop, whereas the aspartic acid cannot.

The Ca^{2+} -oxygen distances of E101D- Ca^{2+} were intermediate between hexacoordinated Mg^{2+} -oxygen distances and septacoordinated Ca^{2+} -oxygen distances (Table 1). When rmsd values were calculated over just the binding-loop region (residues 90–101), PVEF- Ca^{2+} most closely resembled the wild-type- Ca^{2+} conformation (with an rmsd of $0.3 \text{ \AA}$), followed by E101D- Ca^{2+} ($0.45 \text{ \AA}$), with E101D- Mg^{2+} ($0.71 \text{ \AA}$) showing the greatest deviation. The orientation of residue 101 in each of these mutants is shown relative to the wild-type structure in Figure 6c.

The residue at $-Z$ has been previously identified as crucial in transforming the radius of the coordinating sphere when different cations are bound through adjustments in the sidechain torsion angles [22,24]. Moreover, this residue modulates the number of ligands available to the metal ion, through the change from bidentate to monodentate

coordination. The glutamic acid at this position in the loop was reported as 92% conserved over 567 EF-hand sequences compared by Falke *et al.* [8]. In the 8% of loops where the residue at $-Z$ was not a glutamic acid, it was reported as an aspartic acid. Aspartic acid occurs naturally at $-Z$ in some myosin regulatory light chains (RLCs). Coordinates have been published for the RLC of scallop myosin, and the authors have determined that the site with aspartic acid at $-Z$ contains Mg^{2+} , although both Mg^{2+} and Ca^{2+} were present in the crystallization buffer [23]. It has also been shown that a glutamic acid for aspartic acid substitution at the $-Z$ position of chicken smooth muscle RLC converts this $\text{Ca}^{2+}/\text{Mg}^{2+}$ site into a Ca^{2+} -specific site [25]. The substituted glutamic acid residue was postulated to be sterically blocked from shifting into the monodentate configuration required for Mg^{2+} binding. In this case, the loop defined by the specific residues intrinsic to the myosin RLC was too constricted for a glutamic acid at $-Z$ to bestow both types of metal-ion binding. In the case of the E101D parvalbumin mutant, the loop radius seems to be too large for the aspartic acid to provide the preferred bidentate coordination of a Ca^{2+} ion, causing the Ca^{2+} off-rates to escalate and the Ca^{2+} affinity to decrease. In each circumstance, the interplay between the $-Z$ residue and the plasticity of the loop imposes the limits on the species of cations that are allowed to bind. Clearly, the residue at position $-Z$ has a pivotal role in determining which cation will coordinate with the lowest energy geometry allowed within the constraints of the loop structure.

Biological implications

Parvalbumin is a Ca^{2+} buffer found primarily in fast-muscle tissue and fast-firing nerve cells. It is currently widely used as a developmental neuronal marker and has been implicated in the hyperexcitability of fast muscle in mutant mice [26]. Recent work by Idrizbegovic *et al.* [27] suggests that parvalbumin and calbindin may be expressed as a rescue response when neurons located in the cochlear nucleus are exposed to auditory trauma. However, its primary value in this work is its close structural relationship to larger regulatory proteins.

The responses to changes in Ca^{2+} levels exhibited by individual members of the EF-hand family are quite diverse, and it is principally the metal-ion-binding parameters that determine these responses. Calmodulin, troponin C, calpain, myosin light chains, Ca^{2+} -dependent protein kinase, caltractin and recoverin are all EF-hand proteins that are involved in a wide variety of physiological processes, including signaling, cell cycle regulation, second messenger production, muscle contraction, microtubule organization and vision (reviewed in [3]). Because of their involvement in an overwhelming number of signal cascades, Ca^{2+} -binding proteins have also been implicated in certain serious disease states such as Alzheimer's disease [28] and other age-related cognitive defects [29],

diseases of the nervous system [30], leukemia [31] and various forms of cancer [32,33]. These regulatory proteins, because of their increased complexity of structure and function, are universally more difficult to express and crystallize than parvalbumin.

Using site-directed mutagenesis we can alter parvalbumin binding sites to confer new cation-binding properties, and we can examine these mutants crystallographically to determine the structural basis of the new properties. In addition to furthering the understanding of Ca^{2+} signal transduction, a better understanding of the mechanisms by which these proteins make the fine distinctions necessary for proper function would aid in identifying drug-targeting strategies and in designing Ca^{2+} -binding sites for protein-engineering purposes.

Materials and methods

Site-directed mutagenesis

Mutations were introduced into wild-type DNA or DNA containing other mutations using synthetic oligonucleotides to introduce single or multiple amino acid changes, and the DNA was amplified by either *Taq* or *Pfu* DNA polymerase using thermal cycling (PCR) [34]. The oligonucleotides carrying the nucleotide changes were designed to encompass a unique restriction site common to wild-type DNA to facilitate ligation of the PCR products with appropriate restriction fragments and the plasmid. Each 5' or 3' oligonucleotide was used as a primer and paired with a complementary primer that spans another restriction site, thus allowing correct orientation and ligation into the desired site. The mutated DNA was cloned into the *NcoI*–*Bam*HI cloning site of the pET 3d expression plasmid. The complete sequences of all cDNAs were verified by the standard dideoxy method using Sequenase II reagents (USB).

Expression and purification of parvalbumin mutants

Expression and purification of parvalbumin and its mutants were performed as previously described by Pauls and Berchtold [35], with some modifications: DNA in the pET 3d plasmid with confirmed sequences was used to transform *Escherichia coli* strain BL21 (DE3) cells and expression was checked on SDS–PAGE gels. One ml of a log-phase subculture of the best expressor was inoculated into 2 l enriched tryptone-yeast medium with M-09 salts and 100 $\mu\text{g}/\text{ml}$ ampicillin. Cultures were incubated in a dry rotary shaker at $A_{600} = 0.6$ – 1.0 and isopropylthiogalactoside was added to 0.4 mM. After 4 h, cells were pelleted at 6370 g for 5 min at 4°C, and resuspended in 20 mM imidazole, pH 8.0, containing 1 μM leupeptin, pepstatin and 10 μM phenylmethanesulfonate at 30 ml/l original culture. The cell suspension was sonicated in wet ice for 6 min in 60 s bursts with 90 s rests at level 8 with a Heat Systems Sonicator. The sonicate was centrifuged for 30 min at 48,400 g. The supernatant, containing ~95% of the parvalbumin, was precipitated with 70% saturation ammonium sulfate, and stirred overnight at 4°C. Contaminants were pelleted along with a small amount of parvalbumin at 48,400 g for 30 min and the supernatant, rich in parvalbumin, was dialyzed extensively against 10 mM imidazole pH 8.0 to remove salts. When the conductivity reached that of 10 mM imidazole, the dialyzed parvalbumin was eluted with a 0 to 500 mM NaCl gradient from a DEAE-Sephacel column equilibrated with the 10 mM imidazole solution at pH 8.0. The purified fractions were dialyzed against one change of 50 volumes of 1 mM EDTA (to remove Ca^{2+}) in 5 mM ammonium bicarbonate, and three changes of 50 volumes of 5 mM ammonium bicarbonate, then lyophilized to dryness to be stored at -20°C . The yields for these parvalbumin mutants were 40–60 mg/l original culture.

Crystallization

The F102W mutant was crystallized under the wild-type crystallization protocol employing the vapor diffusion method in hanging drops,

pH 5–5.5, 0.05–0.10 M CaCl_2 , and using 3.1–3.2 M ammonium sulfate as the precipitant [36]. Crystals of F102W grew at both room temperature and at 4°C. PVEF crystallized at 4°C in 50 mM HEPES, 50 mM CaCl_2 , pH 7 with 40% PEG 4000 as the precipitant. The PVEF-E101D– Mg^{2+} crystals required identical conditions to those for PVEF except that 50 mM CaCl_2 was replaced by 200 mM MgCl_2 . The PVEF-E101D– Ca^{2+} crystals were also grown at 4°C in 50 mM HEPES, 50 mM CaCl_2 , pH 7 with 40% PEG 4000 as the precipitant. The space groups and unit cell parameters for each mutant are shown in Table 2.

Data collection and processing

Data were collected on the F102W mutant at room temperature. The other three mutants were flash frozen in a cryostream and maintained at ~100K throughout data collection. No additional cryoprotectant was required because of the presence of 40% PEG 4000 in the mother liquor. Data for F102W, PVEF-E101D– Mg^{2+} and PVEF-E101D– Ca^{2+} were collected using a Rigaku RAXIS IIC detector using $\text{CuK}\alpha$ radiation. X-ray diffraction data collection for the PVEF data was performed at the National Synchrotron Light Source Beamline X4A at Brookhaven National Laboratory on a manual-scanning Fuji imaging plate at a wavelength of 1.74332 Å. In order to achieve both adequate spot separation and good resolution, the detector was offset by 20° and one 40 frame data set was collected with $\chi = 0^\circ$, then a second set of 40 frames was collected at $\chi = 90^\circ$. All data sets were indexed using DENZO and merged with SCALEPACK [37]. Processing statistics are given in Table 2.

Refinement

The structures were determined by molecular replacement using the coordinates for wild-type β -carp parvalbumin [17]. X-PLOR [38] was used for the F102W and PVEF-E101D– Mg^{2+} refinements. CNS [39] was used to refine the PVEF and PVEF-E101D– Ca^{2+} structures. The PVEF-E101D– Ca^{2+} structure was refined in CNS to R and R_{free} values of 19.70 and 22.61, respectively, and then all subsequent refinement was carried out with SHELX-97 using the Konnert–Hendrickson conjugate-gradient (CGLS) algorithm [40]. The PVEF-E101D– Mg^{2+} was refined initially in X-PLOR to R and R_{free} values of 25.78 and 33.40, then further refined in SHELX-97 using the CGLS algorithm. After reaching convergence using the CGLS algorithm, bond length estimated standard deviations (ESDs) were calculated by SHELX-97 for the metal ion–oxygen bond distances in both PVEF-E101D– Mg^{2+} and PVEF-E101D– Ca^{2+} by performing one round of full-matrix least-squares refinement, with damping set to zero in order to estimate ESDs without applying shifts [40]. Ca^{2+} –oxygen distances were not restrained during refinement in any of the structures. SIGMAA-weighted $2F_o - F_c$ and $F_o - F_c$ maps were viewed in Chain [41] for the X-PLOR refinements and in O [42] for maps from CNS and SHELX-97. See Table 2 for refinement statistics.

Structure analysis and figures

Superposition of structures was performed using the Kabsch algorithm [43], or in Quanta97 [44]. Quanta97 was used to calculate the rmsd values between structures and homologous regions of structures. All figures were created in Ribbons [45]. Delta distance calculations for inter-residue distances were calculated from an edited FORTRAN program originally written by Mike Quillin. The distances between the alpha carbons of each residue are calculated for two different structures, and then those inter-residue distances are compared by subtracting one set from the other. The differences were plotted at an absolute value cut-off of 1 Å or greater using Matlab® 4.2a [46].

Tryptophan fluorescence

Ca^{2+} titrations were performed for the mutant PVEF based on the FORTRAN program RECIPC [47], which calculated the Ca^{2+} aliquot required for each pCa value relative to the buffer content, the temperature and the pH. Protein samples were placed in a buffer containing 120 mM MOPS, 2 mM EDTA, and 135 mM KCl; and the pH of the buffer was adjusted to 7 by addition of a few drops of 10 mM NaOH solution before addition of the protein sample. Steady-state fluorescence was measured on an AMINCO Bowman Series 2 Luminescence Spectrometer at an excitation wavelength of 290 nm and an emission wavelength of 324 nm.

Accession numbers

Structure factor and coordinate files have been deposited in the Protein Data Bank with accession codes 1b8c (E101D–Mg²⁺), 1b8l (E101D–Ca²⁺), 1b8r (F102W–Ca²⁺) and 1b9a (PVEF–Ca²⁺).

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