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Oligomerization of an avian thymic parvalbumin

Chemical evidence for a Ca^{2+}-specific conformation

Raymond C. Hapak, Hongmei Zhao, Michael T. Henzl*

Biochemistry Department, 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211, USA

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Abstract

CPV3, the third parvalbumin isoform to be identified in the chicken, is produced exclusively in the thymus gland. Although parvalbumins are typically cysteine-deficient, CPV3 contains two cysteine residues, at positions 18 and 72. The reported three-dimensional parvalbumin structures suggest that the side chain of cysteine-72 should be solvent-accessible. Accordingly, we find that CPV3 readily forms disulfide-linked oligomers in the absence of reducing agents. The reaction, employing either O_2 or ferricyanide ion as the oxidant, is apparently restricted to the Ca^{2+}-bound form of the protein. The differing reactivity of the Ca^{2+}, Mg^{2+}, and apo-forms has significant structural implications.

Key words: Parvalbumin; Ca^{2+}-binding protein; Disulfide bond

1. Introduction

Parvalbumins are small, vertebrate-specific proteins that bind two mols of Ca^{2+} with high affinity [1,2]. The metal ion-binding sites display the helix–loop–helix structural motif common to all members of the calmodulin superfamily [3,4]. Parvalbumins are abundant in select skeletal myofibrils and neurons and are widely believed to serve as cytosolic Ca^{2+} buffers. The parvalbumin family encompasses two sub-lineages: β-parvalbumins (pI < 5.0) and α-parvalbumins (pI > 5.0) [5].

Whereas fish and amphibians express multiple skeletal muscle isoforms (e.g. [6,7]), postnatal mammals express a single muscle isoform that is also expressed in other tissue settings [8]. Early in mammalian development, however, an additional parvalbumin isoform is produced in the fetal placenta [9]. This protein, discovered by MacManus [10], has been labeled oncomodulin due to its frequent reappearance in neoplastic tissues (≈80% of all mammalian tumors regardless of origin, [11]) and its conjectured role as a Ca^{2+}-dependent modulator early in embryological development.

The pattern of parvalbumin expression in avian species differs from that observed in lower vertebrates or in postnatal mammals. Although birds, like mammals, express a single muscle parvalbumin, two distinct parvalbumin isoforms have been identified in the avian thymus gland. Avian thymic hormone (or ATH[1]) was the first to be identified [12]. Its name derives from a capacity to stimulate differentiation of T-cell precursors in vitro [13]. A second thymic parvalbumin, labeled CPV3, was recently discovered by this lab [14]. The cDNAs for both proteins have been cloned [15,16]. Both are β-parvalbumins and show 52% sequence identity. The CPV3 sequence is identical to that of oncomodulin at 73 of 108 positions.

Although parvalbumins are characteristically deficient in cysteine, CPV3 contains two cysteine residues, at positions 18 and 72. The side-chain of Cys^{18}, a characteristic feature of β-parvalbumins [17], is part of the hydrophobic core. Due to its relative inaccessibility [18–20], Cys^{18} is unreactive toward sulfhydryl reagents in the Ca^{2+}-bound forms of oncomodulin [21], carp parvalbumin [22,23], and perch parvalbumin [24]. However, inspection of the existing β-parvalbumin X-ray structures [18–20,25,26] suggests that Cys^{72}, on the other hand, should be solvent-accessible. In view of the potential role of CPV3 as an extracellular signaling factor, we have investigated its capacity for forming disulfide-linked dimers. Our results indicate that the reactivity of the Ca^{2+}-bound forms differs significantly from that of the apo- or Mg^{2+}-bound forms.

2. Materials and methods

Chicken thymus glands were purchased from Pel-Freez Biologicals. The following supplies were purchased from Sigma Chemical Co.: Sephadex G-25, Sephadex G-75, acrylamide, *N,N'*-methylene-*bis*-acrylamide, *N,N,N',N'*-tetramethyle thylenediamine, ammonium

*Corresponding author.

Abbreviations: ATH, avian thymic hormone; CPV3, chicken parvalbumin isoform 3; EGTA, [ethylene-*bis*-(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; DTNB, 5,5'-dithio-*bis*-(2-nitrobenzoate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CD site, the metal ion-binding site in the parvalbumins flanked by the C and D helical segments; EF site, the metal ion-binding site in the parvalbumins flanked by the E and F helical segments.

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persulfate, DTNB, HEPES, MES, DTT, ampicillin, LB broth, lysozyme, phenylmethylsulfonyl fluoride, 2-mercaptoethanol, and xylenol orange. SDS, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, Na_2EDTA, and a standard Ca^{2+} solution were obtained from Fisher Scientific. Protein molecular weight standards were purchased from Bio-Rad. $TbCl_3$ was purchased from Aldrich Chemical Co.

CPV3 was isolated from chicken thymus tissue as described elsewhere [27]. Expression of CPV3 in *Escherichia coli* DH5α_was driven by the *lac* promoter of Bluescript (Stratagene). High level expression of oncomodulin has been achieved in this laboratory employing a Bluescript-based vector that we call pLD2 [28]. Reasoning that CPV3 would be expressed at similarly high levels in this construction, we shuttled the CPV3 coding sequence into the pLD2 vector, preserving the relative placement of the promoter elements, the Shine–Dalgarno sequence, and the initiator codon. The resulting plasmid is called pHZ1. Routine recombinant DNA manipulations were performed in accordance with Maniatis et al. [29].

Isolation of the recombinant protein was carried out as described previously for the tissue-derived protein [27]. One gram of cell paste yields 2–3 mg of purified protein. The purified protein contains 1.9–2.0 equivalents of Ca^{2+}, as determined by atomic absorption.

Thiol-protecting reagents (dithiothreitol or 2-mercaptoethanol) were removed from the CPV3 preparations by gel-filtration on Sephadex G-25 in 0.15 M NaCl, 0.025 M HEPES-NaOH, pH 7.4. Prior to loading the 1.0 ml protein sample (10 mg/ml), the column (1.0 cm × 28 cm bed, equipped with flow adapter) was equilibrated with buffer that had been thoroughly deoxygenated with N_2. The eluate was monitored at 280 nm. Reductant-free CPV3 eluted well ahead of the excess reductant and was collected in a nitrogen-filled serum bottle.

The sulfhydryl content of the CPV3 preparation was determined with Ellman's reagent [28], as described previously [21]. CPV3 concentrations were determined by titration with Tb^{3+}, monitoring the sensitized emission that accompanies binding of the lanthanide. Since the titrations level off abruptly upon addition of two molar equivalents of Tb^{3+}, the protein concentration can be determined to within 5%. The Tb^{3+} titrant was standardized by titration with EDTA at pH 6.0 [31].

Unless otherwise specified, the oligomerization reactions were studied at room temperature. Samples were quick-frozen and stored at −80°C prior to electrophoresis. Although CPV3 is isolated as the Ca^{2+} complex, additional Ca^{2+} (1.0 mM) was generally added when examining the behavior of the Ca^{2+}-bound species, to insure that both sites were completely occupied. To study the behavior of apo-CPV3, excess EDTA (2 mM) was included in the dimerization solution. The Mg^{2+}-bound form of the protein was produced by adding 2 mM EGTA and 20 mM Mg^{2+} ion.

Dimerization was monitored by SDS-PAGE. Following a pre-electrophoresis step (to remove residual persulfate ion), the 15 μl samples were underlayered with a syringe. Reductant was omitted from the SDS sample buffer, and the protein samples were not boiled prior to loading. The reservoir buffer was 0.025 M Tris, 0.20 M glycine, and EDTA (0.001 M) was included in the anode buffer. Gels were stained with Coomassie brilliant Blue R250.

3. Results

The translated cDNA sequence provided the first suggestion that CPV3 might contain a reactive cysteine residue [16]. In addition to Cys^{18}, a characteristic of β-parvalbumin isoforms, CPV3 also displayed a cysteine residue at position 72. The reported parvalbumin structures [18–20,25,26] indicate that the side-chain of this residue should be solvent-accessible. For purposes of illustration, the conformation of oncomodulin in the vicinity of Ser^{72} is displayed in Fig. 1A. It is clear from this representation that Ser^{72} resides in a β-turn at the end of helix D and that the side-chain hydroxyl is oriented toward the solvent. Based on its high degree of sequence identity with oncomodulin (73 of 108 residues), we would expect the polypeptide chain of CPV3 to adopt a similar conformation, rendering Cys^{72} solvent-accessible and potentially capable of participating in a disulfide-mediated dimerization reaction.

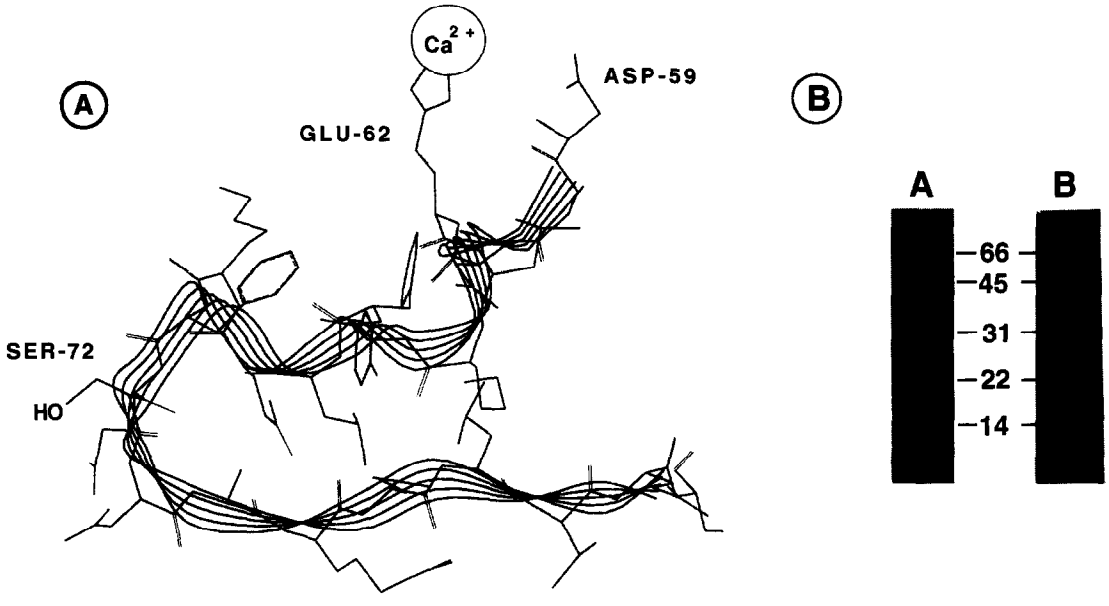

Fig. 1. (A) The side-chain of residue 72 is solvent-accessible. The conformation of oncomodulin in the vicinity of residue 72 is displayed above. The X-ray crystallographic structure of the Ca^{2+}-bound form of oncomodulin (20) was downloaded from the Brookhaven Protein Data Bank and displayed with SYBYL (Tripos Associates, St. Louis, MO). In CPV3, Serine-72 is replaced by cysteine. (B) Dimerization of CPV3 during storage at −20°C. This reductant-free preparation of CPV3 had been stored at −20°C for a period of several months prior to analysis by SDS-PAGE. *Lane A*, 2 μg of CPV3, untreated. *Lane B*, 2 μg of CPV3, treated with 10 mM DTT for 10 min at room temperature prior to electrophoresis. The observed mobilities for select molecular weight standards are indicated at center.

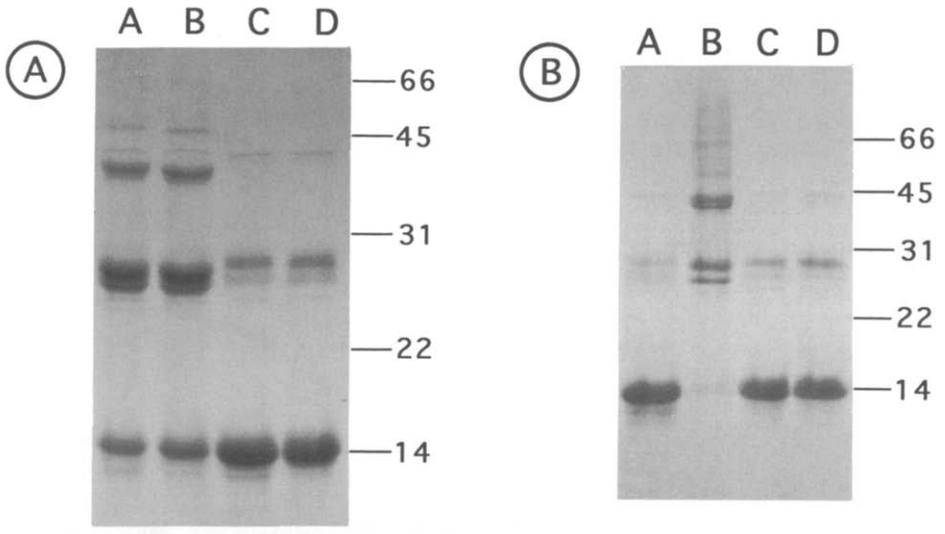

Fig. 2 (A) Oxygen-mediated oligomerization of CPV3. Samples of reductant-free CPV3 (200 μM) were incubated at room temperature for 48 h under conditions designed to yield either the apo-, Mg^{2+}-, or Ca^{2+}-bound forms of the protein. 6 μl aliquots were then analyzed by SDS-PAGE. *Lane A*, no addition; *lane B*, 1.0 mM Ca^{2+}; *lane C*, 2.0 mM EDTA; *lane D*, 2.0 mM EGTA plus 20 mM Mg^{2+}. (B) Ferricyanide-mediated oligomerization of CPV3. Potassium ferricyanide was added to samples of CPV3 (200 μM) to afford a final concentration of 5 mM. Solution conditions were chosen to yield either the apo-, Mg^{2+}, or Ca^{2+}-bound forms of the protein. After 5 min at room temperature, 6 μl samples were resolved by SDS-PAGE. *Lane A*, no ferricyanide added; *lane B*, ferricyanide plus 1.0 mM Ca^{2+}; *lane C*, ferricyanide plus 2.0 mM EDTA; *lane D*, ferricyanide plus 2.0 mM EGTA and 20 mM Mg^{2+}.

We, therefore, examined a CPV3 solution that had been stored at −20°C in the absence of reductant for a prolonged period of time. The majority of the preparation displayed an M_r of about 30,000, consistent with formation of a covalent dimer (Fig. 1B, lane A). Treatment with DTT resulted in quantitative conversion back to the monomeric species (Fig. 1B, lane B), indicating that a disulfide linkage provided the covalent cross-link.

Initial attempts to monitor the dimerization reaction were plagued by irreproducibility. We subsequently deduced that the variability was correlated with a reduced sulfhydryl content. Presumably, the diminished sulfhydryl content resulted from side-reactions, notably the production of cysteic acid, between the reductant-free CPV3 and atmospheric O_2. To minimize these putative side-reactions, excess reductant was removed by gel-filtration on columns that had been thoroughly equilibrated with deoxygenated buffer, and the reductant-free CPV3 was collected in N_2-filled serum bottles. Moreover, to avoid exposure of the protein to high O_2 concentrations at the air-solution interface, the CPV3 was introduced via syringe into the incubation vessel under a layer of mineral oil. The latter precaution insured a gradual introduction of O_2 into the CPV3 solution.

With these measures taken, the dimerization behavior became reproducible. The reductant-free protein collected after passage through the G-25 column contains 2.0 mol of -SH. After gradual exposure to oxygen, virtually all of the protein is converted into higher molecular weight species. The results of a typical experiment are presented in Fig. 2A. In this experiment, reductant-free CPV3 was incubated for periods up to 48 h under conditions designed to afford either apo-, Ca^{2+}, or Mg^{2+}-bound forms of the protein (see above).

In the presence of 1 mM Ca^{2+}, nearly all of the monomeric CPV3 is converted to higher molecular weight species after 48 h (Fig. 2A, lane B). In fact, it is not necessary to introduce additional Ca^{2+} into the reaction to promote disulfide-bond formation. As isolated, the protein contains roughly 2.0 equivalents of Ca^{2+}, and this level is sufficient to facilitate oligomerization (Fig. 2A, lane A) . By contrast, in the presence of excess EDTA (Fig. 2A, lane C) or Mg^{2+} (Fig. 2A, lane D), the protein remains almost entirely monomeric. These data indicate that the Ca^{2+}-bound form of the protein dimerizes much more readily than either the apo- or Mg^{2+}-bound forms of the protein.

We have also examined the relative rates of CPV3 dimerization employing ferricyanide as the oxidant. Ferricyanide is a relatively mild oxidant that is often used to promote disulfide-bond formation during protein refolding experiments [32]. The results, displayed in Fig. 2B, are consistent with the O_2-driven oxidation studies discussed above. The rate of reaction is greatly increased, but the pattern of reactivity is identical. Intermolecular disulfide bridges are formed to a significant extent only in the presence of free Ca^{2+} (Fig. 2B, lane B). The levels of dimer present in the apo- (lane C) and Mg^{2+}-bound (lane D) samples of CPV3 are comparable to that seen in the untreated control (lane A).

Notice that trimers and higher multimers are evident as well as dimers on the acrylamide gels shown in Fig. 2. Initially, we thought that these species arose during electrophoresis because they are more prominent

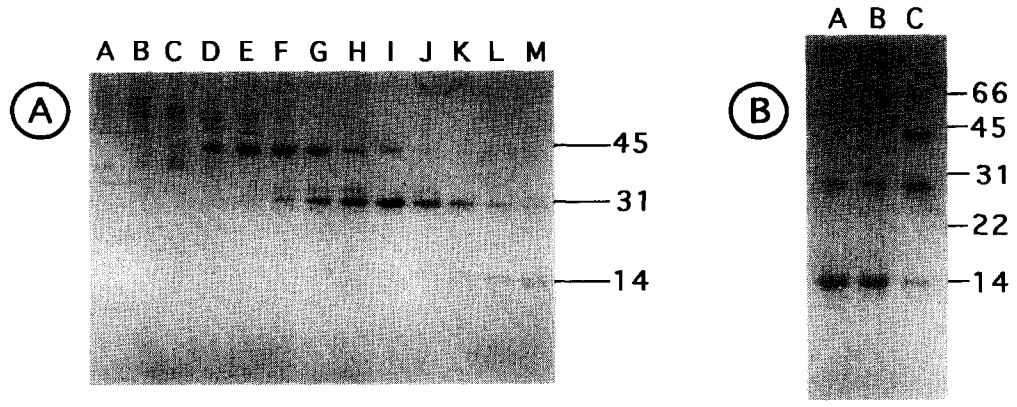

Fig. 3. (A) Gel-filtration of oxidized CPV3 preparation. A 1.0 ml sample of reductant-free CPV3 (180 μM) that had been incubated for 72 h at room temperature was loaded onto a G-75 column (1.5 × 47 cm) that had been equilibrated with 0.15 M NaCl, 0.025 M HEPES-NaOH, pH 7.4, and eluted at 20 ml/h with the same buffer. 10 μl aliquots of the 1.0 ml fractions were immediately analyzed by SDS-PAGE. Lanes A–M correspond to fractions 49 through 61. (B) Effect of temperature on the extent of oligomerization. Samples of reductant-free CPV3 (180 μM) were incubated either at −20°C (lane A), 4°C (lane B), or 23°C (lane C) for 48 h in the presence of 1.0 mM Ca^{2+}. 2.0 μl aliquots were removed for analysis by SDS-PAGE.

when the pre-electrophoresis is omitted. However, if an oxidized CPV3 preparation is subjected to gel-filtration chromatography on Sephadex G-75, the fractions (Fig. 3A, lanes A–F) eluting just ahead of the dimeric material (lanes F–K) display substantial amounts of higher molecular weight complexes. We conclude from this result that the multimeric species are present in the oxidized preparation prior to electrophoresis.

If CPV3 oxidation is allowed to occur at lower temperatures, oligomerization is halted at the dimer stage. Samples of CPV3 that had been incubated for 48 h at either −20°C, 4°C or room temperature (23°C) were examined by SDS-PAGE (Fig. 3B). The samples that had been incubated at the lower temperatures display only monomer and dimer bands (lanes A and B, respectively). By contrast, the sample incubated at room temperature (lane C) displays a prominent band with a molecular weight corresponding to a crosslinked trimer, in addition to the dimer band.

Parenthetically, the doublets that are visible on the polyacrylamide gels may reflect modification of cysteine and/or methionine residues by oxygen or by reactive species in the polyacrylamide matrix, either during the incubation period or transit through the stacking gel. These non-specific oxidative events could alter the SDS content of the SDS–protein micelles, thereby modulating their electrophoretic mobilities. The detergent content of SDS–parvalbumin complexes is clearly sensitive to amino acid sequence. For example, the three known chicken parvalbumin isoforms, although very similar in molecular weight, exhibit apparent molecular weights ranging from 12,000 to 15,000 by SDS-PAGE [14,16].

4. Discussion

The experiments described above reveal that the thymus-specific chicken parvalbumin called CPV3 is capable of forming intermolecular disulfide crosslinks. At low temperatures (either 4° or −20°C), disulfide-mediated crosslinking terminates at the dimer stage (Fig. 3B). Presumably, this species reflects formation of a disulfide bridge between the Cys^{72} sulfhydryl groups of two CPV3 monomers, symbolized (18*72)-(72*18). Interestingly, however, exposure of reductant-free CPV3 to oxygen at room temperature results in the appearance of trimers and higher multimers.

We surmise that these larger complexes are produced by disulfide-bond formation between the Cys^{72} sulfhydryl of a CPV3 monomer and one of the Cys^{18} residues in the dimer. Although Cys^{18} is largely inaccessible, the data of Ahmed et al. [20] indicate that the sulfhydryl group of that residue is connected to the protein surface by a narrow channel roughly 6 Å deep. This channel may allow the sulfhydryl of Cys^{72} to approach sufficiently closely for reaction to occur. The putative structure of the trimeric species afforded by this reaction is symbolized (18*72)-(72*18)-(72*18). Note that the predominant dimeric and trimeric species lack free Cys^{72} sulfhydryl groups and, thus, cannot condense to form larger complexes. Rather, they can only serve as acceptors in the polymerization process. In all likelihood, depletion of the monomer pool limits chain-elongation largely to the trimer stage. The absence of multimeric species at the reduced incubation temperatures may reflect reduced conformational mobility and/or insufficient thermal energy for the reaction between Cys^{18} and Cys^{72}.

Significantly, CPV3 forms oligomers only in the presence of Ca^{2+}. The apo-and Mg^{2+}-bound forms of the protein both exhibit a very limited capacity for intermolecular crosslinking. The contrasting behavior observed for the apo- and Ca^{2+}-bound forms is not unexpected. UV difference spectroscopy [33], circular dichroism [24], proton magnetic resonance [34], chemical modification

[21–24], and differential scanning calorimetry [35] all indicate that the apo-protein is less ordered than the Ca^{2+}-bound form. The loss of structure that accompanies Ca^{2+} removal in the β-parvalbumins is more prominent than in the α-isoforms [36] and may reflect the greater electrostatic repulsion of the more acidic β-isoforms.

A priori, there are two potential explanations for the difference in oligomerization behavior observed for the Ca^{2+} and Mg^{2+}-bound forms of CPV3. It is possible that Mg^{2+} ion is not bound at one or both sites. Alternatively, the Ca^{2+}-bound conformation of the protein may differ significantly from the Mg^{2+}-bound form of the protein. Unpublished work from this lab (Zhao, H., Hapak, R.C. and Henzl, M.T., unpublished data) indicates that both sites will be occupied at the concentration of Mg^{2+} employed in the oligomerization studies. Thus, the disulfide crosslinking experiments lend strong support to spectroscopic data indicating that Ca^{2+} and Mg^{2+}-bound forms of parvalbumins may adopt perceptibly different conformations.

This subject has been a topic of considerable interest over the years. Early UV difference measurements [33] and proton magnetic resonance [34] experiments revealed perceptible spectroscopic alterations upon Ca^{2+}–Mg^{2+} exchange. The crystal structure of pike parvalbumin (pI 4.1) complexed with Mg^{2+} at the EF site, reported by Declercq et al. [25], offered a structural basis for these differences. Specifically, Glu^{101} is converted from a bidentate ligand in the Ca^{2+} complex to a monodentate ligand in the Mg^{2+} complex. Similar differences were recently observed in solution by Blancuzzi et al. in their detailed 2D-NMR analysis of pike parvalbumin III [37].

The extent of the structural changes that accompany Ca^{2+}–Mg^{2+} exchange remains a matter of conjecture. Although the chemical shift differences observed by Blancuzzi et al. [37] are largely confined to the CD and EF ion-binding loops, there are pronounced changes in the amide proton shifts for Lys^{64} and Glu^{103}. These residues are located in the D and F helices, respectively, immediately adjacent to the ion-binding loops. The observed chemical shift alterations suggest that their secondary structures differ perceptibly in the Ca^{2+} and Mg^{2+}-complexed forms of the protein and that the difference in Ca^{2+} and Mg^{2+}-coordination geometry is relayed, at least, to the first several residues of the flanking C-terminal helical element. In this context, Hutnik et al. [38] noted that the fluorescence behavior of Trp^{102} in cod parvalbumin differs markedly in the Ca^{2+}- and Mg^{2+}-bound forms.

The oligomerization studies described herein suggest that, at least for the β-parvalbumin known as CPV3, the conformational alteration accompanying Ca^{2+}-Mg^{2+} exchange is transmitted the entire length of the D helix to the β-turn involving residues 71–74.

Since the biological function of CPV3 is unknown, we cannot state whether the observed oligomerization reactions have any physiological significance. Inside the cell, CPV3 would undoubtedly be present in monomeric form, due to the high intracellular concentration of glutathione. In the extracellular milieu, however, dimerization could occur and would, in fact, be favored by the high Ca^{2+} concentrations. It has been suggested that ATH plays an extracellular signaling role. If CPV3 functions similarly, then the intermolecular crosslinking reaction could have some biological consequence. It is known, for example, that S100β forms a disulfide-linked dimer and that the dimeric form of the protein is a potent stimulator of nerve growth [39]. However, the parallel between CPV3 and the S100 system is not exact. Whereas the S100 proteins normally exist as non-covalent dimers, parvalbumins are monomeric. The physiological relevance, if any, of the oligomerization reaction is a subject for future investigations.

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