



Contents lists available at [SciVerse ScienceDirect](#)

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca



The impact of alpha-N-acetylation on structural and functional status of parvalbumin

Sergei E. Permyakov^{a,b,*}, Alisa A. Vologzhannikova^a, Victor I. Emelyanenko^a,
Ekaterina L. Knyazeva^a, Alexei S. Kazakov^a, Yulia S. Lapteva^a, Maria E. Permyakova^a,
Andrei P. Zhadan^a, Eugene A. Permyakov^{a,b}

^a Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Moscow region, 142290 Russia

^b Pushchino State Institute of Natural Sciences, Pushchino, Moscow region, 142290 Russia

ARTICLE INFO

Article history:

Received 10 May 2012

Received in revised form 30 May 2012

Accepted 1 June 2012

Available online xxx

Keywords:

N-terminal acetylation

Thermodynamics

Protein stability

Metal binding

Calcium binding proteins

Parvalbumin

Oncomodulin

ABSTRACT

The effect of alpha-N-acetylation (Nt-acetylation) on the properties of parvalbumin (PA), a Ca²⁺-binding relaxing factor of skeletal muscles and major food allergen, has been explored. Intact PA contains an N-terminal acetyl group which is absent in the protein expressed in *Escherichia coli* (rWT), as confirmed by mass spectrometry. Compared to intact pike α -PA, its rWT form exhibits essentially altered profile of thermal unfolding, lowered α -helicity, and decreased affinities to Ca²⁺ and Mg²⁺. The structural destabilization of the rWT protein results in lowered resistance to chymotryptic digestion and increased propensity to oligomerization. The rate constants of Ca²⁺ dissociation from the rWT PA are markedly increased, which indicates that Nt-acetylation modifies functional status of the protein. Rat α -PA demonstrates similar properties for intact and rWT forms. The drastic difference in the effects induced by Nt-acetylation in the PA orthologs can be rationalized by higher disorder level of AB domain in pike PA. Though evolution of PA's genes resulted in the protein sequences with highly divergent properties, Nt-acetylation unifies their functional properties. The structural stability conferred to PA by Nt-acetylation may contribute to its allergenicity. Overall, Nt-acetylation is shown to be a prerequisite for maintenance of structural and functional status of some parvalbumins.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

N-terminal acetylation (Nt-acetylation) is a universal irreversible co- or post-translational enzymatic protein modification involving a transfer of an acetyl group from acetyl-coenzyme A to the α -amino group of the first amino acid of a protein [1–9].

Abbreviations: Nt-acetylation, N-terminal acetylation; NAT, N-terminal acetyltransferase; PA, parvalbumin/oncomodulin; α -PA, α isoform of parvalbumin; intact PA, parvalbumin isolated from muscles; rWT α -PA, recombinant wild-type α -parvalbumin; IPTG, isopropyl β -D-1-thiogalactopyranoside; Tris, tris(hydroxymethyl)aminomethane; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; TCA, trichloroacetic acid; GdmCl, guanidine hydrochloride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; DSC, differential scanning calorimetry; CD, circular dichroism spectroscopy; NMR, nuclear magnetic resonance.

* Corresponding author at: Institute for Biological Instrumentation of the Russian Academy of Sciences, Institutskaya str., 7, Pushchino, Moscow region, 142290 Russia. Tel.: +7 4967 31 89 72; fax: +7 4967 33 05 22.

E-mail address: permyakov.s@gmail.com (S.E. Permyakov).

While Nt-acetylation is rarely reported in prokaryotes [10–12], about 57% of yeast proteins [7], 70% of proteins in *Drosophila melanogaster* [13], and 84% in humans [7] are α -N-acetylated. Nt-acetylation is catalyzed by several N-terminal acetyltransferases (NATs) with unique substrate selectivity in regard to several N-terminal amino acids (except for RimL in *Escherichia coli*, which seem to recognize also some other protein segments, and eukaryotic NatD, substrate specificity of which lies within the first 30–50 residues) [4,7,8,11,14]. In *E. coli* single-chain NATs (RimL, RimJ, RimM) act likely post-translationally. Eukaryotic NAT machinery is more sophisticated: single or multi-subunit NATs (NatA to NatE, and NatF in higher eukaryotes) associated with ribosomes (found in humans also in non-ribosomal form) act primarily cotranslationally. The catalytic subunit Naa10p of human NatA may also act post-translationally. Furthermore, plants possess a dedicated pathway for post-translational Nt-acetylation of organelle-targeted proteins [15]. Higher eukaryotes have a more complex system of NatA (the major NAT) subunits than found in lower eukaryotes. Compared to yeast, at least four combinations of human NatA complex are possible [4]. Besides, different mammalian Naa10p (subunit of NatA) variants are produced by alternative splicing [16]. Recently an acetylation of ϵ -amino group was reported for human

Naa10p [17–19], thereby establishing a link between NATs and lysine acetyltransferases.

The existence of a specialized Nt-acetylation system, which acts in concert with other N-terminal processing systems, suggests that it serves some vital function(s). Indeed, a single S37P mutation in human Naa10p subunit gives rise to a lethal disorder of infancy [20]. Furthermore, knockdown of the subunits of human NATs triggers apoptosis in human cell lines (NatA [21]), disrupts normal cell-cycle progression and induces growth inhibition in human cell cultures (NatB [22]), causes p53-dependent cell death and reduced growth of human cell lines (NatC [23]). Though Nt-acetylation is evidently essential for normal proliferation and survival of eukaryotic cell, multiple attempts to establish the primary function of this modification at the molecular level had revealed surprisingly complicated and partly contradictory picture.

Since ubiquitination of N-terminal α -amino group may trigger degradation of a protein [24], a blocking of this group *via* acetylation should prevent from ubiquitin-mediated protein degradation. Indeed, *in vivo* studies evidence that Nt-acetylation inhibits degradation of some proteins by the ubiquitin proteolytic system [25]. The ability of N-terminal acetyl group to confer resistance to proteolysis by exopeptidases (e.g., [26–31]) should further impede the ubiquitin-dependent protein degradation. Finally, Nt-acetylation may also inhibit the ubiquitin-independent proteasomal degradation [32]. Surprisingly, recent findings favor the opposite view: Nt-acetylation may act as a degradation signal (degron), targeted by the Doa10 ubiquitin ligase [33]. Presumably the effect of Nt-acetylation (degradation or stabilization) depends upon particular protein and cellular conditions.

Other functions demonstrated for N-terminal acetyl group include inhibition of protein targeting to the endoplasmic reticulum [34], mediation of protein–protein [9,35–42] and protein–membrane [43,44] interactions. The ability of Nt-acetylation to affect protein–protein interactions (partly due to the loss of positive charge of the α -amino group) may be a general phenomenon, causing multiple alterations in the cellular interaction networks. In this case the functional consequences of Nt-acetylation may greatly vary depending upon a particular protein and its respective target. Since some proteins normally subjected to Nt-acetylation do not require this modification for their functional activity [45–47], Nt-acetylation seems to be a functional prerequisite only for a specific subset of proteins.

Calcium signaling *via* numerous Ca^{2+} -binding proteins affects every aspect of cell's life and death [48–52]. Many of the Ca^{2+} -binding proteins are Nt-acetylated (e.g., annexins, calbindin, calmodulin, calpain, myosin regulatory light chains, parvalbumin, protein kinase C, S100 proteins, troponin C), but functional significance of this modification remains unclear. Here we present a detailed study of the effect of Nt-acetylation on structural and functional properties of a classical Ca^{2+} -buffering protein, parvalbumin [53–55]. Parvalbumin (PA) is a vertebrate-specific cytosolic small acidic protein (106–113 residues, pI 3.9–5.5) of the EF-hand superfamily of Ca^{2+} -binding proteins, which is expressed in fast-twitch muscles (mainly in skeletal, but sometimes in cardiac), specific neurons, certain kidney and endocrine glands cells, Corti's cells, and others. While function of PA in non-muscle tissues has not been firmly established, multiple studies showed that PA serves as a soluble relaxing factor accelerating the Ca^{2+} -mediated relaxation in the fast muscles [55–59]. Though molecular details of this process are not fully clear, the kinetics of Ca^{2+} and Mg^{2+} association and dissociation of their complexes with PA are believed to reflect the functional status of the protein. Parvalbumin is a major cross-reactive fish allergen [60–65]. It represents the second largest animal food allergen family. In sensitized individuals producing IgE antibodies against PA a contact with or consumption of fish-related products can lead to urticaria and dermatitis, angioedema, diarrhea,

asthma, systemic anaphylactic reactions and death. The high structural stability of Ca^{2+} -loaded PAs seems to promote sensitization of patients despite cooking and exposure to the gastrointestinal tract.

Regardless of the phylogenetic lineage (α or β), parvalbumin consists of three 30-residue-long subdomains, each containing a central loop flanked by short amphipathic α -helices (labeled A–F) [53,66]. Helices C,D and E,F with their corresponding loops form two EF-hand type $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding motifs (defined in PDOC00018 entry of PROSITE database), termed CD and EF subdomains. The AB subdomain is non-functional because of a two-residue deletion in the AB-loop. The N-terminal residue (mostly, Ala or Ser) of PA is α -N-acetylated [66–69]. Since comparative studies of intact and recombinant PA forms, differing by presence of the N-terminal acetyl group in the intact protein, did not reveal marked difference in their IgE-binding properties [70], it is generally assumed that structural consequences of the Nt-acetylation are negligible. Here we show that Nt-acetylation is able to induce drastic changes in the key physico-chemical properties of some PAs, including structural stability, protease resistance, propensity to oligomerization, and equilibrium and kinetic parameters of metal binding. Since this effect was not observed in an orthologous PA, one may conclude that evolution of PA's genes resulted in the protein sequences with highly divergent properties, but the ancient covalent modification unifies their properties to ensure proper functional status. Thus, Nt-acetylation seems to be a functional prerequisite for parvalbumin. Besides, the structural stabilization and protease resistance conferred to PA by Nt-acetylation suggests that this process contributes to allergenicity of parvalbumin.

2. Materials and methods

2.1. Materials

Ultra-grade HEPES, H_3BO_3 and glycine were from Sigma–Aldrich Co., Fluka and Merck Biosciences, respectively. Biochemical grade Tris was purchased from Panreac Química, S.A. Biochemistry grade guanidinium chloride was from Merck Biosciences. DEAE-Sephacel and SP Sepharose FF resins were from Amersham Biosciences. Toyopearl SuperQ-650M was purchased from TOSOH Bioscience. Sephadex G-25 was a product of Pharmacia LKB. Calcium and magnesium chloride standard solutions (Ca^{2+} content of 0.0005%) were from Fluka. EDTA and EGTA acids were from Sigma–Aldrich Co. and Fluka, respectively. Analytical grade PMSF was purchased from Amresco. Premium grade α -chymotrypsin from bovine pancreas was from Sigma–Aldrich Co. Molecular mass markers for SDS-PAGE and sodium chloride were purchased from Helicon (Moscow, Russia). Silver staining of electrophoresis gels was carried out using Amersham Biosciences PlusOne protein silver staining kit. Other chemicals were reagent grade or higher. All buffers and other solutions were prepared using ultrapure water (Millipore Simplicity 185 system). Plastics or quartz ware were used instead of glassware, to avoid contamination of protein samples with Ca^{2+} . SnakeSkin dialysis tubing (3.5 kDa MWCO) from Thermo Scientific was used.

2.2. Isolation of parvalbumins

Northern pike (*Esox lucius*) skeletal muscle α -PA was isolated as described earlier [69,71,72]. Previously we have found two variants of pike α -PA, α_1 and α_2 , differing by three residues [69]. In the present work the major α_1 -isoform (>99%; will be referred to as “ α -PA”) was studied.

The rat (*Rattus norvegicus*) α -PA was isolated and purified using the described earlier procedures [61,71,72] with the following modifications. 750 g of rat skeletal muscles were homogenized in

blender in 30 mM Tris–HCl buffer, 100 mM NaCl, 0.5 mM PMSF, 1 mM DDT, 1 mM EDTA, pH 8.0. The homogenate (volume of 2.2 L) was periodically stirred at 4 °C for 1 h, followed by centrifugation at 10,000 × g for 20 min at 4 °C. The supernatant was filtered through cotton wool and 2 mM CaCl₂ was added. The solution was heated for 20 min up to 70 °C, kept at 70 °C for 5 min, cooled using ice-water bath and filtered through cotton wool. 10% TCA was added to the solution, followed by incubation at 4 °C for 30 min. The resulting suspension was centrifuged at 14,000 × g for 20 min at 4 °C. The pellet was resuspended in 1 M Tris–HCl buffer, pH 8.0, and loaded onto Sephadex G-75 column (5 cm × 23 cm) equilibrated with 20 mM Tris–HCl, 60 mM NaCl, pH 8.0. The elution rate was 2 ml/min; the fractions with PA were collected and precipitated by TCA as described above. The resulting pellet was dissolved in 20 mM Tris–HCl pH 8.0 buffer (buffer A), and 3–4-fold dialyzed against buffer A at 4 °C. The dialyzed solution was loaded onto Toyopearl SuperQ-650M column (0.9 cm × 5 cm) equilibrated with buffer A. The column was flushed by buffer A containing 15 mM NaCl. The protein was eluted with buffer A and 45 mM NaCl; elution rate was 1 ml/min. The fractions with PA (*ca* 250 mg) were collected, exhaustively dialyzed against distilled water at 4 °C, freeze-dried and stored at –18 °C.

The synthetic *E. coli* codon optimized gene encoding Northern pike (*E. lucius*) α-PA, the major α₁-isoform [69], was cloned into the pET28b vector (Novagen) between the *Xba*I and *Xho*I restriction sites. The plasmid was transformed into *E. coli* strain BL21(DE3). The cells were grown at 22 °C in 2-YT media (3–4 L) with 50 μg/ml kanamycin and 0.4% D-glucose. PA expression was induced by 1 mM IPTG. The cells were grown for 3 h, collected by centrifugation at 5000 × g for 15 min at 4 °C, and stored at –18 °C. Purification of pike α-PA was based on the procedure described in [69,71,72]. The cells were lysed in 50 mM Tris–HCl, 1 mM PMSF, 1 mM DTT, pH 7.4, using a French press (IBI RAS, Pushchino, Russia). 1 mM CaCl₂ was added to the lysate and the mixture was heated up to 80 °C (kept for 3 min), then cooled in ice-water bath and centrifuged at 15,500 × g for 30 min at 4 °C. The supernatant was dialyzed at 4 °C first against distilled water and then against 5 mM HEPES, pH 7.4 (buffer B). The dialyzed solution was loaded onto DEAE-Sephacel column (2.6 cm × 10 cm) equilibrated with buffer B. PA was eluted with a linear gradient of CaCl₂ (300 ml of buffer B – 300 ml of 300 mM CaCl₂); elution rate was 1 ml/min. The fractions with PA were collected, concentrated *via* freeze-drying, and dialyzed against 20 mM Tris–HCl, 60 mM KCl, 2 mM EDTA, pH 8.0 (buffer C), at 4 °C. The dialyzed solution was loaded onto Toyopearl SuperQ-650M column (0.9 cm × 7.5 cm) equilibrated with buffer C. The protein was eluted with 20 mM Tris–HCl, 20 mM CaCl₂, pH 8.0; elution rate was 0.5 ml/min. The fractions with PA were collected, exhaustively dialyzed against distilled water at 4 °C, freeze-dried and stored at –18 °C. The yield was 40–60 mg of protein per liter of cell culture.

Rat (*R. norvegicus*) α-PA gene was re-cloned from the pRV6 vector (derivative of pBlueScript II SK(+) vector) into the pET29a vector (Novagen) between the *Xba*I and *Xho*I restriction sites (the pRV6 plasmid was kindly provided by Prof. Michael T. Henzl, University of Missouri, USA). The plasmid was transformed into *E. coli* strain BL21(DE3). rWT rat α-PA was purified similarly to rWT pike α-PA, with the following modifications. The DEAE-Sephacel column was replaced by SP Sepharose FF column (0.9 cm × 7.5 cm) equilibrated with 10 mM sodium acetate buffer, 0.5 mM CaCl₂, pH 4.5. The protein was eluted with 10 mM Tris–HCl pH 8.0 buffer; elution rate was 1 ml/min. The yield was 40–60 mg of protein per liter of cell culture.

The PA samples were homogeneous as judged by native and SDS-PAGE. Their UV absorption and fluorescence spectra were characteristic for Phe residues, since α-PA lacks Trp/Tyr residues. Protein concentrations were estimated spectrophotometrically

using molar extinction coefficients of $\epsilon_{259\text{ nm}} = 1810\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{258\text{ nm}} = 1600\text{ M}^{-1}\text{ cm}^{-1}$ for pike [73] and rat [74] α-PAs, respectively.

2.3. The purification of protein samples from metal ions

Metal depletion from protein samples was performed using a combination of the TCA protein precipitation method [75] and the Sephadex G-25 gel-filtration method [76]. Both methods were combined for preparation of metal-free states of pike α-PAs, for Mg²⁺-titrations of rat α-PAs, and chemical crosslinking experiments. In other cases only the Sephadex G-25 gel-filtration method was employed.

2.4. Mass spectrometry measurements

Protein molecular masses were determined by electrospray ionization mass spectrometry (ESI-MS). The sample of 0.1–0.5 μM PA in 50:50 (v/v) mixture of 1 mM formic acid and acetonitrile was directly infused into LCMS-2010EV (Shimadzu Co.) mass detector, ESI probe coupled to a single quadrupole detector. Mass spectrometry spectra were collected in positive mode from 600 to 1200 amu at scan speed 128 amu/s, interface voltage 4.5 kV, and nebulizing nitrogen flow 1.5 L/min.

2.5. The scanning calorimetry measurements

The scanning calorimetry studies were carried out on a Nano DSC microcalorimeter (TA Instruments) at a 1 K/min heating rate and excess pressure of 4 bar (pH 9.0–9.2, 10 mM H₃BO₃–KOH or 20 mM glycine–KOH buffer). PA concentrations were 0.6–2.1 mg/ml. The protein specific heat capacity (C_p) was calculated as described by Privalov and Potekhin [77]. The partial molar volume of PA and specific heat capacity of the fully unfolded protein were estimated according to Häckel et al. [78] and Makhatadze and Privalov [79], respectively. The temperature dependence of C_p was analyzed according to either the *cooperative two-state model* [I] or the *model of two successive transitions* [II]:

$$N_n \leftrightarrow D_n \quad \text{[I]}$$

$$N \leftrightarrow I \leftrightarrow D, \quad \text{[II]}$$

where N , I and D denote native, intermediate and denatured protein states, respectively; n is a number of molecules involved in the transition (cooperativity of thermal transition). n is equivalent to the ratio of van't Hoff enthalpy to calorimetric enthalpy ($\Delta H_{\text{vH}}/\Delta H_{\text{cal}}$). The experimental data were fitted using Microcal OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA) software, using the equations derived earlier [80]. The heat capacity change accompanying a transition (ΔC_p) was supposed to be independent of temperature. ΔC_p , mid-transition temperature (T_0), enthalpy of protein denaturation at temperature T_0 (ΔH_0), and n for the model [I] were used as fitting parameters (in some cases n was set to 1). Double set of the fitting parameters was used for the model [II].

2.6. Refolding of pike parvalbumin samples

The 6 μM protein solution in 10 mM H₃BO₃–KOH pH 9.0 (buffer D) with addition of 1.5 mM EDTA and 6 M GdmCl (the conditions ensuring complete unfolding of intact pike PA [81]) was dialyzed at room temperature against buffer D containing 1.5 mM EDTA, then against buffer D, and finally against buffer D with 1 mM CaCl₂. The ratio of volumes of the buffers used for dialysis and the protein solution was about 10. Finally, the protein was exhaustively dialyzed against distilled water, freeze-dried and stored at –18 °C.

2.7. Circular dichroism measurements

Circular dichroism (CD) studies were carried out with a J-810 spectropolarimeter (JASCO, Inc.), equipped with a Peltier-controlled cell holder. The instrument was calibrated with an aqueous solution of d-10-camphorsulfonic acid according to the manufacturer's instruction. The cell compartment was purged with nitrogen (dew-point of -40°C). The quartz cell with pathlength of 1.00 mm was used for far-UV region measurements. Protein concentration was 6–10 μM . Buffer conditions: 10 mM H_3BO_3 , pH 8.9, 1.5 mM EDTA or 1 mM CaCl_2 (for apo- and Ca^{2+} -loaded PA states, respectively), or 10 mM Tris-HCl, 1 mM EGTA, 1 mM MgCl_2 , pH 7.3 (for Mg^{2+} -loaded PA). The small contribution of buffer was subtracted from experimental spectra. Band width was 2 nm, averaging time 2 s, and accumulation 3. Quantitative estimates of the secondary structure contents were made using the CDPPro software package [82] (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>). The experimental data in 190–240 nm range were treated by SELCON3, CDSSTR and CONTIN algorithms, using SDP48 and SMP56 reference protein sets. The final secondary structure fractions represent averaged values.

2.8. Limited proteolysis by α -chymotrypsin

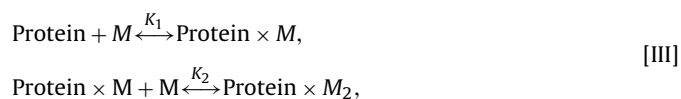
5 μl of bovine α -chymotrypsin solution (0.1 mg/ml) in 10 mM HEPES-KOH buffer, 1 mM CaCl_2 , pH 7.3 were added to 100 μl of PA solution (0.91–0.99 mg/ml) in the same buffer. The reaction proceeded overnight at 37°C and was stopped by addition of 1 mM PMSF in 2-propanol. The proteolytic mixture was analyzed by SDS-PAGE (15% resolving gel; 4 μg of protein per lane). The gel was stained with Coomassie Brilliant Blue R-250 and scanned using Molecular Imager PharoFX Plus System (Bio-Rad Laboratories, Inc.).

2.9. Fluorescence measurements

Fluorescence studies were performed on a Cary Eclipse spectrofluorimeter (Varian, Inc.), equipped with a Peltier-controlled cell holder. Quartz cells with pathlength of 10 mm were used. Protein concentrations were 4–16 μM . Fluorescence of Phe residues of parvalbumin was excited at 258–259 nm.

2.10. Estimation of equilibrium and kinetic metal association/dissociation constants

The binding of $\text{Ca}^{2+}/\text{Mg}^{2+}$ to parvalbumin can be successfully described by the sequential metal-binding scheme [80,83,84]:



where M denotes metal ion, K_1 and K_2 are equilibrium metal-binding constants for the two active EF-hands of the protein. The metal affinity of PA was estimated from the spectrofluorimetric titration of the metal-depleted protein with a $\text{CaCl}_2/\text{MgCl}_2$ standard stock solution, followed by a titration of the metal-loaded protein with EDTA potassium salt standard solution. Protein concentration was 9–16 μM . Buffer conditions: 10 mM HEPES-KOH, pH 8.2 (Ca^{2+} -binding experiments) or 10 mM HEPES-KOH, 1 mM EGTA, pH 7.3 (Mg^{2+} -binding experiments). Fluorescence intensities were corrected for the dilution effect. Estimation of the metal association constants was performed taking into account a

competition between protein and chelator for the metal ion, which is described by the scheme [III] and the equilibrium [IV]:



The metal association constant for EDTA, K_{EDTA} , was calculated according to Schwarzenbach and Flaschka [85]. The data were globally fitted according to the schemes [III] and [IV] using FluoTitr v.1.4 software (IBI RAS, Pushchino, Russia), implementing nonlinear regression algorithm by Marquardt [86]. K_1 and K_2 were used as fitting parameters. The accuracy of the K_1 and K_2 estimates was about $\pm 1/4$ orders of their magnitudes.

Stopped-flow fluorescence measurements were performed using RX.2000 Rapid Mix Accessory (Applied Photophysics Ltd., Leatherhead, UK) with a dead time of 6 ms. Temperature of all solutions was kept at 25°C using NESLAB RTE 7 bath circulator (Thermo Fisher Scientific Inc.) and Peltier-controlled cell holder. Equal volumes (0.3 ml) of 8–10 μM parvalbumin (Ca^{2+} to protein molar ratio of 2:1, or 50–60 μM MgCl_2 was added to the metal-depleted protein) and 1–2 mM EGTA/EDTA in 10 mM HEPES-KOH buffer, pH 8.2 (Ca^{2+} dissociation experiments) or pH 7.3 (Mg^{2+} dissociation), were mixed using pneumatic drive. The excitation wavelength was 259 nm and emission was measured at 284 nm. Each experiment was repeated 6 times. The experimental kinetic curves were described by the following equation:

$$F(t) = \frac{F_1[P](t) + F_2[P \times M](t) + F_3[P \times M_2](t)}{P_0}, \quad (1)$$

where $[P]$, $[P \times M]$ and $[P \times M_2]$ are concentrations of apo-protein, and its states with either one or two metal ions bound, respectively; F_i ($i = 1, \dots, 3$) are fluorescence intensities of these protein states; P_0 is a total protein concentration. The values of protein concentrations were determined by solving the following set of equations, describing the sequential metal-binding scheme [III] and scheme [IV], using Runge-Kutta-Fehlberg method [87]:

$$\begin{aligned} \frac{d[P]}{dt} &= k_{\text{off}1}[P \times M] - k_{\text{on}1}[P][M] \\ \frac{d[P \times M_2]}{dt} &= k_{\text{on}2}[P \times M][M] - k_{\text{off}2}[P \times M_2] \\ \frac{d[E]}{dt} &= k_{\text{off}3}[E \times M] - k_{\text{on}3}[E][M] \\ P_0 &= [P] + [P \times M] + [P \times M_2] \\ E_0 &= [E] + [E \times M] \\ M_0 &= [M] + [P \times M] + 2[P \times M_2] + [E \times M] \end{aligned} \quad (2)$$

Here, $[E]$ and $[E \times M]$ are concentrations of free and metal-bound chelator, respectively; E_0 and M_0 are total concentrations of chelator and metal ions, respectively; $k_{\text{off}i}$ and $k_{\text{on}i}$ ($i = 1, \dots, 3$) are rate constants of metal dissociation and association for P , $P \times M$ and E , respectively. The $k_{\text{on}i}$ ($i = 1, 2$) values were expressed via the K_i values ($k_{\text{on}i} = k_{\text{off}i}K_i$, $i = 1, 2$) estimated from spectrofluorimetric titrations (see above). The $k_{\text{off}3}$ values for Ca^{2+} and Mg^{2+} were taken from the works [88] and [89], respectively. The corresponding $k_{\text{on}3}$ values were calculated from the equilibrium metal-binding constants for EGTA/EDTA calculated according to Schwarzenbach and Flaschka [85]. The fitting parameters, $k_{\text{off}1}$, $k_{\text{off}2}$, and F_i ($i = 1, \dots, 3$), were estimated using nonlinear least-squares method by Marquardt [86].

2.11. Chemical crosslinking of proteins

Crosslinking of proteins with glutaric aldehyde was performed in 30 mM H_3BO_3 buffer, pH 9.2. 0.02% glutaric aldehyde was added to 1 mg/ml solution of PA (either metal-depleted or with addition

of 1 mM $\text{CaCl}_2/\text{MgCl}_2$). After incubation at 37 °C for 1 h the reaction was terminated by addition of SDS sample buffer. The samples were subjected to SDS-PAGE (5% concentrating and 15% resolving gels) and staining with Coomassie Brilliant Blue R-250. The gel was scanned using Molecular Imager PharoSFX Plus System (Bio-Rad Laboratories, Inc.) and analyzed by Quantity One software.

2.12. Predictions of intrinsic disorder

The predictions of the intrinsic disorder propensity of PAs were performed using PONDR analysis v.1 software (IBI RAS, Pushchino, Russia) as described earlier [90].

3. Results and discussion

3.1. Isolation and mass spectrometric characterization of intact and rWT parvalbumins

The samples of intact Northern pike (*E. lucius*) α -PA and rat (*R. norvegicus*) α -PA were isolated from skeletal muscles. Tag-free recombinant pike and rat α -PAs were expressed in *E. coli* strain BL21(DE3). All samples were homogeneous as judged by native and SDS-PAGE. The absence of Trp/Tyr contribution in their UV absorption and fluorescence spectra (data not shown) confirms the lack of contaminating Trp/Tyr-containing proteins in the samples.

Since the intact and recombinant PAs could be subjected to different protein processing resulting in noticeable structural differences between them, molecular masses of the PA samples were estimated by electrospray ionization mass spectrometry (ESI-MS; Supplement 1). The major fraction (>95%) of intact pike α -PA has a mass of 11,821 Da, which is consistent with mass of Nt-acetylated form of the previously described α_1 -isoform [69]. The bacterial expression does not favor Nt-acetylation of proteins [10–12]. Indeed, compared to the intact protein, mass of the primary fraction (>95%) of rWT pike α -PA is 43 Da lower (11,778 Da), which is close to the contribution of an acetyl group (42.04 Da). Thus, the mass spectrometry data indicate that rWT pike α -PA differs from the intact protein by the lack of N-terminal acetyl group. Similar situation is observed for rat α -PAs (Supplement 1). The mass of the intact rat protein is consistent with typical for PAs processing, namely excision of the initiating Met residue and Nt-acetylation. The mass of the major fraction (50%) of rWT rat α -PA is 42 Da lower, indicating the lack of N-terminal acetyl group. The mass of the second large fraction (40%) of rWT rat α -PA exceeds that of the major fraction by 513 Da, which may be both due to covalent modification(s) and/or binding of unknown ligand(s). Other minor fractions of the PA samples mostly correspond to metal-bound or oxidized states of the forms mentioned above.

3.2. Thermal denaturation of intact and rWT parvalbumins

To assess the effect of Nt-acetylation on thermodynamic properties of the PAs, differential scanning calorimetry (DSC) was used as a technique providing direct information on thermodynamics of protein thermal denaturation. The temperature dependencies of specific heat capacities for apo- and metal-loaded states of the PA samples, derived from DSC data, are shown in Fig. 1. Similarly to the intact protein, apo-state of rWT pike α -PA exhibits the lack of marked heat sorption peak, and its heat capacity closely approaches that calculated for the fully unfolded protein according to Makhatadze and Privalov [79] (Fig. 1a), which is indicative of absence of rigid tertiary structure, as previously shown for intact pike α -PA [81]. At the same time, Ca^{2+} - and Mg^{2+} -loaded states of pike α -PA demonstrate distinct heat sorption peaks. The Mg^{2+} -bound (1 mM MgCl_2) state of rWT pike α -PA exhibits 26 °C lower

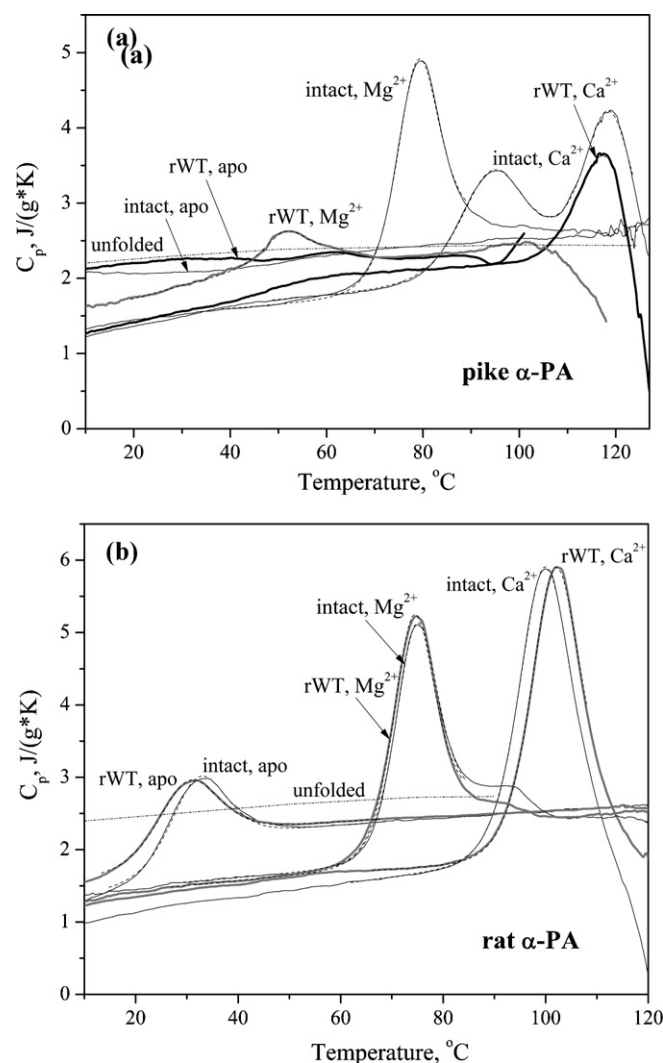


Fig. 1. Temperature dependencies of specific heat capacities of apo- and metal-bound states for intact and rWT α -PAs of pike (a) and rat (b), calculated from DSC data (pH 9.0–9.2, 10 mM H_3BO_3 –KOH or 20 mM glycine–KOH; heating rate was 1 K/min). Protein concentration was 0.6–2.1 mg/ml. PA was saturated by Ca^{2+} by addition of 1 mM CaCl_2 . Apo-form of pike α -PA was prepared using combination of the TCA protein precipitation method [75] and the Sephadex G-25 gel-filtration method [76] (1 mM EGTA), while for rat α -PA only the last method was employed. The metal-depleted protein (without EGTA) was saturated by Mg^{2+} by addition of 1 mM MgCl_2 . The analysis of DSC data for Ca^{2+} -bound intact pike α -PA was performed according to the model of two successive transitions [II]; in other cases the cooperative two-state model [I] was used (dashed curves). The resulting thermodynamic parameters are presented in Table 1. Dotted curve corresponds to the heat capacity of fully unfolded protein, as estimated according to Makhatadze and Privalov [79].

thermal stability and notably increased cooperativity of the thermal unfolding ($n = 5.6$) (Table 1), which points out oligomerization of the protein. The Ca^{2+} -loaded (1 mM CaCl_2) state of rWT pike α -PA demonstrates the absence of well-defined first (low-temperature) heat sorption peak, characteristic for the intact protein [81] (mid-transition temperature, $t_{1/2}$, of 93 °C). Instead, a low enthalpy heat sorption peak at around 60 °C is observed. This behavior may be a sign of partial population of certain protein states. To shift the equilibrium between these hypothetical protein states, DSC studies were performed at various pH values (9.0–10.3), ionic strength conditions (up to 1 M KCl) and Ca^{2+} concentrations (1–50 mM CaCl_2). In all the experiments the low enthalpy heat sorption peak did not disappear (data not shown). Therefore it is likely that this peak corresponds to the melting of a subdomain of the protein. Small size of this subdomain could rationalize the small enthalpy

Table 1

Thermodynamic parameters describing thermal denaturation of the various PAs, estimated from the DSC data shown in Fig. 1 according to either the *cooperative two-state model* [I] or the *model of two successive transitions* [II]. For designations of the parameters, see Section 2. The indices 1 and 2 refer to the parameters describing the first (low-temperature) and second heat sorption peaks, respectively.

Protein	Protein form	Protein state	T ₀ (°C)		ΔH ₀ (J/g)		ΔC _p (J/(gK))		n
			T _{0,1}	T _{0,2}	ΔH _{0,1}	ΔH _{0,2}	ΔC _{p,1}	ΔC _{p,2}	
Pike α-PA	Intact	Ca ²⁺	92.9	120.7	21.3	28.8	0.39	−0.86	–
		Mg ²⁺	77.8		29.3		0.93		1.0
	rWT	Ca ²⁺	120.0		25.6		−0.75		1.0
		Mg ²⁺	52.2		4.5		−0.06		5.6 ^a
Rat α-PA	Intact	Ca ²⁺	100.0		54.3		0.02		0.53 ^a
		Mg ²⁺	73.9		30.7		0.83		1.0
		apo	29.7		15.7		0.69		1.0
	rWT	Ca ²⁺	101.4		46.7		0.59		0.67 ^a
		Mg ²⁺	73.4		31.4		0.87		1.0
		apo	27.6		14.5		0.59		1.0

^a n value is used as an additional fitting parameter.

changes accompanying its melting. Overall, Nt-acetylation of pike α-PA drastically changes thermal denaturation behavior of the metal-bound protein states. Opposite to pike PA, rat α-PA exhibits only minor differences in thermal denaturation behavior between intact and rWT protein forms (Fig. 1 b): the differences between *t*_{1/2} values and enthalpies of protein denaturation are within 2 °C and 16%, respectively, regardless of the metal content (Table 1). Similarly to the intact protein (*n*=0.53 [81]), Ca²⁺-loaded state of rWT rat α-PA exhibits marked deviation from the simplest two-state mechanism of thermal denaturation (*n*=0.67). Summing up, Nt-acetylation induces slight (rat α-PA) to dramatic (pike α-PA) changes in thermal behavior of the PA orthologs.

In fact, the unexpectedly marked difference between thermodynamic properties of intact and rWT forms of pike α-PA could arise not because of the covalent modification of the protein, but due to differences in mechanisms of protein folding in prokaryotic and eukaryotic organisms (for example, processing by NATs; prolyl isomerases should be excluded since pike α-PA does not contain Pro residues). To address this question, we have tried bacterial co-expression of pike α-PA with *E. coli* NATs RimJ or RimI, but it gave unacceptably low protein yield. Therefore we carried out *in vitro* refolding of pike α-PAs. Apo-states of the intact and rWT forms were unfolded by GdmCl, and then stepwise folded in the course of dialysis against solutions with gradually increasing Ca²⁺ concentration (see Section 2). The DSC studies of the refolded protein samples did not reveal any significant changes in their thermal denaturation behavior (data not shown). Thus, the peculiarities of *in vivo* conditions of protein folding are unlikely to cause the observed differences between thermodynamic properties of intact and rWT pike α-PAs. Hence, these differences seem to be explained by the presence of N-terminal acetyl group in the intact protein, which could affect protein conformation directly or *via* alteration in the process of protein folding.

The Nt-acetylation is expected to affect, first of all, the structure of the AB domain of PA. According to the structural interpretation of DSC data for intact pike α-PA proposed earlier [80], its AB domain unfolds in the first thermal transition. In this case the Nt-acetylation is likely to affect the behavior of the first heat sorption peak. Our DSC data (Fig. 1 a) corroborate these considerations.

3.3. Comparison of secondary structure contents for intact and rWT parvalbumins

The changes in thermodynamic properties of pike α-PA induced by Nt-acetylation (Fig. 1a, Table 1) should be reflected in changes of the secondary and tertiary structures of the protein, which can be monitored by circular dichroism (CD) spectroscopy. The far-UV CD spectra of PAs were analyzed using the CDPro software [82].

The resulting estimates of secondary structure contents for different states of pike and rat PAs are collected in Table 2. As expected, rWT rat α-PA does not differ from the intact protein form irrespective of the metal content. The CD estimates for apo-state of rWT rat α-PA are very close to those derived from an analysis of its tertiary structure (2JWW PDB entry, NMR) using DSSP program [91]. Meanwhile, compared to the CD data both X-ray structures of Ca²⁺-loaded states of rat α-PAs (1RTP and 1RWY) reveal 7% higher α-helicity and 3% lower β-sheets content, which can be explained by structural perturbations caused by the crystallization process. The analogous comparison for Ca²⁺-loaded state of intact pike α-PA shows that CD estimate of the protein's α-helicity is in between the values derived from its X-ray and NMR structures, while the fraction of β-sheets is elevated by 1–3%.

In contrast to rat α-PA, the secondary structure of rWT pike α-PA notably differs from that for the intact protein, regardless of the metal content (Table 2). The difference in α-helicity between the intact and rWT forms of pike α-PA increases in the order: Ca²⁺-bound, Mg²⁺-bound, and apo-state (7%, 10%, and 24%, respectively). Hence, the metal binding reduces the structural differences between the intact and rWT proteins. The metal depletion is accompanied by drastic loss of α-helical structure in the rWT pike α-PA, which is partly compensated by an increase in the β-sheets content. The intact protein exhibits similar but much less pronounced changes. Similarly, previous CD studies of Ca²⁺-loaded states of intact and recombinant forms of Atlantic cod (*Gadus morhua*) PA have shown somewhat lower α-helicity of the recombinant protein [70].

Summarizing the CD data, one can conclude that the secondary structure changes induced in the PAs by Nt-acetylation well correlate with the changes in thermodynamic properties of the proteins observed by DSC, except for the denatured apo-state of pike α-PA.

3.4. Limited proteolysis of α-parvalbumins

The destabilized secondary structure of rWT pike α-PA (Table 2) suggests an increased overall mobility of its polypeptide chain, which could result in an increase of protein's susceptibility to enzymatic proteolysis. To ascertain this suggestion the Ca²⁺-bound (1 mM CaCl₂) states of intact and rWT forms of pike α-PA were subjected to limited proteolysis by bovine α-chymotrypsin (Fig. 2a). While the intact protein remains largely undigested in these conditions, the rWT protein exhibits effective conversion of the monomeric form into the fragments with molecular masses below 5 kDa. Thus, Nt-acetylation of pike α-PA contributes to its resistance to proteolytic digestion by the serine protease. The protective effect was not observed for rat α-PA (Fig. 2b). Since the resistance of parvalbumin to degradation by human digestive system is considered

Table 2

The secondary structure fractions estimated from CD data for apo- (1.5 mM EDTA), Mg²⁺- (1 mM EGTA, 1 mM MgCl₂) and Ca²⁺-loaded (1 mM CaCl₂) intact and rWT forms of α -PA at 3 °C. Protein concentration was 6–10 μ M. Buffer conditions: 10 mM H₃BO₃-KOH, pH 8.9 (for apo- and Ca²⁺-loaded PA states) or 10 mM Tris-HCl, pH 7.3 (for Mg²⁺-loaded PA). The estimates calculated with CDPro software [82] are compared with the values derived from analysis of tertiary structures using DSSP program [91].

α -PA	Protein state	α -Helices (%)	β -Sheets (%)	3D structure (%)		PDB entry, method
				α -Helices	β -Sheets	
Pike α -PA						
Intact	apo	42.8	11.0	n.a.	n.a.	n.a.
rWT		18.9	20.0	n.a.	n.a.	n.a.
Intact	Mg ²⁺	56.5	4.7	n.a.	n.a.	n.a.
rWT		46.2	5.7	n.a.	n.a.	n.a.
Intact	Ca ²⁺	54.8	4.8	56.8	1.8	1PVA, X-ray
				50.5	1.8	2PAS, NMR
				50.5	3.7	3PAT, NMR
rWT		47.9	5.3	n.a.	n.a.	n.a.
Rat α -PA						
Intact	apo	54.4	5.5	n.a.	n.a.	n.a.
rWT		53.1	5.3	54.1	5.5	2JWW, NMR
Intact	Mg ²⁺	54.3	5.9	n.a.	n.a.	n.a.
rWT		53.0	5.9	n.a.	n.a.	n.a.
Intact	Ca ²⁺	52.6	6.5	59.6	3.7	1RTP, X-ray
rWT		52.4	6.9	59.6	3.7	1RWY, X-ray

n.a., data not available.

to be a factor contributing to its allergenicity, the observed protective effect of N-terminal acetyl group of pike α -PA against the enzyme of digestive system indicates that this group may serve as one of the structural determinants of PA's allergenicity.

3.5. Metal-binding properties of intact and rWT parvalbumins

Since apo- and metal-loaded states of rWT pike α -PA differ from the appropriate states of the intact protein in secondary structure content (Table 2) and thermodynamic properties (Fig. 1, Table 1), one may expect that metal-binding properties of the recombinant protein may differ from those of its intact counterpart. Spectrofluorimetric titrations of the metal-depleted PA samples by Ca²⁺/Mg²⁺, followed by competitive metal removal by EDTA (Fig. 3a), were analyzed according to the *sequential metal-binding scheme* [III], taking into account competition between the protein and chelator for the metal ion. The resulting equilibrium metal association constants for the two active EF-hands of PA, K_1 and K_2 , are shown in Table 3. The K_i values for Ca²⁺-binding to intact pike α -PA are close to the previous estimates [81]. Calcium affinities of both metal-binding sites of rWT pike α -PA are dramatically (1.0–3.6 orders of magnitude) lowered compared with those for the intact protein. The impaired Ca²⁺ affinity of the rWT protein is clearly seen from the abrupt decrease in its intrinsic fluorescence intensity upon EDTA titration of the Ca²⁺-loaded protein (Fig. 3a). Magnesium affinities of the rWT protein follow the same trend, being lowered by 1.7–2.8 orders of magnitude. The ratio of total free energy of Mg²⁺ association ($\Delta G = -RT(\ln K_1 + \ln K_2)$) and the same value for Ca²⁺ association

with rWT pike α -PA is very close to that ratio for the intact protein (0.64 versus 0.70, respectively). It implies that Nt-acetylation is accompanied with nearly identical shrinkage of the energetic scales of the metal binding. This fact confirms the self-consistency of our estimates of the metal-binding constants.

It should be mentioned that one of the factors previously suggested to be of importance for stabilization of the intermediate state of Ca²⁺-loaded intact pike α -PA, which is populated in the process of its thermal unfolding (Fig. 1 a, 105–110 °C), is strong Ca²⁺-binding. Since calcium affinity of its rWT form is significantly decreased, this intermediate state is expected to be destabilized. Probably this effect results in disappearance of the well-defined low-temperature heat sorption peak observed for the intact protein (Fig. 1a).

In contrast to pike α -PA, the rWT rat protein does not reveal any significant Ca²⁺/Mg²⁺ affinity differences from its intact form (Table 3), which agrees with the minor effects of Nt-acetylation monitored by other methods.

In accord with lowered calcium affinity of rWT pike α -PA, it exhibits 1.6–38-fold faster Ca²⁺ dissociation, as evidenced by fluorimetric stopped-flow measurements (Fig. 3b, Table 4). At the same time, the association of Ca²⁺ slows down 10–57-fold (Table 4). Overall, calcium affinity of rWT pike α -PA decreases mostly due to the lowering of Ca²⁺ association rate (the effect is more prominent for the EF-hand being filled first). Magnesium dissociation from rWT pike α -PA occurs within the dead time of the stopped-flow instrument (6 ms). At least 2–16-fold faster compared to the intact protein Mg²⁺ dissociation from rWT pike α -PA (Table 4)

Table 3

The values of equilibrium Ca²⁺ and Mg²⁺ association constants at 20 °C for various α -PA forms, estimated spectrofluorimetrically according to the *sequential metal-binding scheme* [III]. Protein concentration was 9–16 μ M. Buffer conditions: 10 mM HEPES-KOH, pH 8.2 (Ca²⁺-binding experiments) or 10 mM HEPES-KOH, 1 mM EGTA, pH 7.3 (Mg²⁺-binding). Excitation wavelength was 258–259 nm.

Protein	Protein form	Ca ²⁺		Mg ²⁺	
		K_1 (M ⁻¹)	K_2 (M ⁻¹)	K_1 (M ⁻¹)	K_2 (M ⁻¹)
Pike α -PA	Intact	9×10^8	9×10^9	2×10^6	9×10^5
	rWT	8×10^7	2×10^6	3×10^3	2×10^4
Rat α -PA	Intact	3×10^{9a}	2×10^{9a}	9×10^5	2×10^6
	rWT	5×10^{9a}	2×10^{9a}	4×10^5	8×10^5

^a Measured at 15 °C.

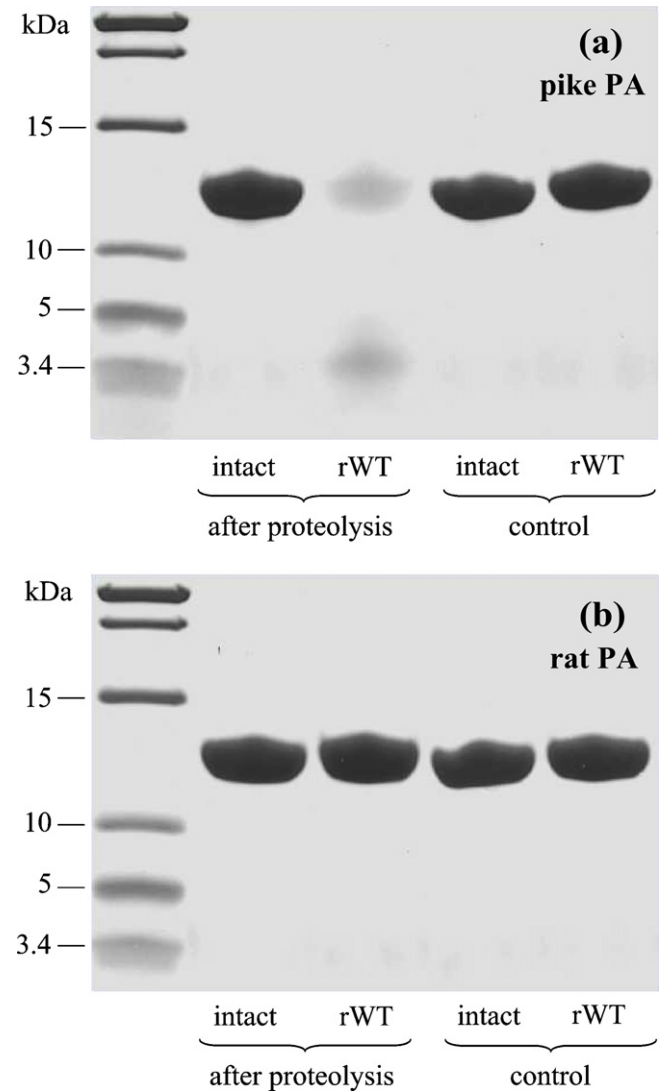


Fig. 2. Limited proteolysis of Ca^{2+} -bound states of intact and rWT forms of pike (a) and rat (b) α -PA by bovine α -chymotrypsin, monitored by SDS-PAGE. Buffer conditions: 10 mM HEPES–KOH buffer, 1 mM CaCl_2 , pH 7.3. The enzyme to substrate weight ratio was 1:200. PA concentration was 0.87–0.94 mg/ml. The PA samples not subjected to proteolysis were used as controls. 4 μg of protein was loaded per lane. The numbers in the left-hand column indicate molecular masses of markers in kDa.

is in agreement with its lowered Mg^{2+} affinity. Though the values of rate constants of $\text{Ca}^{2+}/\text{Mg}^{2+}$ dissociation from pike PA were not previously reported, they are rather close to those for orthologous PAs (for review, see [53]). To the best of our knowledge, the rate constants of metal dissociation from PA estimated within the

Table 4

The parameters of Ca^{2+} and Mg^{2+} binding for intact and rWT forms of pike α -PA at 25 °C, estimated from fluorimetric experiments. The values of equilibrium metal association constants, K_1 and K_2 , and rate constants of metal dissociation, $k_{\text{off}i}$ ($i = 1, 2$), were estimated according to the sequential metal-binding scheme [III] from equilibrium and stopped-flow experiments, respectively. The rate constants of metal association, $k_{\text{on}i}$ ($i = 1, 2$), were calculated from the estimates of K_i and $k_{\text{off}i}$. Protein concentration was 4–16 μM . Buffer conditions: 10 mM HEPES–KOH, pH 8.2 (Ca^{2+} -binding/dissociation experiments) or pH 7.3 (Mg^{2+} -binding/dissociation). The Mg^{2+} titration experiments were performed in the presence of 1 mM EGTA. In kinetic experiments Ca^{2+} to protein molar ratio was of 2:1, or 50–60 μM MgCl_2 was added to the metal-depleted protein; the competitive metal removal was initiated by addition of 0.5–1 mM EGTA/EDTA. Excitation wavelength was 258–259 nm.

Protein form	K_1 (M^{-1})	K_2 (M^{-1})	$k_{\text{on}1}$ ($\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{on}2}$ ($\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{off}1}$ (s^{-1})	$k_{\text{off}2}$ (s^{-1})
Ca^{2+}						
Intact	7×10^8	4×10^9	$(3.3 \pm 0.5) \times 10^8$	$(3.5 \pm 0.4) \times 10^8$	0.49 ± 0.08	0.099 ± 0.012
rWT	4×10^7	2×10^6	$(3.2 \pm 0.4) \times 10^7$	$(6.1 \pm 1.0) \times 10^6$	0.80 ± 0.11	3.8 ± 0.6
Mg^{2+}						
Intact	4×10^5	1.3×10^6	$(3.5 \pm 0.9) \times 10^7$	$(1.3 \pm 0.3) \times 10^7$	80 ± 20	10 ± 2
rWT	8×10^3	8×10^3	$>1.2 \times 10^6$	–	>160	–

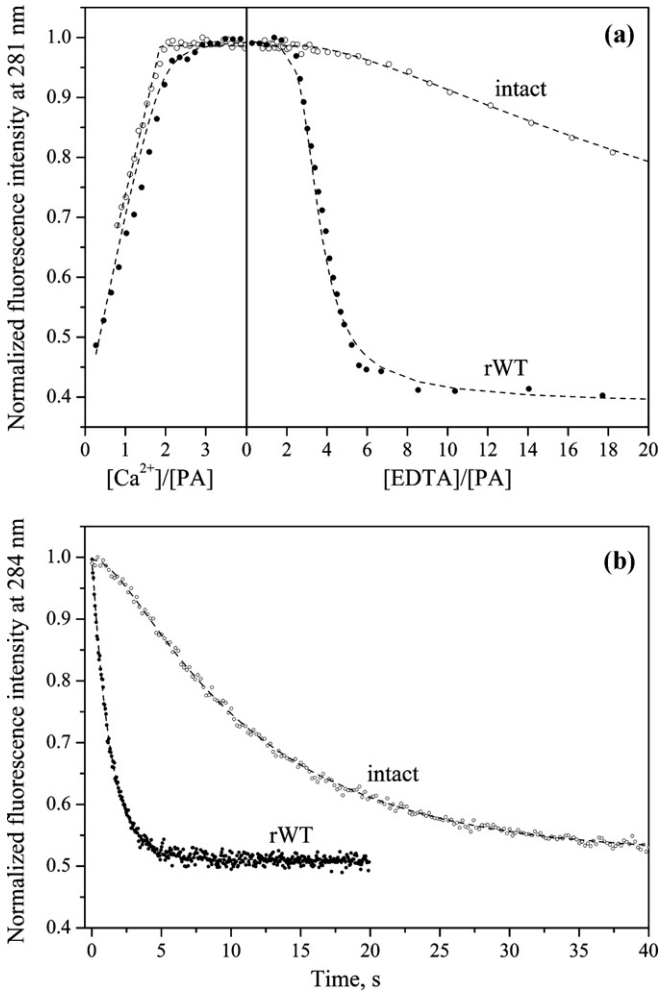


Fig. 3. Fluorimetric Ca^{2+} - and EDTA-titrations of intact and rWT forms of pike α -PA (a) and time courses of EGTA-induced Ca^{2+} removal monitored by fluorescence stopped-flow technique (b) at 25 °C. Buffer conditions: 10 mM HEPES–KOH, pH 8.2. Protein concentration was 4–16 μM . Apo-PA was prepared using combination of the TCA protein precipitation method [75] and the Sephadex G-25 gel-filtration method [76]. Ca^{2+} to protein molar ratio in kinetic experiments was 2:1; competitive Ca^{2+} removal was initiated by addition of 0.5–1 mM EGTA. Excitation wavelength was 258–259 nm. Points are experimental, dashed curves are theoretical fits computed according to the sequential metal-binding scheme [III].

sequential metal-binding scheme are reported here for the first time.

It is noteworthy that Nt-acetylation of pike α -PA induces characteristic changes in its K_i ($i = 1, 2$) values. For Ca^{2+} association with the intact protein $K_1 < K_2$, but for rWT protein $K_2 < K_1$ (Table 3). The similar regularity is valid for Mg^{2+} binding as well. Furthermore, the same tendency is observed for the Ca^{2+} dissociation rate constants

(Table 4). These facts indicate that Nt-acetylation is accompanied with reversal of the order of filling of the protein's EF-hands with $\text{Ca}^{2+}/\text{Mg}^{2+}$.

Summing up, in contrast to rat α -PA, Nt-acetylation of pike α -PA significantly modifies both equilibrium and kinetic properties of Ca^{2+} and Mg^{2+} dissociation/association, which should affect functioning of the protein as a soluble relaxing factor of skeletal muscles. The ability of N-terminal acetyl group to alter metal-binding properties of PA is in line with previous reports that AB domains of pike α -PA and rat PA modulate their Ca^{2+} -binding properties [92,93].

3.6. The structural rationale for the effects caused by Nt-acetylation

Previous study has shown that AB domain of intact pike α -PA is electrostatically inactive [81], which suggests that the charge decrease accompanying Nt-acetylation is unlikely to affect stability of the protein. Meanwhile, since pK_a values of α -amino group in proteins mostly lie in the range from 7.2 to 8.2 [94], the α -amino group of rWT pike α -PA is likely deprotonated at pH values used in our DSC experiments (9.0–9.2). The structural stabilization induced by Nt-acetylation of pike α -PA can be attributed to a change in hydrophobicity of its N-terminus. Alternatively, the N-terminal acetyl group could affect oligomeric state of the protein, giving rise to pike α -PA oligomers with drastically different physico-chemical properties. This possibility has been examined by chemical crosslinking of pike α -PA samples (1 mg/ml) by 0.02% glutaric aldehyde (Supplement 2). Though results of these experiments are dependent on protein concentration, reaction time, temperature and other factors, they enable us to reveal differences in behavior of various PAs and their states. In contrast to the intact protein, apo and Mg^{2+} -bound states of the rWT protein exhibit notable fraction (67–72%) of high molecular weight oligomers and/or aggregates, which are unable to penetrate the 5% concentrating or 15% resolving gels. These protein forms are not detected for Ca^{2+} -bound state of pike α -PA. They are also absent for rat α -PA (Supplement 3). Furthermore, centrifugation of rWT pike α -PA (1 mg/ml) at $20,000 \times g$ for 15 min shows precipitation of 1–4% of apo (1 mM EGTA, pH 9.0) and Mg^{2+} -bound (1 mM EGTA, 2 mM MgCl_2) protein states. The oligomerization/aggregation of Mg^{2+} -loaded rWT pike α -PA accounts for the high cooperativity of its thermal denaturation ($n = 5.6$, Table 1). The association processes of the protein are likely related to its lowered structural stability (Tables 1 and 2), accompanied by solvent exposure of protein's hydrophobic groups. Thus, Nt-acetylation of pike α -PA protects the protein from oligomerization and aggregation by means of a structural stabilization of the protein. In the case of Ca^{2+} -bound pike α -PA, exhibiting the same distribution of oligomeric forms regardless of the presence of the N-terminal acetyl group (Supplement 2), the structural effects induced by Nt-acetylation seem to be due to either direct structural stabilization of the protein or caused by changes in its folding.

Notably, Ca^{2+} -loaded rWT rat α -PA demonstrates noticeable conversion of the monomeric state into a set of oligomeric forms (Supplement 3). Nevertheless, its physico-chemical properties are virtually indistinguishable from those for the intact protein. This circumstance is especially intriguing considering that about 40% of the protein sample corresponds to an unknown protein form (Supplement 1). If Nt-acetylated form of rat α -PA would differ from the intact protein by its properties, these changes would be detected by the biophysical and biochemical techniques applied in the present work. Hence, the experimental data evidence negligible structural effect of Nt-acetylation upon rat α -PA.

The higher with respect to the rat protein structural sensitivity of pike α -PA to Nt-acetylation can be rationalized by higher disorder level of its AB domain (residues 1–37), as manifested by its

higher average VSL2P score (0.73 versus 0.57, for pike and rat α -PAs, respectively), and confirmed by CH-plot analysis. The same impact on more disordered structure is likely to induce more prominent structural consequences, so pike α -PA is expected to be more vulnerable to any structural interventions, including Nt-acetylation.

4. Conclusions

The detailed characterization of apo- and metal-bound states of various forms of orthologous α -parvalbumins by means of combination of thermophysical, spectral and biochemical techniques shows wide range of differences in physico-chemical properties of intact and rWT forms of the protein. While these forms of rat α -PA practically do not differ from each other, intact and rWT pike α -PAs demonstrate surprisingly noticeable differences in their structural and functional properties. The rWT pike protein is significantly destabilized, as evidenced by lowered α -helicity (Table 2), thermal stability (Fig. 1, Table 1), and reduced resistance to enzymatic proteolysis (Fig. 2a). The lowered structural stability of rWT pike α -PA well correlates with its increased propensity to formation of high molecular weight oligomeric forms (Supplement 2). Finally, metal-binding ability of the rWT protein, which is related to functioning of PA as a metal-buffer in relaxation of skeletal muscles, is dramatically lowered, by ca 1–4 and 2–3 orders of magnitude, for Ca^{2+} and Mg^{2+} , respectively (Table 3, Fig. 3a). The respective rate constants of metal dissociation/association are accordingly increased/decreased (Table 4, Fig. 3b). For example, the rate constants of Ca^{2+} association of the rWT pike protein are 10–57-fold lower than the same values for the intact protein. Since mass spectrometry data evidence that intact and rWT forms of the α -PAs studied differ just by N-terminal acetyl group (Supplement 1), the observed differences in their physico-chemical properties are attributed to this group. Since chemical refolding of intact and rWT pike α -PAs did not change their thermal denaturation behavior, it can be concluded that peculiarities of protein folding in eukaryotic and prokaryotic organisms cannot explain the differences in their properties. Nevertheless, it is unclear whether these differences arise due to direct structural perturbation of the correctly folded protein by N-terminal acetyl group or they are mostly due to alterations in the process of protein folding caused by this group. In either case the N-terminal acetyl group is shown to be a structural and functional prerequisite for some of parvalbumins.

The fact that minor structural modifications of N-terminus are able to induce major changes in protein's physico-chemical properties has been previously documented for another Ca^{2+} -binding protein, α -lactalbumin [95]. An additional initiating Met residue of recombinant bovine α -lactalbumin seriously impaired both thermal stability and Ca^{2+} affinity of the protein. On the contrary, genetic removal of Glu1 residue markedly improved the same properties of the protein. The experimental data evidence that N-terminal region of a protein can greatly affect its structural stability. This conclusion is in line with the observation that dynamic properties of N-terminus are important for conformational stability of proteins [96].

The stabilizing action of N-terminal acetyl group documented for pike α -PA is not observed for rat α -PA (sequence identity of 60%), which could be related to lower disorder propensity of its AB domain, which makes it more resistant to structural perturbations. Since other EF-hand proteins were predicted to be balancing between ordered and disordered states (for example, S100 proteins [90]), Nt-acetylation is likely to affect their structural stability. Comparing properties of different forms of rat and pike α -PAs, it can be concluded that evolution of PA's genes leads to the protein sequences possessing highly divergent properties, but Nt-acetylation unifies their properties thereby ensuring their proper functional status.

The increased resistance to proteolytic digestion conferred to pike α -PA by Nt-acetylation evidences that this modification may be a factor contributing to allergenicity of parvalbumins, since the resistance to degradation by human digestive system is considered to be important for its allergenicity. The example of pike α -PA points out the possibility that structural stabilization induced by Nt-acetylation may give rise to increased resistance of Nt-acetylated proteins toward action of proteases, different from aminopeptidases. Furthermore, since proteins of higher organisms are more prone to disordering compared to their counterparts in lower organisms (for review, see [97]), the structural stabilization of proteins from higher organisms induced by this common covalent modification could serve as a mechanism of partial compensation of their higher disorder level. Although our data indicate that the stabilizing effect of Nt-acetylation observed for pike α -PA is not of electrostatic nature, the structural origins of this phenomenon remain to be established.

Acknowledgements

We are indebted to Prof. Michael T. Henzl (University of Missouri, USA) for providing the plasmid encoding rat α -PA. This work was supported by a grant from the Program of the Russian Academy of Sciences “Molecular and Cellular Biology” (P.E.A.), and a grant RFBR 11-04-00860-a from the Russian Foundation for Basic Research (S.E.P.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ceca.2012.06.002>.

References

- [1] K. Narita, Isolation of acetylpeptide from enzymic digests of TMV-protein, *Biochimica et Biophysica Acta* 28 (1958) 184–191.
- [2] H. Jornvall, Acetylation of protein N-terminal amino groups structural observations on alpha-amino acetylated proteins, *Journal of Theoretical Biology* 55 (1975) 1–12.
- [3] B. Persson, C. Flinta, G. von Heijne, H. Jornvall, Structures of N-terminally acetylated proteins, *European Journal of Biochemistry* 152 (1985) 523–527.
- [4] K.K. Starheim, D. Gromyko, R. Velde, J.E. Varhaug, T. Arnesen, Composition and biological significance of the human Nalpha-terminal acetyltransferases, *BMC Proceedings* 3 (Suppl. 6) (2009) S3.
- [5] J. Soppa, Protein acetylation in archaea, bacteria, and eukaryotes, *Archaea* 2010 (2010), <http://dx.doi.org/10.1155/2010/820681>.
- [6] T. Arnesen, Towards a functional understanding of protein N-terminal acetylation, *PLoS Biology* 9 (2011) e1001074.
- [7] T. Arnesen, P. Van Damme, B. Polevoda, K. Helsens, R. Evjenth, N. Colaert, J.E. Varhaug, J. Vandekerckhove, J.R. Lillehaug, F. Sherman, K. Gevaert, Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans, *Proceedings of the National Academy of Sciences of the United States of America* 106 (2009) 8157–8162.
- [8] K.K. Starheim, K. Gevaert, T. Arnesen, Protein N-terminal acetyltransferases: when the start matters, *Trends in Biochemical Sciences* 37 (2012) 152–161.
- [9] P. Van Damme, T. Arnesen, K. Gevaert, Protein alpha-N-acetylation studied by N-terminomics, *FEBS Journal* 278 (2011) 3822–3834.
- [10] J.D. Jones, C.D. O'Connor, Protein acetylation in prokaryotes, *Proteomics* 11 (2011) 3012–3022.
- [11] B. Polevoda, F. Sherman, N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins, *Journal of Molecular Biology* 325 (2003) 595–622.
- [12] J.P. Waller, The N₂-terminal residues of the proteins from cell-free extracts of *E. coli*, *Journal of Molecular Biology* 7 (1963) 483–496.
- [13] S. Goetze, E. Qeli, C. Mosimann, A. Staes, B. Gerrits, B. Roschitzki, S. Mohanty, E.M. Niederer, E. Laczko, E. Timmerman, V. Lange, E. Hafen, R. Aebersold, J. Vandekerckhove, K. Basler, C.H. Ahrens, K. Gevaert, E. Brunner, Identification and functional characterization of N-terminally acetylated proteins in *Drosophila melanogaster*, *PLoS Biology* 7 (2009) e1000236.
- [14] B. Polevoda, F. Sherman, Composition and function of the eukaryotic N-terminal acetyltransferase subunits, *Biochemical and Biophysical Research Communications* 308 (2003) 1–11.
- [15] W.V. Bienvenut, D. Sumpton, A. Martinez, S. Lilla, C. Espagne, T. Meinel, C. Giglione, Comparative large-scale characterisation of plant vs. mammal proteins reveals similar and idiosyncratic N-alpha-acetylation features, *Molecular and Cellular Proteomics* 11 (2012), M111.015131, <http://dx.doi.org/10.1074/mcp.M111.015131>.
- [16] K.H. Chun, S.J. Cho, J.S. Choi, S.H. Kim, K.W. Kim, S.K. Lee, Differential regulation of splicing, localization and stability of mammalian ARD1235 and ARD1225 isoforms, *Biochemical and Biophysical Research Communications* 353 (2007) 18–25.
- [17] J.H. Seo, J.H. Cha, J.H. Park, C.H. Jeong, Z.Y. Park, H.S. Lee, S.H. Oh, J.H. Kang, S.W. Suh, K.H. Kim, J.Y. Ha, S.H. Han, S.H. Kim, J.W. Lee, J.A. Park, J.W. Jeong, K.J. Lee, G.T. Oh, M.N. Lee, S.W. Kwon, S.K. Lee, K.H. Chun, S.J. Lee, K.W. Kim, Arrest defective 1 autoacetylation is a critical step in its ability to stimulate cancer cell proliferation, *Cancer Research* 70 (2010) 4422–4432.
- [18] J.H. Lim, J.W. Park, Y.S. Chun, Human arrest defective 1 acetylates and activates beta-catenin, promoting lung cancer cell proliferation, *Cancer Research* 66 (2006) 10677–10682.
- [19] D.H. Shin, Y.S. Chun, K.H. Lee, H.W. Shin, J.W. Park, Arrest defective-1 controls tumor cell behavior by acetylating myosin light chain kinase, *PLoS One* 4 (2009) e7451.
- [20] A.F. Rope, K. Wang, R. Evjenth, J. Xing, J.J. Johnston, J.J. Swensen, W.E. Johnson, B. Moore, C.D. Huff, L.M. Bird, J.C. Carey, J.M. Opitz, C.A. Stevens, T. Jiang, C. Schank, H.D. Fain, R. Robinson, B. Dalley, S. Chin, S.T. South, T.J. Pysher, L.B. Jorde, H. Hakonarson, J.R. Lillehaug, L.G. Biesecker, M. Vandell, T. Arnesen, G.J. Lyon, Using VAAST to identify an X-linked disorder resulting in lethality in male, *American Journal of Human Genetics* 89 (2011) 28–43.
- [21] T. Arnesen, D. Gromyko, F. Pendino, A. Rynningen, J.E. Varhaug, J.R. Lillehaug, Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-alpha-acetyltransferase complex, *Oncogene* 25 (2006) 4350–4360.
- [22] K.K. Starheim, T. Arnesen, D. Gromyko, A. Rynningen, J.E. Varhaug, J.R. Lillehaug, Identification of the human N(alpha)-acetyltransferase complex B (hNatB): a complex important for cell-cycle progression, *Biochemical Journal* 415 (2008) 325–331.
- [23] K.K. Starheim, D. Gromyko, R. Evjenth, A. Rynningen, J.E. Varhaug, J.R. Lillehaug, T. Arnesen, Knockdown of human N alpha-terminal acetyltransferase complex C leads to p53-dependent apoptosis and aberrant human Arl8b localization, *Molecular and Cellular Biology* 29 (2009) 3569–3581.
- [24] A. Ciechanover, R. Ben-Saadon, N-terminal ubiquitination: more protein substrates join in, *Trends in Cell Biology* 14 (2004) 103–106.
- [25] A. Hershko, H. Heller, E. Eytan, G. Kaklij, I.A. Rose, Role of the alpha-amino group of protein in ubiquitin-mediated protein breakdown, *Proceedings of the National Academy of Sciences of the United States of America* 81 (1984) 7021–7025.
- [26] D.S. Jayawardene, C. Dass, The effect of N-terminal acetylation and the inhibition activity of acetylated enkephalins on the aminopeptidase M-catalyzed hydrolysis of enkephalins, *Peptides* 20 (1999) 963–970.
- [27] R.J. Wallace, Acetylation of peptides inhibits their degradation by rumen microorganisms, *British Journal of Nutrition* 68 (1992) 365–372.
- [28] H.P. Driessen, F.C. Ramaekers, W.T. Vree Egberts, H.J. Dodemont, W.W. de Jong, G.I. Tesser, H. Bloemendal, The function of N alpha-acetylation of the eye-lens crystallins, *European Journal of Biochemistry* 136 (1983) 403–406.
- [29] H. John, E. Maronde, W.G. Forssmann, M. Meyer, K. Adermann, N-terminal acetylation protects glucagon-like peptide GLP-1-(7–34)-amide from DPP-IV-mediated degradation retaining cAMP- and insulin-releasing capacity, *European Journal of Medical Research* 13 (2008) 73–78.
- [30] H. Paradis, Y. Langelier, J. Michaud, P. Brazeau, P. Gaudreau, Studies on in vitro proteolytic sensitivity of peptides inhibiting herpes simplex virus ribonucleotide reductases lead to discovery of a stable and potent inhibitor, *International Journal of Peptide and Protein Research* 37 (1991) 72–79.
- [31] E.M. Berger, G. Cox, L. Weber, J.S. Kenney, Actin acetylation in *Drosophila* tissue culture cells, *Biochemical Genetics* 19 (1981) 321–331.
- [32] M.M. Pena, S.P. Melo, Y.Y. Xing, K. White, K.W. Barbour, F.G. Berger, The intrinsically disordered N-terminal domain of thymidylate synthase targets the enzyme to the ubiquitin-independent proteasomal degradation pathway, *Journal of Biological Chemistry* 284 (2009) 31597–31607.
- [33] C.S. Hwang, A. Shemorry, A. Varshavsky, N-terminal acetylation of cellular proteins creates specific degradation signals, *Science* 327 (2010) 973–977.
- [34] G.M. Forte, M.R. Pool, C.J. Stirling, N-terminal acetylation inhibits protein targeting to the endoplasmic reticulum, *PLoS Biology* 9 (2011) e1001073.
- [35] R. Behnia, B. Panic, J.R. Whyte, S. Munro, Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p, *Nature Cell Biology* 6 (2004) 405–413.
- [36] S.R. Setty, T.I. Strohlic, A.H. Tong, C. Boone, C.G. Burd, Golgi targeting of ARF-like GTPase Arl3p requires its Nalpha-acetylation and the integral membrane protein Sys1p, *Nature Cell Biology* 6 (2004) 414–419.
- [37] D.C. Scott, J.K. Monda, E.J. Bennett, J.W. Harper, B.A. Schulman, N-terminal acetylation acts as an avidity enhancer within an interconnected multiprotein complex, *Science* 334 (2011) 674–678.
- [38] R. Caesar, A. Blomberg, The stress-induced Tfs1p requires NatB-mediated acetylation to inhibit carboxypeptidase Y and to regulate the protein kinase A pathway, *Journal of Biological Chemistry* 279 (2004) 38532–38543.
- [39] J. Mima, T. Kondo, R. Hayashi, N-terminal acetyl group is essential for the inhibitory function of carboxypeptidase Y inhibitor (I(C)), *FEBS Letters* 532 (2002) 207–210.
- [40] S.E. Hitchcock-DeGregori, R.W. Heald, Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in *Escherichia coli*, *Journal of Biological Chemistry* 262 (1987) 9730–9735.

- [41] A.T. Coulton, D.A. East, A. Galinska-Rakoczy, W. Lehman, D.P. Mulvihill, The recruitment of acetylated and unacetylated tropomyosin to distinct actin polymers permits the discrete regulation of specific myosins in fission yeast, *Journal of Cell Science* 123 (2010) 3235–3243.
- [42] T. van Welsem, F. Frederiks, K.F. Verzijlbergen, A.W. Faber, Z.W. Nelson, D.A. Egan, D.E. Gottschling, F. van Leeuwen, Synthetic lethal screens identify gene silencing processes in yeast and implicate the acetylated amino terminus of Sir3 in recognition of the nucleosome core, *Molecular and Cellular Biology* 28 (2008) 3861–3872.
- [43] R. Behnia, F.A. Barr, J.J. Flanagan, C. Barlowe, S. Munro, The yeast orthologue of GRASP65 forms a complex with a coiled-coil protein that contributes to ER to Golgi traffic, *Journal of Cell Biology* 176 (2007) 255–261.
- [44] A. Murthi, A.K. Hopper, Genome-wide screen for inner nuclear membrane protein targeting in *Saccharomyces cerevisiae*: roles for N-acetylation and an integral membrane protein, *Genetics* 170 (2005) 1553–1560.
- [45] M.T. Ryan, D.J. Naylor, N.J. Hoogenraad, P.B. Hoj, Affinity purification, overexpression, and characterization of chaperonin 10 homologues synthesized with and without N-terminal acetylation, *Journal of Biological Chemistry* 270 (1995) 22037–22043.
- [46] H.M. Kang, G. Kassam, S.E. Jarvis, S.L. Fitzpatrick, D.M. Waisman, Characterization of human recombinant annexin II tetramer purified from bacteria: role of N-terminal acetylation, *Biochemistry* 36 (1997) 2041–2050.
- [47] J.O. Hoog, M. Weis, M. Zeppezauer, H. Jorvall, H. von Bahr-Lindstrom, Expression in *Escherichia coli* of active human alcohol dehydrogenase lacking N-terminal acetylation, *Bioscience Reports* 7 (1987) 969–974.
- [48] D.E. Clapham, Calcium signaling, *Cell* 131 (2007) 1047–1058.
- [49] E. Carafoli, Calcium signaling: a tale for all seasons, *Proceedings of the National Academy of Sciences of the United States of America* 99 (2002) 1115–1122.
- [50] E. Carafoli, Calcium—a universal carrier of biological signals. Delivered on 3 July 2003 at the special FEBS meeting in Brussels, *FEBS Journal* 272 (2005) 1073–1089.
- [51] E.A.K.R.H. Permyakov, Calcium Binding Proteins, John Wiley & Sons, Inc., Hoboken, New Jersey, 2010.
- [52] A.J. Laude, A.W. Simpson, Compartmentalized signalling: Ca²⁺ compartments, microdomains and the many facets of Ca²⁺ signalling, *FEBS Journal* 276 (2009) 1800–1816.
- [53] E.A. Permyakov, Parvalbumin, Nova Science Publishers, Inc., New York, 2006.
- [54] T.L. Pauls, J.A. Cox, M.W. Berchtold, The Ca²⁺(-)-binding proteins parvalbumin and oncomodulin and their genes: new structural and functional findings, *Biochimica et Biophysica Acta* 1306 (1996) 39–54.
- [55] M.W. Berchtold, H. Brinkmeier, M. Muntener, Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease, *Physiological Reviews* 80 (2000) 1215–1265.
- [56] C.W. Heizmann, M.W. Berchtold, A.M. Rowleron, Correlation of parvalbumin concentration with relaxation speed in mammalian muscles, *Proceedings of the National Academy of Sciences of the United States of America* 79 (1982) 7243–7247.
- [57] M. Muntener, L. Kaser, J. Weber, M.W. Berchtold, Increase of skeletal muscle relaxation speed by direct injection of parvalbumin cDNA, *Proceedings of the National Academy of Sciences of the United States of America* 92 (1995) 6504–6508.
- [58] B. Schwaller, J. Dick, G. Dhoot, S. Carroll, G. Vrbova, P. Nicotera, D. Pette, A. Wyss, H. Bluethmann, W. Hunziker, M.R. Celio, Prolonged contraction–relaxation cycle of fast-twitch muscles in parvalbumin knockout mice, *American Journal of Physiology* 276 (1999) C395–C403.
- [59] B. Schwaller, The use of transgenic mouse models to reveal the functions of Ca²⁺ buffer proteins in excitable cells, *Biochimica et Biophysica Acta* 1820 (2012) 1294–1303.
- [60] A. Bugajska-Schretter, L. Elfman, T. Fuchs, S. Kapiotis, H. Rumpold, R. Valenta, S. Spitzauer, Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca²⁺ depletion, *Journal of Allergy and Clinical Immunology* 101 (1998) 67–74.
- [61] T. Van Do, S. Elsayed, E. Florvaag, I. Hordvik, C. Endresen, Allergy to fish parvalbumins: studies on the cross-reactivity of allergens from 9 commonly consumed fish, *Journal of Allergy and Clinical Immunology* 116 (2005) 1314–1320.
- [62] S. Elsayed, K. Aas, Characterization of a major allergen (cod). Observations on effect of denaturation on the allergenic activity, *Journal of Allergy* 47 (1971) 283–291.
- [63] A. Bugajska-Schretter, M. Grote, L. Vangelista, P. Valent, W.R. Sperr, H. Rumpold, A. Pastore, R. Reichelt, R. Valenta, S. Spitzauer, Purification, biochemical, and immunological characterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin, *Gut* 46 (2000) 661–669.
- [64] S.L. Taylor, J.L. Kabourek, S.L. Hefle, Fish allergy: fish and products thereof, *Journal of Food Science* 69 (2004) 175–180.
- [65] J.A. Jenkins, H. Breiteneder, E.N. Mills, Evolutionary distance from human homologs reflects allergenicity of animal food proteins, *Journal of Allergy and Clinical Immunology* 120 (2007) 1399–1405.
- [66] C.E. Nockolds, R.H. Kretsinger, C.J. Coffee, R.A. Bradshaw, Structure of a calcium-binding carp myogen, *Proceedings of the National Academy of Sciences of the United States of America* 69 (1972) 581–584.
- [67] C. Gerday, The primary structure of the parvalbumin II of pike (*Esox lucius*), *European Journal of Biochemistry* 70 (1976) 305–318.
- [68] J.P. MacManus, D.C. Watson, M. Yaguchi, The complete amino acid sequence of oncomodulin—a parvalbumin-like calcium-binding protein from Morris hepatoma 5123tc, *European Journal of Biochemistry* 136 (1983) 9–17.
- [69] S.E. Permyakov, A.S. Karnoup, A.G. Bakunts, E.A. Permyakov, Sequence microheterogeneity of parvalbumin pl 5.0 of pike: a mass spectrometric study, *Biochimica et Biophysica Acta* 1794 (2009) 129–136.
- [70] Y. Ma, U. Griesmeier, M. Susani, C. Radauer, P. Briza, A. Erler, M. Bublin, S. Alessandri, M. Himly, S. Vazquez-Cortes, I.R. de Arellano, E. Vassilopoulou, P. Saxoni-Papageorgiou, A.C. Knulst, M. Fernandez-Rivas, K. Hoffmann-Sommergruber, H. Breiteneder, Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp, *Molecular Nutrition & Food Research* 52 (Suppl. 2) (2008) S196–S207.
- [71] K.S. Rao, C. Gerday, Low molecular weight proteins of pike (*Esox lucius*) white muscles. I. Extraction and purification-polymorphism, *Comparative Biochemistry and Physiology. Part B: Comparative Biochemistry* 44 (1973) 931–937.
- [72] J. Haiech, J. Derancourt, J.F. Peche, J.G. Demaille, A new large-scale purification procedure for muscular parvalbumins, *Biochimie* 61 (1979) 583–587.
- [73] K.S. Rao, C. Gerday, Low molecular weight proteins of pike (*Esox lucius*) white muscles. II. Chemical and physical properties, *Comparative Biochemistry and Physiology. Part B: Comparative Biochemistry* 44 (1973) 1113–1125.
- [74] M.T. Henzl, J.D. Larson, S. Agah, Influence of monovalent cations on rat alpha- and beta-parvalbumin stabilities, *Biochemistry* 39 (2000) 5859–5867.
- [75] M. Yazawa, M. Sakuma, K. Yagi, Calmodulins from muscles of marine invertebrates, scallop and sea anemone, *Journal of Biochemistry* 87 (1980) 1313–1320.
- [76] H.E. Blum, P. Lehky, L. Kohler, E.A. Stein, E.H. Fischer, Comparative properties of vertebrate parvalbumins, *Journal of Biological Chemistry* 252 (1977) 2834–2838.
- [77] P.L. Privalov, S.A. Potekhin, Scanning microcalorimetry in studying temperature-induced changes in proteins, *Methods in Enzymology* 131 (1986) 4–51.
- [78] M. Häckel, H.J. Hinz, G.R. Hedwig, Partial molar volumes of proteins: amino acid side-chain contributions derived from the partial molar volumes of some tripeptides over the temperature range 10–90 degrees C, *Biophysical Chemistry* 82 (1999) 35–50.
- [79] G.I. Makhatazde, P.L. Privalov, Heat capacity of proteins. I. Partial molar heat capacity of individual amino acid residues in aqueous solution: hydration effect, *Journal of Molecular Biology* 213 (1990) 375–384.
- [80] S.E. Permyakov, A.G. Bakunts, M.E. Permyakova, A.I. Denesyuk, V.N. Uversky, E.A. Permyakov, Metal-controlled interdomain cooperativity in parvalbumins, *Cell Calcium* 46 (2009) 163–175.
- [81] S.E. Permyakov, A.G. Bakunts, A.I. Denesyuk, E.L. Knyazeva, V.N. Uversky, E.A. Permyakov, Apo-parvalbumin as an intrinsically disordered protein, *Proteins* 72 (2008) 822–836.
- [82] N. Sreerama, S.Y. Venyaminov, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis, *Analytical Biochemistry* 287 (2000) 243–251.
- [83] E.A. Permyakov, V.N. Medvedkin, L.P. Kalinichenko, E.A. Burstein, Comparative study of physicochemical properties of two pike parvalbumins by means of their intrinsic tyrosyl and phenylalanine fluorescence, *Archives of Biochemistry and Biophysics* 227 (1983) 9–20.
- [84] E.A. Permyakov, Luminescent Spectroscopy of Proteins, CRC Press, Inc., Boca Raton, Ann Arbor, London, Tokyo, 1993.
- [85] G. Schwarzenbach, H. Flaschka, Die Komplexometrische Titration, Ferdinand Enke Verlag, Stuttgart, 1965.
- [86] D.W. Marquardt, An algorithm for least-squares estimation of nonlinear parameters, *Journal of the Society for Industrial and Applied Mathematics* 11 (1963) 431–441.
- [87] G.E. Forsythe, M.A. Malcolm, C.B. Moler, Computer Methods for Mathematical Computations, Prentice-Hall, 1977.
- [88] P.D. Smith, G.W. Liesegang, R.L. Berger, G.P.R.J. Czerlinski, A stopped-flow investigation of calcium ion binding by ethylene glycol bis(b-aminoethyl ether)-N,N-tetraacetic acid, *Analytical Biochemistry* 143 (1984) 188–195.
- [89] E.S. Hyman, A study of the rate of chelation of magnesium by CDTA and EDTA in the ATP (Na²⁺ K⁺)-ATPase system, *Biochimica et Biophysica Acta* 600 (1980) 553–570.
- [90] S.E. Permyakov, R.G. Ismailov, B. Xue, A.I. Denesyuk, V.N. Uversky, E.A. Permyakov, Intrinsic disorder in S100 proteins, *Molecular BioSystems* 7 (2011) 2164–2180.
- [91] W. Kabsch, C. Sander, Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features, *Biopolymers* 22 (12) (1983) 2577–2637.
- [92] E.A. Permyakov, V.N. Medvedkin, Y.V. Mitin, R.H. Kretsinger, Noncovalent complex between domain AB and domains CD*EF of parvalbumin, *Biochimica et Biophysica Acta* 1076 (1991) 67–70.
- [93] M.T. Henzl, S. Agah, J.D. Larson, Association of the AB and CD-EF domains from rat alpha- and beta-parvalbumin, *Biochemistry* 43 (2004) 10906–10917.
- [94] G.R. Grimley, J.M. Scholtz, C.N. Pace, A summary of the measured pK values of the ionizable groups in folded proteins, *Protein Science* 18 (2009) 247–251.
- [95] D.B. Veprintsev, M. Narayan, S.E. Permyakov, V.N. Uversky, C.L. Brooks, A.M. Cherskaya, E.A. Permyakov, L.J. Berliner, Fine tuning the N-terminus of a calcium binding protein: alpha-lactalbumin, *Proteins* 37 (1999) 65–72.
- [96] N.N. Khechinashvili, M.V. Fedorov, A.V. Kabanov, S. Monti, C. Ghio, K. Soda, Side chain dynamics and alternative hydrogen bonding in the mechanism of protein thermostabilization, *Journal of Biomolecular Structure and Dynamics* 24 (2006) 255–262.
- [97] V.N. Uversky, A.K. Dunker, Understanding protein non-folding, *Biochimica et Biophysica Acta* 1804 (2010) 1231–1264.

SUPPLEMENTARY MATERIALS

Supplement 1. The molecular masses of intact and recombinant PA samples estimated by ESI-MS, and their assignments.

<i>Protein</i>	<i>Protein form</i>	<i>Molecular mass, Da</i>	<i>Content, %</i>	<i>Mass assignment</i>	<i>Calculated average mass, Da</i>
pike α -PA	intact	11821	>95	Nt-acetylated α_1 -isoform, described earlier [69]	11820.43
	rWT	11778	>95	α_1 -isoform, described earlier [69]	11778.39
rat α -PA	intact	11836	>95	acetylated P02625 entry of Swiss-Prot database, lacking initiating Met residue	11836.38
	rWT	11794	50	P02625 entry of Swiss-Prot database, lacking initiating Met residue	11794.35
		12307	40	unknown PA form	-

Supplement 2. Distributions of oligomeric forms for various states of pike α -PA as judged from chemical crosslinking experiments (0.02% glutaric aldehyde). Protein concentration was 1 mg/ml. Buffer conditions: 30 mM H₃BO₃, pH 9.2. PA sample was either metal-depleted or 1 mM CaCl₂/MgCl₂ was added. The fractions of oligomeric PA forms were estimated from SDS-PAGE data. The gels were stained with Coomassie Brilliant Blue R-250, scanned using Molecular Imager PhorosFX Plus System (Bio-Rad Laboratories, Inc.) and analyzed by Quantity One software.

<i>Pike α-PA</i>	<i>Protein state</i>	<i>12 kDa band, %</i>	<i>24 kDa band, %</i>	<i>48 kDa band, %</i>	<i>66 kDa band, %</i>	<i>High molecular weight fraction[*], %</i>
intact	apo	53	38	9	-	-
	Mg ²⁺	80	20	-	-	-
	Ca ²⁺	39	33	18	10	-
rWT	apo	23	5	-	-	72
	Mg ²⁺	25	8	-	-	67
	Ca ²⁺	35	31	20	14	-

^{*}protein oligomers/aggregates not penetrating the gel

Supplement 3. Distributions of oligomeric forms for various states of rat α -PA as judged from chemical crosslinking experiments. For details refer to legend to the Supplement 2.

<i>Rat α-PA</i>	<i>Protein state</i>	<i>12 kDa band, %</i>	<i>24 kDa band, %</i>	<i>48 kDa band, %</i>	<i>66 kDa band, %</i>
intact	apo	70	30	-	-
	Mg ²⁺	53	33	14	-
	Ca ²⁺	55	22	11	12
rWT	apo	82	15	3	-
	Mg ²⁺	72	18	10	-
	Ca ²⁺	25	28	27	20