Functional Characterization of Calsequestrin from the Killifish *Fundulus heteroclitus*

A Thesis submitted
to the Graduate School
Valdosta State University

in partial fulfillment of requirements
for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
in the College of Arts & Sciences

May 2012

Tatyana Ekaterina Nienow

BS, University of Georgia, 2006

This thesis, "Functional Characterization of Calsequestrin from the Killifish *Fundulus heteroclitus,*" by Tatyana Ekaterina Nienow, is approved by

Thesis Chair

Theresa J Grove
Theresa J. Grove, Ph.D.
Assistant Professor of Biology

Committee Member

Timothy J. Fort, Ph.D.
Assistant Professor of Biology

Brian G. Miller, Ph.D.
Assistant Professor of Chemistry & Biochemistry
Florida State University

Dean of the Graduate School

Alfred F. Fuciarelli, Ph.D.
Professor of Chemistry

FAIR USE

This thesis is protected by the Copyright Laws of the United States (Public Law 94-553, revised in 1976). Consistent with fair use as defined in the Copyright Laws, brief quotations from this material are allowed with proper acknowledgement. Use of the material for financial gain without the author's expressed written permission is not allowed.

DUPLICATION

I authorize the Head of Interlibrary Loan or the Head of Archives at the Odum Library at Valdosta State University to arrange for duplication of this thesis for educational or scholarly purposes when so requested by a library user. The duplication shall be at the user's expense.

Signature Tatyana E. Nixon

I refuse permission for this thesis to be duplicated in whole or in part.

Signature ______________________________

ABSTRACT

Calsequestrin (CSQ) is the main calcium-binding protein that regulates calcium release from the sarcoplasmic reticulum in both skeletal and cardiac muscles. CSQ undergoes dramatic conformational changes upon binding and releasing calcium. While the properties of CSQ have been well studied in homeotherms, less work has been done in poikilothermic organisms. In this study skeletal muscle CSQ from the killifish *Fundulus heteroclitus* (FCSQ1) was sequenced, expressed, and characterized. The calculated molecular weight of FCSQ1, excluding its signal sequence, is approximately 49 kDa. The primary structure of FCSQ1 is highly conserved when compared to CSQs from other vertebrates, although its overall sequence similarity, theoretical pI (3.94), and tail length, are more similar to the cardiac isoform rather than the skeletal muscle isoform. At 15°C, pH 7.5, the K_D of FCSQ1 is 0.10 ± 0.006 mM in the absence of KCl and 0.14 ± 0.018 mM in the presence of 0.1 M KCl as calculated from a fluorescence spectroscopy-based assay. This is lower than the generally reported K_D of 1 mM, but is similar to the results obtained using a similar technique by Hidalgo *et al.* (1996). FCSQ1 also exhibits pH independence and moderate temperature dependence, suggesting that there is partial adaptation to the varying thermal environment in which *F. heteroclitus* is found.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank Dr. Gretchen Bielmyer of Valdosta State University for verifying that my purified protein and buffer solutions were decalcified. I would also like to thank my committee chair, Dr. Theresa J. Grove, and my committee members, Drs. Timothy J. Fort, and Brian G. Miller, for their assistance, advice, and support. This work was supported by National Science Foundation grant IOS 0817805.

Chapter I

INTRODUCTION

Changes in temperature affect all biological processes by altering the functional properties of proteins, lipids, and other macromolecules. While proteins can be found that are adapted to various temperature extremes, any specific protein will generally only function within a relatively narrow temperature range, due to the influence of temperature upon stability and flexibility (Somero, 1995). Low temperatures (above freezing) provide stability at the cost of flexibility, while high temperatures provide flexibility at the cost of stability. Loss of flexibility or functional structure at thermal extremes will impact the functions of and interactions between proteins that are necessary to maintain physiological processes.

Organisms have evolved multiple strategies of overcoming physiological challenges due to the variable temperatures of their environments (Hochachka and Somero, 2002; Somero, 1995). Homeothermic organisms avoid these physiological challenges by maintaining a relatively constant temperature within their bodies regardless of the external temperature. In contrast, the body temperatures of poikilothermic organisms do vary with environmental temperatures, and thus their physiological processes are potentially affected by changes in temperature. The fact that many metabolic pathways and proteins involved in these pathways are conserved across diverse organisms underscores the importance of physiological adaptations in species found in very different thermal environments.

There are three “strategies” by which ectothermic organisms can alter their protein expression and function in response to changes in physiological temperature (Hochachka and Somero, 2002). First, the level of protein expression can be increased or decreased to compensate for alterations in the efficacy of the protein’s catalytic or binding sites. Second, the cellular environment can change in response to temperature fluctuations resulting in changes in pH and the concentration of intracellular osmolytes that maintain the normal function of proteins. Finally, selective pressure on protein-encoding genes can, by changing the primary structure of the protein, optimize the trade-off between flexibility and stability, resulting in proteins that are better suited for function at a specific physiological temperature. Changes in the amino acid sequence that are a result of mutations may alter the interactions and bonds responsible for a protein’s secondary, tertiary, and quaternary structures. Ionic interactions and hydrogen bonds between amino acid side chains stabilize the protein at low temperatures, while hydrophobic effects and amino acids with bulky side chains increase stability at high temperatures.

Poikilothermic fishes offer the opportunity to study how alterations in protein structure and function contribute to their adaptation to different environmental temperatures. There is a significant amount of research on the relationship between protein structure and function of enzymes in ectothermic animals; A_4-lactate dehydrogenase (A_4-LDH) in particular has been well-studied. A_4-LDH shows temperature dependence and adaptation in several species of tuna and cold-adapted Antarctic notothenoids, and only a few amino acid substitutions distant to the catalytic site or loop regions can result in enzymatic adaptation the physiological temperature of an organism (Low *et al.*, 1973; Graves and Somero, 1982; Holland *et al.*, 1997; Fields and Somero, 1998).

There has also been a significant amount of research on the relationship between temperature and function of many proteins involved in muscle contraction in fishes. The ryanodine receptor, which releases calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR), tends to become stuck in an "open" conformation at lower temperatures (Sitsapesan *et al.*, 1991), which interferes with calcium cycling. The bluefin tuna (*Thunnus thynnus*) that is adapted to colder waters has an increased number of sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps in the SR membrane to compensate for the reduced activity of SERCA at low temperature (Landiera-Fernandez *et al.*, 2003). While much is known about the structure-function relationships of enzymes in ectotherms, comparatively little research has been conducted on non-enzymatic proteins in muscle. The research presented here increases our understanding of the function of non-enzymatic proteins, specifically, the function of calsequestrin (CSQ) in the eurythermal teleost, *Fundulus heteroclitus*.

Poikilothermic killifish in the family Fundulidae are adapted to several different thermal regimes and serve as a model system for studying the molecular mechanisms involved in thermal adaptation. The members of this family inhabit a wide range of environments along the eastern coasts of North and Central America and can be found inhabiting subtropical (*F. similis* and *F. grandis*) and temperate (*F. heteroclitus* and *F. majalis*) waters. The mummichog, *F. heteroclitus,* is found along the eastern coast of North America and experiences fluctuating temperatures during both seasonal and daily tidal cycles (Chidester, 1920; Lotrich, 1975; Sidell *et al.*, 1983). This species can be subdivided into two genetically distinct populations: a northern population found from Newfoundland to Maryland, and a southern population found from Maryland to Florida, with a mixing of the two populations in the contact zone (Powers *et al*., 1986; Ropson *et al*., 1989; Bernardi *et al*., 1993; Gonzalez-Villaseñor and Powers, 1990). The temperature cline along the eastern coast varies 1°C per degree latitude (Powers and Place, 1978); therefore, the northern population of *F. heteroclitus* experiences and has adapted to lower mean annual temperatures than the southern population. Populations of northern *F. heteroclitus* found off the coast of Maine can experience temperatures that range between -1°C to 15°C over the course of a year due to seasonal variations (Sidell *et al*., 1983). When trapped in tidal pools, the temperature can reach as high as 30°C or more during summer months, and temperature changes can vary by 14°C within an hour due to rapid tidal fluctuations (Sidell *et al*., 1983). Even during these dramatic temperature changes, individuals appear to be unaffected, as they are still able to maintain position and even swim and move against flows that can be as great as 40 cm s^{-1}, or 9-13 body lengths s^{-1} (Sidell *et al*., 1983).

CSQ is a calcium-binding protein found in the SR of muscle. CSQ has two isoforms (CSQ1 and CSQ2), both of which have been isolated from a diverse variety of vertebrates, including mammalian, avian, and amphibian organisms (Damiani *et al.*, 1986). CSQ2 is expressed only in cardiac and slow-twitch muscles, while CSQ1 is found in both skeletal (fast-twitch) and cardiac muscles (Damiani *et al.,* 1990); no other isoforms have been found to date. Early research showed that CSQ binds 40-50 mol Ca^{2+} per mol CSQ (MacLennnan and Wong, 1971; Ikemoto *et al.,* 1972); more recent findings have shown that calcium binding capacity is actually greater for the two isoforms, 80 mole Ca^{2+} per mol CSQ1 and 60 mol Ca^{2+} per mol CSQ2 (Park *et al.,* 2004). Both CSQ isoforms bind calcium with a medium affinity ($K_D \cong 0.5$-1 mM) (MacLennnan and Wong, 1971; Ikemoto *et al.,* 1972). As a high capacity, medium affinity calcium-binding protein, CSQ binds large quantities of calcium, keeping the concentration of free calcium ions within the SR at low levels, and CSQ also releases calcium quickly in order to trigger muscle contractions. Quantitative studies of the amounts of CSQ and Ca^{2+} in muscle fibers have shown that CSQ quantity and binding capacity is sufficient to account for a large proportion of Ca^{2+} ions stored in the SR (Murphy *et al.*, 2009). Thus, CSQ is the main calcium storage protein in both cardiac and skeletal muscles.

Calsequestrin is highly charged with 33-46% amino acid residues being acidic (aspartic acid or glutamic acid) (Watabe *et al.*, 1991; Caudwell *et al.*, 1978; Slupsky *et al.,* 1987). CSQ does not bind calcium using EF-hand calcium-binding domains that are present in other calcium-binding proteins (e.g., troponin C and parvalbumin). Instead, the acidic residues of CSQ form clusters of two to three negatively charged amino acids that bind positively charged calcium ions (Yano and Zarain-Herzberg, 1994). The crystal structures for both isoforms of CSQ from rabbit skeletal and cardiac muscles have been solved (Wang *et al.*, 1998; Park *et al.*, 2004). As a folded monomer, CSQ has three thioredoxin-like domains. In each domain, a β-sheet is sandwiched between four α-helices, one pair on each side. Short interdomain loops connect these domains together. When the domains are folded, the aspartic and glutamic acid residues form an acidic, hydrophilic exterior surrounding the hydrophobic core of each domain. As CSQ folds during calcium binding, the helical content increases, and conserved aromatic residues move to the interior, which possibly offsets the instability caused by the large number of negatively charged residues (Park *et al.,* 2004). The inter-domain space is also filled with acidic residues, making the center of the protein hydrophilic and therefore unstable without the presence of cations (e.g., Ca^{2+}) (Wang *et al.,* 1998).

Isoforms CSQ1 and CSQ2 are very similar in sequence and structure, differing mainly in their C-terminal regions. The C-terminal tail is highly variable in length among all reported calsequestrin sequences; however, there is a trend that the aspartic acid-rich tail in CSQ2 is longer than in CSQ1. In spite of this, CSQ2 has a smaller overall negative charge than CSQ1 and binds fewer calcium ions (Park *et al.,* 2004). The tail, normally disordered, is hypothesized to be involved in back-to-back dimerization through the formation of a large electronegative pocket between the two proteins; this would naturally be stabilized by the formation of cross-bridges caused by calcium binding (MacLennan *et al.*, 2002; Wang *et al.*, 1998).

When the muscle cell is relaxed, calcium ions are stored within the lumen of the SR. The large calcium gradient between the lumen of the SR and sarcoplasm is maintained both by the continual action of SERCA, which pump calcium into the SR from the sarcoplasm and CSQ, which binds calcium in the SR to keep free calcium levels low. CSQ has a storage capacity of $\geq$ 40-50 mol Ca^{2+} per mol CSQ (MacLennnan and Wong, 1971; Ikemoto *et al.,* 1972; Park *et al.*, 2004), leaving the concentration of free calcium in the lumen at 1 mM, the inhibitory level of the SERCA pump (Fryer and Stephenson, 1996). CSQ undergoes dramatic conformational changes during contraction and relaxation cycles. When calcium is absent, or at very low concentrations (< 0.01 mM) CSQ exists as a random coil, with no ordered structure and low alpha-helical content (Ikemoto *et al.,* 1972; Ostwald and MacLennan, 1974; Ikemoto *et al.*, 1974;

Cozens and Reithmeier, 1984; Aaron *et al.*, 1984). It is only upon binding of calcium cations that CSQ begins to adopt a folded structure. While CSQ initially folds into a monomer, at calcium concentrations of ~1 mM CSQ begins to dimerize in a front-to-front manner. When luminal concentrations of calcium are high (≥ 3 mM), CSQ forms long polymer chains that associate with the ryanodine receptors at the terminal cisternae of the SR via interactions with two other proteins, junctin and triadin (Franzini-Armstrong *et al.*, 1987; Glover *et al.*, 2002). This complex serves as a negative regulator of the activity of the ryanodine receptor, inhibiting activity at low levels of luminal Ca^{2+} (Beard *et al.*, 2002; Györke *et al.*, 2004), while CSQ's location near the ryanodine receptors enables calcium to rapidly leave the SR when these calcium channels open during contraction.

Although CSQ has been studied in homeothermic organisms, its function in amphibians, fishes, and other poikilotherms is less understood. The bodies of poikilothermic fishes living in intertidal zones, such as killifish from the family Fundulidae, must not only overcome the physiological challenges associated with temperature changes caused by seasonal fluctuations, but also the more dramatic and rapid changes associated with daily tidal cycles. To test the hypothesis that CSQ from southern *Fundulus heteroclitus* is functionally adapted to the physiological temperatures and pH within skeletal muscle, the full-length sequence of skeletal muscle calsequestrin from the killifish *Fundulus heteroclitus* (FCSQ1) was determined, and the function of recombinant FCSQ1 was examined with respect to temperature and pH.

Chapter II

METHODS

Collection of Specimens

Fundulus heteroclitus were caught off the coast of Jekyll Island, Georgia, USA (31°2'27.37"N and 81°25'21.66"W) with minnow traps. Fish were transported to Valdosta State University and held in recirculating seawater (28%) tanks until sacrificed. Fish were anesthetized with Finquel (MS-222), followed by spinal transection and pithing using protocols approved by Valdosta State University's Institutional Animal Care and Use Committee (for approval letters, see Appendix A). Glycolytic skeletal muscle was dissected and immediately used for RNA isolation or frozen at -80°C until use.

RNA Isolation, cDNA Preparation, and Sequencing

Total RNA from *F. heteroclitus* was isolated using TRIzol reagent (Invitrogen, Grand Island, NY) following manufacturer's instructions. Single-strand cDNA was made from total RNA (5 µg) using oligo (dT) primer (Table 2.1) and Superscript III (Invitrogen) as per manufacturer's protocol. cDNA was stored at -20°C until used to amplify *F. heteroclitus* CSQ1 (FCSQ1) cDNA.

Initial amplifications of partial FCSQ1 single-strand cDNA were performed in 50 µL reaction volumes containing 100 ng/µL cDNA, 1X Advantage 2 polymerase buffer, 1X Advantage 2 polymerase mix (Clontech, Mountain View, CA), 200 µM dNTPs, and 500 nM degenerate primers (Table 2.1), which were based on an amino acid alignment of published CSQ1 and CSQ2 sequences. Reactions were as follows: initial denaturation of 2 min at 94°C, followed by 35 cycles of 45 s at 94°C with an annealing step of 1 min at 62°C and an elongation step of 45 s at 72°C; the reaction concluded with a final 5 min elongation at 72°C. Purified PCR products were ligated in pCR®2.1 TOPO vector, and TOP10 One Shot® cells were transformed as per manufacturer's instructions (Invitrogen). Purified plasmids were digested with EcoR1 to determine insert size, and uncut plasmids containing inserts were sequenced at the Florida State University Sequencing Facility using M13 primers (Table 2.1). Sequences were analyzed and aligned using DNASTAR LaserGene 8.0.

The 5' and 3' RACE-ready cDNAs from FCSQ1 were prepared using the SMARTer RACE Amplification Kit (Clontech) following the manufacturer's instructions. Based on partial FCSQ1 nucleotide sequences, gene specific primers CSQAF and CSQBR (Table 2.1) were designed for use in 5' and 3' RACE-PCR, respectively. Amplification was performed with Advantage 2 DNA polymerase with the gene specific primer and Universal primer. The 5' and 3' cDNA samples were prepared under the following conditions in 50 µL reactions: 2.5 µL first-strand cDNA, 1X Universal Primer Mix (UPM), 200 µM gene specific primer, 1X Advantage 2 polymerase buffer, 200 µM dNTPs, and 1X Advantage 2 DNA polymerase mix. Touchdown PCR was performed as follows: 5 cycles of denaturation at 94°C for 30 s and annealing and extension at 72°C for 3 min; 5 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 30 s, and extension at 72°C for 3 min; and 25 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 3 min. Samples were cloned, sequenced, and aligned as previously described. The full-length coding sequence of

Table 2.1. Primers used for amplification of *Fundulus heteroclitus* CSQ1 cDNA sequences.

[a]Primer Name	[b]Primer Sequence	[c]DNA positions
Degenerate primers		
CSQ8F	5’-GCWGCYCARGTCTTGGARGA-3’	241 → 260
CSQ11R	5’-CCWGAMAGRACATCYTCKATCCA-3’	1084 ← 1106
Fundulus specific primers		
CSQAF	5’-GAGGAGCCAGTGGAGGTCATTG-3’	430 → 451
CSQBR	5’-AGATGATGCTGAGGTCAGGAAGG-3’	895 ← 917
CSQF1	5’-ACATTTCTCCATCCCTTCCAAG-3’	-112 → -91
CSQR1	5’-ATTTCATTTTGACCCCACCA-3’	1505 ← 1524
CSQF2	5’-ATGGAAAAGGGCCTGGAG-3’	58 → 76
CSQR2	5’-TTACTCATCATCATCATCATCATCA-3’	1280 ← 1305
Other primers		
Oligo (dT)	5’-GCTTTTTTTTTTTTTTTTTTTT-3’	poly(A) tail
M13F	5’-GTAAAACGACGGCCAG-3’	sequencing
M13R	5’-CAGGAAACAGCTATGAC-3’	sequencing
Petblueup	5’-GTCACGACGTTGTAAAACGAC-3’	sequencing
Petbluedown	5’-GTTAAATTGCTAACGCAGTCA-3’	sequencing

[a] F indicates forward primers; R indicates reverse primers

[b] M = A or C; R = A or G; Y = C or T; W = A or T; K = G or T

[c] Locations of primers are based on cDNA consensus sequence of *Fundulus heteroclitus* CSQ. Position 1 indicates the putative start site (ATG). Negative positions indicate positions upstream of the cDNA start site.

FCSQ1 cDNA was then amplified with gene specific primers CSQF1 and CSQR1. Reaction mixtures were identical to those used to obtain partial FCSQ1 sequence, and PCR reactions were similar except the extension time for each cycle was 75 s. PCR products were cloned and sequenced as previously described.

Sequence Comparisons

Amino acid sequences were compared among CSQ isoforms from a variety of different vertebrates. The following sequences were obtained from Genbank: rabbit *Oryctolagus cuniculus* CSQ1 (GenBank accession no. NP_001075737) and CSQ2 (NM_001101691); human *Homo sapiens* CSQ1 (AAH22289) and CSQ2 (NM_001232); dog *Canis familiaris* CSQ1 (XP_850097) and CSQ2 (XM_540252); zebra fish *Danio rerio* CSQ1 (NP_001070192) and CSQ2 (AAH75775). Amino acid analysis, molecular mass, and isoelectric point calculations were conducted using ProtParam (Gasteiger *et al.*, 2005). Identities and similarities were calculated using The Sequence Manipulation Suite (Stothard, 2000). The SR targeting sequence was identified using SignalP (Peterson *et al.*, 2011).

Expression and Purification of Recombinant Teleost CSQ1

Full-length coding sequence of FCSQ1 minus the predicted signal sequence was amplified via PCR as previously described using the gene specific primers CSQF2 and CSQR2 (Table 2.1). PCR products of expected length were ligated into PetBlue-1AccepTor Vector (Novagen, Madison, WI). Colonies containing plasmid with correct insert were identified by digestion with XmnI, and the sequence was confirmed by sequencing. Tuner(DE3)pLacI cells (Novagen) were transformed with plasmid containing FCSQ1 as per manufacturer's instructions. LB broth containing 100 µg/mL ampicillin and 1% glucose was inoculated and grown at 37°C with agitation until OD_{600} of 1. Temperature of the cultures was decreased to 15°C prior to induction of expression with 500 µM IPTG for 24 hr. Cells were harvested by centrifugation and stored at -80°C until FCSQ1 purification.

CSQ was purified from *E. coli* following the protocol described in Cala and Jones (1983), with minor modifications. Briefly, cells were resuspended in Buffer A (10 mM MOPS, 0.1 mM EGTA, 500 mM NaCl, 1 mM DTT, pH 7.0) and lysed by intermittent sonication for 15-20 sec with cooling on ice after each sonication. The homogenate was centrifuged for 10 min at 14,000 x g, and the supernatant applied to a phenyl sepharose column (2 x 13 cm) equilibrated with Buffer A. The column was washed with 100 mL Buffer A. Protein was eluted with 100 mL Buffer A containing 10 mM $CaCl_2$ followed by 100 mL 10 mM MOPS, pH 7.0. Fractions containing recombinant calsequestrin were identified using 10% Tris/Glycine SDS-PAGE; gels were stained with Coomassie Blue. Fractions containing partially purified FCSQ1 were combined and dialyzed exhaustively against 0.1 M potassium phosphate, 1 mM EGTA, pH 7.1 and applied to a DEAE sepharose column (2.5 x 8 cm) equilibrated with 0.1 M potassium phosphate, 1 mM EGTA, pH 7.1. The column was washed with the same buffer, and FCSQ1 was eluted using a linear gradient of 0 to 1.0 M NaCl in 0.1 M potassium phosphate, 1 mM EGTA, pH 7.1.

Fractions containing FCSQ1 were identified using 10% Tris/Glycine SDS-PAGE as previously described and combined; purified FCSQ1 was concentrated using Amicon Ultra-15 centrifugal filter devices with a 10,000 Da cutoff (Millipore, Billerica, MA). FCSQ1 identity and purity was verified by 10% Tris/Glycine SDS-PAGE and subsequent staining with either Stains-All, which stains calcium-binding proteins blue (King and Morrison, 1976; Campbell *et al.*, 1983), or Coommassie Blue. In addition, N-terminal Edman sequencing of purified recombinant FCSQ1 was conducted at Florida State University's Analytical Lab and Research Facility. Molecular weight was estimated from the electrophoretic mobility of FCSQ1 compared to the mobility of known standards.

Decalcification of FCSQ1 and Buffer Solutions

Divalent cations were removed from recombinant FCSQ1 by dialysis against 10 mM Tris-HCl, pH 7.5 containing 5% Chelex (w/v) at 4°C for 4 days, changing buffer every 24 hr, with continuous stirring. Divalent cations were removed from buffer used in the fluorometric assay by the addition of 5% Chelex with continuous stirring at 4°C for 4 days, changing Chelex every 24 hr. Because decalcification with Chelex increases pH of buffers, the pH was adjusted immediately prior to assay using either 1 N or 0.5 N HCl in 10 mM Tri-HCl (or 10 mM MOPS) that had been decalcified with 5% Chelex. Decalcification of the protein was confirmed by atomic absorption spectroscopy at Valdosta State University's Molecular Advanced Spectroscopy and Biotechnology Facility.

Intrinsic Fluorescence Assay and Calculation of K_D

Concentration of decalcified FCSQ1 was determined using bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, St. Louis, MO) prior to assays. An intrinsic fluorescence assay that relies on tryptophan fluorescence was modified from Park *et al.* (2003) and Hidalgo *et al.* (1996). Calcium chloride standard (0.1 M; Thermo Fisher Scientific, Beverly, MA) was added stepwise to 15 μg/mL FCSQ1 in 10 mM Tris-HCl (pH 6.6, 6.8, 7.2, or 7.5). For the temperature dependence experiments, the pH of 10 mM

Tris-HCl was adjusted to 7.5 at 25°C and allowed to vary with the temperature of the assay (10, 15, 25, or 35 °C). For the fluorescence assay that examined the effects of increased ionic strength (pH 7.5, 15°C), KCl standard (4 M; Thermo Fisher Scientific) was added to the assay for a final concentration of 0.1 M KCl. The sample was allowed to equilibrate five minutes after each addition of calcium. Emission fluorescence was measured with an LS 55 Fluorescence Spectrometer (Perkin-Elmer, Norwalk, CT). Excitation wavelength was 282 nm, and emission wavelength was 331 nm; 5 nm slit width was used for both excitation and emission.

A minimum of three FCSQ1 preparations (two with 0.1 M KCl) were used for each experimental condition, and assays using each of the protein preparations were run in triplicate. Estimates for K_D were calculated using the following equation (Goutelle *et al*. 2008), where K_D is equal to $[K_H]^n$:

$$Y = \frac{Y_{\max} * [Ca]^n}{[K_H]^n + [Ca]^n}$$

Where K_H represents the Hill constant, n represents the index of cooperativity, and [Ca] represents the calcium concentration. When n = 1 there is no cooperativity, and when there is perfect cooperativity n = the number of ligand binding sites. The Hill constant indicates the concentration of ligand that accounts for one half of the maximal response; in the case of fluorescence assays, the concentration calcium (mM) that results in half maximal fluorescence is due to fluorescence of intrinsic tryptophans. K_D was calculated as $K_D = [K_H]^n$. Standard error was calculated from the averages obtained from each FCSQ1 protein preparation. Comparisons between groups were performed with one-way analysis of variance (ANOVA), followed by a Tukey test to identify statistically significant groups. Significance was accepted for $p < 0.05$.

Chapter III

RESULTS

Molecular Characterization of FCSQ1

The full-length sequence of CSQ1 from glycolytic skeletal muscle of southern population of *F. heteroclitus* (FCSQ1) was obtained by PCR (Figure 3.1). FCSQ1 has an open-reading frame of 1,305 bases, corresponding to 434 amino acids. The first 20 amino acids are predicted to be the signal sequence that directs the protein to the SR, which is presumably cleaved after import into the SR. The functional protein that includes the tail is 414 amino acids with a calculated molecular weight of 48,474 Da. Including the tail, its predicted isoelectric point is 3.97, which is similar to that of CSQ1 in other organisms (Table 3.1). One hundred and thirty-seven of the 414 amino acid residues (33%) are acidic residues, aspartic acid and glutamic acid. The aspartic acid tail of FCSQ1 is longer (61 amino acids) than the acidic tail of CSQ1 from mammalian organisms (e.g., 14 amino acids in rabbit).

Overall the sequence of FCSQ1 shares a high percent identity and similarity with other reported CSQ sequences (Tables 3.2 and 3.3). Although the CSQ sequence was determined from the glycolytic skeletal muscle of *F. heteroclitus*, it has a greater sequence identity to the cardiac muscle isoform than skeletal muscle, over 64% identity with CSQ2 compared to 58-61% identity with CSQ1 isoforms.

```
1    ATGCTCTCCTTGTGGTTGTTCCTGCTCCCTTGCCTGAGCCTTGTGAGCGTGGCGCCGGCC    60
     M  L  S  L  W  L  F  L  L  P  C  L  S  L  V  S  V  A  P  A
61   GAAAAGGGCCTGGAGTTTCCGCAGTACGACGGGAAGGACCGTGTTCTCGACATCAGTGAT   120
     E  K  G  L  E  F  P  Q  Y  D  G  K  D  R  V  L  D  I  S  D
121  AAGAATTACAAGAAGGCCTTGAAGAAGCACAGCATGCTCTGCCTGTTCTACCACCAGCCG   180
     K  N  Y  K  K  A  L  K  K  H  S  M  L  C  L  F  Y  H  Q  P
181  GTGCCGGACAATAAGGAGCTGCAGAAACAGCACCAGATGACCGAGCTGGTGCTGGAGCTT   240
     V  P  D  N  K  E  L  Q  K  Q  H  Q  M  T  E  L  V  L  E  L
241  GTAGCTCAAGTCATGGAGGAGAAGGAGATTGGGTTTGGAATGGTTGACTCGCTCAAAGAT   300
     V  A  Q  V  M  E  E  K  E  I  G  F  G  M  V  D  S  L  K  D
301  GCAAAAGTTGCAAAGAAACTGGGCTTGGAGGAAGAGGGAAGTGTATATGTTTTTAAAGAT   360
     A  K  V  A  K  K  L  G  L  E  E  E  G  S  V  Y  V  F  K  D
361  GACAGAATGATCGAGTTCGATGGCTTGCTGTCTGCTAACACTCTGGTGGAGTTCTTGTTG   420
     D  R  M  I  E  F  D  G  L  L  S  A  N  T  L  V  E  F  L  L
421  GACCTTTTGGAGGAGCCAGTGGAGGTCATTGAAAACGCTCTGGAGCTCAAAGCCTTTGAT   480
     D  L  L  E  E  P  V  E  V  I  E  N  A  L  E  L  K  A  F  D
481  AGGATGCAAGAGGATATTCGCATAATTGGTTACTTCAAGGGTGAAGACACTGAGCATTAT   540
     R  M  Q  E  D  I  R  I  I  G  Y  F  K  G  E  D  T  E  H  Y
541  GAAGCGTTCAAAGAGGCTGCGGAGCACTTTCAGCCCTACATTAAATTCTTCGCCACATTT   600
     E  A  F  K  E  A  A  E  H  F  Q  P  Y  I  K  F  F  A  T  F
601  GAAAAATCAGTGGCCAAGGAACTGACTCTGAAGCTGAATGAGGTGGACTTTTATGAGCCC   660
     E  K  S  V  A  K  E  L  T  L  K  L  N  E  V  D  F  Y  E  P
661  TTCATGGATGAGCCCGTCACCATCCCAGGAAGGCCTCACTCCGAGAAGCAGCTGGTGAAA   720
     F  M  D  E  P  V  T  I  P  G  R  P  H  S  E  K  Q  L  V  K
721  TTCATTACTGAACACAGACGACCAACTCTGCGGAAGCTGCGTGCAGAAGACATGTTTGAA   780
     F  I  T  E  H  R  R  P  T  L  R  K  L  R  A  E  D  M  F  E
781  ACCTGGGAGGATGACATCGAGGGCATCCACATTGTGGCCTTTGCAGAGGAAGAAGACCCC   840
     T  W  E  D  D  I  E  G  I  H  I  V  A  F  A  E  E  E  D  P
841  GATGGTTATGAGTTCCTAGAGATCTTGAAGGAGGTAGCCAGAGACAACACTCACCTTCCT   900
     D  G  Y  E  F  L  E  I  L  K  E  V  A  R  D  N  T  H  L  P
901  GACCTCAGCATCATCTGGATTGACCCTGATGACTTCCCCCTGCTGATTCCTTACTGGGAG   960
     D  L  S  I  I  W  I  D  P  D  D  F  P  L  L  I  P  Y  W  E
961  AAGACCTTTAAGGTGGATCTGTTCAGGCCGCAGATTGGAGTCGTCAATGTTACGGATGCG  1020
     K  T  F  K  V  D  L  F  R  P  Q  I  G  V  V  N  V  T  D  A
1021 GACAGCATATGGTTGGAAATGGAGAACGAGGAGGACCTGCCCACGGCTCAGCAGCTGGAG  1080
     D  S  I  W  L  E  M  E  N  E  E  D  L  P  T  A  Q  Q  L  E
1081 GACTGGATCGAAGATGTTCTTTCAGGCAAAGTAAACACTGAAGATGATGATGATGATGAT  1140
     D  W  I  E  D  V  L  S  G  K  V  N  T  E  D  D  D  D  D  D
1141 GATGAAGACACAGATGATGATGATGATGATGATGACGATGATGATGACGACGACAAAGAT  1200
     D  E  D  T  D  D  D  D  D  D  D  D  D  D  D  D  D  D  K  D
1201 GATGAGGATGATGACGATGATGACGACGATGAGGACGACGATGAGGACAACGATGAGGAC  1260
     D  E  D  D  D  D  D  D  D  D  E  D  D  D  E  D  N  D  E  D
1261 GACGAGGAAGAGAACGAAGAGGATGATGATGATGATGATGAGTAA                1305
     D  E  E  E  N  E  E  D  D  D  D  D  D  E
```

Fig. 3.1. Nucleotide and deduced amino acid sequence of the coding region of CSQ1 cDNA from southern *Fundulus heteroclitus*. Sequences were reconstructed from overlapping sequences of PCR amplified fragments. Primer sequences were removed prior to alignment. The putative SR targeting sequence is shaded.

Table 3.1. Comparison of % acidic residues, theoretical pI calculated using the ProtParam server (Gasteiger *et al*., 2005), and tail length of selected CSQ sequences. The predicted signal sequence was not included in calculations.

	% Acidic a.a.'s		pI		Tail length	
	CSQ1	CSQ2	CSQ1	CSQ2	CSQ1	CSQ2
Fundulus heteroclitus	33%	Not available	3.97	Not available	61	Not available
Danio rerio	33%	30%	3.85	3.99	60	36
Canis familiaris	30%	28%	3.91	4.08	14	38
Oryctolagus cuniculus	30%	27%	3.92	4.11	14	37
Homo sapiens	28%	27%	3.95	4.14	9	27

Table 3.2. Percent identity (top number) and percent similarity (bottom number) comparison of skeletal muscle (CSQ1) amino acid sequences (excluding signal sequence) of select vertebrate species. Calculations were made using The Sequence Manipulation Suite (Stothard, 2000).

	Fundulus heteroclitus	*Danio Rerio*	*Canis familiaris*	*Oryctolagus cuniculus*	*Homo sapiens*
Fundulus heteroclitus	ID	57.68 72.81	60.39 70.05	61.11 70.77	58.45 68.12
Danio rerio		ID	56.97 69.27	58.16 69.50	56.97 67.85
Canis familiaris			ID	96.73 98.64	95.91 97.55
Oryctolagus cuniculus				ID	95.10 96.73
Homo sapiens					ID

Amino acid groups: GAVLI, FYW, CM, STNQ, KRH, DE, P

Table 3.3. Percent identity (top number) and percent similarity (bottom number) comparison of FCSQ1 from *Fundulus heteroclitus* with cardiac muscle CSQ2 amino acid sequences (excluding signal sequence) of select vertebrate species. Calculations were made using The Sequence Manipulation Suite (Stothard, 2000).

	Fundulus heteroclitus	*Danio Rerio*	*Canis familiaris*	*Oryctolagus cuniculus*	*Homo sapiens*
Fundulus heteroclitus	ID	76.81 83.82	64.01 76.57	64.98 76.33	63.77 74.88
Danio rerio		ID	71.61 81.84	71.61 81.84	72.05 82.56
Canis familiaris			ID	93.61 96.16	90.84 94.64
Oryctolagus cuniculus				ID	92.07 94.12
Homo sapiens					ID

Amino acid groups: GAVLI, FYW, CM, STNQ, KRH, DE, P

The results of purification of the recombinant FCSQ1 are shown in Figure 3.2. All bands in the purified sample stained blue with Stains-All (data not shown). The large band (between 50 and 75 kDa) was identified as the recombinant CSQ through Edman degradation (data not shown). The molecular weight of FCSQ1 was estimated from the SDS-PAGE gels to be 55 kDa, which is larger than its predicted molecular weight calculated from the sequence. The smaller bands seen just beneath become more apparent when the sample is boiled prior to running on the gel and are likely degradation products (data not shown).

Temperature Sensitivity of FCSQ1 in the Absence of KCl

The effect of temperature on the activity of FCSQ1 was examined. The dissociation constant (K_D) of FCSQ1 for calcium is moderately sensitive to temperature (Figure 3.3), ranging from 0.10 ± 0.006 mM at 15°C to 0.24 ± 0.023 mM at 35°C. There is a statistically significant difference in K_D of the lowest two temperature points, 10°C and 15°C with 35°C ($p = 0.004$). The temperature coefficient ($Q_{10(10^\circ C - 35^\circ C)}$) is 1.3 for the range of temperatures tested, and the $Q_{10(15^\circ C - 35^\circ C)}$ is 1.5.

pH Sensitivity of FCSQ1 in the Absence of KCl

The sensitivity of FCSQ1 to pH was also examined (Figure 3.4). The K_D at pH 7.2 is significantly different from pH 6.6 ($p = 0.027$) and 7.5 ($p = 0.004$), but not 6.8 ($p = 0.271$). None of the other data points vary significantly from each other, according to one-way ANOVA ($p = 0.005$). Below pH 6.2, the change in fluorescence due to calcium binding varied too greatly to produce consistent and reliable results (data not shown).

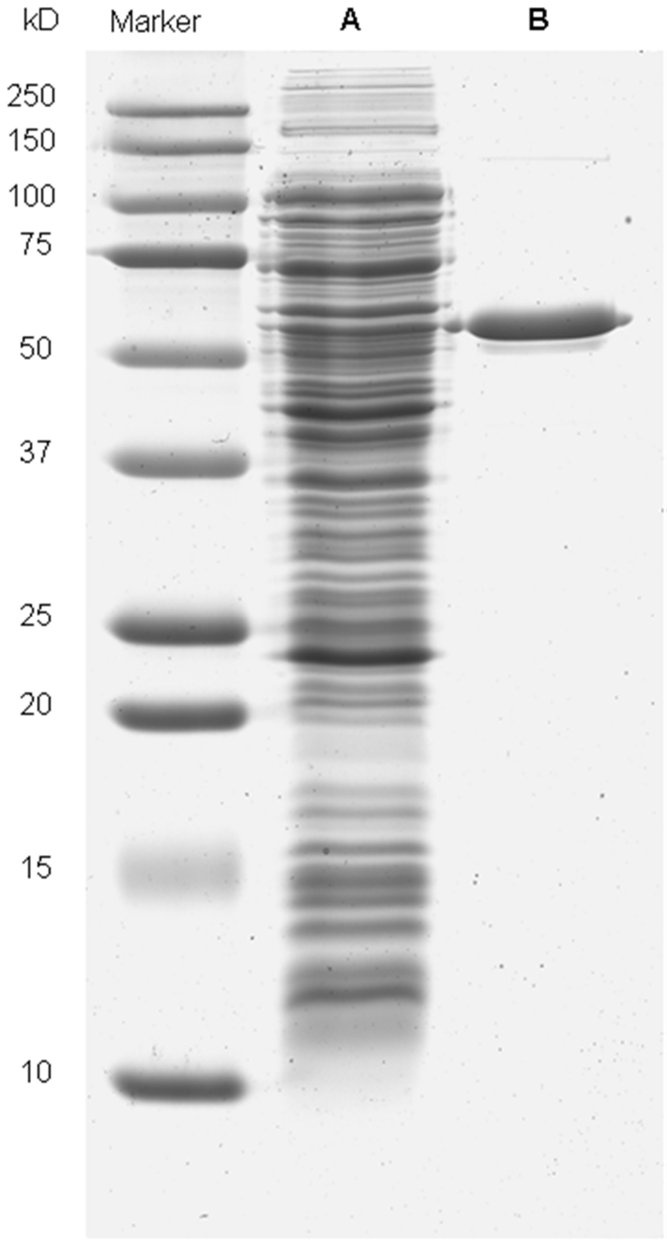

Figure 3.2. 10% Tris/glycine gel. Lane A contains *E. coli* homogenate before purification; lane B shows the final result after purification through both phenyl and DEAE sepharose columns, followed by combining and concentrating the fractions from the DEAE column. 20 µg total protein was loaded in lane A and 0.75 µg protein in lane B.

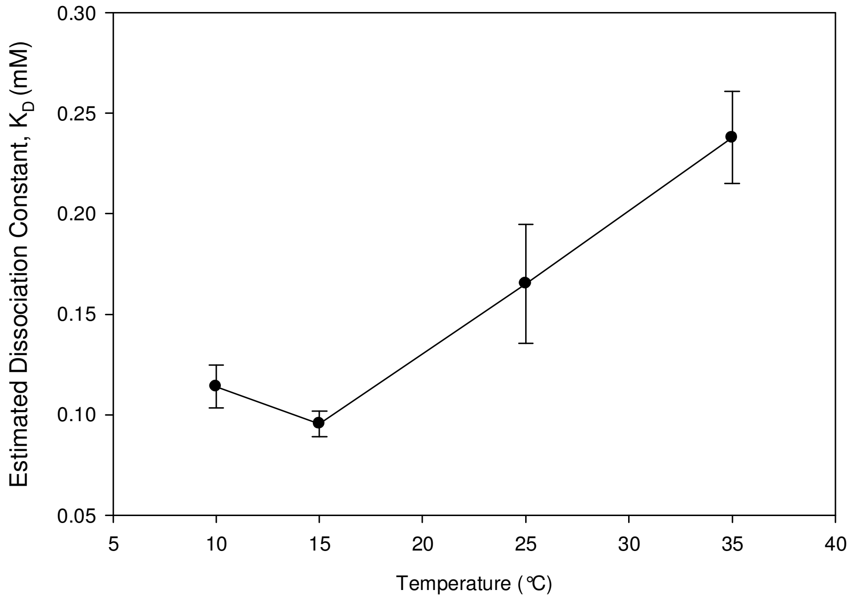

Figure 3.3. Effect of temperature on the apparent dissociation constant (K_D) of FCSQ1. Each data point represents three protein preparations run in triplicate. Error bars represent S.E.M.

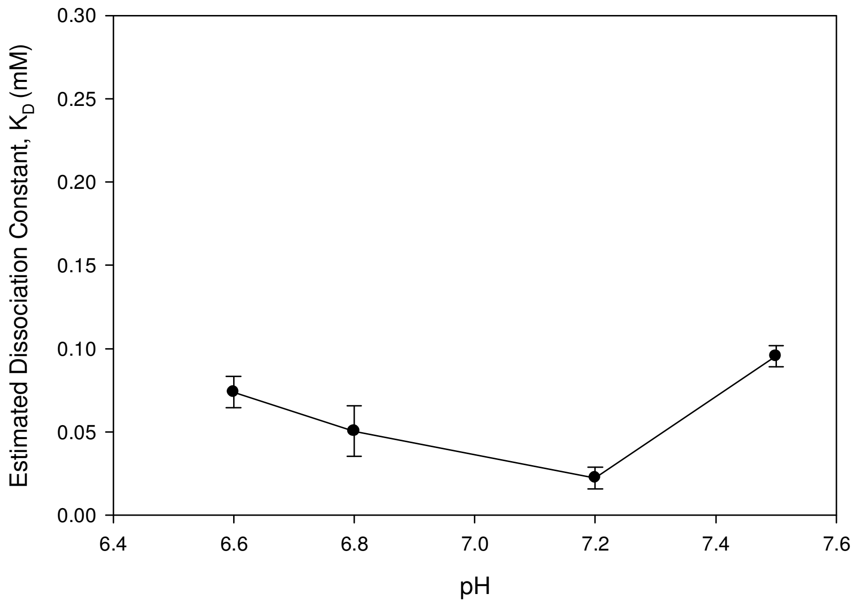

Figure 3.4. Effect of pH on the apparent dissociation constant (K_D) of FCSQ1. Each data point represents three protein preparations run in triplicate. Error bars represent S.E.M..

FCSQ1 Activity in the Presence of 0.1 M KCl

In the absence of KCl, the K_Ds for FCSQ1 are lower than previously published reports of mammalian CSQ. At 15°C, pH 7.5, in the presence of 0.1 M KCl, the K_H for CSQ is 0.65 ± 0.004 mM, which is comparable to the reported K_H of CSQ (0.57 mM) (Hidalgo *et al.*, 1996). The K_D and K_H for CSQ in the presence of 0.1M KCl are compared to the K_Ds and K_Hs of CSQs from other species under similar conditions in Table 3.4. The K_D for FCSQ1 is lower than the K_D calculated for other organisms, but both K_D and K_H are similar to the values calculated by Hidalgo *et al.* (1996).

Table 3.4. K_D and K_H of FCSQ1 compared to other CSQ in the presence of KCl.

CSQ	K_D	K_H
Rabbit, skeletal[a]	0.82 mM	0.89 mM
Rabbit, skeletal[b]	1 mM	Not available
Dog, cardiac[c]	0.4 mM	Not available
Carp, skeletal[d]	0.4 mM	Not available
Rabbit, skeletal[e]	0.2 mM	0.57 mM
F. heteroclitus, skeletal[f]	0.14 ± 0.018 mM	0.65 ± 0.004 mM
Rabbit, skeletal[g]	0.8 mM	Not available

Unless otherwise noted, values given were obtained in 0.1M KCl, 20°C, pH 7.5.

[a] from Aaron *et al*; 1984 (24°C)

[b] from MacLennan and Wong, 1971

[c] from Slupsky *et al.*, 1987

[d] from Watabe *et al.*, 1991

[e] from Hidalgo *et al.*, 1996 (temperature not given, 0.15M KCl)

[f] this study (15°C)

[g] from Ostwald and MacLennan, 1974

Chapter IV

DISCUSSION

Much of the work investigating CSQ has focused on the structural and functional characteristics of this protein from mammals. This study expands our understanding of CSQ1 from a eurythermal teleost, *Fundulus heteroclitus.* FCSQ1 is highly conserved, with over 50% identity to other, more distantly related, vertebrate animals. Interestingly, the identity and similarity are greater when compared to the cardiac isoform, rather than skeletal muscle CSQ. To date, only CSQ1 has been reported to be present within skeletal muscle, so it is unlikely that FCSQ1 is the cardiac isoform.

The difference in size between the calculated molecular weight and its apparent size in SDS is a known characteristic of CSQ. The mobility of CSQ using SDS-PAGE is sensitive to pH, and when using buffers at an alkaline pH, CSQ typically runs larger than its calculated size, at about 55 kDa or more (Cala and Jones, 1983; Campbell *et al.,* 1983; Cozens and Reithmeier, 1984). There is a faint band at 150 kDa, which is likely the result of polymerization due to the high concentration of purified CSQ in high salt. The smaller bands seen below FCSQ1 (Figure 3.2) stain blue in Stains-All and become more prominent when the protein sample is boiled during sample preparation and therefore, are likely degradation products of FCSQ1 (data not shown).

The sequence of FCSQ1 is very similar to other CSQs; CSQ in general is very highly conserved, even between highly divergent species. This suggests there is strong evolutionary pressure towards conservation of function, presumably due to the need for

CSQ to retain many aspartic and glutamic acid residues in order to bind large quantities of Ca^{2+} ions. FCSQ1 is highly acidic; 33% of its sequence consists of acidic residues (Table 3.1). The CSQ1 isoform tends to be slightly more acidic than the CSQ2 isoform, with a lower isoelectric point (Table 3.1), despite that the acidic tail tends to be longer in CSQ2. An exception to this trend is seen in *Danio rerio*: the acidic tail for CSQ1 is much longer than mammalian CSQ1, and it is twice as long as the tail for CSQ2. The cardiac muscle isoform for *D. rerio*, however, still remains more similar to CSQ2 in mammalian organisms. Both FCSQ1 and CSQ1 and CSQ2 for *D. rerio* are more acidic than the mammalian CSQs, with longer tails and lower isoelectric points, so it is possible that greater acidity, in particular longer aspartic acid tails, is a property unique to fishes.

This is the first study to examine the sensitivity of recombinant CSQ1 from the teleost *Fundulus heteroclitus* (FCSQ1) to temperature and pH under low ionic strength conditions. The temperatures examined are within the range of physiological temperatures experienced by this temperate fish species; southern populations of *F. heteroclitus* experience yearly temperatures that range on average from ~13°C to ~30°C over the course of the year (Locarnini *et al.*, 2010). Temperature coefficients (Q_{10}) were used to examine the effects temperature on calcium binding by FCSQ1. Usually Q_{10} values of ~2 are determined for biological functions when examined within the normal range physiological temperatures. The results of the fluorescence assay indicate that FCSQ1 is moderately temperature-dependent, with a temperature coefficient of 1.3 across the range of temperatures examined in this study (10°C-35°C) and a K_D of 0.11 mM to 0.24 mM. FCSQ1 showed greater temperature sensitivity between the assay temperatures of 15°C-35°C (Q_{10} 1.5) than between 10°C-35°C (Q_{10} 1.3). Some thermal dependence of CSQ function is not unexpected for poikilothermic organisms. Interestingly, using an isotopic assay Watabe *et al.* (1991) found that the maximal amount of Ca^{2+} bound by CSQ1 from carp (*Cyprinus carpio*) decreased as temperature decreased, while the Ca^{2+} binding ability remained unchanged. In contrast, the Ca^{2+} binding capacity of rabbit CSQ1 showed little temperature dependency (Watabe *et al.*, 1991).

The function of FCSQ1 is largely independent of pH until it approaches pH 6.2 or lower, which corresponds to conditions of muscle fatigue *in vivo* (Fitts, 1994). At pH 6.2, consistent K_D values could not be obtained (data not shown). Rabbit CSQ1 showed an increase in K_H below pH 6.0 in the absence of KCl (Hidalgo *et al.*, 1996). At pH 6.0, it appears that the abundance of protons compete with Ca^{2+} in binding to CSQ. This has been seen in previous reports, both in experiments measuring CSQ binding directly (Hidalgo *et al.*, 1996) and the calcium binding to skeletal muscle triads (Donoso *et al.*, 1996). Rather than showing an increase in K_D, below pH 6.2 the fluorescence assay in this study produced widely divergent results. As there are several histidines (pKa 6.0) present in the predicted amino acid sequence, it is possible that if these residues were protonated below pH 6.2, they interfered with Ca^{2+} binding.

Although this study primarily focused on the functional characterization of FCSQ1 with minimal interference from confounding factors, many previous studies of CSQ function have been carried out under conditions of relatively high ionic strength, $\geq$ 0.1 M KCl. The dissociation constant of FCSQ1 is conserved when compared to values that were determined using the same experimental method. The K_D of FCSQ1 was determined to be 0.14 mM in the presence of 0.1 M KCl, which is similar to the reported K_D of rabbit CSQ1 (Hidalgo *et al.*, 1996). These K_Ds were determined using the fluorometric assay that monitors the intrinsic fluorescence of conserved tryptophans. However, the assays using FCSQ1 were conducted at 15°C, which is a physiologically relevant temperature for *F. heteroclitus,* and Hidalgo *et al.* (1996) did not state the assay temperature, but it is likely that a temperature of 25°C was likely used.

The K_D values determined for FCSQ1 at pH 7.5 are similar to those determined by Hidalgo *et al.* (1996), both in the presence and absence of KCl. The activity of FCSQ1 is also similar to CSQ from other vertebrates. The calculated K_D values for FCSQ1, as well as those reported in Hidalgo *et al.* (1996), are lower than previously reported K_Ds for CSQ1 under similar conditions temperatures, pH, and KCl concentrations (Table 3.4). The majority of the values available for CSQ were obtained through isotopic assays that measured the total amount of Ca^{2+} bound (MacLennan and Wong, 1971; Watabe *et al.*, 1991; Slupsky *et al.*, 1987). One K_D value for CSQ from rabbit skeletal muscle was obtained through Ca^{2+} titrations using NMR (Aaron *et al.*, 1984). The differences in K_D values between FCSQ1 and other CSQs could be a result of the different methods used.

The experiments in this study focused primarily on characterizing the functional properties of skeletal muscle calsequestrin from a eurythermal teleost, without interference from salts or other potentially confounding variables. In general, FCSQ1 shares many characteristics with other calsequestrins, even from highly diverged species. Because of this overall high degree of similarity between diverged species, differences in structure, sequence and function between more closely related species may be important for adaptation. To date, most research into CSQ has been limited to mammalian organisms, and very little attention has been paid to the importance of temperature on the function of CSQ. Only one other study on the functional properties of CSQ from a teleost has been published, that of skeletal muscle CSQ from *C. carpio* (Watabe *et al.,* 1991). Future studies involving related *Fundulus* species adapted to different thermal environments would lead to greater insight into the adaptation of CSQ in poikilothermic organisms.

REFERENCES

Aaron, B. M., Oikawa, K., Reithmeier, R. A. F., & Sykes, B. D. (1984). Characterization of Skeletal Muscle Calsequestrin by 1H NMR Spectroscopy. *The Journal of Biological Chemistry, 259*(19), 11876-11881.

Beard, N. A., Sakowska, M. N, Dulhunty, A. F., & Laver, D. R. (2002). Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channels. *Biophysical Journal, 82*(1), 310-320.

Bernardi, G., Sordino, P., & Powers, D. A. (1993). Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences USA, 90*, 9271-9274.

Cala, S. E., & Jones, L. R. (1983). Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca2+-dependent elution from phenyl-sepharose. *Journal of Biological Chemistry, 258*, 11932-11936.

Campbell, K. P., MacLennan, D. H., & and Jorgensen, A. O. (1983). Staining of the Ca2+-binding Proteins, Calsequestrin, Calmodulin, Troponin C, and S-100, with the Cationic Carbocyanine Dye “Stains-all.” *The Journal of Biological Chemistry, 258*(18), 11267-11273.

Caudwell, B., Antoniw, J. F., & Cohen, P. (1978). Calsequestrin, Myosin, and the Components of the Protein-Glycogen Complex in Rabbit Skeletal Muscle. *European Journal of Biochemistry, 86*(2), 511-518.

Chidester, F. E. (1920). The behavior of *Fundulus heteroclitus* on salt marshes of New Jersey. *American Naturalist. 54*, 551-557.

Cozens, B., & Reithmeier, R. A. F. (1984). Size and shape of rabbit skeletal muscle calsequestrin. *Journal of Biological Chemistry, 259*, 6248-6252.

Damiani, E., Salvatori, S., Zorzato, F., & Margreth, A. (1986). Characteristics of skeletal muscle calsequestrin: comparison of mammalian, amphibian and avian muscles. *Journal of Muscle Research and Cell Motility, 7*, 435-445.

Damiani, E., Volpe, P., & Margreth, A. (1990). Coexpression of two isoforms of calsequestrin in rabbit slow-twitch muscle. *Journal of Muscle Research and Cell Motility*, *11*, 522-530.

Donoso, P., Beltrán, M., & Hidalgo, C. (1996). Luminal pH regulates calcium release kinetics in sarcoplasmic reticulum vesicles. *Biochemistry, 35*, 13419-13425.

Fields, P. A. and G. N. Somero. (1998). Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenoid fishes. *Proceedings of the National Academy of Sciences USA,* 95: 11476-11481.

Fitts, R. H. (1994). Cellular mechanisms of muscle fatigue. *Physiological Reviews, 74*(1), 49-95.

Franzini-Armstrong, C., Kenney, L. J., & Varriano-Marston, E. (1987). The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study. *The Journal of Cell Biology, 105*, 49-56.

Fryer, M. W., & Stephenson, D. G. (1996). Total and sarcoplasmic reticulum calcium contents of skinned fibres from rat skeletal muscle. *Journal of Physiology, 493*, 357-370.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. In J. M. Walker (Ed.), *The Proteomics Protocols Handbook* (pp. 571-607). Totowa, NJ: Humana Press.

Glover, L., Quinn, S., Ryan, M., Pette, D., & Ohlendieck, K. (2002). Supramolecular calsequestrin complex: Protein-protein interactions in chronic low-frequency stimulated muscle, postnatal development and ageing. *European Journal of Biochemistry, 269*, 4607-4616.

Gonzalez-Villaseñor, L. I., & Powers, D. A. (1990). Mitochondrial-DNA restriction-site polymorphisms in the teleost *Fundulus heteroclitus* support secondary intergradation. *Evolution, 44*, 27-37.

Goutelle, S., Maurin, M., Rougier, F., Barbaut, X., Bourguignon, L., Ducher, M., & Maire, P. (2008). The Hill equation: a review of its capabilities in pharmacological modelling. *Fundamental Clinical Pharmacology, 22*, 633-648.

Graves, J. E., & Somero, G. N. (1982). Electrophoretic and functional enzymic evolution in four species of eastern pacific barracudas from different thermal environments. *Evolution, 36*, 97-106.

Györke, I., Hester, N., Jones, L. R., & Györke, S. (2004). The Role of Calsequestrin, Triadin, and Junctin in Conferring Cardiac Ryanodine Receptor Responsiveness to Luminal Calcium. *Biophysical Journal, 86*, 2121-2128.

Hidalgo, C., Donoso, P., & Rodriguez, P. H. (1996). Protons induce calcium conformational changes. *Biophysical Journal, 71*(4), 2130-2137.

Hochachka, P. W., & Somero, G. N. (2002). *Biochemical adaptation: mechanism and process in physiological evolution*. New York, NY: Oxford University Press, Inc.

Holland, L. Z, McFall-Ngai, M., & Somero, G. N. (1997). Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus *Sphyraena*) from different thermal environments: differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site. *Biochemistry, 36*, 3207-3215.

Ikemoto, N., Bhatnagar, G.M., Nagy, B., & Gergely, J. (1972). Interaction of divalent cations with the 55,000-dalton protein component of the sarcoplasmic reticulum. Studies of fluorescence and circular dichroism. *Journal of Biological Chemistry, 247*, 7835-7837.

Ikemoto, N., Nagy, B., Bhatnagar, G. M., & Gergely, J. (1974). Studies on a Metal-binding Protein of the Sarcoplasmic Reticulum. *The Journal of Biological Chemistry, 249*(8), 2357-2365.

King, L. E., & Morrison, M. (1976). The visualization of human erythrocyte membrane proteins and glycoproteins in SDS polyacrylamide gels employing a single staining procedure. *Analytical Biochemistry, 71*(1), 223-230.

Landiera-Fernandez, A. M., Morrissette, J. M., Blank, J. M., & Block, B. A. (2003). Temperature dependence of the Ca^{2+}-ATPase (SERCA2) in the ventricles of tuna and mackerel. *The American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 286*, R398–R404.

Locarnini, R. A., Mishonov, A. V., Antonov, J. I., Boyer, T. P., Garcia, H. E., Baranova, O. K., Zweng, M. M., & Johnson, D. R. (2010). In S. Levitus (Ed.), *World Ocean Atlas 2009, Volume 1: Temperature* (pp. 1-184). Washington, DC: NOAA Atlas NESDIS 68, U.S. Government Printing Office.

Lotrich, V. A. (1975). Summer home range and movements of *Fundulus heteroclitus* (Pisces: Cyprinodontidae) in a tidal creek. *Ecology, 56*, 191-198.

Low, P. S., Bada, J. L., & Somero, G. N. (1973). Temperature adaptation of enzymes: roles of the free energy, the enthalpy, and the entropy of activation. *Proceedings of the National Academy of Sciences of the United States of America, 70*(2), 430-432.

MacLennan, D. H., Abu-Abed, M., & Kang, C. H. (2002). Structure-Function Relationships in Ca^{2+} Cycling Proteins. *Journal of Molecular Cell Cardiology, 34*, 897-918.

MacLennan, D. H., & Wong, P. T. S. (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proceedings of the National Academy of Sciences, USA*, *68*(6), 1231-1235.

Murphy, R. M., Larkins, N. T., Mollica, J. P., Beard, N. A., & Lamb, G. D. (2009). Calsequestrin content and SERCA determine normal and maximal Ca^{2+} storage levels in sarcoplasmic reticulum of fast- and slow-twitch fibres of rat. *Journal of Physiology, 587*(2), 443-460.

Ostwald, T. J., & MacLennan, D. H. (1974). Isolation of a High Affinity Calcium-binding Protein from Sarcoplasmic Reticulum. *The Journal of Biological Chemistry*, *249*(3), 974-979.

Park, H., Park, I. Y., Kim. E., You, B., Fields, K., Dunker, A. K., & Kang, C. (2004). Comparing skeletal and cardiac calsequestrin structures and their calcium binding. *Journal of Biological Chemistry, 279*, 18026-18033.

Park, H., Wu, S., Dunker, A. K., & Kang, C. (2003). Polymerization of calsequestrin. *Journal of Biological Chemistry, 278*, 16176-16182.

Peterson, T. N., Brunak, S., von Heijne, G., & Nielson, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods, 8,* 787-786.

Powers, D. A., & Place, A. R. (1978). Biochemical genetics of *Fundulus heteroclitus* (L.). I. Temporal and spatial variation in gene frequencies of Ldh-B, Mdh-A, Gpi-B, and Pgm-A. *Biochemical Genetics, 16*, 593-607.

Powers, D. A., Ropson, I., Brown, D. C., Van Beneden, R., Cashon, R., Gonzalez-Villaseñor, L. I., & DiMichele, J. A. (1986). Genetic variation in *Fundulus heteroclitus:* Geographic distribution. *American Zoologist, 26*, 131-144.

Ropson, I. J., Brown, D. C., & Powers, D. A. (1989). Biochemical genetics of *Fundulus heteroclitus* (L.), VI. Geographical variation in the gene frequencies of 15 loci. *Evolution, 44*, 16-26.

Sidell B. D., Johnston, I. A., Moerland, T. S., & Goldspink, G. (1983). The eurythermal myofibrillar protein complex of the mummichog: adaptation to a functional thermal environment. *Journal of Comparative Physiology, 153*, 167-173.

Sitsapesan, R., Montgomery, R. A. P., MacLeod, K. T., & Williams, A. J. (1991). Sheep cardiac sarcoplasmic reticulum calcium release channel: modulation of conductance and gating by temperature. *Journal of Physiology, 434*, 469-488.

Slupsky, J. R., Ohnishi, M., Carpenter, M. R., & Reithmeier, R. A. F. (1987). Characterization of Cardiac Calsequestrin. *Biochemistry, 26*, 6539-6544.

Somero, G. N. (1995). Proteins and Temperature. *Annual Reviews of Physiology, 57,* 43-68.

Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques, 28*, 1102-1104.

Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K., & Kang, C. H. (1998). Crystal Structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. *Nature Structural Biology, 5*(6), 476-483.

Watabe, S., Ushio, H., & Hashimoto, K. (1991). Purification and Characterization of a Calsequestrin-like Calcium-binding Protein from Carp (*Cyprinus carpio*) Sarcoplasmic Reticulum. *Comparative Biochemistry, 99B*(3), 545-552.

Yano, K., & Zarain-Herzberg, A. (1994). Sarcoplasmic reticulum calsequestrins: Structural and functional properties. *Molecular Cellular Biochemistry, 135*, 61-70.

APPENDIX A:

IACUC Approval

VALDOSTA
STATE
UNIVERSITY
Building for Our Next Century
1906-2006

MAR 26 2008

26 March, 2008

Dr. Theresa Grove
Department of Biology
Valdosta State University
Valdosta, GA 31698

Dear Dr. Grove:

The Institutional Animal Care and Use Committee (IACUC) review of your proposal IACUC number 00014-2008 – "Thermal Adaptation in Killifish: The Role of Calsequestrin in Glycolytic Skeletal Muscle" is complete and the committee has approved the proposal. The attending veterinarian has signed this document and a copy is being provided to you. If you have any questions or would like any other information, please do not hesitate to contact me during the day at (229) 333-5767 or by email at jmlockha@valdosta.edu.

Sincerely,

J. M. Lockhart

J. Mitchell Lockhart
Chair, Institutional Animal Care and
Use Committee

cc: Barbara Gray, Institutional Official, VSU-IACUC

Department of Biology
College of Arts and Sciences
Address 1500 N. Patterson St. • Valdosta, GA 31698–0015
Phone 229.333.5759 • **Fax** 229.245.6585

VALDOSTA
STATE
UNIVERSITY
Building for Our Next Century

July 8, 2011

Dr. Theresa Grove
Department of Biology
Valdosta State University

RE: AUP-00038-2011
Muscle Function in fish Species
Adapted to Different Thermal
Regimes

Dear Dr. Grove

Your Animal Use Protocol referenced above has been approved by the Institutional Animal Care and Use Committee under Animal Welfare Assurance Number A4578-01. This approval is for the period of July 8, 2011 through July 7, 2014. Each year, an annual review and report request must be submitted to the IACUC to keep your protocol active. You will be contacted by the IACUC Administrator in the Office of Sponsored Programs & Research Administration approximately two months before the annual review request and report is due.

Please remember that you must obtain IACUC approval before amending or altering the scope or procedures of the protocol. You are also required to report to the Attending Veterinarian, the IACUC Chair, and/or the IACUC Administrator any unanticipated problems with the animals, which become apparent during the course, or as a result of, the research activity.

You will find the IACUC's Standard Operating Procedures and helpful resources on the Office of Sponsored Programs & Research Administration website at http://www.valdosta.edu/ospra. However, if you have any questions, please contact the IACUC Administrator at iacuc@valdosta.edu or 333-7837.

Sincerely,

Barbara Gray

Barbara H. Gray, Director
Office of Sponsored Programs
& Research Administration
IACUC Administrator

Cc: Dr. Phil Gunter, Institutional Official
Dr. Theresa Grove, IACUC Chair
Dr. Teresa Doscher, Attending Veterinarian
Dr. Robert Gannon, Biology Department Head