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Molecular and Functional Characterization of Parvalbumin in the Atlantic Sharpnose Shark, Rhizoprionodon Terraenovae

Neil Dominic Sanscrainte
MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF PARVALBUMIN IN THE ATLANTIC SHARPNOSE SHARK, RHIZOPRIONODON TERRAENOVAE

By

NEIL DOMINIC SANSCRAINTE

A Thesis submitted to the Department of Biological Science in partial fulfillment of the requirements for the degree of Master of Science

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The members of the Committee approve the Thesis of Neil Sanscrainte defended on June 26, 2006.

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Joseph Travis, Dean, College of Arts and Science

The Office of Graduate Studies has verified and approved the above named committee members.
This thesis is dedicated to all who have helped me through this transitional time in my life, both professionally and personally. Especially to my parents, Gerald and Marlene Sanscrainte, and my fiancée, Christine Glenn, I thank you and love you for the support and motivation you have given me.
ACKNOWLEDGEMENTS

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ABSTRACT

Parvalbumin (PV), a soluble protein of skeletal muscle that facilitates muscular relaxation, is expressed across many phyla and has highly conserved Ca\(^{2+}\) binding sites. These features make it ideal for comparisons among organisms that contain highly diverse internal osmolarities. To maintain function in the presence of high internal levels of urea, certain elasmobranch multimeric proteins are stabilized by organic solutes (such as trimethylamine N-oxide, TMAO), which counteract urea. While normal intracellular levels of urea (~400 mM) and TMAO (~200 mM) are not hypothesized to affect calcium-binding protein function in elasmobranch muscle, no studies have determined whether elasmobranch PV is structurally modified to function in the absence of TMAO. This study is the first to address the function of PV in a coastal shark, the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). Sequencing of PV from RNA was performed with PCR amplification, cloning, and plasmid sequencing. Parvalbumin from *R. terraenovae* and a teleost fish, the common carp (*Cyprinus carpio*) was purified using AMS fractionation, followed by size-exclusion and anion-exchange chromatography. Ca\(^{2+}\) K\(_D\) of purified shark and carp PV was measured in the presence of a 2:1 ratio of urea/TMAO and a 2:1 ratio of urea/mannitol by a fluorometric competitive binding assay. Additionally, PV isoform distribution, size, and concentration were determined for three additional coastal sharks by SDS-PAGE, Western blot, and digital comparison to SDS-PAGE standards. *R. terraenovae* Ca K\(_D\) averaged 6 nM, and no significant difference for K\(_D\) was observed between species, or between urea/TMAO and urea/mannitol treatments for both shark and carp. PV titer of the major carp isoform was nine fold higher than the average shark PV titer. It is hypothesized that the monomeric nature of PV may be less susceptible than multimeric proteins to perturbation by urea, as interactions between subunits are less stable than interactions forming protein tertiary structure. The markedly different PV concentrations in the elasmobranch and teleost species used in this study
may be indicative of varying properties of fish fast muscle that merit future comparative studies into fast muscle performance.
**INTRODUCTION**

**Osmoregulation in Elasmobranch and Teleost Fish**

The subclass Elasmobranchii is a group of mostly marine cartilaginous fishes composed of around 800 extant species, most notably the sharks, skates, and rays (Ballantyne, 1997). Elasmobranchs and marine teleosts maintain drastically different internal osmotic pressures and consequently use different osmoregulatory methods. Teleosts are osmoregulators and maintain an internal pressure ~300 milliosmolar (mOsm). To remain hypoosmotic while maintaining internal ion concentrations lower than that of the environment, teleosts must constantly counteract the loss of body water and influx of ions (osmotic pressure ~1000 mOsm). Lost water is replenished by drinking seawater, as the majority of Na\(^+\) and Cl\(^-\) ions ingested are actively transported out of the body by specialized chloride cells in the gills. Ingested divalent ions (Ca\(^{2+}\), Mg\(^{2+}\), SO\(_4^{2-}\)) pass through the feces, while excess divalent salts are secreted in urine. Additionally, gills serve as sites of copious nitrogenous waste removal (Wilmer et al., 2000).

Elasmobranchs are urea-osmoconformers and maintain an osmotic pressure slightly above seawater. The bulk of this osmotic strength comes from high organic osmolyte concentrations rather than elevated ionic levels, as is seen in true osmoconformers such as marine invertebrates and the hagfish. Internal ion concentrations in sharks and rays are comparable to that of teleosts (Table 1). While elasmobranch internal osmolarity precludes the need to counter the influx of seawater, salts still diffuse into the body and are secreted in urine, through the rectal gland, and across the gill epithelium (Haywood, 1973).

**Properties of Urea and TMAO**

Intracellular organic osmolytes in elasmobranchs vary according to tissue type and species, but the two most common in sharks are urea and trimethylamine N-oxide (TMAO). Urea is the highest concentrated molecule found in sharks, second only to water, with intracellular and tissue concentrations ranging from 350-600 mM.
(Ballantyne, 1997; Steele et al., 2005). While ammonia is the nitrogen donor for most urea synthesis in mammals, glutamine is the source of nitrogen for urea synthesis in elasmobranchs and some teleosts (Figure 1a) (Ballantyne, 1997). The final step in the urea cycle is the splitting of arginine into ornithine and urea by arginase, and the synthesis of urea is often referred to as the ornithine urea cycle (OUC). While urea synthesis has long been known to occur in liver mitochondria, recent work by Steele et al. (2005) suggests that the OUC may also occur in skeletal muscle of skates. The level of hepatic urea production is estimated to be more than 150 times that in muscle, however, suggesting that the muscle OUC may be involved in ammonia reuptake from metabolic activity rather than the synthesis of large quantities of urea (Steele et al., 2005).

<table>
<thead>
<tr>
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<th>K</th>
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Table 1. Intracellular free ion and osmolyte concentrations (mM) found in muscle of a sample teleost and elasmobranch species. As ion levels are only slightly elevated in elasmobranches, organic osmolytes comprise the bulk of internal osmotic strength.

Urea acts as a protein denaturant, and millimolar concentrations have been shown to affect the function of certain proteins \textit{in vitro} (Yancey and Somero, 1980). Understandably, most vertebrates maintain low intracellular urea levels (<1 mM). While incompletely understood, the denaturing mechanism of urea has been intensely studied. Molar concentrations of urea perturb protein tertiary structure by interacting directly with the peptide backbone as well as disrupting the solvent properties of water (Salvi et al., 2005). Recent molecular dynamics simulations by Bennion and Daggett (2003) suggest a mechanism connecting urea’s direct and indirect roles in the denaturation process. In the
Figure 1. Urea and TMAO cycles in elasmobranchs. a) Simplified hepatic urea cycle in elasmobranchs. CPS = carbamoyl phosphate synthetase; OTC = ornithine transcarbamylase; AGS = argininosuccinate synthetase; AGL = argininosuccinate lyase; ARS = arginase (Modified from Ballantyne, 1997). b) Simplified TMAO cycle in elasmobranchs. Tmase = trimethylamine oxidase.
presence of urea, hydrogen bonding between water molecules is disrupted and water diffusion is decreased. This disruption of the normal solvation properties of water results in increased water-protein hydrogen bonding (particularly in residues around the hydrophobic core), thereby making water the initial denaturant. After disruption of secondary structure, water and urea enter the hydrophobic core where urea then interacts directly with the peptide backbone, stabilizing the denatured state of the protein (Bennion and Daggett, 2003). Though the effect of urea on proteins appears to be universal, urea may interact variably with different protein structures. Crystal studies show that urea interacts with specific sites on lysozymes, but the bacterial ribonuclease barnase, however, shows less site-specific interactions (Salem et al., 2006).

Methylamines are another osmolyte found in high intracellular concentrations in elasmobranchs, averaging around 200 mM (Yancey and Somero, 1980). In many teleost fish TMAO is involved in equalizing intra- and extra-cellular osmolarity (Raymond and DeVries, 1998). Choline from the diet is oxidized to trimethylamine (TMA) in the gut, and hepatic or digestive gland trimethylamine oxidase (Tmase) then oxygenates TMA into TMAO (Figure 1b) (Seibel and Walsh, 2002). Most marine fish and some elasmobranchs are known to synthesize TMAO, and Tmase activity has been reported in several elasmobranch species including dogfish, lemon sharks, and silky sharks (Schlenk and Li-Schlenk, 1994; Ballantyne, 1997). While nurse shark Tmase has been shown in vivo to directly synthesize TMAO from choline, this mechanism is not yet understood (Goldstein and Funkhouser, 1972). The hydrolysis of phosphatidylcholine (a phospholipid common in cell membranes) releases a free choline, which Seibel and Walsh (2002) suggest may be an endogenous source for increased TMAO synthesis in elasmobranchs. Interestingly, the resulting diacylglycerol from this reaction results in a “fatty liver” condition in mammals, and might be an explanation for the large amount of lipids present in shark livers (Seibel and Walsh, 2002).

The methylamine TMAO has been shown to be a protein stabilizing agent. Interactions between TMAO and the denatured peptide backbone are thermodynamically unfavorable, and therefore drive the protein towards the native folded conformation (Wang and Bolen, 1997). This is best evidenced by Baskakov and Bolen (1998) who forced thermodynamically unfolded RNase mutants into a functional tertiary
conformation by the addition of TMAO. Although several natural osmolytes can induce protein folding, TMAO is the most effective (Baskakov and Bolen, 1998). High concentrations of methylamines such as TMAO may detrimentally impact protein structure, as the stabilizing effect may make protein structure too rigid or lower binding constants to suboptimal levels (Yancey et al., 1982).

**Urea and TMAO as Counteracting Solutes**

Yancey and Somero (1979; 1980) conclusively showed that while urea concentrations found in elasmobranch tissues disrupt the function of many purified enzymes *in vitro*, physiological concentrations of TMAO in sharks offset urea’s effects on enzyme function in an equal, yet opposite, manner. For this reason, urea and TMAO are described as counteracting solutes (Somero et al., 1982). Comparative studies have shown that a roughly 2:1 ratio of urea to methylamine maximally preserves native protein function. This ratio has been observed consistently in elasmobranch tissues with urea ~400 mM and TMAO ~200 mM (Yancey and Somero, 1980; Ballantyne, 1997). Under experimental conditions, the equilibrium constant \( K_m \) increases in the presence of urea for creatine kinase, pyruvate kinase, and lactate dehydrogenase, but decreases for argininosuccinate lyase. For each enzyme, the addition of TMAO in a 1:2 ratio with urea has an equal and opposite effect on \( K_m \), effectively restoring function (Yancey and Somero, 1980). Only a few proteins, such as elasmobranch lactate dehydrogenase, have been shown to require urea for optimal function; most proteins sensitive to physiological urea in elasmobranchs rely on the stabilizing effect of TMAO (Yancey and Somero, 1979). TMAO has recently been shown also to counteract the effects of hydrostatic pressure on proteins of deep-sea teleosts (Yancey et al., 2001).

**Parvalbumins**

Studies have shown the effects of intracellular urea on multimeric catalytic proteins, but the effect of these urea levels on monomeric ion binding proteins has not been systematically explored. Parvalbumins (PVs) are well characterized low-molecular-weight calcium binding proteins (10-12,000 Da) found in many vertebrate and invertebrate endocrine systems, nervous systems, and voluntary muscles (Eberhard and
Erne, 1994; Rall, 1996). These soluble proteins are of the EF-hand family, named for the ability to represent the characteristic calcium binding domains (alpha helix–loop–alpha helix) with the index finger and thumb (Kretsinger and Nockolds, 1973). Parvalbumin contains two such motifs (CD and EF) and one nonfunctional motif (AB). The CD and EF motifs bind Ca\(^{2+}\) with a high affinity (association constant \(K_A \sim 10^8 \text{ M}^{-1}\)) and Mg\(^{2+}\) with a lower affinity \(K_A \sim 10^4 \text{ M}^{-1}\) (Eberhard and Erne, 1994; Pauls et al., 1996). The ion binding loops are twelve amino acids in length, with six residues involved in both Ca\(^{2+}\) and Mg\(^{2+}\) binding. All coordinating ligands in PV are oxygen, with dichotomous ion affinity arising from glutamate, the last coordinating residue, acting as a bidentate ligand in Ca\(^{2+}\) binding and a monodentate ligand in Mg\(^{2+}\) binding (Cates et al., 1999).

In skeletal myofibers, PV is located in the sarcoplasm and is involved with muscle relaxation. As part of the myofiber calcium buffer system, PV has a calcium affinity greater than that of troponin C, but in resting muscle Mg\(^{2+}\) concentrations are much higher than Ca\(^{2+}\) concentrations, resulting in PV being saturated by Mg\(^{2+}\). This prevents Ca\(^{2+}\) released from the SR during excitation from immediately binding to PV, and instead Ca\(^{2+}\) binds to troponin C and muscle contraction is initiated. Calcium levels rise during multiple contractions, and Mg\(^{2+}\) slowly disassociates from PV as a result of PV’s higher affinity for Ca\(^{2+}\) (disassociation rate for Mg\(^{2+}\) \(\sim 0.1 \text{ s}^{-1}\)), which leaves troponin C and binds to PV (Hou et al., 1991). From there calcium is sequestered away from PV by the SR Ca-ATPase pump, returning Ca\(^{2+}\) to the SR and ending the relaxation cycle (Rall, 1996).

Thus, PV as a calcium buffer has an important roll in relaxation. Several experiments in mammalian systems have provided evidence that sarcomere relaxation rates are a function of PV titer. Direct injection of PV cDNA significantly shortened half-relaxation time in rat soleus (slow skeletal) muscle, though contraction time was unaffected (Muntener et al., 1995). The induced expression of PV in isolated rat cardiac myocytes, which do not normally express PV, returned relaxation rates in diseased cells to normal, and PV gene transfer has showed similar results in aged rat hearts in vivo (Wahr et al., 1999; Schmidt et al., 2005; Michele et al., 2004). These experiments demonstrate the potential role PV may play in the treatment of diseases involving relaxation dysfunction. Chen et al. (2001) confirmed earlier observations that PV knockout mice showed no difference in fast muscle myosin isoforms, troponins, SR Ca\(^{2+}\)-
ATPase activity, or Ca\(^{2+}\) release from the SR (Schwaller et al., 1999). Fast muscle in these knockout mice did however have contraction/relaxation cycles and mitochondria levels similar to that found in slow muscle. These data indicate that PV is essential for fast muscle contractions, as myocyte plasticity drives fast muscle to express slow muscle characteristics in the absence of PV (Chen et al., 2001).

The molecular properties of PV have been previously described for a number of teleost fish. Generally, multiple isoforms of PV are present at high concentrations in white (fast-twitch) skeletal muscle. The toadfish swimbladder, the fastest vertebrate muscle, has the highest PV concentration known (Klug et al., 1988; Rome, 2006). While PV expression in red (slow-twitch) or pink (mixed fiber type) fibers has historically been considered nonexistent or inconsequential, recent work by Wilwert et al. (2006) reports that relaxation rate correlates to PV expression in red muscle along the axis of two teleost fishes.

Aside from the crystal structure of leopard shark muscle PV and a partial sequence of dogfish neural PV, few published data pertain to PV from elasmobranchs (Roquet et al., 1992; Conlon et al., 1992). This study is the first to explore the function of elasmobranch PV and the effects of urea and urea/TMAO on ligand affinity. The dissociation constant (K\(_D\)) for Ca\(^{2+}\) was determined for purified PV from the Atlantic sharpnose shark *Rhizoprionodon terraenovae* in the presence of physiological levels of urea and TMAO and in the absence of TMAO. For comparison, similar experiments were performed on the major PV isoform from the common carp *Cyprinus carpio*. It was hypothesized that shark PV plays a similar role in elasmobranchs as in other vertebrates, and would therefore exhibit a similar K\(_D\) for Ca\(^{2+}\) in physiologically relevant levels of urea and TMAO. The effects of urea on PV function were previously unknown, but the presence of urea-rich tissues in elasmobranchs suggested that shark PV may be more functionally robust than carp PV in the presence of a denaturant. This study shows that urea in the mM range has no effect on the Ca\(^{2+}\) K\(_D\) for shark or carp PV. Parvalbumin from three additional coastal sharks was also identified and molecular size and PV concentrations were estimated. Parvalbumin expression in carp was on average nine times higher than in the four shark species examined.
METHODS

Animals

Mature *R. terraenovae* from several sample sites in the Gulf of Mexico were captured in variable mesh size gill net (3.0-5.5 inches). White muscle for PV purification was removed and initially stored at –20°C for 24-72 hours, transported on dry ice, and stored at –80°C (Figure 2). Post-dorsal white muscle samples were flash-frozen in liquid nitrogen for PV sequencing. White muscle samples were collected for comparative molecular analyses from several other coastal shark species: *Carcharhinus limbatis* (blacktip shark), *Carcharhinus isodon* (finetooth shark), and *Sphyrna tiburo* (bonnethead). All species were selected based on availability.

*C. carpio* are a teleost whose PV has been well characterized and were acquired through electroshock from Lake Talquin, FL. Animals were sacrificed by spinal cord severing and white muscle samples were taken immediately post-mortem. All procedures using vertebrates were approved by the FSU Animal Care and Use Committee.

Identification of PV, MW Estimation, and PV Quantification

Crude homogenates (20% w/v) and homogenate supernatants from four species of shark (*R. terraenovae, C. limbatis, C. isodon,* and *S. tiburo*) and one teleost (*C. carpio*) were loaded onto one-dimensional SDS-PAGE as described by Schägger and von Jagow (1987) modified with 13.6% tricine-SDS ployacrylamide gels. Western blots were used to identify all isoforms of PV present in whole muscle samples. The primary antibody used was monoclonal mouse anti-frog PV (Sigma-Aldrich) and the secondary antibody was goat anti-mouse IgG conjugated to alkaline phosphatase (BioRad). Bands were visualized using stabilized Western Blue® substrate (Promega). To estimate MW, samples were run on SDS-PAGE concurrently with Precision Plus Protein™ Standards (BioRad). Digitization and MW estimation was performed using QuantityOne v4.6 (BioRad) and Un-Scan-It (Silk Scientific, Inc.) software. Known concentrations of purified *R. terraenovae* PV were used as a standard curve for SDS-PAGE analysis to determine PV concentrations from white muscle homogenate supernatants. Homogenate
Figure 2. Cross section of mature *R. terraenovae*. White muscle (WM) fills most of the body interior. Red muscle (RM) forms an exterior layer just under the denticles. SC = spinal cord.
pellets were also analyzed with SDS-PAGE to confirm minimal loss of PV after centrifugation. Quantification was performed with QuantityOne v4.6 (BioRad) and Un-Scan-It (Silk Scientific, Inc.) software. Parvalbumin titer was calculated from:

\[ \frac{mg\ PV}{g\ wet\ weight} = \frac{mg\ PV\ in\ lane}{mL\ sample\ in\ lane} \times \frac{mL\ of\ buffer}{g\ tissue} \]

Isoelectric point (pI) was estimated with two-dimensional SDS-PAGE standards (BioRad). Two-dimensional SDS-PAGE was performed with the ZOOM® IPGRunner™ System (Invitrogen) using isoelectric focusing strips pH 3-10.

**Purification of PV**

Parvalbumins from *R. terraenovae* and *C. carpio* were purified using modifications of Laney et al. (1997) as follows.

**Preparation of skeletal muscle homogenate**

A 20% w/v homogenate of skeletal muscle was diced in MOPS buffer (20 mM MOPS, 240 mM KCl, 1 mM DTT, pH 7.5) at 4°C and sonicated on ice with a Fisher 50 Sonic Dismembrator. A protease inhibitor (1.5 mM benzamidine hydrochloride) was added to the homogenate prior to dicing. Homogenate was then centrifuged at 12,000 g for 30 minutes. Ammonium sulfate (AMS) fractionation to 62% was performed on *R. terraenovae* supernatant and 70% on *C. carpio* supernatant; fractionation percentage was experimentally determined to yield minimal PV precipitation in this step. Fractionation was followed by centrifugation at 16,000 g for 2 minutes and supernatant was then brought to 100% AMS fractionation and stirred at 4°C for 2 hours to allow complete precipitation of proteins. Centrifugation at 16,000 g for 30 minutes yielded a protein rich pellet used for gel filtration chromatography. All supernatants were kept at neutral pH during AMS fractionations.

**Size exclusion chromatography**

The pellet from AMS fractionation was reconstituted with a minimal volume of gel filtration column eluant (100 mM NH₄HCO₃, pH 7.8) and loaded onto a size
exclusion column (S-100 Sephacryl, column size: 2.5 x 90 cm) and collected in fractions of 120 drops at a flow rate of 0.3-0.4 mL/minute. Fractions containing PV were detected using ultraviolet spectroscopy scanning at wavelength ratio 260/280, SDS-PAGE, and Western blot as previously described.

**Ion exchange chromatography**

Fractions identified as containing PV were pooled and either fractionated at 100% AMS and centrifuged at 16,000 g for 30 minutes or condensed using ultrafiltration cells (Amicon) with a 1,000 NMWL membrane (Millipore). Concentrated sample was dialyzed against two 1 L changes of ion exchange column eluant (25 mM MES, pH 5.7) for 12 hours each in 6-8000 MWCO regenerated cellulose tubing (Spectra). Sample was then injected onto a DEAE column (DEAE-cephacel, column size: 2.5 x 10 cm) and washed with 50 mL MES buffer. Parvalbumin was eluted off column with a 0–0.2 M linear NaCl gradient for *C. carpio* and a 0–0.1 M linear NaCl gradient for *R. terraenovae*. Western blot and SDS-PAGE confirmed purification, and isoform purity was established by two-dimensional SDS-PAGE.

**Removal of Divalent Cations from PV and Solutions**

Divalent cations were removed from purified PV by a two-step procedure using modifications of Eberhard and Erne (1994) and Erickson et al. (2005). Aliquots of 0.5 mL purified PV were injected into 3,500 MWCO Slide-A-Lyzer\textsuperscript{®} dialysis cassettes (Pierce) and were dialyzed at 4°C against 0.5 L of the 1\textsuperscript{st} decalcification buffer (20 mM Hepes, 150 mM K\textsuperscript{+}, 40 µM EDTA, 6 M urea, 5% chelex (BioRad), pH 7.2) for 4 hours. Removal of EDTA from the cassette was performed by dialysis in the 2\textsuperscript{nd} decalcification buffer (20 mM Hepes, 150 mM K\textsuperscript{+}, 5% chelex, pH 7.2) for 2 hours at 4°C. Testing of the 2\textsuperscript{nd} decalcification buffer with Fluo-3 after this step confirmed the reduction of EDTA to non-competitive trace amounts. This method improves upon previous protocols by reducing six-fold the time protein is kept at 4°C. Final PV concentrations were determined using the Micro-Biuret method as described by Itzhaki and Gill (1964). Cations were removed from solutions by treatment with 5% chelex for 1 hour. Chelex was then compacted by centrifugation, thereby allowing removal of de-ionized buffer.
Solution pH was adjusted by adding several microliters of molecular grade HCl (Sigma-Aldrich) and measuring the pH of buffer subsamples, which were then discarded to avoid possible Ca\(^{2+}\) contamination. CaCl\(_2\) solutions were made by diluting a 0.1 M calcium standard (Orion) with decalcified Hepes buffer. Disposable or acid-washed polypropylene and acid-washed stir bars were used for mixing of all solutions.

**Fluo-3 Competition Assay**

All fluorometric assays were performed on a Varian Cary Eclipse 3E fluorescence spectrophotometer with temperature control. Competition assay methods were modified from Eberhard and Erne (1991; 1994). The fluorescent calcium binding indicator Fluo-3 (Molecular Probes-Invitrogen) was reconstituted using DMSO and stored in 1 mM aliquots at –80\(^\circ\)C. For experimental assays, Fluo-3 was diluted to 50 µM with decalcified Hepes buffer (20 mM Hepes, 150 mM K\(^+\), pH~7.2). Assays included 50 µL of diluted Fluo-3 added to 1.95 mL of experimental buffer for a final Fluo-3 concentration of 1.25 µM. For experiments with both protein and Fluo-3, final PV concentrations of 1.25-1.45 µM was used to ensure competition of calcium binding species. To explore the effect of urea levels in elasmobranchs on PV binding, experiments were performed in 20 mM Hepes buffer containing TMAO and urea in their physiologically appropriate concentrations and ratio (360 mM urea and 180 mM TMAO) as well as Hepes buffer containing a similar ratio of urea and mannitol (360 mM urea and 180 mM mannitol). Osmotic strength has been shown to affect mitochondrial respiration in sharks, and Ballantyne (1997) notes the necessity of performing elasmobranch protein assays in physiological osmolyte levels to ensure native function (Lewiston et al., 1979). Therefore, mannitol was used in experiments without urea to preserve osmotic strength. Aliquots of 0.1 mM CaCl\(_2\) were added to the assay and stirred thoroughly, with fluorescence measurements (F) taken after each aliquot when the system reached equilibrium (experimentally determined to be ~ 1 minute). Maximum fluorescence (F\(_{\text{max}}\)) of Fluo-3 was measured after the addition of several aliquots of 0.1 M CaCl\(_2\) to ensure saturation of Fluo-3. Excitation was set to 505 nm, emission spectrum to 525-535 nm, and F readings taken at 530 nm. Although *R. terraenovae* can occur in a wide range of water temperatures (up to 30\(^\circ\)C), PV function is conserved over the physiological
temperature range in the eurythermal fish *Fundulus heteroclitus*, and all experiments were done at 20°C, a native water temperature for both *C. carpio* and *R. terraenovae* (J. Carlson, unpublished data; Wallace, 2005).

The equilibrium constant $K_D$ was calculated for Fluo-3 both in the presence of urea/TMAO and in the presence of urea/mannitol using non-linear regression as calculated by SigmaPlot v8.0 (SPSS Inc.). The concentration of Fluo-3 bound to calcium ($[Ca^{2+}*Fluo-3]$) was calculated by multiplying [Fluo-3] by the fraction of Fluo-3 bound to calcium ($F/F_{max}$). $[Ca^{2+}_{free}]$ was then calculated by $[Ca^{2+}_{total}]-[Ca^{2+}*Fluo-3]$. $K_D$ of Fluo-3 was calculated by a least squares fit of $[Ca^{2+}_{free}]$ to $[Ca^{2+}*Fluo-3]$ using the hyperbolic equation:

$$[Ca^{2+} * Fluo - 3] = \frac{F_{max}x[Ca^{2+}_{free}]}{K_{D Fluo-3} + [Ca^{2+}_{free}]}$$

*C. carpio* and *R. terraenovae* PV $K_D$s were calculated similarly, with $[Ca^{2+}_{free}]$ and $[Ca^{2+}*PV]$ derived from the known binding parameters of Fluo-3:

$$[Ca^{2+}_{free}] = \frac{K_{D Fluo-3}x[Ca^{2+} * Fluo - 3]}{[Fluo - 3]-[Ca^{2+} * Fluo - 3]}$$

$$[Ca^{2+} * PV] = [Ca^{2+}_{total}]-[Ca^{2+} * Fluo - 3]-[Ca^{2+}_{free}]$$

All fluorescence data was normalized for non-linear regression fits, which had minimal effect on PV $K_D$ calculations. One-way ANOVA was used to test for differences in PV $Ca^{2+}$ $K_D$ between osmolyte treatments and species.

**Sequencing of PV**

Total RNA was isolated using TRIzol reagent (Invitrogen) and first strand cDNA was synthesized by RT-PCR (Invitrogen). Amplification of PV cDNA was done with degenerate primers designed from conserved binding regions in PV and partial R. terraenovae PV sequences determined by Edman degradation. PCR product was run out on an agarose gel and purified using QIAquick® Gel Extraction Kit (Qiagen). Purified
PCR product was cloned using the TOPO TA Cloning® kit and pCR® 2.1-TOPO® vector. Cloned vectors were purified using QIAprep® Spin Miniprep Kit and submitted for direct sequencing to the FSU DNA Sequencing Facility.
RESULTS

Identification of PV, MW Estimation, and PV Quantification

The results of PV identification by Western blot are shown in Figure 3. Anti-PV antibodies identified single isoforms in all shark species tested and three isoforms in *C. carpio*. Figure 4a shows white muscle homogenates and *R. terraenovae* PV standard curve SDS-PAGE. Sizes and titer of PV are shown in Table 2. Molecular weight size estimation showed that shark PVs examined averaged 19% larger (mean MW = 12.8 kDa) than the major carp isoform II (10.7 kDa). Parvalbumin titer in carp isoform II was over 9 times higher (2.9 mg/g) than the average shark PV titer (mean = 0.315 mg/g). Western blot of two-dimensional SDS-PAGE confirmed *R. terraenovae* isoform purity (Figure 5b). *R. terraenovae* PV isoelectric point was estimated to be 4.6 by comparison with 2D SDS-PAGE standards. This is similar to PV pI estimates by two Expasy Proteomics Server tools (www.expasy.org) from partial sequence data for *R. terraenovae*, this study (pI ~ 4.9); *C. carpio*, GenBank accession number CAC83659 (pI ~ 4.5); and the leopard shark, *T. semifasciata*, GenBank accession number P30563 (pI ~ 5.0).

Purification of PV

Parvalbumin from *R. terraenovae* and the major PV isoform from *C. carpio* were isolated to near purity (Figure 5a). Of note, a slightly smaller band appeared with Western blot analysis during size-exclusion chromatography on *R. terraenovae* proteins. Whether this is a much less expressed 2nd PV isoform detected only when proteins become concentrated from the chromatography process, a PV fragment, or a false PV antibody reaction is unclear.

Fluo-3 Competition Assay

The equilibrium constant $K_D$ was determined separately for Fluo-3 and PV in urea/TMAO buffer and urea/mannitol buffer. Mean $K_D$ values ± SD for Fluo-3, *R. terraenovae* PV, and *C. carpio* PV are reported in Table 3. Data fit the assumptions of
Figure 3. Identification of PVs. *R. terraenovae* (RT); *C. limbatus* (CL); *C. isodon* (CI); *S. tiburo* (ST); *C. carpio* (CC). a) Stained SDS-PAGE; PV isoforms are indicated by arrowheads. b) Montage of Western blots for PV isoforms. PV band from *S. tiburo* (ST) required additional development.
y = 0.0016x + 0.0365
\[ R^2 = 0.9935 \]

Figure 4: PV quantification. a) Montage of stained SDS-PAGE showing *R. terraenovae* PV standard curve and muscle homogenate supernatants. *R. terraenovae* (RT), *C. limbatus* (CL), *C. isodon* (CI), *S. tiburo* (ST), *C. carpio* (CC).  b) Standard curve used to calculate PV concentrations.
Figure 5. Purification and 2D SDS-PAGE of PV. a) SDS-PAGE of purified *R. terraenovae* (RT) and *C. carpio* (CC) PV. Purity was confirmed by Western blot. b) 2D SDS-PAGE of *R. terraenovae* PV. 2D SDS-PAGE standards revealed pI \( \approx 4.6 \).
Table 2. MW and PV concentration in shark and carp skeletal muscle. MW sizes were estimated from SDS-PAGE standards. PV titer was calculated from concentrations derived from a known PV standard curve.

<table>
<thead>
<tr>
<th></th>
<th>MW (kD)</th>
<th>PV titer (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. terraenovae</em></td>
<td>13.1</td>
<td>0.19</td>
</tr>
<tr>
<td><em>C. limbatus</em></td>
<td>12.4</td>
<td>0.28</td>
</tr>
<tr>
<td><em>C. isodon</em></td>
<td>12.9</td>
<td>0.68</td>
</tr>
<tr>
<td><em>S. tiburo</em></td>
<td>12.5</td>
<td>0.26</td>
</tr>
<tr>
<td><em>C. carpio I</em></td>
<td>10.1</td>
<td>0.29</td>
</tr>
<tr>
<td><em>C. carpio II</em></td>
<td>10.6</td>
<td>2.90</td>
</tr>
<tr>
<td><em>C. carpio III</em></td>
<td>12.3</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Table 3. Ca$^{2+}$ $K_D$ of PV from *R. terraenovae* and *C. carpio* in the presence of urea/TMAO and urea/mannitol. Experiments were performed at 20°C in 150 mM K$^+$ ions at pH ~ 7.2 with 360 mM urea and either 180 mM TMAO or 180 mM mannitol. All values are nM ± SD. No significant difference in $K_D$ values was found between species or treatments (p=0.537).

<table>
<thead>
<tr>
<th></th>
<th>Fluo-3</th>
<th><em>R. terraenovae</em></th>
<th><em>C. carpio</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>urea/TMAO</td>
<td>634±41 nM (n=8)</td>
<td>5.48±1.21 nM (n=5)</td>
<td>6.27±.86 nM (n=6)</td>
</tr>
<tr>
<td>urea/mannitol</td>
<td>611±36 nM (n=7)</td>
<td>6.19±1.27 nM (n=4)</td>
<td>6.22±.46 nM (n=5)</td>
</tr>
</tbody>
</table>
Figure 6. Representative curves from competitive binding assays. [Fluo-3] and [PV] = 1.25 µM. a) Replicate titrations of Fluo-3 with Ca\(^{2+}\) in 360 mM urea, 180 mM TMAO, and 150 mM K\(^+\) at pH ~ 7.2 and 20\(\degree\)C. b) Titrations of Fluo-3 and *R. terraenovae* PV with Ca\(^{2+}\) in the presence (closed circles) and absence (open circles) of TMAO. Titrations of Fluo-3 alone are shown for comparison (triangles). c) Ca\(^{2+}\) binding to *R. terraenovae* PV in the presence of urea and the absence of TMAO. PV parameters were derived from measured Fluo-3 parameters in competitive assays.
one-way ANOVA, and no significant difference was found between species or treatment (p=0.537). Representative curves from the competitive binding assays as well as from PV Ca$^{2+}$ K$_D$ calculations are shown in Figure 6. Carp PV Ca$^{2+}$ K$_D$s are similar to values calculated by Erickson et al. (2005) in the absence of both urea and TMAO, indicating that these osmolytes do not affect PV K$_D$ at the concentrations present in the assay.

**Sequencing of PV**

Edman degradation and degenerate primers yielded 97 amino acids from *R. terraenovae* PV. Several rounds of PCR were required to express adequate product for gel purification and cloning, possibly from low expression levels of PV mRNA. Sequences were confirmed as PV from NCBI Blast search ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and results are shown in Figure 8.
DISCUSSION

This study is the first to measure the effects of physiologically relevant levels of urea in elasmobranchs on the function ($K_D$) of an ion binding protein. Parvalbumin Ca$^{2+} K_D$ from both sharpnose and carp in 360 mM urea showed no difference whether in a 2:1 ratio with TMAO or mannitol. Thus, physiologically relevant levels of urea in elasmobranchs do not influence Ca$^{2+}$ binding in PV. While myosin regulatory light chains have one functional EF hand domain (Ca$^{2+} K_D \sim 10^{-5}$ M), Ortiz-Costa et al. (2002) measured only the catalytic ATPase function in experimental assays containing K$^+$ EDTA. No change in ATPase function was observed in [urea] up to 1 M, but as these measurements were taken in non-physiological ionic parameters, it is unknown if normal muscle myosin Ca$^{2+}$ binding or ATPase activity is affected by levels of urea found in elasmobranchs.

Competitive binding assays show *R. terraenovae* Ca$^{2+} K_D$ in the presence of urea/TMAO and urea/mannitol to be \(~6 \text{ nM}, very similar to that of rat and carp PV (Eberhard and Erne, 1994; Erickson et al., 2005). This suggests that PV in white skeletal muscle plays a similar role as a Ca$^{2+}$ buffer for shark fast muscle contraction-relaxation as in other vertebrates. This is not surprising, as Ca$^{2+}$ binding proteins, such as PV, are vital to proper vertebrate skeletal muscle function.

**PV Expression is Higher in Teleost White Muscle than Elasmobranch White Muscle**

This study is the first to look at levels of PV expression in elasmobranchs. While ion affinity, sequence, and pI of shark PV are similar to those of known teleost PVS, the major PV isoform in the teleost from this study is expressed over four fold higher than the highest elasmobranch PV expression observed. Since muscle samples used for PV quantification were not taken from identical locations for all species, this study does not rigorously address PV expression from specific muscle regions. Though Thys et al. (2001) has shown that PV expression can vary along the longitudinal axis of the largemouth bass *Micropterus salmoides* by up to 25%, the marked increase in PV expression in carp compared to shark is clearly shown. Analysis by SDS-PAGE of
Figure 7. *R. terraenovae* PV expression compared to several teleost species. RT = *R. terraenovae*; BS = *Boreogadus saida* (Arctic cod); FH = *Fundulus heteroclitus* (mummichog); CC = *C. Carpio*; GG = *Gobionotothen gibberifrons* (Antarctic Notothenioid). Stained SDS-PAGE; PV isoforms are indicated by arrowheads. Note relative levels of PV expression between shark and teleost PV.
whole muscle homogenates from several other teleost species showed similar results (Figure 7). While concentrations of minor carp PV isoforms were more in-line with shark PV concentrations and markedly less than isoform II levels in carp (Figure 3 and Table 2), the functional parameters (and consequently the contribution to muscle relaxation) of isoforms I and III have not been determined.

Previous work has established an inverse relationship between PV expression and muscle relaxation rates. As the elasmobranch and teleost species in this study were chosen based on availability, there is limited predictive power in extending observations to wider taxa. Still, it is intriguing to speculate on the functional consequences of the drastically differing levels of PV between teleosts and elasmobranchs. Recent work has focused heavily on the function and comparison of red muscle in sharks and the teleost family Scombridae (Bernal et al., 2006a; Bernal et al., 2006b), but very little is known about comparisons between shark and teleost white muscle function. As noted by Altringham and Ellerby (1999), comparisons of swimming kinematics, while extensively studied in fish, are difficult due to a lack of uniform measurements. Bone (1978) notes that teleosts swim faster than elasmobranchs of comparable size, and swimming speed (as measured by tail beat frequency) would be a good indicator of such. Comparative studies of white muscle function in elasmobranchs and teleosts are worthy of future study, as PV in fish may determine certain characteristics or limitations of fast muscle swimming performance.

The Monomeric Nature of PV May Explain its Resistance to Urea Perturbation

As this study has shown, PV function is not altered in the presence of urea, and the monomeric structure may be in part responsible. Yamashita et al. (2005) have shown that metals bind to proteins in areas of hydrophilic atoms surrounded by hydrophobic atom groups, though whether these areas of high hydrophobicity contrast contribute to perturbation resistance by denaturing agents is not known. Interestingly, detection of several EF hand proteins, including troponin C and PV, is possible by performing \(^{45}\)Ca audioradiography, or as more recently described using the fluorescent Ca indicator quin2, on protein transferred from SDS-PAGE to a PVDF membrane (Maruyama et al., 1984; Tatsumi et al., 1997). This method uses SDS and β-mercaptoethanol and shows that the
EF hand is to some degree still functional in a denatured state. Thus, function of EF hand proteins such as PV in the presence denaturing agents such as urea or SDS may be due to structural durability in this protein family. Resistance to urea perturbation is one of several experimental parameters that do not affect PV function; nominal changes in ionic strength (± 50 mM) and pH have also shown to minimally affect PV $K_D$s in several teleost species (Erickson et al., 2005; Erickson and Moerland, 2006).

Alteration of protein function by urea levels in elasmobranchs has only been shown to occur in elasmobranch and mammalian multimeric proteins. Studies dating back to Bonaventura et al. (1974) show that lower concentrations of urea are needed to disrupt the quaternary structure of proteins than to unfold individual monomers (Griessler et al., 2003). Subunits of multimeric enzymes are non-covalently joined into a quaternary structure by hydrophobic interactions and hydrogen bonds, both of which have been shown to be targets of urea denaturation. Additionally, the catalytic sites of multimeric proteins may be located where two subunits interface (Whitford, 2005). Quaternary structure is often required for function of multimeric proteins, and urea levels in elasmobranchs may be high enough to perturb subunit interactions and catalytic sites. Urea in the molar range is often required to disrupt tertiary structure, and this may explain why urea levels in the millimolar range do not affect PV, but do alter multimeric protein function. Interestingly, there are reports of elasmobranch hemoglobin (HB) to remain functional in molar concentrations of urea, while human HB subunits split into monomers at much lower urea levels (Bonaventura et al., 1974). Solved crystal structures indicate that certain shark HB sequences differ from human HB in areas of subunit interface, indicating that structural modification may be necessary for multimeric proteins to maintain function in the presence of urea (Naoi et al., 2001).

**Comparison of PV Sequences**

In PV, six amino acids coordinate $\text{Ca}^{2+}$ in the CD binding pocket and five amino acids coordinate $\text{Ca}^{2+}$ in the EF pocket (Figure 8). As predicted from conservation of PV function in *R. terraenovae*, these coordinating residues are identical to coordinating residues in other teleosts as well as mammalian PV. Interestingly, the only skate PV sequence in the literature, *Raja clavata*, shows a tyrosine substitution at coordinating
residue 56 for phenylalanine. As PV function in Rajiformes is unexplored, it is unknown if this substitution of a neutral hydrophobic residue for a polar hydrophobic residue affects Ca$^{2+}$ binding.

**Summary**

This study has shown that Ca$^{2+}$ binding in *R. terraenovae* and *C. carpio* is unaffected by urea levels in elasmobranchs. PV is therefore not dependant on the counteracting solute system that has been shown to be necessary for several enzymes to function in urea ~400 mM. This is the first study to explore elasmobranch ion-binding protein function, and whether this resistance to urea perturbation is related to the monomeric structure of PV or to the properties of protein metal-binding sites deserves further study. Great strides have been made in revealing the mechanism of protein denaturation, and proteins such as PV may illuminate certain motifs that exhibit resistance to chaotropic agents. Additionally, the comparatively low levels of PV expression in shark white skeletal muscle may be indicative of differences between elasmobranch and teleost white muscle function. The recent attention given to the role of PV in slow muscle makes the internalized red muscle of the endothermic lamnid sharks of particular interest. As the role of PV in elasmobranch muscle kinetics and contraction has not been addressed, the characterization of PV in various shark body types may reveal new insights into muscle function in cartilaginous fishes.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
<th>Sequence 4</th>
<th>Sequence 5</th>
</tr>
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<tbody>
<tr>
<td><em>R. terraenovae</em></td>
<td>PMTKVLKEDD INKALSAFKD PGTFDYKRFF QLVGLKGKSE AQVKEVFIEL</td>
<td>PMTKVLKADD INKAlSAFKD PGTFDYKRFF HLVGLKGKD AQVKEVFIEL</td>
<td>SSKITSILNP ADITKALEQC AAGFHHTAFF KASGLSKKSD AELAEIFNVL</td>
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<td>SMTDLLNAED IKAVGAFSA TDSFDKFF QMVGLKKSA DDVKKVFHML</td>
</tr>
<tr>
<td><em>T. semifasciata</em></td>
<td>DKDRSGFIEE EBLKSVLKGF SAHGRDSLDS TTAKALEAGD SDHDGKI</td>
<td>DKDSGFIEE EBLKGVLKGF SAHGRDLNDT ETAKALLAGD SDHDGKIGAD FFAKMVAQA</td>
<td>DGDQSGYIEV EBLKNFKCF SGARVLNDD ETSLNAGSD SDGDHGKILD EFKSMKMT</td>
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<td>DQDSGFEIEE DELKFLQNF MLAGARALTDG ETKFLSAGD SDGDHGKIGVD EFTAVLK</td>
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<tr>
<td><em>R. clavata</em></td>
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<td>DQDKSFMLIEE DELKLFQNF MLDARALTDG ETKFLKGQAD SDGDGKIGVD EFTALVKA</td>
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<td>DQDSGFEIEE DELKFLQNF MLAGARALTDG ETKFLSAGD SDGDHGKIGVD EFTAVLK</td>
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<tr>
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<td>DQDKSFMLIEE DELKLFQNF MLAGARALTDG ETKFLSAGD SDGDHGKIGVD EFTAVLK</td>
<td>DQDSGFEIEE DELKFLQNF MLAGARALTDG ETKFLSAGD SDGDHGKIGVD EFTAVLK</td>
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</tr>
<tr>
<td><em>D. rerio</em></td>
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<td>DKDSGFIEE EBLKSVLKGF SAHGRDSLDS TTAKALEAGD SDHDGKI</td>
<td>DKDSGFIEE EBLKSVLKGF SAHGRDSLDS TTAKALEAGD SDHDGKI</td>
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<td>DKDSGFIEE EBLKSVLKGF SAHGRDSLDS TTAKALEAGD SDHDGKI</td>
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<tr>
<td><em>H. sapiens</em></td>
<td>SMTDLLNAED IKAVGAFSA TDSFDKFF QMVGLKKSA DDVKKVFHML</td>
<td>SMTDLLNAED IKAVGAFSA TDSFDKFF QMVGLKKSA DDVKKVFHML</td>
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<td>SMTDLLNAED IKAVGAFSA TDSFDKFF QMVGLKKSA DDVKKVFHML</td>
</tr>
</tbody>
</table>

Figure 8. Comparison of PV sequences. CD, and EF motifs are marked and coordinating residues are in yellow. *R. terraenovae*, this study; *T. semifasciata* (leopard shark), GenBank accession# P30563; *R. clavata* (thornback ray), GenBank accession# P02630; *D. rerio* (zebrafish), GenBank accession# CAK05381; *H. sapiens* (human), GenBank accession# CAA44792.
REFERENCES


Neil Sanscrainte was born in 1981 and raised in Toledo, OH. After graduating from Toledo Christian High School, he attended the University of North Carolina at Wilmington, NC. He graduated from UNCW in 2003 with a B.S. in Marine Biology and an English minor, earning University and Departmental Honors (Honors thesis: “Feeding ecology of neonate Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*): field and laboratory observations”). Neil pursued his Master’s in Biology at Florida State University, graduating in 2006. His Master’s work involved the molecular and functional characterization of parvalbumin in the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. Neil and his fiancée Christine M. Glenn will wed in August 2006 and move to Gainesville, FL, where Neil will work as a Molecular Biology Laboratory Technician for the USDA Center for Medical, Agricultural, and Veterinary Entomology’s Mosquito and Fly Research Unit.