



Parvalbumin characterization from the euryhaline stingray *Dasyatis sabina*

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ABSTRACT

The Atlantic stingray, *Dasyatis sabina*, found along the Gulf of Mexico and southeastern Atlantic coasts, is a euryhaline species of elasmobranch. This species is able to osmotically compensate for changing environmental salinity by altering plasma and intracellular solutes, including urea and counteracting methylamines (betaine and TMAO). Parvalbumin (PV) is an intracellular protein that facilitates muscle relaxation by sequestering calcium. Determining the effects that *in situ* concentrations of urea (146 mM), betaine (62 mM), and TMAO (11 mM) have on PV function in marine and freshwater adapted populations of *D. sabina* could provide insight into intracellular correlates of euryhaline tolerance for this species. PV from marine and freshwater populations of *D. sabina* was identified and purified by SDS-PAGE, western blot analysis, and full amino acid sequence analysis. Both populations exhibited two PV isoforms, PV I (~12.18 kDa mw) and PV II (11.96 kDa mw). PV dissociation constants (K_D) were determined in the presence and absence of physiological concentrations of urea, betaine, and TMAO by fluorescence spectroscopy using the fluorescent Ca^{2+} indicator fluo-3 which competes with PV for Ca^{2+} . Functional studies revealed PV I showed no significant changes in calcium binding from *in situ* muscle conditions, except in the presence of betaine. In contrast, PV II's ability to bind calcium was increased relative to physiological conditions in the presence of each osmolyte independently. Thus, it appears that organic osmolytes have isoform specific effects on PV function.

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1. Introduction

Muscle contraction and relaxation involves rapid cycling of calcium in muscle by way of the sarcoplasmic reticulum. Intracellular proteins, including members of the EF hand family are vital to this process. Though members of this super family possess diverse cellular functions, they all maintain the characteristic helix–loop–helix binding motif specialized for divalent cation binding, specifically calcium and magnesium (Nakayama and Kretsinger, 1994; Kawasaki et al., 1998). Troponin C, calmodulin, and parvalbumin are a few of the EF hand proteins known to be directly involved in the muscle contraction–relaxation cycle.

Parvalbumin is a soluble intracellular calcium buffer that is found in relatively high concentrations in fast twitch muscle fibers and low concentrations in slow twitch muscle fibers (Rall, 1996). It is a low molecular mass protein ranging from approximately 10.5–12.2 kDa in size. Parvalbumin is thought to function in a manner that accelerates muscle relaxation. During the contraction phase of muscle activity, parvalbumin exchanges bound magnesium for calcium. Calcium is slowly sequestered back into the sarcoplasmic reticulum (SR) during muscle relaxation via the SR Ca^{2+} -ATPase pump allowing magnesium

to rebound to parvalbumin (Rall, 1996). Parvalbumin from many phyla has been well characterized, but the structure and function of this protein from elasmobranchs has not been closely investigated.

The majority of phyla studied have been found to possess more than one isoform of parvalbumin. There are four known isoforms of PV (α , β , β lineage isoform 1, and β lineage isoform 2) falling into two distinct lineages, α and β (Nakayama and Kretsinger, 1994; Pauls et al., 1996). These isoforms are known to possess differences not only in divalent cation binding but also in function. However, not all PV isoforms are believed to be solely involved in muscle relaxation. One isoform of parvalbumin is thought to be involved in protection of non-muscle cells, particularly neurons, from calcium-induced cell damage (Pauls et al., 1996). While most parvalbumins possess two cation binding domains, oncomodulin, the mammalian β lineage of parvalbumin, is known to possess a single calcium specific binding site (Pauls et al., 1993; Pauls et al., 1996). Across the animal kingdom there is diversity in the number of parvalbumin isoforms found in muscle and throughout the body. Teleosts, for instance, can have up to five different isoforms of parvalbumin; while most elasmobranchs, with few exceptions, studied thus far have been found to possess a single isoform (Huriaux et al., 1997; Roquet et al., 1992; Sanscrainte, 2006). Carp and humans are known to possess parvalbumin from both lineages while the thornback ray, *Raja clavata*, has been shown to only possess the β lineage.

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Marine elasmobranchs are osmoconformers that employ a combination of solutes to maintain extracellular and intracellular osmotic strength close to or slightly above that of their marine environment. Intracellular and extracellular osmoregulation in marine elasmobranchs involves high concentrations of urea (350–600 mM) (Ballantyne, 1997). Euryhaline elasmobranchs possess intracellular concentrations of urea between 140–200 mmol/L, and plasma concentrations of approximately 195 mmol/L (Piermarini and Evans, 1998; Treberg et al., 2006). Urea is known to disrupt hydrophobic interactions necessary for proper function and structure of proteins. Due to these destabilizing effects, an *a priori* expectation is that the elevated levels of urea characteristic of elasmobranchs should be deleterious to protein function (Evans, 1993). In elasmobranchs, however, these denaturing effects are counteracted by protein-stabilizing methylamines, including betaine and TMAO, in a net 2:1 ratio with urea (Yancey and Somero, 1979). However, urea in concentrations characteristic of elasmobranch intracellular conditions has also been shown to be essential for some protein and organelle function in elasmobranchs (Ballantyne, 1997). For example, shark heart mitochondria has been shown to have evolved optimal function in the presence of the physiologically high urea content of elasmobranchs (Lewiston et al., 1979).

Dasyatis sabina is a euryhaline stingray, with one population that has diverged to solely inhabit the freshwater lakes and rivers of the St. Johns River System, Florida (Piermarini and Evans, 1998). In freshwater, this species is known to possess a lower concentration of urea, methylamines, and salts in order to maintain a lower plasma osmolality (621 mOsm kg⁻¹) (Piermarini and Evans, 1998; De Vlaming and Sage, 1973). During saltwater acclimation, this species has been found to increase levels of urea, methylamines, and salt in order to increase their plasma osmolality to that characteristic of most marine elasmobranchs (953 mOsm kg⁻¹) (Piermarini and Evans, 1998). The euryhaline stingray is believed to use a common osmoregulatory strategy in freshwater to reduce osmotic pressure of their serum/plasma similar to their marine counterparts (Piermarini and Evans, 1998). *D. sabina* has also been found to possess a higher concentration of the methylamine, betaine, than TMAO to counteract the destabilizing effects of urea as seen in the majority of marine elasmobranchs (Treberg et al., 2006).

Previous research on *D. sabina* has focused mainly on the dynamics of urea, betaine, TMAO and plasma osmolality during the salt water to freshwater transitions. The effects of changing intracellular conditions on protein function brought about by possible changes in these organic osmolytes have not been investigated extensively. This study addressed the intracellular side of the puzzle by investigating how function of an intracellular muscle protein, PV, is effected by *in situ* relevant concentrations of urea (146 mM), TMAO (11 mM) and betaine (62 mM) found in the freshwater population of *D. sabina* (Treberg et al., 2006). While differences in PV characteristics are not expected between freshwater and marine populations of *D. sabina*, this study focused on the inherent molecular and functional differences that may be present between the two PV isoforms.

Determining the effects that urea, betaine, and TMAO have on parvalbumin could provide insight into *D. sabina*'s adaptation to its environment. Results from this investigation might also provide valuable information on the adaptability of muscle protein functionality in the face of environmental variation. PV is known to be a robust protein and therefore is expected to maintain optimal function across osmotic regimes. Maintenance of proper function in the presence of these protein destabilizers and stabilizers could provide insight into how this protein is able to increase muscle relaxation during environmental stress. This investigation has shown PV's ability to bind calcium is increased in the presence of urea.

Additionally, this research investigated the calcium binding properties of parvalbumin from a freshwater teleost, *Cyprinus carpio* (carp), like all freshwater teleosts, are osmoregulators and maintain higher internal ion concentrations than that of the surrounding environment. PV in *C. carpio* has been well characterized in the

literature, making this species an ideal comparison for studying how robust parvalbumin is from another organism, not normally exposed to destabilizing osmolytes: urea, betaine, and TMAO.

2. Materials and methods

2.1. Animal collection

Mature freshwater *D. sabina* (Dasyatidae) ($n=3$) from Lake Monroe of the St. Johns River System were obtained by hook and line. White muscle samples from the pectoral fins used for RNA analysis were immediately flash frozen in liquid nitrogen then transferred to -80°C . The remaining white muscle was transferred on dry ice to -80°C .

Mature marine *D. sabina* ($n=6$) from Apalachicola Bay, Florida were obtained by gill net. White muscle samples obtained were transferred on dry ice to -80°C .

Carp was used in this study as a control during primary experiments. *C. carpio* (Cyprinidae) ($n=5$) from Lake Talquin, Florida were collected by electroshock. White muscle samples were extracted post-mortem and stored at -80°C .

All animal use was in accord with the current NIH/NRC Guide for Care and Use of Laboratory Animals. Procedures and protocols used during this study were approved by Florida State University's Institutional Animal Care and Use Committee (IACUC).

2.2. Purification of parvalbumin

PV from carp and stingray was isolated by protocols from Laney et al. (1997) and Erickson et al. (2005), with the following modifications. The two isoforms found in *D. sabina* white muscle were eluted on a DEAE-cellulose anion exchange column by application of a shallow linear gradient: 0–0.1 M NaCl in 25 mM MES pH 5.7 followed by 0.1–0.2 M NaCl in 25 mM MES pH 5.7 at 4°C . DEAE chromatography was unable to completely separate the two isoforms of PV found in *D. sabina*. Therefore, HPLC was performed on the samples by application to a reverse phase chromatography column. The two isoforms of PV were separated on a Jupiter C4 analytical column (Phenomenex, $250\times 4.6\text{ mm}$) with a mobile phase A=0.1% TFA in water and B=0.075% TFA in acetonitrile. Both PV isoforms were loaded onto the reverse phase chromatography column at 39% B. The samples were run in the presence of 39% B for five minutes followed by a linear gradient of 39–45% B over a period of 60 minutes at a flow rate of 0.7 mL/min. The effluent from the column was monitored continuously at 220 and 280 nm. Fractions containing the pure peaks for PV isoforms I and II were pooled and subsequently concentrated down by vacuum centrifugation. A Beckman System Gold equipped with a model 126 solvent delivery system, a model 168 diode array detector, a Rheodyne 7125 sample injection valve, and a Bio-Rad model 2128 fraction collector were used to perform all HPLC separations. Following purification and identification all samples were stored at -80°C until decalcification studies were performed.

Protein identity and purity were confirmed by one-dimensional SDS-PAGE and western blot analysis, using protocols from Schagger and von Jagow (1987) and Erickson et al. (2005). The primary antibody was a monoclonal mouse anti-frog PV (Sigma), followed by a secondary antibody, goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad). The bands were visualized with Western Blue substrate (Promega). Isoelectric focusing was performed using two-dimensional gel electrophoresis according to Invitrogen protocol. *C. carpio* expressed three PV isoforms; the major isoform (II ~10.5 kDa) was used for further analysis. *D. sabina* expressed two PV isoforms which were both analyzed further.

2.3. Decalcification of parvalbumin and buffer solutions

Divalent cations were removed from buffer solutions by addition of 5% chelex (Bio-Rad) and subsequent stirring at room temperature for 12–18 h. Centrifugation was performed at 3500 g for 15 min to collect chelex

at the bottom to allow de-ionized buffer to be extracted. Molecular grade HCl (Sigma) was used to bring buffers to pH 7.2. To prevent calcium contamination, subsamples were taken from the buffer to test pH. Calcium solutions (0.1, 1.0, and 10.0 μ M) were made by dilution of a 0.1 M calcium standard (Orion) with appropriate decalcified buffer.

PV isoforms from *C. carpio* and *D. sabina* were stripped of divalent cations in a three-step process using protocols modified from Erickson et al. (2005) and Eberhard and Erne (1994). Purified PV was dialyzed for 18 h in 1 L of Hepes buffer (20 mM Hepes, 150 mM KCl) with 6 M urea, 40 μ M EDTA, 40 μ M EGTA and 5% chelex. Urea allowed the protein to denature for full removal of cations by chelex, EDTA, and EGTA. PV was then dialyzed for 8 h in 500 mL of Hepes buffer (20 mM Hepes, 150 mM KCl) with 40 μ M EDTA, 40 μ M EGTA and 5% chelex allowing the protein to refold in a calcium-free environment. PV solutions were then dialyzed for 18 h in Hepes buffer (20 mM Hepes, 150 mM KCl) with chelex present to ensure all EDTA contamination would be removed from the protein solution. This protocol was repeated for each subsequent buffer solution. Parvalbumin concentration was determined by microbiuret assay or spectrophotometric assay at a wavelength of 562 nm using a bicinchoninic acid protein assay kit (Sigma).

2.4. Determination of PV K_D s for calcium

Fluorescent competition assays used for determination of calcium dissociation constants (K_D) were modified from the Eberhard and Erne protocol (1991). Fluo-3 (Molecular Probes™; Invitrogen Corporation, Eugene, OR) was used as the fluorescent indicator at a final concentration of 1.25 μ M by dilution in the appropriate decalcified buffer (pH 7.2). Standard curves were determined for calcium versus raw fluorescence in 2 mL of decalcified buffer solution containing 1.25 μ M Fluo-3 with addition of 5 μ L aliquots of varying calcium concentrations (0.1–1.0 mM).

Functional assays were performed in solutions to mimic the physiological ionic strength of muscle from a freshwater teleost (20 mM Hepes, 150 mM KCl) and the freshwater population of *D. sabina*, (20 mM Hepes, 150 mM KCl, 146 mM urea, 62 mM betaine, 11 mM TMAO) (Treberg et al., 2006). While the exact intracellular concentrations are uncertain for the freshwater population of *D. sabina*, the concentrations used here were guided by the results of Treberg et al. (2006) and represent a valid first approximation under these conditions. Assays including Fluo-3 and PV were performed in 2 mL decalcified solutions with 1.25–1.50 μ M PV with addition of 5 μ L aliquots of calcium solutions. Experiments testing the effects of ionic strength found in freshwater *D. sabina* muscle in the absence of the organic osmolytes contained Hepes buffer (20 mM Hepes, 150 mM KCl) with 219 mM mannitol. Experiments testing effects of total methylamines on PV contained: Hepes buffer, 146 mM mannitol, 62 mM betaine, and 11 mM TMAO. The presence of individual osmolytes were tested in buffers containing: Hepes buffer with 208 mM mannitol, 11 mM TMAO; Hepes buffer with 157 mM mannitol and 62 mM betaine and Hepes buffer with 73 mM mannitol and 146 mM urea. Each experiment consisted of four cuvettes: two control (Fluo-3 with only decalcified buffer) and two with parvalbumin. All experiments were repeated six times and run at 15 °C, the physiologically relevant temperature of *D. sabina*. The fluorescence changes were observed on a Varian Cary Eclipse 3E fluorescence spectrophotometer with internal temperature control set at an excitation wavelength of 505 nm and an emission wavelength of 530 nm.

Fluorescence values determined for the two cuvettes with parvalbumin present were compared to the control values. Calcium K_D measurements were determined by non-linear regression analysis calculated by SigmaPlot v8.0. *D. sabina* and *C. carpio* parvalbumin K_D s were determined in the presence and absence of each osmolyte: urea, betaine, and TMAO. One-way ANOVA was performed on PV K_D s both between treatments and species. Significant differences were determined by $p \leq 0.05$.

2.5. Sequence determination of *D. sabina* parvalbumin

Preliminary sequence data was determined by N-terminal sequence analysis and in-gel digest. Purified PV was run on a SDS-PAGE gel, and the section containing the desired PV isoform was excised and placed in a pre-washed 1.5 mL microcentrifuge tube. Samples were washed in varying concentrations of acetonitrile and ammonium bicarbonate solutions (50% acetonitrile for 15 min, 50% acetonitrile/50 mM ammonium bicarbonate for 30 min, and 50% acetonitrile/10 mM ammonium bicarbonate for 30 min) and dried by vacuum centrifugation. In-gel digests were performed in a solution of 10 mM ammonium bicarbonate with 0.2 μ g of sequencing-grade trypsin. Peptide extraction was performed in a solution containing 5% trifluoroacetic acid and 60% acetonitrile. Direct sequencing was then performed using reversed phase HPLC. This provided determination of partial amino acid sequence of the individual PV, which was used to design degenerate primers for reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA was isolated from *D. sabina* white muscle using TRIzol Reagent according to the manufacturers (Invitrogen) protocol. First-strand cDNA was synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo (dT) primer according to Invitrogen's Superscript III protocol. The degenerate primers were used to amplify PV from total cDNA. Gene specific primers (PVI: CAAGGTGTTCTTCGACATGGTGG, CTCCACATCAGTCTTCGCCTC; PVII: GTTCTCTGCCGGTGCTCG, CCGTCGTGGTCAGTGTCTCC) were designed to complete the amplification of PV cDNA from *D. sabina* using rapid amplification of cDNA ends (RACE-PCR). Products from PCR were cloned and transformed according to the TOPO TA Cloning kit and pCR 2.1-TOPO vector. Positive clone vectors were purified by use of QIAprep Spin Miniprep Kit (Qiagen) and submitted for sequencing by the FSU DNA Sequencing Facility.

2.6. PV quantification and molecular mass

Molecular mass was estimated from the electrophoretic mobility of PV in crude homogenates and purified PV samples. Estimates of PV titer in *D. sabina* white muscle were derived from quantification of PV bands in SDS gels; 10% and 20% crude homogenate supernatants were run along with Precision Plus Protein Standards (Bio-Rad), and previously determined concentrations of *C. carpio* PV isoform II were used as a standard curve for determination of PV concentrations in *D. sabina*. Quantification and molecular weights were performed with QuantityOne v4.6 (Bio-Rad) and UnSCAN-It (Silk Scientific, Inc.) software. PV concentrations from 10% homogenates were calculated based on the formula below:

$$\text{PV (mg)} \times \text{wet mass (g)}^{-1} = \frac{\text{PV (mg)}}{\text{Total PV loaded in lane (mL)}}$$

Statistical differences between isoforms were determined by $p \leq 0.05$ by use of a Student's *t*-test.

Molecular weight was also determined by MALDI-TOF mass spectrometry. Both isoforms of PV found in *D. sabina* were analyzed on an Axima CFR+ MALDI-TOF Mass Spectrometer, using a sinapinic acid matrix.

3. Results

3.1. Molecular characterization of parvalbumin

Two isoforms of PV, denoted I and II in order of their relative electrophoretic mobility, were detected in *D. sabina* white muscle and their identity was confirmed by western blot analysis (Fig. 1). Estimates of *in situ* PV expression by quantification of SDS gels show that the titer of PV I is roughly twice that of PV II in all samples of *D. sabina* muscle

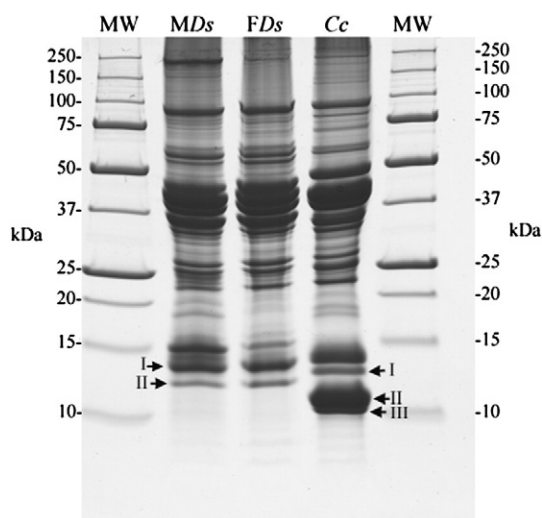


Fig. 1. Identification of PV in *D. sabina* and *C. carpio*. SDS-PAGE gels of 10% white muscle homogenates for freshwater (FDs) and marine (MDs) *Dasyatis sabina* and (Cc) *Cyprinus carpio*. Bio-Rad unstained precision markers are shown to the left and right of homogenates.

examined (Table 1). Further, comparison of freshwater and marine samples by a Student's *t*-test shows no statistically significant differences in the apparent expression of the two isoforms, indicating that PV titer is not a function of environmental salinity in this species (Table 1). For comparison, samples from the common carp (*C. carpio*) also were quantified by SDS-PAGE (Fig. 1). The sum of PV I and II expression in *D. sabina* white muscle is roughly half that of the sum of the three isoforms expressed in *C. carpio* (I=0.20 mg/g wet mass, II=2.03 mg/g wet mass, III=0.47 mg/g wet mass).

The molecular weight of *D. sabina* PV I and II was estimated from SDS-PAGE gels, MALDI-TOF mass spectrometry, and calculated from the full amino acid sequence (Table 2). Comparison of the electrophoretic mobility of PVs I and II with Precision Plus Protein Standards on SDS-PAGE gels like those shown in Fig. 1 yielded molecular mass estimates of 13.55 ± 0.36 kDa and 12.52 ± 0.24 kDa for PV I and II, respectively, from freshwater samples of *D. sabina*. Similarly, estimates from marine samples gave molecular mass estimates for PV I and II of 13.15 ± 0.20 kDa and 12.31 ± 0.20 kDa, respectively. For each isoform, estimates of molecular weight for samples from freshwater and marine populations were not significantly different. MALDI-TOF mass spectrometry showed that the molecular weight of isoform I from marine and freshwater samples was essentially identical, as was the mw of PV II from the two sample types (Table 2 and Fig. 3).

Molecular weights of PV I and II also were calculated from full amino acid sequences (Table 2 and Fig. 4). Comparison of the calculated molecular weights for isoforms I and II with the values determined by MALDI-TOF mass spectrometry show a slight, but finite, difference that is consistent with the post-translational removal of methionine and acetylation of the first amino acid (Table 2). For both isoforms, estimates of molecular weight derived from the comparison of electrophoretic mobility to that of known standards

Table 1
Quantification of PV in white muscle of freshwater and marine *D. sabina*

PV isoform	Freshwater <i>D. sabina</i>	Marine <i>D. sabina</i>	<i>p</i> -value
I	1.00 ± 0.14	0.88 ± 0.23	0.39
II	0.51 ± 0.10	0.48 ± 0.09	0.75

Measurements were performed in 20 mM MOPS, 240 KCl, 1 mM DTT, pH 7.5 at a 20% w/v ($n=3$). All units are in mg/g wet mass with ± 1 S.D.

Table 2
Molecular mass of PV

PV isoform	Amino acid sequence	SDS gels		MALDI-TOF mass spectrometry	
		Freshwater	Marine	Freshwater	Marine
I	12.27 kDa	13.55 ± 0.36 kDa	13.15 ± 0.20 kDa	12.18 kDa	12.19 kDa
II	12.04 kDa	12.52 ± 0.24 kDa	12.31 ± 0.20 kDa	11.96 kDa	11.96 kDa

Molecular mass was determined in three different ways for both freshwater and marine samples of *D. sabina*.

MALDI-TOF mass spectrometry analysis suggests methionine is removed and the next amino acid in the sequence is acetylated.

gave results that are slightly greater than those obtained by calculation and MALDI-TOF mass spectrometry (Table 2).

Isoelectric points of PV I and II from *D. sabina* were estimated from comparison with known standards in 2-dimensional gel electrophoresis (Table 3, Fig. 2). PV from freshwater and marine samples of *D. sabina* were determined to possess identical pI values for both isoforms (I=4.70; II=4.80). Isoelectric points calculated from the full length amino acid sequence analysis were nominally higher than those measured from electrophoretic mobility (I=4.95; II=5.02) (Table 3).

The full amino acid sequence was determined for freshwater and marine *D. sabina* PV I and II (Fig. 4). The sequences were deduced based on Edman degradation and RT-PCR, and NCBI protein blast was used to confirm the sequence as PV. There were no differences in the sequences of either PV I or II between samples from freshwater and marine samples of *D. sabina*. PV I and II from *D. sabina* were found to have 44% total identity and 71% binding pocket identity, confirming that the two isoforms are distinctly different. Full amino acid sequence alignment of freshwater and marine *D. sabina* PV I and II (Fig. 4) were compared and found to possess 50% and 42% identity respectively to the major PV isoform of the freshwater teleost, *C. carpio*, and to two elasmobranchs, with 58% and 45% identity to *T. semifasciata* (leopard shark; NCBI accession no. P30563) and with 39% and 64% identity to *R. clavata* (thornback ray; NCBI accession no. P02630).

3.2. Functional characterization of parvalbumin

Preliminary investigation found PV isolated from freshwater and marine populations of *D. sabina* did not show significant differences in functional characterization (data not shown), therefore, marine samples were used for analysis of PV calcium binding (K_D) in the presence of urea, betaine, TMAO. Dissociation constants (K_D) of calcium were determined for *D. sabina* PV I and II (both isolated from the white muscle of the marine populations), as well as *C. carpio* II PV, in seven buffer systems of different solute composition (methods; Figs. 5 and 6). Carp II PV showed no significant changes in calcium binding between all seven buffer systems. PV I from *D. sabina* behaved similarly to carp II PV in nearly all buffer systems: calcium binding decreased (K_D increased), though not significantly different, in the presence of betaine and TMAO jointly and independently relative to the normal physiological condition. Urea, though not significantly

Table 3
Isoelectric point determination of PV

PV isoform	Amino acid sequence	2-D gels
I	4.95	4.70
II	5.02	4.80

pI quantification for PV isoforms I and II based on full amino acid sequence and 2-D gel electrophoresis.

Freshwater and marine samples were found to be identical for both analyses. Differences between amino acid sequence and 2-D gel analysis of pI proved not to be significantly different (p -value < 0.05).

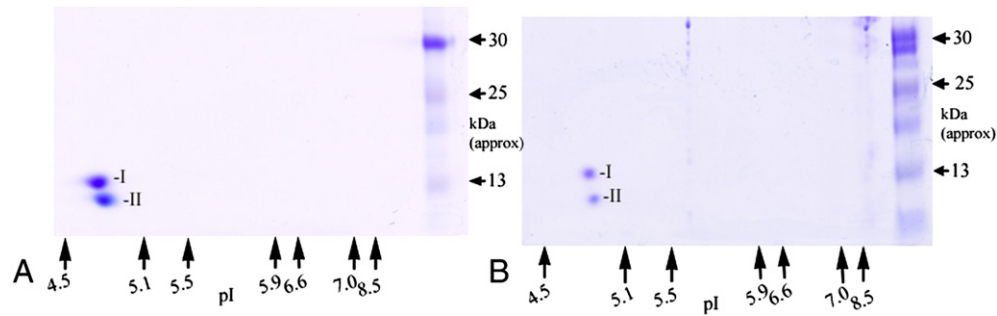


Fig. 2. 2-D gel electrophoresis of PV. A) Freshwater *D. sabina* PV I and II. B) Marine *D. sabina* PV I and II. Kaleidoscope pre-stained markers from Bio-Rad are shown to the right of each gel.

different, does appear to lower K_D and thus allow increased calcium binding in PV I. Only when betaine was tested independently of urea and TMAO (8.23 ± 0.50 nM) was significant difference for PV I found from physiological conditions (6.03 ± 0.50 nM). A distinctly different pattern of response was observed for PV II, relative to that of PV I and carp II PV: as shown in Fig. 5B, the ability of PV II to bind calcium was increased (a decrease in K_D) in the presence of each osmolyte independently, as well as in the presence of total methylamines.

4. Discussion

Molecular characterization of the two PV isoforms in *D. sabina* revealed them to be identical in marine and freshwater populations

(Fig. 1). All elasmobranch PV sequences were aligned to *C. carpio* II PV (Fig. 4). No differences were present among the coordinating residues (relative positions 1,3,5,7,9,12 within each domain) in the CD and EF domains (highlighted in Fig. 4) of PV where calcium binding occurs, with the exception of the 7th position in the CD site. In *C. carpio* and *T. semifasciata*, phenylalanine is found at this position, whereas the rest of the elasmobranchs possess tyrosine at that location. Amino acid substitution at this site changes a non-polar hydrophobic residue to a polar hydrophilic residue, and the additional –OH group found on tyrosine, not present on phenylalanine, logically might be expected to result in differences between PV in *C. carpio* and *D. sabina* ability to bind calcium. However, if the binding ability is similar between the two, the –OH group is not in a position to affect parvalbumin's ability

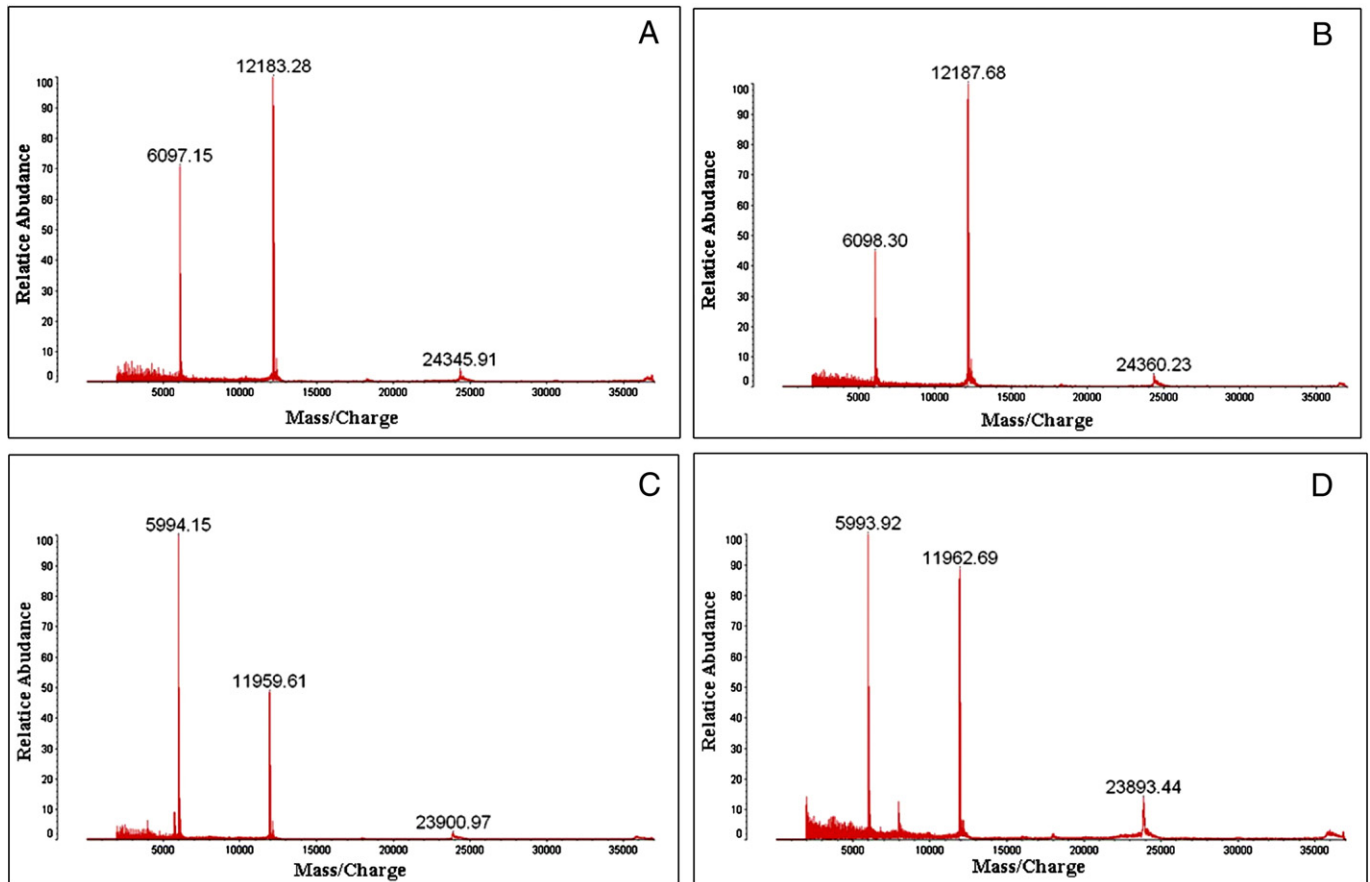


Fig. 3. Determination of *D. sabina* molecular mass by MALDI-TOF mass spectrometry. (A) and (C) Show mass spectrum for freshwater PV I and II, respectively. (B) and (D) Show mass spectrum reading for marine PV I and II, respectively. PV isoforms were separated by HPLC prior to mass spectrometry analysis (matrix, sinapinic acid). MALDI was used to determine if post-translational modifications were found in either isoform of PV. The second peak in each spectrum represents the molecular mass. The third peak in spectrum represents a doublet.

		*	20	*	40	*	60	
<i>C. carpio</i> β	–	MAFAGVLNDADIAAALEACKAADSEFNHKAFFAKVGLT	SKSADDVKKAF	AIIDQKSGFIEEDE	:	63		
<i>T. semifasciata</i>	–	PMTKVLKADDINKAISAFKDPGTFDYKRFHVLVGLKGTDAQVKEVFE	ILDQKSGFIEEEE	:	62			
<i>R. clavata</i>	–	SSKITSILNPADITKALEQCAAG	–FHHTAFFKASGLSKKSDAE	LAEIFNVLDGDSGYIEVEE	:	62		
<i>D. sabina</i> I	–	TITDVLAKDDIKKALDNFQKPGSEFNHKKVFFDMVGLKKKAKT	DVEKVF	KILDKDSGYIEEDE	:	62		
<i>D. sabina</i> II	–	SSDISALLSAKDIEKALAECSAS	–FNHKTFFQTSGLNKKNDAL	TKIFRVLDE	DASGYIEVEE	:	62	
		*	80	*	100	*		
<i>C. carpio</i> β	L	KLFLQNFKADARALTDGETKTFLKAGDS	GDGKIGVDEFTALVKA	–	:	109		
<i>T. semifasciata</i>	L	KGVVLKGFSAHGRDLNDTETKALLAAGDS	HDGKIGVDEFTALVKA	–	:	109		
<i>R. clavata</i>	L	KNFLKCFSDGARVLNDKETSNFLAAGDS	HDGKIGVDEFTALVKA	–	:	109		
<i>D. sabina</i> I	L	KHILQCFAPNGRDLADNETQALLAAGDE	HDGKIGVSEFVNLVQA	–	:	109		
<i>D. sabina</i> II	L	TKFLKRFSAAGARDLNQKETEAF	LAAGDTLHDGKIGVSEFVNLVKA	–	:	108		

Fig. 4. PV sequence alignment. Freshwater and marine *D. sabina* possess identical PV I and II isoforms, therefore one representative sequence for each is provided. Both PVs I and II from *D. sabina* were compared to two other elasmobranch (*T. semifasciata* (leopard shark) and *R. clavata* (thornback ray)) as well as *C. carpio* (a well characterized freshwater teleost). Calcium binding pockets (CD and EF) are highlighted in light grey, dark grey signifies where amino acid are different.

to bind calcium. It can be concluded, based on our present calcium binding studies showing similar K_D values for the major PV isoforms in carp and *D. sabina* (Fig. 5), that the additional –OH group found on tyrosine is most likely not in a position to affect parvalbumin's ability to bind calcium. This result is reinforced by a study (Drake et al., 1997) of the galactose binding protein from *Escherichia coli*, an EF hand-like protein. Here, substitution of glutamine at the 7th binding domain position to glycine had no effect on calcium binding or cation size selectivity.

A suite of low molecular weight organic molecules make up the predominating osmolytes used to establish a fit milieu for protein structure and function and regulation of cell volume (Hochachka and Somero, 2002). Urea and methylamines are among the four groups of predominating osmolytes found in almost all cell types across many taxa for intracellular and extracellular osmoregulation (Hochachka and Somero, 2002).

Urea, betaine, and TMAO independently affect the two isoforms of PV in *D. sabina* in distinctly different ways. PV I in *D. sabina* was found to possess a high affinity for calcium similar to carp II PV with one exception. When PV I was tested in the presence of betaine independently, K_D significantly increased relative to physiological conditions. Thus, this PV isoform was found to be insensitive to the deleterious effects these organic osmolytes have been shown to have on other proteins.

In contrast, PV II was found to show a more classic protein response to these organic osmolytes as seen by Yancey, Somero, and others (Yancey and Somero, 1979; Yancey et al., 1982; Yancey, 1985; Yancey and Somero, 1980). Calcium binding by PV II was significantly affected by urea, betaine, and TMAO. This isoform's ability to function was altered in the presence of these osmolytes independently, but when in the physiological 2:1 ratio characteristic of elasmobranchs methylamines were found to counteract the effects of urea. Instead of increasing K_D , thus decreasing calcium binding, by denaturing the protein, each osmolyte independently appeared to increase the calcium binding ability of this protein. A possible interpretation of this pattern is that it could be a direct result of the effects urea and methylamines have on the water accessible surface area (ASA) of the protein. Urea is preferentially accumulated at the surface of the protein resulting in an increase in the ASA, thereby reducing the stability of the protein (Courtenay et al., 2001). The lower urea levels found in the muscle of the freshwater populations of *D. sabina* may not be as destabilizing to the overall stability of this isoform as seen with the higher concentrations of urea found in solely marine elasmobranchs. Betaine and TMAO are found to be preferentially excluded from the surface of the protein resulting in a reduction in the ASA and stabilizing the protein (Courtenay et al., 2000).

While carp PV II was used in this study initially as a control during functional experiments, it revealed unexpected results in the presence of these organic osmolytes. In the presence of each osmolyte independently and in the 2:1 ratio found in elasmobranchs, the

calcium binding ability of PV was not found to be significantly altered. The most surprising of these results was in the presence of urea, a known protein destabilizer, not normally found in the intracellular compartment of teleosts. However, it is unclear at this time if this pattern is common to all PV isoforms found in carp. Functional studies will need to be conducted on the two minor isoforms from carp in the presence of these osmolytes in order to completely understand their effect on PV from a teleost.

While organic osmolytes are found to alter protein function in a variety of ways, the pattern of response seen specifically in K_D of *D. sabina* PV in the presence of betaine and TMAO independently is believed to be a result of the way each osmolyte is interacting with the two PV isoforms. This response has been seen in other proteins. Myoglobin in the presence of betaine has been shown to maintain greater structural stability against the denaturing effects of high urea concentrations compared to TMAO (Ortiz-Costa et al., 2002). This finding may provide one explanation for the increased concentrations of betaine in *D. sabina* as opposed to TMAO, found in most elasmobranchs, to counteract the destabilizing effects of urea. Betaine is also found to be excluded from the ASA of the protein to a greater extent than TMAO (Courtenay et al., 2000). Thus, betaine may also have a slightly stronger stabilizing effect than TMAO. It has also been suggested that euryhaline stingrays in freshwater have a considerably lower concentration of TMAO in their diet and therefore must compensate by accumulating betaine which is synthesized within the liver (Treberg et al., 2006). Therefore, it is not known why calcium binding of PV I and II vary in the presence of the different organic osmolytes, but it may be due to the way each of these osmolytes affect the destabilization/stabilization of each isoform.

PV I and II titer in the white muscle of freshwater and marine populations of *D. sabina* were not found to be significantly different suggesting it is not a compensatory mechanism for handling variation in salinity. Expression of PV was investigated in samples of *D. sabina* from freshwater and marine environments and subsequently compared to a freshwater teleost, *C. carpio*. PV I in *D. sabina* was found to be approximately one third of the total concentration of isoform II in carp. PV II in *D. sabina* was found to be comparable to carp isoform III and 42% larger than carp isoform I (Fig. 1).

This investigation indicated the number of PV isoforms found in elasmobranchs can vary as seen in teleost fish. *D. sabina* was found to possess two parvalbumin isoforms (Fig. 1). Sharks have been found to possess only a single PV isoform (Roquet et al., 1992; Sanscrainte 2006). Carp, a freshwater teleost, used during this investigation was determined to possess three isoforms of PV. Teleosts generally contain up to five isoforms of PV present in their white muscle (Huriaux et al., 1997).

Some questions still remain about the exact point at which the freshwater population of *D. sabina* diverged to be independent from the marine population. It is known that the freshwater population of *D. sabina* only began to occupy the St. Johns River System less than

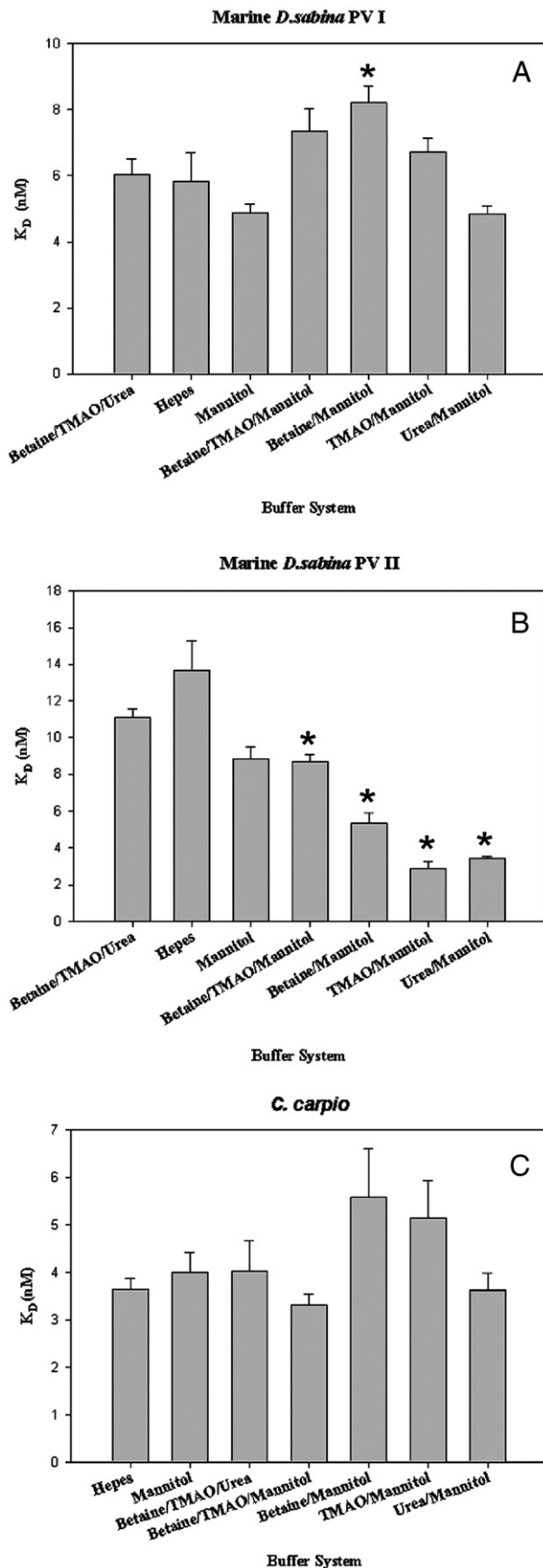


Fig. 5. K_D of PV. (A) *D. sabina* PV I (B) *D. sabina* PV II. (C) Teleost, *C. carpio*, PV II. Physiological conditions for each species are found in the first bar for each graph. Error bars represent ± 1 S.D. ($n=6$) * significant difference in K_D from physiological conditions ($p \leq 0.05$).

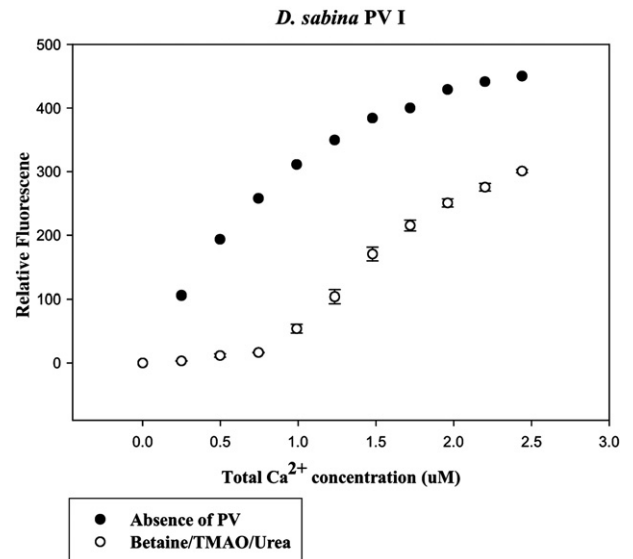


Fig. 6. Representative curves for K_D determination. In this assay, experiments were performed in Hepes buffer containing 146 mM urea, 62 mM betaine, and 11 mM TMAO at 15 °C. Samples containing 25 μ M fluo-3 were titrated with 5 μ L aliquots of calcium solutions in the presence (○) and absence (●) of PV I from marine *D. sabina*.

1 million years ago (Piermarini and Evans, 1998). This study found freshwater and marine PV I and II were not significantly different in molecular characterization, suggesting differences in environmental salinity have not, thus far, required a shift towards either isoform. The main differences were instead found between the two isoforms and how they function in the presence of the organic osmolytes: urea, betaine, and TMAO.

5. Conclusion

This investigation has shown that *D. sabina* possesses two isoforms of PV and that on the basis of molecular and functional characterization; these isoforms are identical in marine and freshwater populations. PV II of *D. sabina* was determined to be more affected by urea, betaine and TMAO than PV I. Urea, betaine, and TMAO independently significantly increased the ability of PV II to bind calcium. Further kinetic studies will be performed to verify if this phenomenon is found in PV in the presence of magnesium, as is the normal state of muscle.

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