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Metal-specific structural changes in parvalbumin

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ABSTRACT

Parvalbumin is a small protein of EF-hand family whose main role is considered to be metal buffering. Recent evidences indicate that parvalbumin also fulfills more complicated functions, which may be determined by the diversity in structural changes in response to the binding of different metal cations. In the present work the conformations of α and β isoforms of pike parvalbumin in the Ca^{2+}- and Mg^{2+}-loaded state were studied by intrinsic fluorescence, circular dichroism and bis-ANS extrinsic fluorescence. We have determined the structural region causing different spectral response on the binding of Mg^{2+}- and Ca^{2+} ions in pike β-parvalbumin. Our data reveal similarity of the metal-bound forms of α-parvalbumin. In contrast, those of β isoform differ significantly in the tyrosine spectral range. We also discuss the possible physiological consequences of the structural rearrangements accompanied Mg^{2+}/Ca^{2+} exchange in pike β-parvalbumin.

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1. Introduction

Parvalbumin is a small (~12 kDa), acidic, cytosolic protein of the EF-hand superfamily [1–3]. It is expressed by vertebrates in fast-twitch muscle cells, specific neurons of the central and peripheral nervous system, sensory cells of the mammalian auditory organ (Corti's cell), and some other cells. PA is widely used as a neuronal marker for a variety of functional brain systems [4]. It is recognized as one of the major animal allergens [5].

The parvalbumin family contains two evolutionary distinct sublineages, α and β (the latest includes oncomodulin), which can be distinguished on the basis of isoelectric point (pI > 5 for α; pI < 5 for β), C-terminal helix length (with few exceptions, one residue longer in α lineage), and several lineage-specific sequence assignments [6,7]. The tertiary structure of PA is sublineage-independent and conserved over a wide phylogenetic range. PA possesses three homologous 30-residue-long subdomains, each containing central loop flanked by short amphipathic α-helices (altogether six helical segments, labeled A–F), limited β-sheet structure, and no disulfides [3]. The loops between the C and D helices and between E and F helices with flanking helices form two EF-hand type Ca^{2+}/Mg^{2+}-binding motifs (CD and EF subdomains). The AB subdomain is non-functional because of a two-residue deletion in the loop between the A and B helices. It covers the hydrophobic surface of the functional domains pair, thereby modulating their calcium affinities [8,9].

The highest concentration of PA (up to several mM) was found in fast-contracting muscle fibers [10], and in the outer hair cells of the organ of Corti [11]. In the fast muscles PA serves as a soluble relaxing factor facilitating the Ca^{2+}-mediated relaxation phase [12,13]. Although major role of PA is assumed to be metal buffering and transport of Ca^{2+}, certain isoforms evidently play additional roles. For example, the mammalian β-PA is secreted by activated macrophages and functions as a potent nerve growth factor for retinal ganglion cells [14]. Nevertheless, the exact function of PA in neuronal and many other tissues is still mainly hypothetical.

Although PA binds different metal cations, only Ca^{2+}- and Mg^{2+}-loaded forms are of undoubted physiological significance. In the cytoplasm of a "resting" cell (e.g. fast-twitch muscle fibers, neurons) with reported intracellular Mg^{2+} concentrations of 0.5–1 mM, the mixed Ca^{2+}/Mg^{2+} sites of PA are occupied largely by Mg^{2+} (80–90%), which needs to dissociate before Ca^{2+} binding can occur. Due to the fact that the rate of Ca^{2+} binding is determined by the rather slow Mg^{2+} off-rate, PA is considered to be a "slow" buffer [15].

The crystal structure of pike β-PA with its EF subdomain loaded with Mg^{2+} has revealed that the coordination number of bound metal ion changes from seven oxygen atoms for Ca^{2+}-bound PA [16] to six oxygen atoms with Mg^{2+} [17]. The main structural feature within the EF site upon Ca^{2+}/Mg^{2+} exchange is considered to be the conformational rearrangement of the Glu101 side chain from the energetically favored conformation in the Ca^{2+}-loaded form to the less favored rotamer in the Mg^{2+}-loaded form. In this rearrangement, the Glul0l side chain located in the EF loop is con-

Abbreviations: PA, parvalbumin; α-PA, α isoform of parvalbumin; β-PA, β isoform of parvalbumin; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; CD, circular dichroism.

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verted from a bidentate ligand in the Ca^{2+}-loaded form to a monodentate ligand in the Mg^{2+}-loaded form [18].

PA possesses a rigid tertiary structure in metal-bound states [3] while the metal-free (apo) PA is intrinsically disordered [19], but the conformational difference between Mg^{2+}- and Ca^{2+}-loaded forms is still largely unclear and contradictory [20–24].

Here we report the results of structural studies of Mg^{2+}- and Ca^{2+}-bound forms of pike PAs.

2. Materials and methods

2.1. Materials

Northern pike (*Esox lucius*) skeletal muscle PAs (α and β isoforms) were isolated as described earlier [25–27]. The purity of the protein samples was confirmed and concentration was determined as in [25–27]. We have previously found two variants of pike α-PA, α_1 and α_2 [27]. All experiments in the present work were performed with the α_1-isoform (referred to as "α-PA").

2.2. Methods

The purification of parvalbumin samples from calcium ions (for experiments with Mg^{2+}-loaded and apo-PAs) was performed using the Sephadex G-25 gel-filtration method described in [28].

Fluorescence studies were performed on a Cary Eclipse spectrofluorimeter (Varian), equipped with a Peltier-controlled cell holder. PA intrinsic fluorescence was excited at 259 nm; bis-ANS fluorescence was excited at 385 nm.

Circular dichroism measurements were carried out with a JASCO J-810 spectropolarimeter (JASCO, Japan), equipped with a Peltier-controlled cell holder. The instrument was calibrated with an aqueous solution of d-10-camphorsulfonic acid (JASCO) according to the manufacturer's instruction. Cuvettes with pathlengths of 10 and 1.00 mm were used for near- and far-UV regions, respectively. Protein concentrations were 125–135 μM and 5–10 μM for near- and far-UV regions, respectively. A small contribution of buffer was subtracted from experimental spectra. Band width was 2 nm, averaging time 1–2 s, and accumulation 3. Quantitative estimations of the secondary structure contents were made using the CDPro software package [29]. The experimental data in 204–240 nm range were treated by CDSSTR and CONTIN algorithms, using SDP48 and SMP56 reference protein sets. The final secondary structure fractions reported here represent the averaged values.

Protein sequence alignment was performed using MUSCLE [30,31] (default parameters) for all discussed parvalbumins.

3. Results

3.1. Intrinsic fluorescence

Fig. 1 shows intrinsic fluorescence spectra of Mg^{2+}- and Ca^{2+}-bound pike PAs. Pike α-PA exhibits phenylalanine fluorescence (main emission maxima at 276, 284 and 292 nm) and β-PA exhibits tyrosine fluorescence (emission maximum at 303 nm). The changes in fluorescence quantum yield are sensitive to the environment properties of the chromophores in a macromolecule (i.e. ultimately to the protein structure), but the position and shape of fluorescence spectra of both PA do not change in response to the changes of the environment due to the absence of tryptophan residue.

Intensity of intrinsic fluorescence of pike α- and β-PA in Mg^{2+}-saturated form is by 8% and 14% higher than that in Ca^{2+}-loaded state, respectively. In modern literature one can find controversial data on difference in conformations for Ca^{2+}- and Mg^{2+}-loaded forms of parvalbumins. No changes in protein phenylalanine fluorescence were detected for Mg^{2+}/Ca^{2+} exchange in common mirror carp pI 4.25 PA [20], perch PA [21] and rat α-PA [22]. At the same time, binding of Ca^{2+} but not Mg^{2+} induced a significant conformational change in the rat β-PA [22]. Conformation of Ca^{2+}-bound avian thymic hormone (β-PA) differs significantly from that of Mg^{2+}-bound state, that more closely resembles apo-protein [23].

Our data suggest that the environment of at least some of aromatic residues of pike PAs is sensitive to the Ca^{2+}/Mg^{2+} exchange in the protein.

3.2. Far-UV CD

Far-UV CD spectra did not reveal any significant differences between Mg^{2+}- and Ca^{2+}-bound PAs (Fig. 2(A)). Estimations of the secondary structure contents for the Mg^{2+}- and Ca^{2+}-saturated PAs using a CDPro software package [29] show a ~6% lower α-helical content in Mg^{2+}-loaded β-PA compared with the Ca^{2+}-saturated form, whereas the difference in content of the elements of secondary structure between Mg^{2+}- and Ca^{2+}-bound forms of α-isoform is negligible (~1.5% for α-helices). Similarly, only slight differences were previously observed in far-UV CD spectra for Mg^{2+} and Ca^{2+}-bound forms of avian thymic hormone [23] and far-UV CD

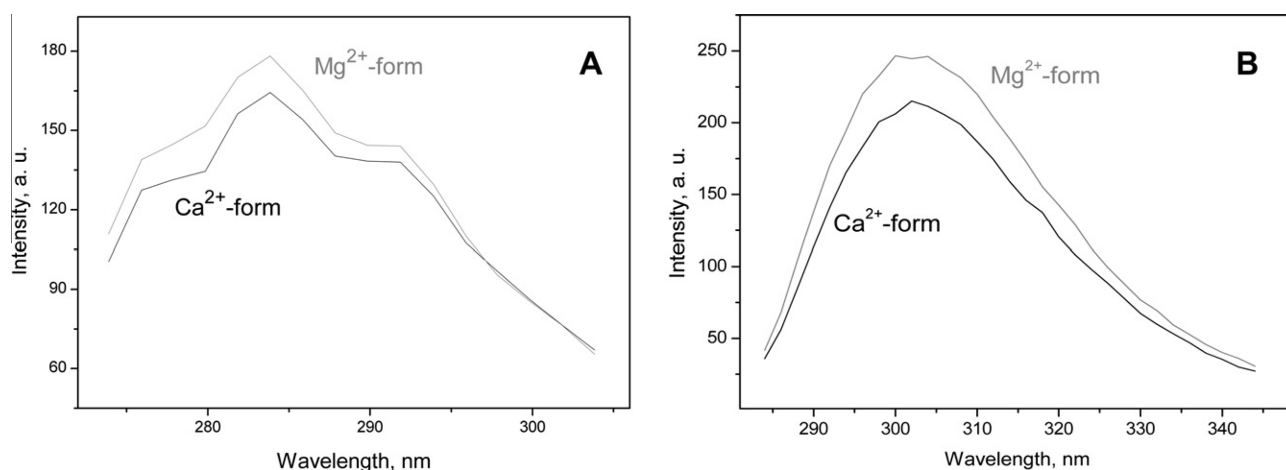

Fig. 1. Fluorescence spectra for Mg^{2+}-saturated and Ca^{2+}-saturated pike α-PA (A) and β-PA (B) at 20 °C. Protein concentrations were 8 μM, cation to protein molar ratios ⩾3. Buffer conditions: 10 mM HEPES–KOH, pH 7.5.

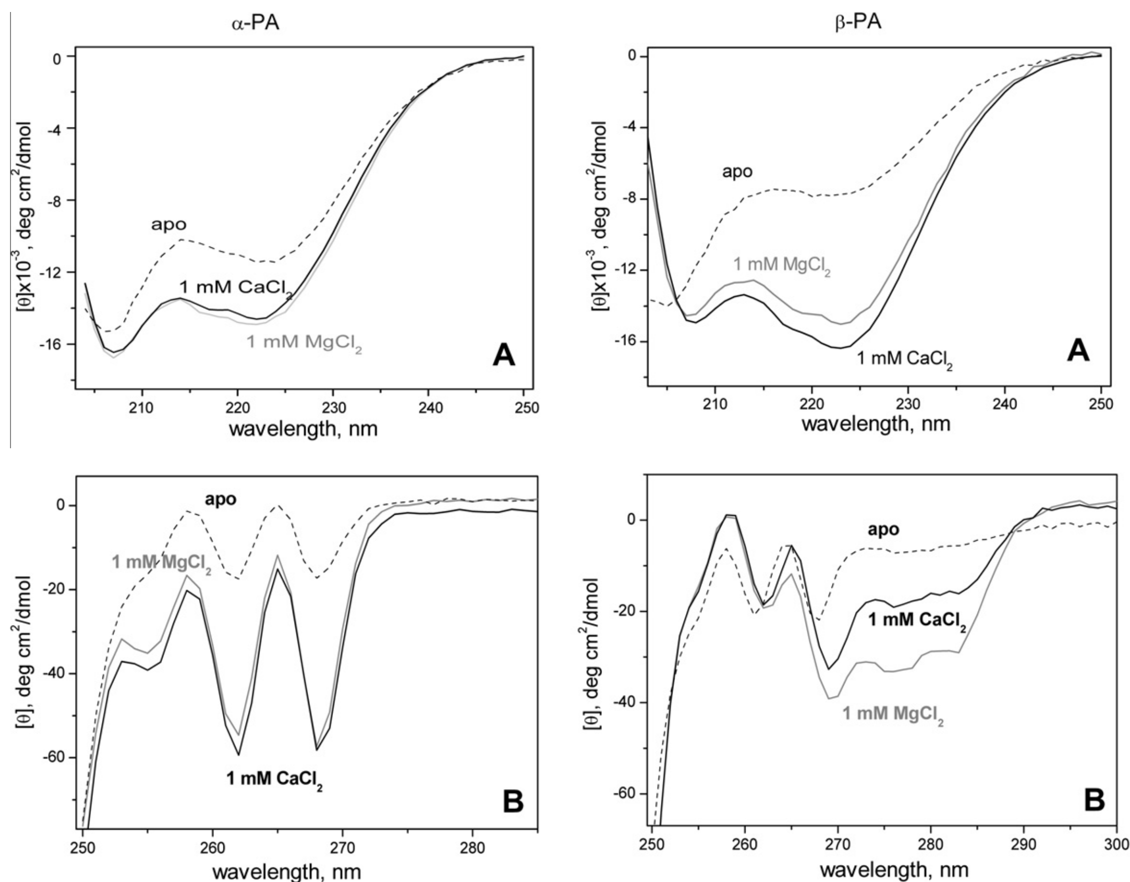

Fig. 2. Far- (A) and near- (B) UV CD spectra for apo-, Mg^{2+}-loaded (1 mM $MgCl_2$) and Ca^{2+}-loaded (1 mM $CaCl_2$) pike α-PA and β-PA at 20 °C. Buffer conditions for far UV: 10 mM H_3BO_3, 1.5 mM EDTA, pH 8.8 (for apo-PAs), 10 mM H_3BO_3, pH 8.8 (for Ca^{2+}-loaded PAs) or 6 mM HEPES, pH 8.2 (for Mg^{2+}-PAs); for near UV: 16.5 mM Tris, 3 mM EDTA, pH 8.3 (for apo-PAs) or 10–20 mM HEPES–KOH, pH 8.2 (for Mg^{2+}- and Ca^{2+}-loaded PAs).

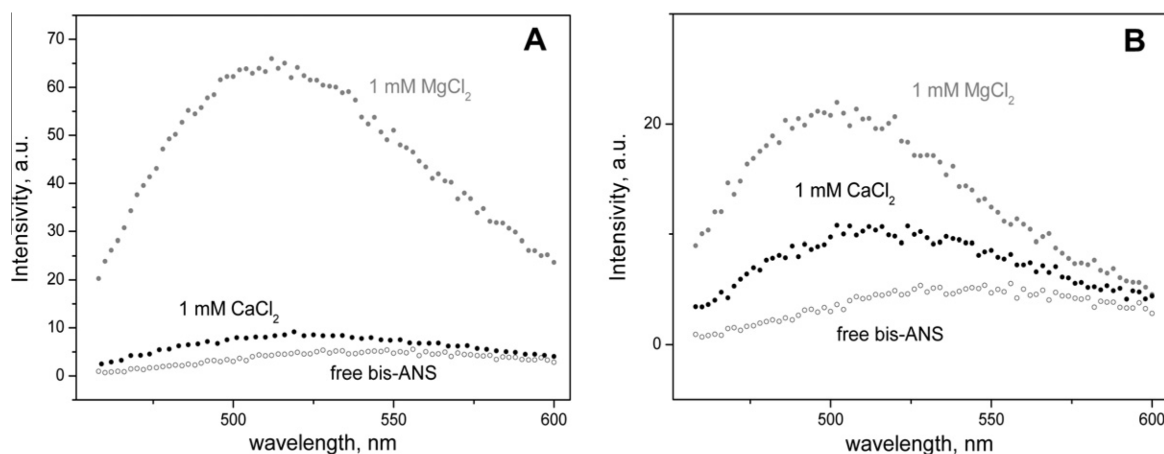

Fig. 3. Fluorescence spectra of bis-ANS in the presence of Mg^{2+}-loaded (1 mM $MgCl_2$) and Ca^{2+}-loaded (1 mM $CaCl_2$) pike α-PA (A) and β-PA (B) at 20 °C. Buffer conditions: 10–20 mM Gly-KOH, pH 9.3 (for Mg^{2+}-bound PAs) and 10 mM H_3BO_3-KOH, pH 8.8 (for Ca^{2+}-bound PAs).

spectra of these forms of PA II from perch were indistinguishable [21]. According to our results the secondary structure of the pike β-PA demonstrates more serious difference between the Mg^{2+} and Ca^{2+} bound forms than that of α-isoform. This is in line with our previous results indicating more prominent changes of secondary structure content during the binding of Ca^{2+} ions to β- than to α-PA (α-helical content increases by 30% and 8%, respectively, compared with apo-proteins [19]).

Overall, Mg^{2+}-saturated pike PAs as well as their Ca^{2+}-bound forms are characterized by high (⩾48%) content of α-helical structure and the secondary structure of β-PA is more sensitive to the substitution of the bound cation though the differences remain moderate.

3.3. Near-UV CD

It was previously shown that apo-forms of pike PAs are characterized by the absence of rigid tertiary structure [19]. The near-UV CD spectra of Mg^{2+}- and Ca^{2+}-loaded forms of pike α-PA are nearly identical and characterized by a large negative molar ellipticity in

the near-UV region (Fig. 2(B)). At the same time, β-isoform demonstrates more interesting behavior (Fig. 2(B)). The magnitude of the near-UV CD spectrum of the Mg^{2+}-bound form of β-PA is small in the Phe spectral range and is comparable to that for apo-form, which suggests that Phe residues in the Mg^{2+}-saturated β-PA (as well as those of its Ca^{2+}-saturated form; [19]) are located in a quite mobile symmetrical environment. Importantly, the environment of the single Tyr in β-PA becomes less symmetrical in the Mg^{2+}-loaded form. The difference in the magnitude of near-UV CD signal between Mg^{2+}- and Ca^{2+}-bound forms at 275 nm (absorption maximum wavelength for Tyr) is even more significant than that between Ca^{2+}-loaded state and unfolded apo-form. Since pike β-PA contains only one Tyr residue per molecule the structural region causing the different near-UV CD response upon the binding of Mg^{2+}- and Ca^{2+} can be easily determined: Tyr49 is located at a C-terminal region of helix C which is a part of the CD subdomain.

A comparison of the structure of fully Ca^{2+}-loaded pike β-PA with the structure of this protein with the EF site occupied by Mg^{2+} ion and CD site occupied by Ca^{2+} [17] shows that such exchange does not affect the position of Tyr, indicating that it is sensitive only to the ion substitution in the CD site.

3.4. Bis-ANS fluorescence

The interaction of hydrophobic dye bis-ANS with Ca^{2+}- and Mg^{2+}-bound pike α-PA at 20 °C causes 2- and 20-fold increase in intensity of fluorescence of the dye, respectively (Fig. 3(A)). The difference in bis-ANS fluorescence intensity for Ca^{2+}- and Mg^{2+}-loaded forms of β-PA is less pronounced: 3- and 7-fold increase in probe fluorescence intensity, respectively (Fig. 3(B)). Taking into consideration that in the presence of apo-PA bis-ANS fluorescence intensity increases by 150- and 50-fold for α- and β-PA, respectively [19], the effects obtained for the metal-saturated forms are moderate. Nevertheless, the experiments with bis-ANS demonstrate that Mg^{2+}-bound forms of pike PAs possess different solvent exposure of hydrophobic residues compared with the Ca^{2+}-loaded forms.

4. Discussion

All experimental approaches employed in the present work indicate the existence of differences between Ca^{2+}- and Mg^{2+}-bound forms of β-PA, and the environment of Tyr49 seems to be the most sensitive to the Ca^{2+}/Mg^{2+} exchange. This residue is not conserved in all PAs but most representatives of the PA family contain an aromatic residue at the C-terminal region of helix C. Importantly, Phe50 of rat β-PA assumes more interior position in the Ca^{2+}-free state (in fact, in the Na^{+}-loaded state, as it appears from the buffer conditions) comparing with that in Ca^{2+}-saturated form, and the residue contributes to a significant increase in contact with helix B [32]. At the same time, in the Ca^{2+}-free state C helix is shortened and Phe50 becomes a part of the CD-loop. The apolar contact area between the AB and CD–EF subdomains decreased from 1501 to 1363 ± 38 $Å^2$ with removal of Ca^{2+}, thus, the difference in hydrophobic contact area between Na^{+}- and Ca^{2+}-loaded forms of rat β-PA is moderate (9.2% ± 2.5%). Recently it has been shown that although the structures of Ca^{2+}-loaded and Ca^{2+}-free (Na^{+}-loaded) forms of chicken PA 3 are generally similar, the conformations of residues located in the C helix, proximal to the CD binding loop (48–51) differ markedly in the two protein forms [33]. However, Phe48 of chicken PA 3 experiences much greater solvent accessibility in response to Ca^{2+} removal. Significantly, Ile50 adopts an interior position in the Na^{+}-bound protein and the realignment of Phe67 and Phe71 facilitates their interaction with Ile50 and reduces their contact with residues in the N-terminal AB domain. Thus, the C-terminal region of helix C seems to be the most sensitive to the metal exchange in the metal-binding sites of PA.

Recently we have shown that PAs in the Ca^{2+}-bound state demonstrate complex mechanism of thermal denaturation, involving at least one intermediate, while the thermal unfolding of Na^{+}- and Mg^{2+}-loaded forms of pike α-PA (as well as that of Mg^{2+}-bound β-PA; unpublished results) represents an “all-or-none” process [34]. The Ca^{2+}-bound state of pike PAs possesses a unique thermal stability; a loss of structural cooperativity and appearance of two separate thermodynamic domains in the Ca^{2+}-bound PA may be caused by impairment of interactions between the protein subdomains. Unfortunately, no 3D structure of fully Mg^{2+}-loaded parvalbumin is available in the PDB data base for any PA. Thereby it is unknown whether the buried Tyr contacts with other hydrophobic residues or forms a hydrogen bond in this protein state, however, tyrosine –OH groups often contribute favorably to protein stability even if they do not form intramolecular hydrogen bonds [35]. Hence, Ca^{2+}/Mg^{2+}-switch of structural cooperativity of thermodynamic domains of β-PA may be due to a coupling of subdomains in Mg^{2+}-loaded form of PA caused by more interior position of aromatic (and/or some other) residues of helix C. This assumption is supported by the fact that the substitution of Ca^{2+} by Mg^{2+} in the EF site of pike 4.10 PA is accompanied by a shortening of about 0.4 Å of the intercationic distance between CD and EF sites [36]. In addition, MacManus et al. inferred that Mg^{2+}-bound form of carp PA could have less random structure than Ca^{2+}-bound, possibly due to the smaller size of Mg^{2+} ion collapsing the binding domain further than Ca^{2+}, leading to a tighter structure (discussed in [22]).

One could suggest that similarly to Tyr49 of pike β-PA Phe48 of α-isoform assumes more interior position in the Mg^{2+}-loaded form causing the changes in fluorescence, but it is not evident from near-UV CD spectra. Pike α-PA contains nine Phe and contribution of one Phe in the spectral changes may not be visible in the total effect especially if these changes are of opposite sign. The position of Phe48 of rat α-PA (the only aromatic residue in the helix C) is nearly the same in solution structure of Na^{+}-bound and crystal structure of Ca^{2+}-loaded form [37]. Notably, one-dimensional NMR of the same protein showed that the line intensities of the ortho proton resonance of Phe48 were exceptionally informative probes of the specific conformational changes that accompany metal binding and metal exchange, though no significant difference was revealed as a whole between Ca^{2+}- and Mg^{2+}-bound forms [38]. Still, position of Phe48 was sensitive to the binding of magnesium, while very slightly difference was observed between K^{+}- and Ca^{2+}-loaded rat α-PA. Another study of rat PAs [22] showed that specific structural rearrangements accompanied Mg^{2+}/Ca^{2+} exchange in rat β-PA, but not in the rat α-isoform. Thus, as there are no clear data in the literature indicating the structural difference between Mg^{2+}- and Ca^{2+}-bound α-PA, the change in solvent accessibility of aromatic residue of the C-terminal region of helix C may be a characteristic of Ca^{2+}-bound β-PA.

The titration of the Mg^{2+}-loaded PA with Ca^{2+} exhibits only a single intermediate species, in which Ca^{2+} is bound in the CD site and Mg^{2+} is bound in the EF site (discussed in [39]). Thus, in muscle cells of pike Mg^{2+}/Ca^{2+} exchange in β-PA following calcium release from the sarcoplasmic reticulum will immediately cause a change in disposition of Tyr49. A typical hallmark for calcium sensors is their Ca^{2+}-dependent conformational changes allowing the interaction with specific ligands and subsequent regulation of downstream effectors [40]. Thus, Ca^{2+}-dependent change of dislocation of Tyr49 in pike β-PA is another evidence for sensor role of PA of β-lineage [22,23] and may suggest an involvement of this residue (and probably the neighboring ones) in complexation with unidentified target(s). Notably, Tyr residues appear much more frequently on the protein surface than expected from their solution properties [41]; moreover, Tyr often makes energetically significant contribu-

tion to protein–protein interaction, thus being one of the most important amino acids in hot spots of the proteins [42,43].

The CD site of β-PA is often considered to be a Ca^{2+}-specific site [15]. Our results have shown that at 20 °C the metal-binding sites of both pike PAs do not differ significant in affinity for Ca^{2+} [19] and Mg^{2+} ions (association constants: $K_1 \sim 7 \times 10^5\ M^{-1}$, $K_2 \sim 2 \times 10^6\ M^{-1}$ for α-PA and $K_1 \sim K_2 \sim 1 \times 10^6\ M^{-1}$ for β-PA; unpublished data). Since the association constants for these cations are very high, both metal-binding sites of pike PAs should be regarded as Ca^{2+}/Mg^{2+} mixed sites. Nevertheless, at least some of residues of CD site of β-PA undergo metal-specific rearrangements upon Ca^{2+}/Mg^{2+} exchange.

An attractive assumption is that the Ca^{2+}-dependent change of accessibility of Tyr49 in pike β-PA is important for posttranslational modifications of the protein, for example, by calcium-dependent tyrosine kinases or by tyrosylprotein sulfotransferase. It seems to be unlikely since we did not reveal any posttranslational modifications of pike PAs by means of mass spectrometry (except for the N-terminal acetylation [27]); moreover, about two decades ago human α- and β-parvalbumin were shown to lack posttranslational modifications as was concluded from electrospray ionization (ESI) mass spectrometry analysis [44]. Furthermore, tyrosine sulfation appears to be impossible since it is a trans-Golgi-specific modification [45] and PA is considered to be a cytosolic protein. However, reports have recently appeared indicating that PA can be detected in the extracellular fluids, for example, PA was found to be secreted by frog skin [46] and rat macrophages [14]. Under standard conditions used for MALDI or ESI mass spectrometry, ionization of proteins and peptides containing sulfoTyr can lead to desulfation [45]. Thus, further studies are required for clarification of this issue.

Notably, previous works that demonstrated the difference between Mg^{2+}- and Ca^{2+}-loaded forms of PA also showed similarity between apo- and Mg^{2+}-bound forms of the protein [22–24]. The significant distinction between apo- and Mg^{2+}-saturated form of pike PAs revealed in the present work is probably explained by much more careful control of the metal ion content in our experiments, ensuring achievement of genuine apo-form of PA [19]. At the same time, we do not argue the assumptions about partial solvent accessibility of apolar surface area of PA in the Mg^{2+}-bound state and its possible role in noncovalent association of the protein with unknown biological target. Although values of specific heat capacity do not show essential difference in the total solvent accessible hydrophobic surface area between Ca^{2+}- and Mg^{2+}-bound pike PAs, the certain accessible apolar groups are probably different as appears from the contradiction between CD data and the results of bis-ANS fluorescence measurements. Thus, the nature of the bound cation can modulate the functions of PA causing change of the binding partner(s) of the protein.

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