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Temperature Dependence of Ion Binding of Parvalbumin from the Eurythermal Teleost, Fundulus Heteroclitus

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TEMPERATURE DEPENDENCE OF ION BINDING
OF PARVALBUMIN FROM THE EURYTHERMAL TELEOST,
FUNDULUS HETEROCLOITUS

By

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ABSTRACT

*Fundulus heteroclitus*, or mummichogs, are small (6-10 cm) teleost fish that are common in salt marshes, bays and estuaries along the East Coast of North America. The distribution of this species is such that populations in the extreme north experience a mean annual temperature of 6°C while those in the south are exposed to a mean annual temperature of 20°C, a thermal gradient of roughly 1°C per degree of latitude. *F. heteroclitus* are extremely polymorphic and several metabolic enzyme-encoding loci (*Ldh-B, Mdh-A, Gpi-B, Idh-A, Est-S*) have been found to vary greatly between northern and southern populations forming distinct clines. It has been suggested that gene frequency variance in isozymes may be a compensatory response to temperature. The non-catalytic ion binding protein, parvalbumin (PV), is thought to increase the rate of relaxation in fast twitch muscle fibers through its high affinity for calcium (Ca\(^{++}\)). The purpose of this study was to determine if PV from *F. heteroclitus* would also exhibit clinal variance between northern and southern populations in response to the steep temperature gradient. The amino acid sequence for the major isoform of PV was determined from four populations of *F. heteroclitus* collected from the East Coast of the United States. These populations represented the observed gene frequency based clinal variation between northern and southern populations of *F. heteroclitus*. The dissociation constants (K\(_D\)) of this PV isoform binding to Ca\(^{++}\) and Mg\(^{++}\) were determined for a temperature regime of 0-25°C. Additionally, the amino acid sequence from a sister species, *Fundulus grandis*, the Gulf killifish, and an outgroup, *Fundulus similis*, the Longnose killifish, were obtained and compared to *F. heteroclitus* to examine divergence within the genus. Due to the northerly invasion of *F. heteroclitus* it was expected that PV from northern populations would maintain function at lower temperatures than southern populations. The major isoform expressed in all populations of *F. heteroclitus* was identical by amino acid sequence analysis, indicating the absence of clinal variance in the expressed gene. Ion binding function of for this PV isoform is relatively insensitive to temperature. K\(_D\) values for PV binding to Ca\(^{++}\) and Mg\(^{++}\) at 0, 5, 15, and 25°C were 7.88 ± 1.073,
7.77 ± 0.5, 7.64 ± 0.633, and 8.25 ± 0.55 and 0.24 ± 0.014, 0.32 ± 0.086, 0.36 ± 0.027, and 0.16 ± 0.019 mM, respectively.
INTRODUCTION

Thermal variance in *F. heteroclitus* habitats

The teleost genus *Fundulus* is established along the length of the East Coast of North America and throughout the Gulf of Mexico (Parenti, 1981). As shown in Figure 1 populations of the species *F. heteroclitus* are found from Newfoundland, Canada to northeastern Florida (Able and Felley, 1986). This distribution encompasses a range of habitats in which temperature can vary over a wide range on a daily and seasonal basis, and as a function of latitude. *F. heteroclitus* are found in bays, saltwater marshes, and tidal creeks, and over the course of one tidal cycle they can be exposed to wide temperature fluctuations. Populations in the extreme north, for example, can experience a 14°C change in less than an hour (Sidell et al., 1983). In addition to acute, daily changes in water temperature populations in northern latitudes experience seasonal cycles in water temperature in excess of 20°C. Finally, the thermal gradient experienced by *F. heteroclitus* along the east coast is also quite dramatic, with a change of 1°C per degree of latitude (Crawford and Powers, 1992). Northern populations are exposed to an annual mean temperature of 6°C while southern populations experience an annual mean temperature of 20°C (Powers et al., 1986).

Biogeographical distribution of *F. heteroclitus*

The *Fundulus* genus is thought to be descended from a common ancestor that also includes those species within the genus *Profundulus* that are native to northern Central America and southern Mexico. Expansion of *Fundulus* occurred in a south to north migratory pattern with *F. heteroclitus* settling along the East Coast of North America (Parenti, 1981).

It has been proposed that settlement of *F. heteroclitus* was by secondary intergradation (Cashon et al., 1981; Powers et al., 1986). The model of secondary intergradation is defined by a period of time in which a barrier to gene flow exists and the existence of such barriers produces
Figure 1. Distribution of *F. heteroclitus* along the east coast of North America (shaded region). Division of populations indicated with arrows and collection sites are denoted with ★.
genetically distinct populations through genetic drift and/or selective forces (Powers et al., 1986). In the case of *F. heteroclitus*, the operative barrier is thought to be associated with glaciation. The last glacial age, ending between 22,000 to 18,000 years ago found most of the north central and northeastern portion of the United States to be under the Laurentide ice sheet (Mickelson et al., 1983). This ice sheet extended south to what is today northern New Jersey, therefore, the northern-most populations of *F. heteroclitus* most likely stopped at southern New Jersey (Ropson et al., 1990).

In an examination of the secondary intergradation model of *F. heteroclitus* distribution, Gonzalez-Villasenor and Powers (1990) examined 48 mitochondrial-DNA (mtDNA) samples via restriction-endonuclease digestion from four populations of *F. heteroclitus*. These populations included one northern representative (Wiscasset, ME), one southern representative (Sapelo Island, GA) and two populations (one in northern New Jersey and one in southern New Jersey) that corresponded to the zone of contact between northern and southern counterparts (Fig. 1). Examination of mtDNA restriction site clones produced two clusters with the northern populations in one cluster and the southern populations in the other. Using the estimated rate of mtDNA sequence divergence for mammals (2% per one million years) Gonzalez-Villasenor and Powers (1990) found the common ancestor of the northern and southern populations to have lived around one million years ago, suggesting the barrier to gene flow occurred well before the last glacial age. While the precise timing and nature of the barrier to gene flow are unknown, temperature is thought to play a pervasive role in the continued deviation between northern and southern populations (Crawford and Powers, 1992; Powers and Place, 1978; Rees et al., 2001; Schulte et al., 2000).

**Clinal variation in *F. heteroclitus* isozymes**

Previous work has shown that *F. heteroclitus* protein encoding loci are extremely polymorphic. Indeed, 70 loci examined showed protein polymorphism to be greater than 50% (Mitton and Koehn, 1974; Powers and Place, 1978). Examination of several polymorphic enzyme-encoding loci within and among populations of mummichogs has shown that *F. heteroclitus* are organized into distinctive clines along the eastern seaboard, with geographically
directional changes in gene frequency (Cashon et al., 1981; Powers et al., 1986; Ropson et al., 1990).

It is often difficult to determine whether polymorphic loci are important to the overall fitness of an organism and thereby subject to selective pressure, or the result of genetic drift. *F. heteroclitus* have proven to be an excellent model organism in which to test the importance of genetic variation across a biogeographical region. Brown and Chapman (1991) found that retention of observed clines could only be maintained outside of natural selection if *F. heteroclitus* exhibited a dispersal distance of less than 1-2 km. Winter movements of *F. heteroclitus* populations have been observed to be at least 2km (Lotrich, 1975). The disparity between mean temperatures to which northern and southern populations are exposed has widely been seen as a candidate for maintenance of geographical clines (Crawford and Powers, 1992; Powers and Place, 1978; Rees et al., 2001; Schulte et al., 2000) suggesting that temperature is a key variable in maintaining clinal variation in mummichog isozymes.

To test temperature as a possible selective agent for the maintenance of clinal gene frequency in *F. heteroclitus*, the function of select rate-limiting enzymes of energy metabolism (*Ldh-B, Mdh-A, Gpi-B, Idh-A* and *B, Est-S*) have been examined across a range of temperatures (Cashon et al., 1981; Powers and Place, 1978). While all of these loci exhibited clinal variation in gene frequency, and several also exhibited functional modification in relation to temperature, none have been as comprehensively examined as cardiac lactate dehydrogenase (*Ldh-B*) (Crawford and Powers, 1989; DiMichele and Powers, 1991; Rees et al., 2001; Schulte et al., 2000), which catalyzes the reversible reaction that transforms pyruvate into lactate. Two codominant alleles of *Ldh-B* have been identified in *F. heteroclitus*; *Ldh-B<sup>a</sup>* predominates in southern populations and *Ldh-B<sup>b</sup>* in northern populations (Cashon et al., 1981; Powers et al., 1991). The *Ldh-B* alleles have greater catalytic efficiency and show conservation of similar *K<sub>m</sub>* values at the physiological temperature in which they are predominant (Powers et al., 1991). In addition to increased efficiency at physiological temperatures northern populations maintain higher *Ldh-B<sup>b</sup>* concentrations vs. *Ldh-B<sup>a</sup>* concentrations in southern populations. The high level of *Ldh-B<sup>b</sup>* is thought to be a compensatory measure in response to colder environmental temperature. *Ldh-B<sup>b</sup>* transcription rates were tested in northern populations acclimated to 25°C and no rate change was observed, indicating that this trait is a genetically based, heritable quality (Crawford and Powers, 1989). Clearly, temperature
has played a significant role in shaping enzyme allele function between northern and southern mummichog populations.

**Temperature sensitivity of proteins**

All physico-chemical processes are inherently sensitive to temperature, and the temperature dependence of protein function is of particular interest in understanding the natural history of poikilothermic organisms (Jaenicke, 1991; Low et al., 1973; Somero, 1995; Somero and Low, 1977). In general, proteins are only marginally stable at physiological temperatures, which is thought to reflect a compromise between structural integrity and the flexibility of conformation needed for catalysis and proper ligand binding (Hochachka and Somero, 2002; Jaenicke, 1991). Examination of protein homologs from populations representing habitats with widely varying temperatures has shown conservation of functional parameters at the organism’s environmental temperature (Erickson et al., 2005; Moerland, 1995; Somero, 1995; Somero and Low, 1977).

Study of protein homologs from varying thermal regimes has illuminated some of the mechanisms employed in response to temperature. These include alterations in protein packing, changes in hydrophobic interactions, protein-solute stabilization, substitutions in protein primary structure, and variation in gene expression (Fields et al., 2001; Jaenicke, 1991; Jaenicke and Bohm, 1998; Somero and Low, 1977; Zavodszky et al., 1998). A generalization that has emerged from these studies is that cold-adapted enzymes differ from their warm-adapted orthologs via amino acid substitutions that increase the flexibility of structures near the binding region. These same amino acid substitutions have been shown to decrease the binding efficiency of cold-adapted enzymes at higher temperatures, suggesting that conformational plasticity is “tuned” to the environmental temperature at which the enzyme functions (Fields and Somero, 1998). Amino acid sequence analysis has shown that a relatively small number of substitutions can create dramatic variations in transcription, stability, and/or function of a protein in relation to temperature (Crawford et al., 1999a; Davis et al., 2004; Schulte et al., 2000). Additionally, in enzyme homologs for which sequence data is known, residue substitutions are almost universally found outside the active site of the protein (Fields and Somero, 1998; Hochachka and Somero, 2002; Holland et al., 1997).
Binding regions in protein homologs exhibit high sequence conservation, likely a reflection of the need to preserve covalent chemistry in ligand coordination by the active site (Jaenicke, 1991; Somero, 1995). Enzyme homologs must retain the ability to coordinate the appropriate reactant and allow for rapid transformation of substrate into product. Substrate transformation was long thought to limit the rate of enzyme catalysis but recent research has implicated conformational changes that occur during the binding event as the rate-limiting step (Hochachka and Somero, 2002). Adaptations in kinetic properties between cold- and warm-adapted homologs appear to be a result of amino acid substitutions that alter conformational plasticity. For example, cold-adapted homologs have residue substitutions that increase the overall flexibility of the protein, a compensatory adaptation to the decreased kinetic motion in a colder environment (Fields and Somero, 1998; Holland et al., 1997). However, when exposed to high temperatures maintenance of structural integrity is compromised resulting in lower catalytic efficiency or even protein degradation. This study focused on the non-catalytic protein parvalbumin (PV) from *F. heteroclitus* to determine the adaptive response it has undergone to maintain ion-binding function across the disparate temperatures to which this species is exposed.

**PV in muscle**

Parvalbumin is a small ($m_w \sim 12,000$), intracellular non-catalytic protein with two ion-binding regions. While present in several invertebrate and vertebrate species, PV is expressed at especially high concentrations in the cytosol of both fish and amphibian fast-twitch skeletal muscle ($\sim 0.75$ mM) (Rall, 1996). Not just a member of the calcium-binding EF-hand family of proteins, PV served as the model that led to the discovery of the EF-hand motif. Two homologous helix-loop-helix binding sites that bind both Ca$^{++}$ and Mg$^{++}$ by ion-specific residue coordination typically characterize EF-hand proteins. In addition to PV, the family includes such regulatory proteins as troponin C (TnC), myosin regulatory light chains and calmodulin (Nakayama and Kretsinger, 1994). EF-hand proteins are involved in signaling, second messenger production, cell cycle regulation and muscle contraction. Parvalbumin contains three domains denoted AB, CD, and EF; of these three only the CD and EF loops are able to coordinate ions with physiologically relevant affinity. Both loops are 12 amino acids in length and coordinate Ca$^{++}$ with 7 or 8 oxygen ligands, octahedral coordination geometry being more common, and Mg$^{++}$ with only 6 ligands (Cates et al., 1999). The truncated loop of the AB domain renders it
unable to coordinate divalent cations, however it has been implicated in stabilization of the tertiary structure of PV (Thepaut et al., 2001).

Parvalbumin is thought to act as a Ca$^{++}$ buffer in the relaxation phase of fast-twitch skeletal muscle contraction (Rall, 1996). Muscle contraction begins when ryanodine receptors located in the membrane of the sarcoplasmic reticulum (SR) open in response to an action potential releasing Ca$^{++}$ into the fiber’s myoplasm. Ionized calcium binds to TnC inducing a conformational shift that allows thin actin filaments to interact with myosin. When ATP binds to the myosin head, cross-bridges form between actin and myosin causing contraction. The relaxation phase begins when Ca$^{++}$ is removed from the cytoplasm by the Ca$^{++}$ ATPase pumps of the sarcoplasmic reticulum (SR). Decreased cytosolic Ca$^{++}$ concentration (~ $10^{-7}$ M) promotes dissociation of Ca$^{++}$ from TnC that allows for the interaction of TnC with tropomyosin causing inhibition of actin and myosin contact (Gillis, 1985).

Parvalbumin non-cooperatively binds two moles of Ca$^{++}$ per one mole of protein with an affinity for Ca$^{++}$ that is about 4 orders of magnitude higher than TnC. Also, PV concentration can

![Figure 2. Illustration of proposed PV function in muscle relaxation (Hou et al., 1993)](image-url)
be ten times more concentrated than TnC within the muscle (Rall, 1996). This leads to a mechanistic contradiction: how can Ca\(^{++}\) bind to TnC while PV is present? Concentration of free Ca\(^{++}\) in a quiescent cell (\(<10^{-8}\) M) is not sufficient to elicit significant binding from EF-hand proteins. Cellular Mg\(^{++}\) concentrations at rest (1mM), however, are observed to be 4 orders of magnitude greater than free Ca\(^{++}\). Parvalbumin, then, remains loaded with Mg\(^{++}\) until the surrounding environment becomes saturated with Ca\(^{++}\). Dissociation of Mg\(^{++}\) from PV is slow relative to a single Ca\(^{++}\) transient and Mg\(^{++}\) does not bind significantly to TnC in a resting muscle fiber, therefore, upon cytosolic excitation and release of Ca\(^{++}\), Ca\(^{++}\) will bind to TnC sites first (Fig. 2). While PV is not a regulatory protein, its function as an aid to muscle relaxation is intricately linked to the Ca\(^{++}\) affinity of TnC which does regulate muscle contraction and resting cellular concentrations of Ca\(^{++}\) and Mg\(^{++}\). Therefore, conservation of PV affinity for Ca\(^{++}\) and Mg\(^{++}\) (measured here as K\(_D\)) becomes essential to preserving contractile function.

Mummichogs are able to maintain muscle function during rapid and extreme temperature change. They have also shown a clear pattern in gene frequency variance across latitude that exposes populations to a 14°C temperature gradient along the east coast of the United States. The function of PV has demonstrated sensitivity to temperature much like what has been observed for binding in catalytic proteins. Dissociation constants for Ca\(^{++}\) and Mg\(^{++}\) consistently increase with increasing temperature and maintain similar values for homologs when tested at environmental temperatures (Eberhard and Erne, 1994; Erickson et al., 2005). In general, the temperature dependence of K\(_D\) is due to faster dissociation rates for Ca\(^{++}\) and Mg\(^{++}\) with increasing temperature (Hou et al., 1992).

Examination of gene frequency variance across biogeographical regions has been limited to catalytic proteins involved in energy metabolism. The present study investigated the characteristics of a non-catalytic protein, PV, to determine whether or not it also demonstrates clinal variance in *F. heteroclitus* populations. Due to the natural variance in temperature to which *F. heteroclitus* populations are exposed, it was hypothesized that PV would function over a wide range of temperatures and that northern populations would retain PV function at lower temperatures than their southern counterparts. The results of this study indicate that, while PV is able to function across all temperatures to which *F. heteroclitus* populations are exposed, the major isoform of this protein does not maintain clinal variance in the expressed gene product or in Ca\(^{++}\) and Mg\(^{++}\) K\(_D\)’s.
MATERIALS AND METHODS

Fish collection

Northern populations of *F. heteroclitus* were obtained from Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine and Marine Biological Laboratory, Woods Hole, Massachusetts. Representatives of the southern populations were obtained from Tex’s Tackle in Wilmington, North Carolina and through the generous donation of Paul Christian at the UGA Marine Extension Service in Brunswick, Georgia (Fig. 1). *F. grandis* and *similis* were collected locally from the Florida State University marine lab located at Turkey Bayou on the Gulf of Mexico. Animals were euthanized with 3-aminobenzoic acid ethyl ester (MS-222) and, if white muscle tissue was not removed immediately for use, stored at –80°C.

Purification of parvalbumin

Parvalbumin was purified according to the methods of Laney et al. (1997) with slight modification. Briefly, homogenized muscle tissue was subjected to 70% and 100% ammonium sulfate (AMS) fractionation. The resultant pellet containing PV was then separated from higher molecular weight proteins by gel permeation chromatography (Sephacryl S100). *F. heteroclitus* has two isoforms of PV that were further separated through ion exchange chromatography (DEAE-cellulose) (Fig. 3). Purity of PV was determined by 17.5 % SDS-PAGE, 2D gel electrophoresis, and Western blots. The primary antibody used for all Western blots was monoclonal anti-parvalbumin (Sigma-Aldrich Co., St. Louis, MO). Positive bands were visualized by ECL Plus Western blotting detection reagents (Amersham Biosciences Corp., Piscataway, NJ) using Anti-mouse IgG peroxidase conjugate (Sigma) or by stabilized Western Blue® substrate (Promega Corp., Madison, WI) using Goat Anti-Mouse IgG-AP (Bio-Rad Laboratories Inc., Hercules, CA). Only the most abundant PV isoform was used in this study.
Figure 3. Panel A depicts sample AMS fractionation. Panel B illustrates the purified major isoform (lanes 1-4) after ion-exchange chromatography. Lane 5 represents combined PV fractions after size-exclusion chromatography.

**Removal of divalent cations**

For measurements requiring the absence of divalent cations, PV was dialyzed against 4M urea, 5% Chelex 100 resin and 10mM EDTA for 24hrs at 4°C. Renaturation of PV and removal
of EDTA was by dialysis against 4L of the appropriate buffer at 4°C. Divalent cations were removed from buffers through filtration after a brief incubation with 10% Chelex 100 resin.

**Kₐ determinations for Ca²⁺ and Mg²⁺**

A fluorescent calcium indicator was used to determine Kₐ for Ca²⁺. The fluorescent indicator Fluo-3 (Molecular Probes Inc., Eugene, OR) was used in a competitive binding assay developed by Eberhard and Erne (1991). Briefly, changes in fluorescence intensity were observed upon addition of CaCl₂ aliquots in the presence of Fluo-3 and a known concentration of

![Figure 4. Sample data set for Kₐ determination of Woods Hole, MA *F. heteroclitus* PV using the fluorescent indicator Fluo-3.](image-url)
cation free PV. Experiments were carried out in a buffer solution containing 20mM Hepes, 150mM KCl, adjusted to an initial pH of 7.2. Fluctuations of pH between 6.5-8.5 do not significantly affect PV $K_D$ (Erickson et al., 2005) therefore pH was allowed to vary with temperature ($\Delta pH \Delta T^{-1} = -0.014 ^\circ C^{-1}$) (Ferguson et al., 1980). Fluo-3 and native PV isolated from the Woods Hole, MA population were added to a final concentration of 1220nM and 155nM, respectively. These measurements were then compared to the fluorescence of Fluo-3 upon addition of Ca$^{++}$ in the absence of PV. Ionized calcium $K_D$ was determined by non-linear least-squares fit of fluorescence intensity vs. [Ca$^{++}$]. Figure 4 depicts a sample data set for Ca$^{++}$ $K_D$. Ionized magnesium $K_D$ was determined by a similar method developed by Erickson and Moerland (personal communication) using Magnesium Green (Molecular Probes) as the fluorescent indicator and Mg$^{++}$ as the ligand.

**Primary sequence analysis of parvalbumin**

Total RNA was isolated from homogenized muscle tissue using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturers directions, which is an extension of the method developed by Chomczynski and Sacchi (1987). First strand cDNA was synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR) using an oligo (dT) primer. Table 1 depicts degenerate primers designed from the aligned amino acid sequences of known teleost ($C. carpio$, $D. rerio$, $G. morhua$, $S. japonicus$, and $S. alpinus$) PV and partial $F. heteroclitus$ PV amino acid sequences. $F. heteroclitus$ partial sequences were obtained through in-gel trypsin digestion of the most abundant isoform of PV and then purified by reversed-phase HPLC. The purified peptides were then sequenced by standard Edman degradation. The degenerate primers were used to amplify a partial PV nucleotide sequence from the synthesized cDNA using PCR. Once a partial sequence specific to $F. heteroclitus$ had been obtained, gene specific primers were designed to amplify PV cDNA from the Woods Hole population using rapid amplification of cDNA ends (RACE-PCR). Primers were then designed to the non-coding regions flanking the 5’ and 3’ ends of PV (Table 1). These primers were then used to amplify full-length PV from all $F. heteroclitus$ populations and two other Fundulus species. Amplified products were cloned using TOPO TA Cloning® reagents (Invitrogen) and transformed into TOP10 cells (Invitrogen) as per manufacturer’s instructions. Transformation products were
grown overnight on rich LB media plates and positive clones were identified with blue/white screening. Positive clones were cultured over night in rich LB media with 50ug/mL of ampicillian. Plasmids were purified using a QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA) according to manufacturer’s instructions. All samples were sequenced with an ABI 3100 using the BDT Version 3.1 sequencing mix (Amersham Biosciences).

Table 1. Primers designed for use in determining PV amino acid sequences for three *Fundulus* species

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<th>Primer Name</th>
<th>Nucleotide sequence</th>
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<tr>
<td>Degenerate Forward</td>
<td>5’- CAYAARGAYTTYTTYGANAA -3’</td>
</tr>
<tr>
<td>Degenerate Reverse</td>
<td>5’- GCRAAYTCRTCNACNCCDATYTT -3’</td>
</tr>
<tr>
<td>Gene-specific Forward</td>
<td>5’- GTCTCGGCATCGGTCAGAGC -3’</td>
</tr>
<tr>
<td>Gene-specific Reverse</td>
<td>5’- CATCAAGAAGGCCCTTCGCGG -3’</td>
</tr>
<tr>
<td>Non-coding region Forward</td>
<td>5’- CATCTGAGCGAATCCACTCT -3’</td>
</tr>
<tr>
<td>Non-coding region Reverse</td>
<td>5’- AGTCAGATGTTGGTCACTCC -3’</td>
</tr>
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RESULTS

PV Ca\textsuperscript{++} K\textsubscript{D}

The Ca\textsuperscript{++} K\textsubscript{D}’s measured for native Woods Hole, MA *F. heteroclitus* PV at assay temperatures of 0, 5, 15, and 25\textdegree C were 7.88 ± 1.073, 7.77 ± 0.5, 7.64 ± 0.633, and 8.25 ± 0.55 nM, respectively (mean ± SD). Figure 5 displays a comparison of this data to other similarly tested teleost species (Erickson et al.) with 95% confidence intervals included for each regression. The species represented by the Antarctic pattern (*Gobionotothen gibberifrons* and *Chaenocephalus aceratus*) are found in waters with a mean temperature of -1.9\textdegree C, those represented by the temperate zone pattern (*Cyprinus carpio* and *Micropterus salmoides*) have a mean habitat temperature of ~20-25\textdegree C, while *F. heteroclitus*, as previously stated, are exposed to varying temperature regimes.

The Ca\textsuperscript{++} K\textsubscript{D} values for *F. heteroclitus* PV were analyzed by one-way analysis of variance (ANOVA) for each temperature and no significant difference was found (P = 0.920). The $\beta_1$ and $r^2$ values obtained from linear regression of all mummichog K\textsubscript{D} values were 0.013 and 0.312, respectively. The low values imply that the relationship between temperature and *F. heteroclitus* PV Ca\textsuperscript{++} K\textsubscript{D} is not strong. For comparison, the $\beta_1$ and $r^2$ values for the Antarctic and temperate zone patterns were 0.472 and 0.993 and 0.238 and 0.980, respectively. The temperature coefficient ($Q_{10}$) for PV Ca\textsuperscript{++} K\textsubscript{D} was 1.02. These data indicate the relative temperature independence of *F. heteroclitus* PV binding to Ca\textsuperscript{++}. Also note the apparent conservation of K\textsubscript{D} between 6 and 9 nM at physiologically relevant temperatures suggesting an advantage in preserving similar binding function at native temperatures.

PV Mg\textsuperscript{++} K\textsubscript{D}

Using Magnesium Green as a fluorescent indicator, Mg\textsuperscript{++} K\textsubscript{D}’s for *F. heteroclitus* at 0, 5, 15, and 25\textdegree C were found to be 0.24 ± 0.014, 0.32 ± 0.086, 0.36 ± 0.027, and 0.16 ± 0.019 mM,
respectively (mean ± SD) (Fig. 6). These values qualitatively support decreased temperature
dependence in Mg$^{++}$ binding to mummichog PV but the data were unable to be analyzed as
described above due to the variance between $K_D$ values.

\[ Ca^{++} K_D \text{ vs. Temperature (°C) } \]

Figure 5. Effects of temperature on Ca$^{++} K_D$ for *F. heteroclitus* PV (error bars include one SD). PV Ca$^{++} K_D$ patterns for Antartic species (dashed line) and temperate zone species (dotted line) (Erickson et al., 2005). 95% confidence limits included for all regressions.
Figure 6. Effects of temperature on Mg$^{++}$ $K_D$ for *F. heteroclitus* PV (error bars include one SD). PV Mg$^{++}$ $K_D$ patterns for the Antarctic species, *C. aceratus* (dashed line) and the temperate zone species *C. carpio* (dotted line) (Erickson, J. R., personal communication). 95% confidence limits included for all regressions.
Primary sequence analysis of *Fundulus* species

Both northern and southern populations of *F. heteroclitus* have identical PV amino acid composition. Binding function was assumed to be identical between all populations due to 100% primary sequence consensus between *F. heteroclitus* populations.

Figure 7 shows *F. heteroclitus* in comparison with two additional *Fundulus* species, *F. grandis* and *F. similis*, obtained in this study. *F. grandis* and *F. similis* differ from *F. heteroclitus* by one and four amino acids respectively. All three species expressed a high degree of sequence conservation in the canonical binding regions of PV. Sequence consensus in comparison to *F. heteroclitus* denoted by a period.

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CD binding site

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EF binding site

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Figure 7. Aligned parvalbumin sequences for *F. heteroclitus*, *F. grandis* and *F. similis.*
DISCUSSION

Previous studies have shown variance with latitude in the gene frequency of catalytic proteins from populations of *F. heteroclitus* (Cashon et al., 1981; Crawford et al., 1999b). The disparity between annual mean temperatures to which northern and southern populations are exposed has widely been seen as a candidate for maintenance of geographical clines (Crawford and Powers, 1992; Powers and Place, 1978; Rees et al., 2001; Schulte et al., 2000). Temperature can have a profound effect on the conformational flexibility of a protein and, in turn, on its binding function. Like other enzyme homologs from cold- and warm-adapted organisms, functional characteristics of northern and southern *F. heteroclitus* isozymes appear “tuned” to their native environmental temperatures. As a general rule, homologs retain similar function when tested at physiologically relevant temperatures and when tested across a suite of temperatures enzyme $K_m$ values (substrate concentration at $\frac{1}{2} V_{\text{max}}$) increase as temperature increases. Additionally, cold-adapted homologs tend to exhibit elevated values in comparison to warm-adapted homologs when tested at common temperatures (Fields and Somero, 1998; Holland et al., 1997; Somero, 1995).

Erickson et al. (2005) demonstrated that the enzyme pattern of functional response to temperature is also seen in PV, a non-catalytic protein. Parvalbumin has been shown to increase the rate of muscle relaxation after contraction. Rapid contraction/relaxation cycles could be extremely advantageous in prey capture and predator avoidance. For example, the high concentration of PV observed in teleost anaerobic muscle could contribute to increased tail beat velocity, thereby increasing burst swimming speed. Conservation of ligand binding parameters at physiologically relevant temperatures, such as the optimization of $K_D$, may be pivotal to the maintenance of rapid contractile function.

Proteins *in vivo* exist at a suite of conformation states permitted by primary structure, not all of them functionally viable. Temperature can impose constraints on the number of possible conformations in addition to determining the rate at which proteins shift among them (Fields and Somero, 1998). Previous studies have identified substitutions in areas of the protein that control
structural movements. Cold-adapted enzymes have employed certain substitutions that alter steric hindrance and charge repulsion to increase flexibility at low temperatures. High conformational plasticity results in an increase in the number of conformational states available, which decreases the amount of binding-competent states. It is this decrease in the proportion of available binding states that leads to higher $K_m$ values when tested at common temperatures with warm-adapted homologs (Fields and Somero, 1998; Holland et al., 1997; Somero, 1995). This pattern would seem a likely candidate for the observed response in $K_D$ values to temperature for the non-catalytic protein PV.

Our study reveals a unique characteristic of Ca$^{++}$ and Mg$^{++}$ binding to PV from *F. heteroclitus*, a eurythermal teleost: binding of Ca$^{++}$ and Mg$^{++}$ is independent of temperature ($Q_{10} = 1.02$) across a range of 0-25°C. The conserved value of $K_D$, while striking in its insensitivity to temperature, remains within the range of conserved $K_D$ values observed at native physiological temperatures for Antarctic and temperate zone fishes (Fig. 5). Similar binding function at native temperatures suggests that conservation of Ca$^{++}$ $K_D$ values between 6 and 9nM might be required for maintaining optimal contractile function.

The importance of this value is illuminated when viewed in the context of Ca$^{++}$ cycling during muscle contraction. Upon release of Ca$^{++}$ into the cytosol from the SR, TnC binds Ca$^{++}$ and contraction begins. Ca$^{++}$-ATPase pumps of the SR initiate relaxation by Ca$^{++}$ uptake. The affinity of PV for Ca$^{++}$ is four orders of magnitude higher than that of TnC and twice as great as that of the Ca$^{++}$-ATPase pumps. However, unlike TnC and Ca$^{++}$-ATPase pumps, PV can bind Mg$^{++}$ under *in vivo* conditions (Gillis 1985). Because PV is prebound to slowly dissociating Mg$^{++}$, the Ca$^{++}$ cycle begins at the Ca$^{++}$ binding sites of TnC. PV assists in relaxation of muscle by acting as a Ca$^{++}$ sink. While the Ca$^{++}$ capacity of PV is limited by the available binding sites, the ATPase pump capacity is relatively infinite due to the release of Ca$^{++}$ into the SR. The Ca$^{++}$-ATPase pumps, then, can lower intracellular Ca$^{++}$ concentration to a point where TnC will no longer bind and Mg$^{++}$ begins to outcompete Ca$^{++}$ for the PV binding sites. Estimates of Ca$^{++}$ $K_D$ values for parvalbumins isolated from several teleost species appear low enough to assist with relaxation in conjunction with the membrane bound SR pumps but high enough to prohibit delay of initiation of the pumps and relaxation.

The apparent temperature insensitivity of binding in *F. heteroclitus* PV is in stark contrast to the usual boundaries imposed by temperature to protein-ligand interactions. Further,
comparing *F. heteroclitus* PV to similarly tested homologs demonstrates the specificity of this quality to mummichogs that is not an inherent trait of teleost PV (Figs. 5 and 6). The temperature insensitive quality of *F. heteroclitus* PV is not only quite distinct from other similarly tested teleosts but is also not seen in the catalytic proteins previously examined in *F. heteroclitus* populations. Enzymes involved in energy metabolism for this species have shown significant variation in allele frequency between northern and southern populations. Amino acid sequence analysis of PV from four populations of *F. heteroclitus* (Fig. 1) established sequence consensus between all populations (Fig. 7) demonstrating a lack of variation in the expressed protein between northern and southern mummichogs. In addition, ion binding function is maintained across the steep thermal gradient to which populations are exposed (Figs. 5 and 6). Binding-competent conformational stability across widely disparate temperatures is an extremely provocative finding. Previous studies have shown that proteins can be engineered in such a way as to significantly increase stability of the three-dimensional shape allowed by primary structure (Somero, 1995). These proteins were not able to maintain comparable function with their native counterparts, presumably due to the inability of the engineered protein to rapidly shift between conformation states required for function. The primary structure of the major isoform of *F. heteroclitus* PV appears constructed so that the effects of temperature have minimal influence over conformational flexibility.

Figure 8 depicts the amino sequence for *F. heteroclitus* PV compared to other known teleost sequences. With one exception, the canonical binding sites of PV are universally conserved in the teleost species. Enzyme homologs have shown a high degree of amino acid sequence conservation at coordinating residues in the active site (Fields and Somero, 1998; Hochachka and Somero, 2002). This observation suggests that sequence consensus in binding regions is required to retain catalytic function. The physiological conditions under which binding occur, however, may vary widely. Enzymes appear “tuned” to the environment native to the organism. However, the need to maintain covalent binding chemistry appropriate to ligand coordination often limits residue substitutions to regions outside of the binding site. This important discovery required that the classic view of enzyme rate limitations be revised. The rate at which the protein can undergo conformational changes, rather than conversion to the activated enzyme complex, appears to be the rate-limiting step (Hochachka and Somero, 2002). Substitutions outside binding regions are thought to stabilize the active three-dimensional
Figure 8. Aligned parvalbumin sequences for *F. heteroclitus*, *grandis* and *similis* (this study); *C. carpio*, common carp (5CPV); *D. rerio*, zebrafish (NP_571591); *H. sapien*, human (NP_002845).
conformation at physiological temperatures (Davis et al., 2004; Holland et al., 1997).

As in catalytic proteins, the binding sites of PV are highly conserved among species. Residue substitutions found outside the active site of *F. heteroclitus* PV appear to be responsible for maintenance of structural integrity across a suite of temperatures. The exception is seen in *F. similis*, which has one amino acid substitution within the binding site, however it is not one of the coordinating residues. Figures 5 and 6 illuminate the striking possibility that certain residue changes can render a protein virtually insensitive to temperature. The amino acid substitutions responsible for this would presumably have to account for both structural integrity at high temperatures and sufficient flexibility at low temperatures.

Interestingly, the non-functional AB domain has been implicated as a stabilizer of the tertiary structure of PV. Evidence suggests that the AB domain forms a hydrophobic face that interacts with the hydrophobic core formed by the C, D, E and F helices (McPhalen et al., 1994; Thepaut et al., 2001). Deletion of the AB domain (residues 1-37) results in decreased Ca$^{++}$ affinity and complete loss of Mg$^{++}$ affinity (Lee et al., 2004; Thepaut et al., 2001). Sequence alignment of PV demonstrates that over half of the residue changes between *F. heteroclitus* and other known teleosts are located in the AB domain (Fig. 8). This observation strongly suggests that residue substitutions in the AB domain play a key role in stabilization of PV in mummichogs across a wide temperature regime.

Protein adaptation to varying temperatures is not limited to conformational plasticity. Post-translational modification, up-regulation of mRNA transcription rates and solute stabilization have all been observed to alter protein function (Hochachka and Somero, 2002; Powers and Schulte, 1998). While potential effects of these factors were not directly addressed in this study, insight into *F. heteroclitus* PV function can be inferred from the experimental conditions employed. Experiments in this study were performed *in vitro* for the most abundant isoform of *F. heteroclitus* PV. Molecular weight of this isoform purified from the Woods Hole, MA population was determined by MALDI/TOF ($m_w\sim11,322$), and found to be virtually identical to the estimate of $m_w (~11,324)$ from the amino acid sequence of the translated gene product. These data suggest that stabilization of PV is not due to post-translational modification. Monovalent cations have been shown to affect Ca$^{++}$ binding to PV. The stability of PV (as measured by melting temperature) increases with high ionic strength while affinity for Ca$^{++}$ decreases (Eberhard and Erne, 1994; Henzl et al., 2000). In this study PV was examined at $I =$
150mM KCl for all temperatures. This concentration of K\(^+\) is too low to significantly affect binding affinity when Ca\(^{++}\) or Mg\(^{++}\) is present (Eberhard and Erne, 1994; Erickson et al., 2005). We conclude that the solution matrix did not acutely affect functional parameters. The ability of mummichog PV to function \textit{in vitro} at constant PV concentrations suggest that this protein has an intrinsic ability to maintain function across temperatures that is separate from any transcriptional rate variance between populations. \textit{F. heteroclitus} PV primary structure, then, appears responsible for the ability of this protein to retain sufficient flexibility at low temperatures, yet remain rigid enough to maintain binding-competent conformations at high temperatures.

Amino acid sequences were determined for two other species from the \textit{Fundulus} genus (Fig. 7). \textit{F. grandis} and \textit{F. similis} differ from \textit{F. heteroclitus} by 1 and 4 residues, respectively. With one exception these residue substitutions are located in the AB domain. At this time no functional data exists for PV from these two species. They do, however, present the possibility of determining exactly which residues can alter PV in such a way as to render it insensitive to temperature and investigating the possible importance of AB domain amino acid substitutions. All three species are members of the monophyletic family Fundulidae (Parenti, 1981). Studies of PV function for each species might make it possible to not only determine the amino acid substitutions of interest but also to gain insight into when these alterations occurred. The possibility of all three species being functionally similar is recognized.

We proposed that the major isoform of \textit{F. heteroclitus} PV would exhibit gene frequency variance along previously established clines. Sequence and functional similarity of PV between northern and southern mummichog populations refutes this hypothesis. They do, however, lead to speculation regarding the successful invasion of this species northward along the East Coast of North America. Muscle function can have a profound influence on prey capture and predator avoidance. PV has been shown to directly increase the rate of muscle relaxation thereby increasing the number of contraction/relaxation cycles possible per unit time (Hou et al., 1991). The data presented here suggest that PV may have expressed the necessary residue substitutions for function at low temperatures before immigration to higher latitudes. The binding characteristics of \textit{F. heteroclitus} PV would be a significant advantage in an organism migrating to habitats with lower annual temperatures thus contributing to their success in invading new habitats.
Natural character variability can often lead to neutral mutations, which can later be coopted for use when an organism is exposed to new environmental conditions. The distinction between “fortuitous” characteristics leading to increased fitness has been clearly separated from those traits shaped by natural selection (Gould and Vrba, 1982). Pre-adaptive features, or exaptations, are commonly identified in organisms that possess a trait not required for reproductive success in their current habitat (Fullard and Dawson, 1997; Gould and Vrba, 1982). It cannot be ruled out that the historical origin of a trait may have been due to its development in an ancestral species. However, the likelihood of a particular trait being selected for can be deduced by the environmental constraints imposed on common ancestors.

The settlement pattern and evolutionary relationships of *Fundulus* species are well characterized (Parenti, 1981; Wiley, 1986) and *Fundulus* species are thought to be descended from a common ancestor native to Central America and Mexico. This region experiences mean temperatures that remain at or above 25°C annually, suggesting that *F. heteroclitus*’ ancestors would most likely have been under selective pressure for function at warmer temperatures than what are currently experienced by northern populations. The apparent temperature insensitivity of PV $K_D$ appears to be a result of genetic variability rather than natural selection. Examination of other species within the genus might elucidate the origin of this unique binding ability as well as the specific structural changes from which it derives.
REFERENCES


BIOGRAPHICAL SKETCH

Rachel Wallace was born May 10, 1979 in Abbeville, Louisiana. She graduated from Delta High School (Delta, Colorado) in 1997 and began her undergraduate studies at Louisiana State University. After completing two years at LSU she transferred to the University of North Carolina at Wilmington where she was awarded a Bachelor’s of Science in Marine Biology in 2001. Rachel served as a Marine Education Intern at the University of Georgia Marine Education Center and Aquarium in Savannah, Georgia before entering the graduate program at Florida State University. Dr. Timothy S. Moerland served as Rachel’s thesis advisor for her Master of Science in Biological Science. Her work focused on the effect of temperature on fish physiology.