Inactivation of Individual Ca^{2+}-Binding Sites in the Paired EF-Hand Sites of Parvalbumin Reveals Asymmetrical Metal-Binding Properties†

Thomas L. Pauls,‡ Isabelle Durussel,§ Martin W. Berchtold,‡ and Jos A. Cox*,§

Institute of Veterinary Biochemistry, University of Zürich-Irchel, 8057 Zürich, Switzerland, and Department of Biochemistry, University of Geneva, CH-1211 Geneva 4, Switzerland

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ABSTRACT: Previously a rat parvalbumin mutant protein PV_{F102W} was constructed with a reporter Trp at position 102 in the middle of the hydrophobic center [Pauls, T. L., *et al.* (1993) *J. Biol. Chem. 268,* 20897–20903]. In the present study three new parvalbumin mutant proteins, derived from PV_{F102W} and containing alterations at positions essential for Ca^{2+} binding in either one of the two Ca^{2+}-binding sites (PV_{-CD} and PV_{-EF}) or in both ($PV_{-CD/-EF}$), were expressed and purified. With the flow dialysis method it was established that both PV_{-CD} and PV_{-EF} bind 1 Ca^{2+} with affinity constants K_{Ca} of 1.1×10^7 and 3.2×10^6 M^{-1}, respectively. Mg^{2+} binding, monitored by equilibrium gel filtration in the absence of Ca^{2+}, showed that both mutants bind 1 Mg^{2+} with $K_{Mg} = 8\ 10^4$ for PV_{-CD} and 3×10^3 M^{-1} for PV_{-EF}. Compared to the parameters of the parent mutant PV_{F102W} (two sites with equal affinities of 2.7×10^7 and 3×10^4 M^{-1} for Ca^{2+} and Mg^{2+}, respectively), these data indicate that inactivation of the EF site, much more than of the CD site, impairs divalent cation binding. The binding of Ca^{2+} and Mg^{2+} is mutually exclusive, indicative of a Ca^{2+}/Mg^{2+} mixed site. However, as for PV_{F102W}, the K_{Mg} values obtained from the competition equation are approximately 40-fold lower than the affinities measured by direct binding. $PV_{-CD/-EF}$ binds neither Ca^{2+} nor Mg^{2+}. Trp fluorimetry revealed that in the three mutant PVs the residue Trp-102 is deeply buried in the hydrophobic core. The fluorescence spectra of the metal-free, Ca^{2+}, and Mg^{2+} forms of PV_{-CD} are very similar to those of the parent mutant PV_{F102W}. In contrast, the spectra of PV_{-EF} indicate the presence of a much less organized hydrophobic core, especially in the metal-free form. The Trp fluorescence of $PV_{-CD/-EF}$ is not sensitive to Ca^{2+} or Mg^{2+} and resembles that of metal-free PV_{-EF}. The comparison of ligand-induced UV difference spectra of PV_{-CD} and PV_{-EF} confirms that Trp-102 is located in a hydrophobic core and that inactivation of the EF loop, but not of the CD loop, strongly destabilizes the protein in the absence of Ca^{2+}, thus explaining the impaired cation affinity of PV_{-EF}. Our data indicate that in rat parvalbumin the EF-hand site is the principal structural nucleus of the paired domain.

High-affinity Ca^{2+} binding to many intracellular proteins occurs at a well organized center called the paired EF-hand domain,[1] which consists of two helix–loop–helix motifs assembled onto a two-site domain with a two-fold symmetry axis. In each EF-hand two amphiphilic helices, oriented at approximately 90°, flank a 12-residue loop, which provides six Ca^{2+}-coordinating oxygen ligands, defined X, Y, Z, –Y, –X, and –Z from the N- to the C-terminus (Kretsinger, 1987). The loop also contains a short β-pleated sheet, which is important for the pairing of two EF-hands (Shaw *et al.*, 1990; Kay *et al.*, 1991). Usually both sites of the paired EF-hand domain are active, with a distance of 11 Å between the two bound Ca^{2+} ions, but this is by no means obligatory: cardiac troponin C (Van Eerd & Takahashi, 1975) and yeast calmodulin (Matsuura *et al.*, 1993) possess a low-affinity site paired to an abortive site; calbindin-D28K (Gross *et al.*, 1993), the regulatory light chain of myosin (Bagshaw & Kendrick-Jones, 1979), and all invertebrate sarcoplasmic Ca^{2+}-binding proteins [for a review, see Cox (1990)] display a high-affinity site paired to an abortive one. The paired configuration has been demonstrated directly in the three-dimensional structure of the sarcoplasmic Ca^{2+}-binding proteins of *Nereis* (Cook *et al.*, 1991) and of amphioxus (Cook *et al.*, 1993) and the regulatory light chain of myosin (Rayment *et al.*, 1993).

In spite of the inherent symmetry of the paired Ca^{2+}-binding domain, the two sites do not necessarily have the same Ca^{2+}-binding characteristics. The paired domain of oncomodulin contains one high-affinity Ca^{2+}/Mg^{2+} mixed site and one Ca^{2+}-specific site (Hapak *et al.*, 1989; Cox *et al.*, 1990). In contrast, the closely homologous parvalbumin (50% sequence identity) has two paired sites, both of the Ca^{2+}/Mg^{2+} mixed type. The structural requirements for the difference in affinity and selectivity of the different types of domains are not known, but small, punctual differences in the sequences of the Ca^{2+}-binding domains influence the metal-binding properties (MacManus *et al.*, 1989; Trevino *et al.*, 1991). There are also indications that the sites within a paired domain influence each other and that this interference is not symmetrical. Studies on mutant forms of α-actinin (Witke *et al.*, 1993) or of calmodulin (Maune *et al.*, 1992) show that the C-terminal site within each paired Ca^{2+}-binding domain is more important in maintaining the tertiary structure of this domain than the N-terminal site, whereas in troponin C it appears to be the opposite (Brito *et al.*, 1993). This raises the question of whether conformational information is directional, *i.e.*, whether it propagates from one defined site to the other, and not *vice versa*. Nothing is known about the hinges and levers in this

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* To whom correspondence should be addressed: Department of Biochemistry 30, Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland. Télefax: 3296102. Electronic mail: cox@cgeuge52.

‡ University of Zürich-Irchel.

§ University of Geneva.

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[1] The term paired EF-hand domain or two-site domain is used to underline the notion that the individual Ca^{2+}-binding sites do not form a stable structural element, but as a pair they form a thermodynamically stable, independent structural unit (Shaw *et al.*, 1990).

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intramolecular information transduction process.

To get more insight into the interactions between two homologous EF-hands in the paired Ca^{2+}-binding domain, we used rat parvalbumin (PV)[2] with its one functional pair of Ca^{2+}/Mg^{2+} mixed sites as a model. In a previous study (Pauls *et al.*, 1993) Phe-102 was replaced by a unique Trp in the central hydrophobic core in rat PV, leading to a mutant protein, PV_{F102W}, with a suitable optical probe inside the protein. Its cation-binding properties were very similar to those of the recombinant unmodified PV_{WT} and of rat wild-type parvalbumin isolated from muscle. In the present study we further modified this mutant by point mutations in order to change essential features of PV, such as its ability to bind two Ca^{2+} ions. The Ca^{2+}- and Mg^{2+}-binding characteristics and ligand-induced conformational changes in two mutant parvalbumins with a single functional Ca^{2+}-binding site are described in this report.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification. Site-directed mutagenesis (Kunkel et al., 1987) was carried out in order to modify the rat PV cDNA in the vector pGEMEX (Promega) which had been used previously for cloning and expression of recombinant rat PV (Pauls & Berchtold, 1993). The DNA template pGEMEX-PV_{F102W} used for mutagenesis contained an initial amino acid replacement Phe-102-Trp for monitoring structural changes upon metal binding (Pauls et al., 1993). On top of this mutation either one or both of the functional Ca^{2+}-binding sites (CD or EF domain) were inactivated. Two synthetic oligonucleotides, $CD_{51/62}$ and $EF_{90/101}$, containing mismatches in codon 51 (GAC → GCC) and codon 62 (GAG → GTG) of the C loop or in codon 90 (GAC → GCC) and codon 101 (GAA → GTG) of the EF loop, respectively, were synthesized: $CD_{51/62}$, 5′-CAC-ATT-CTG-GCC-AAA-GAC-AAA-AGT-GGC-TTC-ATT-GAG-GAG-GAT-GTG-CTG-GGG-TCC-3′; $EF_{90/101}$, 5′-GCT-GCT-GGA-GCC-AAG-GAC-GGG-GAC-GGC-AAG-ATT-GGG-GTT-GAA-GTG-TGG-TCC-ACT-3′. Modified codons in the oligonucleotides are underlined. These oligonucleotides were used for replacing the first and last amino acids, Asp and Glu, in the Ca^{2+}-binding loops by the two nonpolar amino acids Ala and Val, respectively. The resulting PV mutant proteins $PV_{D51A,E62V,F102W}$ and $PV_{D90A,E101V,F102W}$ are named PV_{-CD} and PV_{-EF}. In addition a third PV mutant protein, $PV_{D51A,E62V,D90A,E101V,F102W}$, in which both loops were altered, was produced by mutating PV_{-CD} with the oligonucleotide $EF_{90/101}$ for obtaining the double-defective mutant $PV_{-CD/-EF}$. The mutant proteins were identified by the dideoxy chain termination sequencing method (Sanger *et al.*, 1977).

Expression and purification of all mutant proteins were carried out according to the procedure previously described (Pauls *et al.*, 1993), with some modifications. In the last purification step PV_{-CD} and PV_{-EF} were purified to homogeneity by DEAE-Sephacel ion-exchange chromatography (Pharmacia) with 20 mM imidazole and 2 mM EDTA, pH 7.8, as equilibration buffer. PV_{-CD} and PV_{-EF} eluted as pure proteins during subsequent washing with equilibration buffer. $PV_{-CD/-EF}$ eluted in the flowthrough of this column, which was then dialyzed against 10 mM Tris-HCl, pH 8.5, and 1 mM EDTA and loaded onto a QAE-Sephadex A-25 ion-exchange column (Pharmacia). $PV_{-CD/-EF}$ was eluted in a 0–50 mM NaCl gradient. Pure protein-containing fractions were identified by SDS–PAGE and silver staining. For subsequent studies the pure fractions were dialyzed in H_2O and either lyophilized or stored at −80 °C.

Protein and Metal Ion Determination and Metal Removal. For removal of contaminating metals and for complete equilibration of the protein in the assay buffer, the proteins were precipitated with 3% trichloroacetic acid and then passed through a 40 × 1 cm Sephadex G-25 column equilibrated in 50 mM Tris-HCl, pH 7.5, and 150 mM KCl (buffer A). The assay buffer was freed of contaminating metals by passage over a column of EDTA–agarose (Haner et al., 1984). Total Ca^{2+} and Mg^{2+} concentrations were determined with a Perkin-Elmer Cetus Instruments 2380 atomic absorption spectrophotometer as previously described (Pauls et al., 1993). The protein concentration was determined from the ultraviolet absorption spectrum using molar extinction coefficients at 280 nm of 6400, 6500, and 6900 M^{-1} cm^{-1} for PV_{-CD}, PV_{-EF}, and $PV_{-CD/-EF}$, respectively.

Cation Binding. Ca^{2+} binding was measured at 25 °C by the flow dialysis method of Colowick and Womack (1969) in buffer A containing 0, 1, 5, and 10 mM $MgCl_2$. Protein concentrations were 10–30 μM. Treatment of the raw data was as described by Cox *et al.* (1990). From this and previous studies we estimate that the accuracy of the binding constants obtained from flow dialysis experiments is around 80%. Mg^{2+} binding to parvalbumin in the absence of Ca^{2+} was measured by the equilibrium gel filtration method of Hummel and Dryer (1962). A Sephadex G-25 column (0.7 × 50 cm) was equilibrated in buffer A supplemented with 50 mM EGTA and the indicated concentrations of $MgCl_2$. One-half to 1 mL of 50–200 μM metal-free parvalbumin was applied to the column. In the eluant Mg^{2+} concentrations were determined by atomic absorption, and protein concentrations, by ultraviolet absorption.

The intrinsic metal-binding constants were determined with a Scatchard plot. The Mg^{2+} effect on the Ca^{2+}-binding curves was evaluated with the competition equation $K_{Ca}/K_{Ca.app} = 1 + K_{Mg}[Mg^{2+}]$, where K_{Ca} and $K_{Ca.app}$ are the Ca^{2+}-binding constants in the absence and presence of Mg^{2+}, respectively, and K_{Mg} is the Mg^{2+}-binding constant in the absence of Ca^{2+}.

Spectrofluorimetry. Emission fluorescence spectra were taken with a Perkin-Elmer LS-5B spectrofluorimeter interfaced with a computer. The measurements were carried out on metal-free solutions of 7 μM mutant PVs in buffer A at room temperature with excitation wavelength at 280 nm and slits of 2.5 nm. Two millimolar $MgCl_2$ and 1 mM $CaCl_2$ were added subsequently to obtain the Mg^{2+} and Ca^{2+} forms, respectively. Alternatively, guanidine hydrochloride up to 4 M was added to obtain the fluorescence characteristics of the fully denatured form. All of the spectra were normalized to an optical density of 0.05 at 280 nm. Mg^{2+} titrations of the mutant proteins following the fluorescence change were carried out under experimental conditions identical with those described above. Spectra were recorded after each increment, and the signal change was normalized between 0 (no Mg^{2+} added) and 1 (20 mM Mg^{2+} added).

Conformational Changes Monitored by Near-Ultraviolet Absorption. UV-absorption spectra and difference spectra were measured with a Perkin-Elmer Cetus Instruments Lambda 5 spectrophotometer at room temperature. The metal-free protein was equilibrated in buffer A. Difference spectra were taken on solutions with an optical density at 280 nm of 1, corrected for dilutions and normalized to molar

[2] Abbreviations: PV, parvalbumin; PV_{-CD}, PV with the substitutions D51A/E62V/F102W (abortive CD site); PV_{-EF}, PV with the substitutions D90A/E101V/F102W (abortive EF site); $PV_{-CD/-EF}$, PV with the substitutions D51A/E62V/D90A/E101V/F102W (both sites abortive).

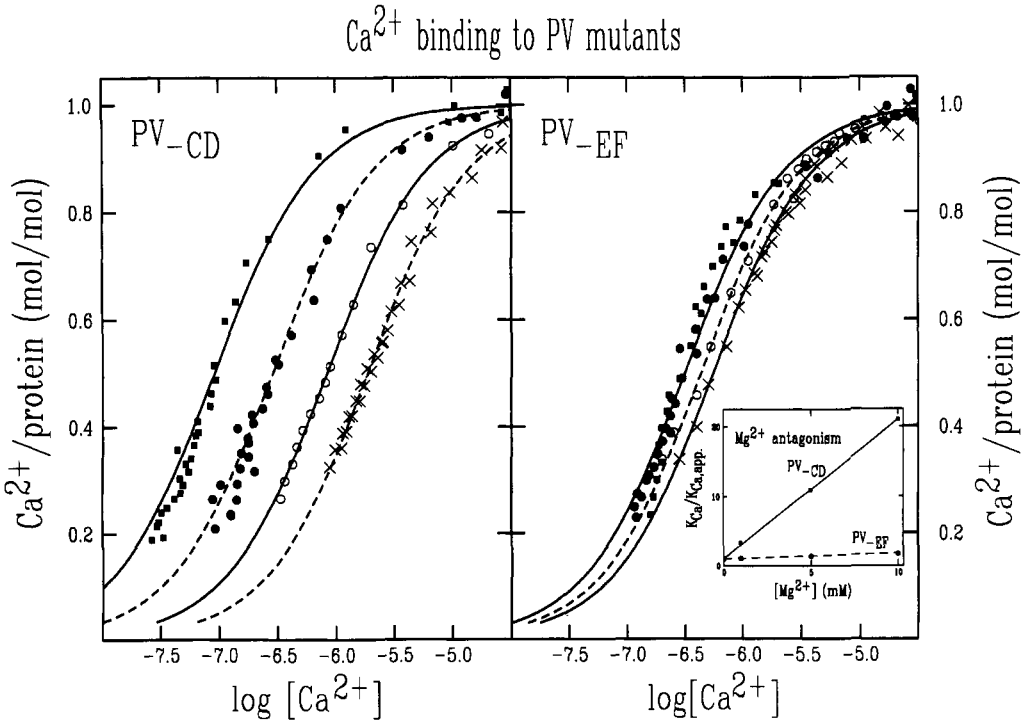

FIGURE 1: Ca^{2+} binding to recombinant PV_{-CD} and PV_{-EF}. Ca^{2+} binding was measured by the flow dialysis method at 25 °C. The PV concentrations were 20–30 μM. Mg^{2+} concentrations were 0 (■), 1 (●), 5 (○), or 10 mM (×). The curves were calculated with the following K_{ass} constants: 1.1×10^7, 3.3×10^6, 1.0×10^6, and 5.2×10^5 M^{-1} for PV_{-CD}; 3.2×10^6, 2.3×10^6 and 1.9×10^6 M^{-1} for PV_{-EF}. Inset: Ratio of the intrinsic Ca^{2+}-binding constant in the absence of Mg^{2+} over the apparent Ca^{2+}-binding constant in the presence of Mg^{2+} as a function of $[Mg^{2+}]$ for PV_{-CD} (■) and PV_{-EF} (●).

concentrations. For spectra involving the metal-free form a correction was carried out to take account of the contamination by Ca^{2+}, assuming a linear dependence of the signal on Ca^{2+}. Ca^{2+} and Mg^{2+} titrations were done in buffer A (in Mg^{2+} titrations 20 μM EGTA was present to complex contaminating Ca^{2+}). The titrations with Ca^{2+} are stoichiometric, and therefore the difference signals $D_{290-298nm}$ and $D_{283-288nm}$ were plotted as a function of added Ca^{2+} per mole of protein; due to the much lower affinity of the proteins for Mg^{2+}, the Mg^{2+} titrations are nonstoichiometric and the signal changes were plotted as a function of the concentration of added Mg^{2+}.

RESULTS

Mutagenesis, Expression, and Purification of Recombinant Proteins. Three different mutant proteins were isolated. Each of the variants contained the initial Phe-102-Trp exchange and additional substitutions in their Ca^{2+}-binding loops, as described under Materials and Methods. Isopropyl D-thiogalactopyranoside induced expression of mutant PVs in *Escherichia coli* strain JM109 (DE3) resulted in protein production similar to that previously described for PV_{WT} and PV_{F102W} (Pauls *et al.*, 1993) with 25–30% mutant protein per total bacterial protein for PV_{-CD} and 20% for PV_{-EF} and $PV_{-CD/-EF}$. Nevertheless, since bacteria expressing PV_{-EF} and $PV_{-CD/-EF}$ grew more slowly, the total amounts of mutant protein per liter of bacterial culture were approximately 2-fold smaller for PV_{-EF} and $PV_{-CD/-EF}$ than for PV_{F102W} and PV_{-CD}. PV_{-EF} and $PV_{-CD/-EF}$ showed a higher mobility in SDS–PAGE when compared to PV_{F102W} (data not shown).

The first steps of purification for all mutant proteins were done as previously described (Pauls *et al.*, 1993) with DEAE-Sephacel ion exchange as the last step. However, the removal of two negative charges in PV_{-CD} and PV_{-EF} resulted in a weaker binding to the ion exchanger. The mutant proteins eluted after approximately 3 column volumes of subsequent washing with 20 mM imidazole and 2 mM EDTA, pH 7.8. The purity of the cleanest fractions was >95% with final yields of 45 and 8 mg/(liter of bacterial culture) for PV_{-CD} and PV_{-EF}, respectively. $PV_{-CD/-EF}$, lacking four negative charges compared to PV_{F102W}, did not bind at all to DEAE-Sephacel. This mutant protein had to be further purified using the stronger ion exchanger QAE-Sephadex A-25 equilibrated in 10 mM Tris-HCl and 1 mM EDTA, pH 8.5. $PV_{-CD/-EF}$ was purified to >95% homogeneity with a final yield of 6 mg/(liter of bacterial culture).

Direct Binding Studies. Figure 1 shows the Ca^{2+}-binding isotherms of PV_{-CD} and PV_{-EF} in the presence of 0, 1, 5, and 10 mM Mg^{2+} as measured by the flow dialysis method. As predicted from the sequence, the mutant proteins display only one site for Ca^{2+}, without indications of an additional low-affinity site (conclusion confirmed by difference spectrophotometry). In the absence of Mg^{2+} the affinity constants for Ca^{2+} are 1.1×10^7 and 3.2×10^6 M^{-1} for PV_{-CD} and PV_{-EF}, respectively. Since the parent mutant PV_{F102W} has two equivalent sites with K_{Ca} of 2.7×10^7 M^{-1} (Pauls *et al.*, 1993), it must be concluded that in the new mutant proteins the pairing of an active site to an abortive one diminishes the Ca^{2+} affinity of the active site to a variable degree, depending on which site was destroyed. This asymmetry is also manifested in the antagonistic effect of Mg^{2+} on Ca^{2+} binding to the residual site in PV_{-CD} and PV_{-EF}. Mg^{2+} affects the Ca^{2+} affinities of the two mutant proteins in very different ways. For PV_{-CD} the apparent constant for Ca^{2+}, $K_{Ca,app.}$, decreases to 5×10^5 M^{-1} in 10 mM Mg^{2+}. Interpreted with the competition equation (see Materials and Methods), the affinity constant for Mg^{2+} amounts to 2×10^3 M^{-1} (inset of Figure 1), which is very similar to the calculated competition constant in PV_{F102W}. In contrast, the affinity of the mutant PV_{-EF} for Ca^{2+} is weakly influenced by Mg^{2+} (Figure 1).

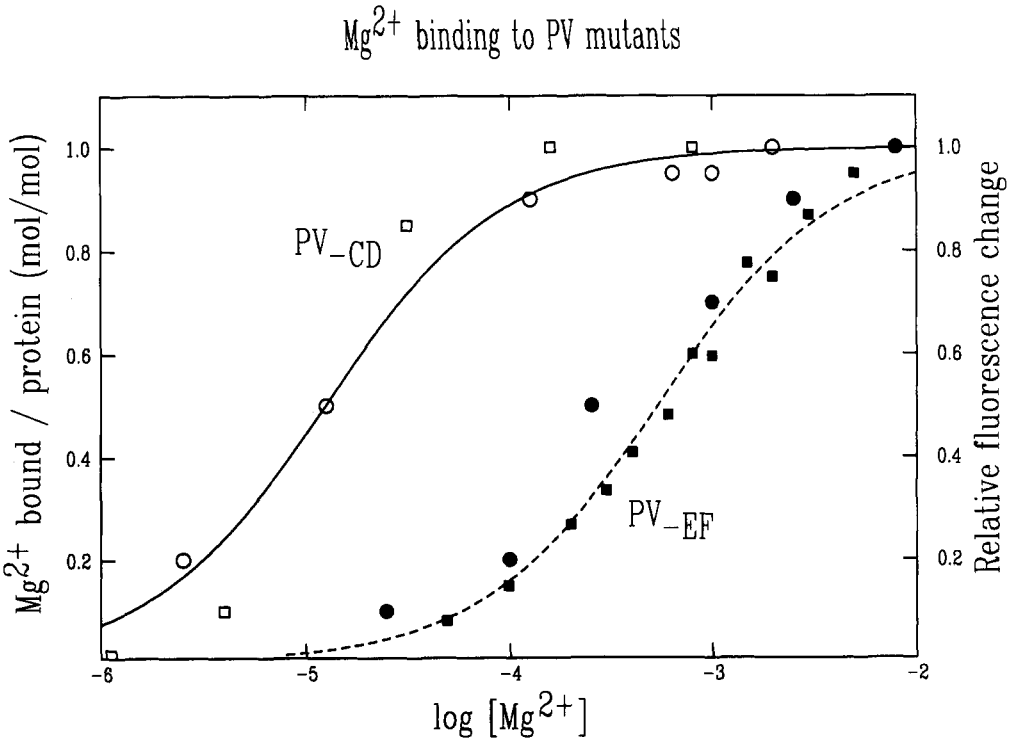

FIGURE 2: Mg^{2+} binding to recombinant PV_{-CD} (open symbols) and PV_{-EF} (closed symbols) measured by the Hummel–Dryer method (○, ●) and by the Trp fluorescence change (□, ■) at room temperature. The following estimates of K_{Mg} were obtained: Hummel–Dryer method, 8×10^4 M^{-1} for PV_{-CD} and 3×10^3 M^{-1} for PV_{-EF}; fluorescence titration, 2×10^3 M^{-1} for PV_{-EF}. The curves were calculated with the K_{ass} constants 8×10^4 (—) and 2×10^3 M^{-1} (- - -), respectively.

With the competition equation one obtains a K_{Mg} value of 75 M^{-1}. This value is even lower than the affinity constant of calmodulin for Mg^{2+} (Milos *et al.*, 1986).

Direct Mg^{2+}-binding studies, carried out with the Hummel–Dryer method, are shown in Figure 2. Both mutant proteins bind 1 Mg^{2+}, and the values of the affinity constants amount to 8×10^4 M^{-1} for PV_{-CD} and 3×10^3 M^{-1} for PV_{-EF}. The magnitudes of these constants have been confirmed by fluorimetric and difference spectrophotometric titrations (see below). The 40-fold higher affinity for Mg^{2+} determined here as compared to the affinity calculated from the competition behavior has also been observed in wild-type parvalbumin and in PV_{F102W} and cannot yet be explained.

The Ca^{2+}- and Mg^{2+}-binding capacity of the mutant $PV_{-CD/-EF}$ was assayed with the Hummel–Dryer method: in the presence of 100 μM free Ca^{2+} and 1 mM free Mg^{2+} no noticeable amount of metal ion was bound to the mutant protein (data not shown).

Fluorescence Characteristics. Trp emission fluorescence spectra of the different forms of PV_{-CD}, PV_{-EF}, and $PV_{-CD/-EF}$ are shown in Figure 3. As previously described for mutant PV_{F102W}, the spectra of the new mutant proteins show strongly blue-shifted maxima and display a high quantum yield, indicating that in these mutant proteins, too, the Trp residue is deeply buried in the hydrophobic core. This Trp residue is completely unmasked in the presence of guanidine hydrochloride (thin solid line in Figure 3). With their maximum at 313 nm, the spectra of the Ca^{2+}, Mg^{2+}, and metal-free forms of PV_{-CD} do not differ much from each other and are very similar in shape and intensity to the corresponding spectra of PV_{F102W}. Those of the PV_{-EF} mutant protein, however, are very different: the metal-free form has a maximum at 325 nm. Upon binding of Mg^{2+}, the maximum moves to 313 nm but the intensity is still 1.5-fold lower than that of Mg^{2+}-loaded PV_{-CD}. Only upon binding of Ca^{2+} did the fluorescence intensity of PV_{-EF} approach that of PV_{F102W} and PV_{-CD}. The Trp fluorescence spectrum of $PV_{-CD/-EF}$ is Ca^{2+}-independent; its shape and intensity suggest that in this inactive mutant protein the environment of the Trp is the same as in metal-free PV_{-EF}.

The Mg^{2+} titration of PV_{-EF} followed by the fluorescence change of Trp (Figure 2) gives an affinity constant, K_{Mg}, of 2×10^3 M^{-1}, which resembles the value from direct binding studies, *i.e.*, 3×10^3 M^{-1}. The Trp fluorescence change for PV_{-CD} is very small (Figure 3), but it is obvious that it occurs in the 10–100 μM range (□ in Figure 2), as expected from the direct binding study.

Difference Spectrophotometry. Figure 4 shows the difference spectra of PV_{-CD} and PV_{-EF} induced by Ca^{2+} and Mg^{2+} and, for the purpose of the analyses, the difference spectra between the metal-free or Ca^{2+} form and the guanidine hydrochloride denatured protein. Qualitatively the spectra resemble those obtained from PV_{F102W}, suggesting a similar overall geometry of the hydrophobic core. Importantly, the signal change of the transition from the unfolded to the metal-free conformation is much greater for PV_{-CD} than for PV_{-EF}, indicating that metal-free PV_{-EF} is much closer to the denatured state than metal-free PV_{-CD}. Conversely, the difference spectra for the transitions from the metal-free to the Ca^{2+}- and Mg^{2+}-loaded forms are smaller for PV_{-CD} than for PV_{-EF}. Thus PV_{-CD} has the characteristics of the mutant PV_{F102W}, whereas PV_{-EF} is much less structured in the metal-free form and recovers the PV_{F102W} structure only upon binding of Ca^{2+}. It is worth noticing that the calculated total signal change from the denatured to the Ca^{2+} form (thin line in Figure 4) is quite similar for the two mutant proteins, thus confirming the fluorescence experiments which indicate that the Ca^{2+}-saturated conformations of both mutant proteins are quite similar. The Mg^{2+} conformations are intermediate between those of the metal-free and the Ca^{2+} form. For

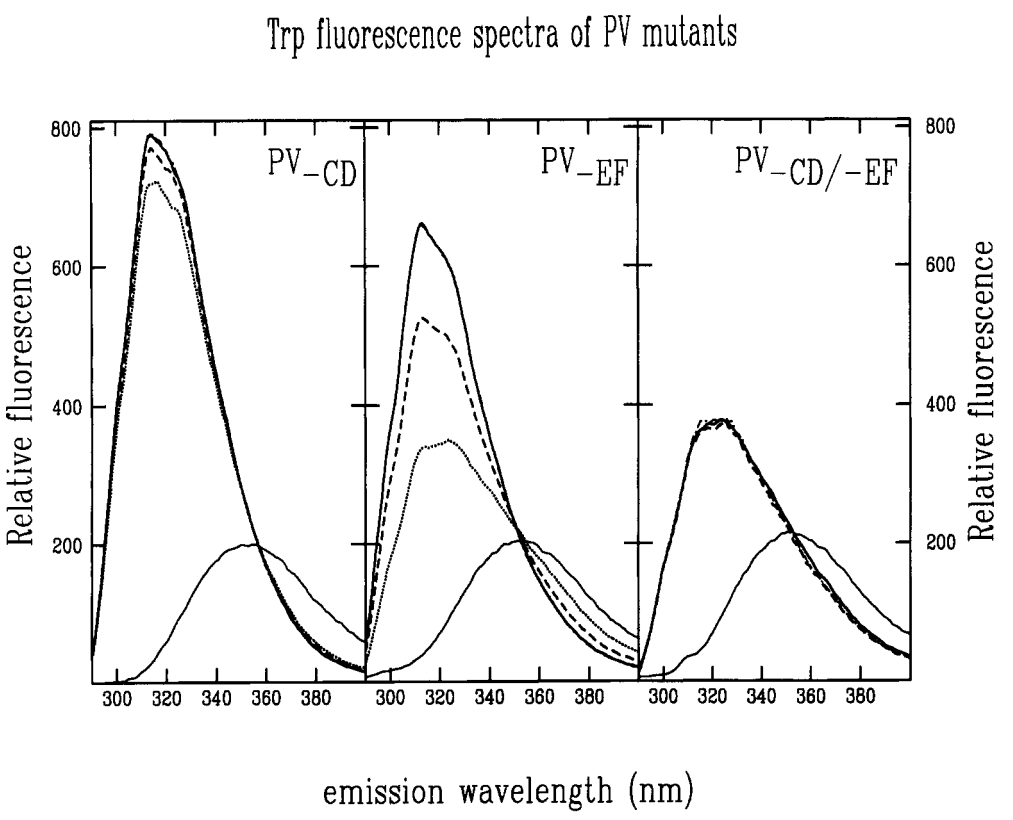

FIGURE 3: Fluorescence spectra of PV_{-CD}, PV_{-EF}, and $PV_{-CD/-EF}$. The protein concentration was 7 μM. Ca^{2+}, Mg^{2+}, or guanidine hydrochloride was added up to 2.5 mM, 5 mM, or 3 M, respectively. The spectra were normalized as described in Materials and Methods. Thick solid line, Ca^{2+} form; dashed line, Mg^{2+} form; dotted line, metal-free form; dashed and dotted line, Ca^{2+} + Mg^{2+} form; thin solid line, guanidine hydrochloride denatured form. Note that the dashed and dotted line nearly coincides with the thick solid line.

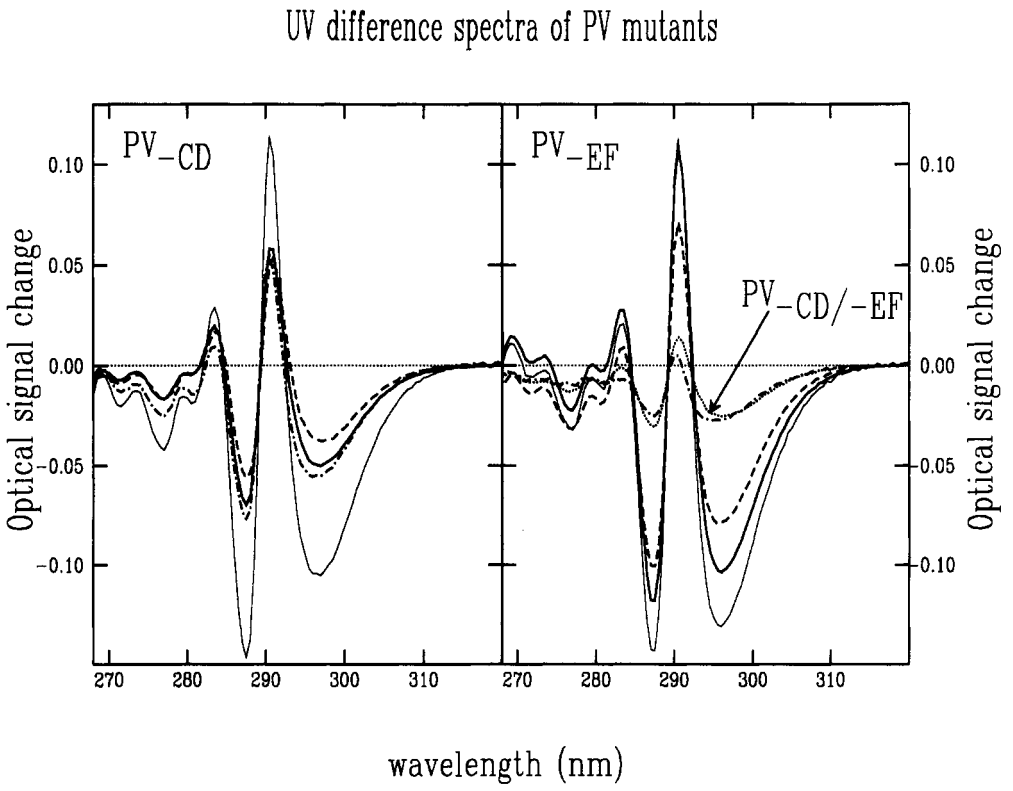

FIGURE 4: Ca^{2+}- or Mg^{2+}-induced difference spectra of the recombinant PV mutant proteins PV_{-CD} and PV_{-EF} at room temperature. The protein concentrations were 150 μM. To the sample cell was added Ca^{2+}, Mg^{2+}, or guanidine hydrochloride up to 2.5 mM, 5 mM, or 3 M, respectively, whereas in the reference cell an identical dilution was performed with buffer A. The spectra were normalized as described in Materials and Methods. Thick solid line, Ca^{2+} form *versus* metal-free form; dashed line, Mg^{2+} form *versus* metal-free form; dashed and dotted line, metal-free form *versus* denatured form; thin solid line, Ca^{2+} form *versus* denatured form (calculated). The spectrum of the metal-free form *versus* the denatured form of the mutant $PV_{-CD/-EF}$ is also shown in the right panel as a thin dotted line (arrow).

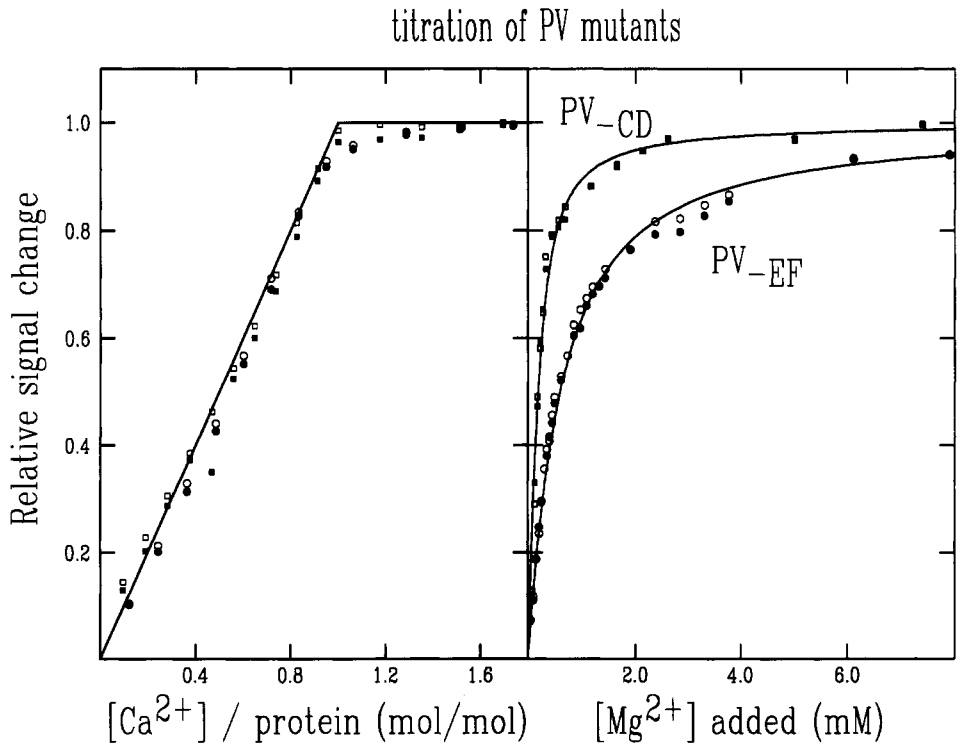

FIGURE 5: Ca^{2+} dependence (A) or Mg^{2+} dependence (B) of the difference signals $D_{290-298nm}$ (open symbols) and $D_{283-288nm}$ (filled symbols) of the mutants PV_{-CD} (□, ■) and PV_{-EF} (○, ●). The measurements were carried out as detailed in Figure 4. In the left panel the optic cell was 0.5 cm wide and the protein concentrations were 391 and 304 μM for PV_{-CD} and PV_{-EF}, respectively. In the right panel the optic cell was 1 cm wide, the protein concentrations were 168 and 181 μM for PV_{-CD} and PV_{-EF}, respectively, and the solid lines were calculated with K_{Mg} values of 2×10^4 and 2×10^3 M^{-1} for PV_{-CD} and PV_{-EF}, respectively.

$PV_{-CD/-EF}$ the UV difference spectral changes from the unfolded to the folded form indicate that this mutant protein resembles closely PV_{-EF} in its less structured, metal-free conformation (Figure 4, dotted line), which is in agreement with the fluorimetric data.

The Ca^{2+} titrations as followed by the relative absorption difference signal (Figure 5A) show that mutants PV_{-CD} and PV_{-EF} bind 1 mol of Ca^{2+} per mole of protein with a high affinity. The Mg^{2+} dependency of the absorption difference (Figure 5B) allows one to calculate the K_{Mg} values, using the following equation:

$$\Delta S/\Delta S_{max} = C_{PV}K_{Mg}[Mg^{2+}]/(1 + K_{Mg}[Mg^{2+}])$$

where ΔS and ΔS_{max} are the measured and maximal signal changes and C_{PV} is the parvalbumin concentration. The K_{Mg} value for PV_{-EF}, *i.e.*, 2×10^3 M^{-1}, is similar to that obtained in Figure 2. The value for PV_{-CD}, 2×10^4 M^{-1}, is rather low when compared to those obtained by direct binding and fluorescence titration, but given the high affinity and protein concentration used, estimation of the upper limit of the K_{Mg} value is not precise.

DISCUSSION

In the present study mutant parvalbumins derived from a previous mutant, PV_{F102W}, and containing either one (PV_{-CD} and PV_{-EF}) or two ($PV_{-CD/-EF}$) abortive Ca^{2+}-binding sites in their C-terminal tandem domain have been analyzed. The highly conserved positions 1 (Asp) and 12 (Glu) in the 12-residue Ca^{2+}-binding loop, which provide essential oxygen ligands for Ca^{2+}, were replaced by the nonpolar residues Ala and Val, respectively. NMR showed that in the loops of pike PV these residues exhibit the biggest changes upon metal binding (Blancuzzi *et al.*, 1993). The same replacements in all three functional loops in yeast calmodulin resulted in weak Ca^{2+} binding when position 12 was changed, whereas no Ca^{2+} binding was detectable when both positions 1 and 12 were substituted (Geiser *et al.*, 1991). Replacements of position 1 alone, D → A, also prevented the binding of Ca^{2+} to yeast mutant calmodulins (Geiser *et al.*, 1991), whereas the same replacement for single sites in troponin C was not enough to inactivate the mutated site (Negele *et al.*, 1992). Therefore, we have chosen to substitute both positions in each Ca^{2+}-binding loop. As expected, PV_{-CD} and PV_{-EF} bind only 1 Ca^{2+} and 1 Mg^{2+}, whereas $PV_{-CD/-EF}$ binds no metal ion at all.

PV_{F102W}, PV_{-CD}, PV_{-EF}, and $PV_{-CD/-EF}$ all contain a single substitution, Phe-102-Trp, with the fluorescent indole group positioned in the center of the protein equally distant from both Ca^{2+}-binding sites. All mutant PVs display the very strong blue-shifted Trp fluorescence spectra with unusually high quantum yield. The difference spectra clearly show that in the new mutant proteins, as in PV_{F102W}, the Trp is hidden in the hydrophobic core; but a positively charged electron-withdrawal group, present in the hydrophobic core, affects the electron density of the indole ring of Trp102 (Ilich *et al.*, 1988). The comparison of the fluorescence and UV difference spectra of PV_{-EF} with those of PV_{-CD} leads to the conclusion that in PV_{-EF} the hydrophobic core surrounding the Trp residue is much less structured than in PV_{-CD}.

One of the most intriguing observations in our study is that, despite the structural symmetry in the two-site domain of parvalbumin, these two sites do not have the same structural importance within the paired EF-hand domain. In the wild-type protein and in PV_{F102W} the two sites appear to be equivalent with the same affinity for Ca^{2+} and the same antagonistic effect of Mg^{2+} on Ca^{2+} binding (Pauls *et al.*, 1993). But important differences show up when either the

N-terminal or the C-terminal site is modified so that it cannot bind Ca^{2+} anymore. When the CD site is inactivated, the remaining EF site still has a high affinity for Ca^{2+} (2.5-fold lower than in parent PV_{F102W}) and for Mg^{2+} (2-fold higher than in PV_{F102W}). Moreover, this mutant protein displays a conformation and metal-induced conformational changes which are very similar to those in PV_{F102W} (Pauls *et al.*, 1993). In contrast, when the EF site is inactivated, the remaining CD site has a 10-fold lower affinity for Ca^{2+} and for Mg^{2+}, the antagonistic effect of Mg^{2+} is strongly decreased, and conformational studies suggest that the hydrophobic core is much more accessible to polar solvent. Apparently, in the paired domain the EF site is structurally much more important than the CD site and constitutes the stable structural nucleus of the paired domain. When this structure is impaired, the CD site binds cations with lower affinity because the metal-free state is much more disorganized than that of the native protein.

The mutant PV_{-EF} is also much less sensitive to Mg^{2+} than the mutant PV_{-CD}, which suggests that when the CD loop is not backed by a functional helix–loop–helix motif, its –Z ligand, Glu-62, is unable to assume the g– $f\chi 1$ rotamer (Blancuzzi *et al.*, 1993), or at least this rotamer is energetically much less favored. Glu-101 in the EF loop can do so, even in the mutant PV_{-CD}. The reason could be that the PV_{-EF} is already much less structured, and going to the highest level of structure is more difficult than for the mutant PV_{-CD}.

An interesting comparison can be made between the structural properties of the mutant parvalbumins with inactive Ca^{2+}-binding sites and those of other EF-hand-containing proteins with similar mutations. Studies on mutant calmodulins with single Ca^{2+}-binding sites in any two of the paired domains (Maune *et al.*, 1992a,b; Gao *et al.*, 1993) show that mutations in sites II and IV prove to be more deleterious for Ca^{2+} binding than mutations in sites I and III. UV difference spectrophotometry of a unique Tyr-138 in site IV of the C-terminal domain indicates that site III mutants can partially reconstitute their hydrophobic environment, whereas this is effectively abolished in site IV mutants. In nonmuscle type α-actinin, too, the inactivation of either one of the paired Ca^{2+}-binding sites revealed the asymmetry in Ca^{2+} binding: the site I mutant protein still binds Ca^{2+} with high affinity, whereas the site II mutant shows a 500-fold lower affinity for Ca^{2+} than the wild-type protein (Witke *et al.*, 1993). In the C-terminal half of cardiac troponin C the site III and site IV mutant proteins bind one Ca^{2+} with a K_{Ca} around 10^6 M^{-1} (Negele *et al.*, 1992), and this is sufficient to promote formation of the short β-sheet between the two sites and large changes in the environment of the aromatic residues (Brito *et al.*, 1993), suggesting symmetry. But Ca^{2+} binding at site III appears more important for the stability of the C-terminal domain (contrary to the case of parvalbumin; this study). Thermodynamic studies on calmodulin and skeletal muscle troponin C have previously shown that site III is the least stable Ca^{2+}-binding site, having an ordered structure only in the presence of bound Ca^{2+} (Tsalkova & Privalov, 1985). In conclusion, even when the affinities for Ca^{2+} of the EF-hand sites in a symmetrical paired two-site domain are the same, inactivation studies systematically reveal a structurally dominant site. In parvalbumin, calmodulin, and α-actinin this dominant site is at the C-terminal end; in troponin C it is at the N-terminal end.

The functional importance of parvalbumin as a buffer of Ca^{2+} and Mg^{2+} can now be evaluated by transfection of these mutant PVs in living cells. Table 1 shows the dissociation constants of the wild-type and mutant proteins for Ca^{2+} and Mg^{2+} under physiological concentrations of Mg^{2+} (taken as 1 mM), revealing that the four proteins display the same sensitivity to the Ca^{2+} signal. PV_{-CD}, PV_{F102W} and PV_{WT}, are supposed to be fully saturated with Mg^{2+} at rest, whereas PV_{-EF} would be mostly in a metal-free state. Therefore, upon an increase of intracellular Ca^{2+} levels the former three proteins would first have to release their bound Mg^{2+} in order to take up Ca^{2+}, whereas PV_{-EF} could immediately bind Ca^{2+}. This allows the effects of Ca^{2+}/Mg^{2+} exchange and of Ca^{2+} buffering to be studied separately. The mutant $PV_{-CD/-EF}$ can yield indications of whether the role of parvalbumin is restricted to the above two actions or includes also divalent cation independent functions. First results with a transfected epidermal cell line show a decrease in mitotic rate, changes in morphology from epithelioid to fusiform, and an increase in motility after expression of PV_{WT} (C. Andressen, personal communication). In contrast, no changes were found in cells expressing the Ca^{2+}-binding-deficient mutant $PV_{-CD/-EF}$ when compared to untransfected cells.

Table 1: Dissociation Constants of PV_{-CD}, PV_{-EF}, PV_{F102W}, and PV_{WT} for Ca^{2+} and Mg^{2+} [a]

	PV_{-CD}	PV_{-EF}	PV_{F102W} [b]	PV_{WT} [b]
Ca^{2+} binding, μM				
in 0 Mg^{2+}	0.1	0.3	0.04	0.04
in 1 mM Mg^{2+}	0.3	0.3	0.2	0.2
Mg^{2+} binding, mM				
from competition	0.5	13	0.5	0.5
from direct methods	0.012	0.3	0.025	0.03

[a] Round numbers were given for all dissociation constants. [b] Taken from Pauls *et al.* (1993).

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