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Cold Body Temperature as an Evolutionary Shaping Force in the Physiology of Antarctic Fishes

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Principal Investigator; University of Maine, Orono

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Final Report for Period: 09/2002 - 08/2006
Submitted on: 11/01/2006
Principal Investigator: Sidell, Bruce D.
Award ID: 0125890
Organization: University of Maine
Submitted By:

Title:
Cold Body Temperature as an Evolutionary Shaping force in the Physiology of Antarctic Fishes

Project Participants

Senior Personnel

Name: Sidell, Bruce
Worked for more than 160 Hours: Yes
Contribution to Project:

Name: Moerland, Timothy
Worked for more than 160 Hours: Yes
Contribution to Project:
Dr. Moerland is a named faculty colleague from Florida State University. He will manage a small subcontract to this award that will begin during the upcoming second year of funding. Dr. Moerland participated as a field team member in our recent Antarctic field season at Palmer Station from April-June 2003.

With his graduate student, Jeffrey Erickson (see below), Moerland is managing the section of our work that focuses on biophysical characterization of parvalbumins from Antarctic fishes.

Name: Vayda, Michael
Worked for more than 160 Hours: Yes
Contribution to Project:
Dr. Vayda is a named senior faculty associate on this grant and receives one month of summer salary in addition to support for materials and supplies.

Dr. Vayda is an expert molecular biologist and assists the PI's laboratory with some molecular biological aspects of the project while also directly managing some other molecular biological aspects of the project in his own laboratory.

Dr. Vayda will be leaving the University of Maine as of 1 June 2004 and will be replaced as a senior faculty associate by Dr. Gregory Mayer, who brings equivalent expertise in molecular biology to our project.

Name: Mayer, Gregory
Worked for more than 160 Hours: Yes
Contribution to Project:
Dr. Mayer is a new faculty member at the University of Maine and is replacing my longtime collaborator and named faculty associate, Dr. Michael Vayda, in our project. Vayda has accepted an administrative position at the University of Vermont and will be leaving the University of Maine as of 1 June 2004. Mayer brings a high level of expertise in molecular biology to the project and has agreed enthusiastically to collaborate with us on the work. During Summer 2004, he will receive one month's summer salary that originally was budgeted for Vayda.

Post-doc

Graduate Student

Name: Hendrickson, Jamie
Worked for more than 160 Hours: Yes
Contribution to Project:

Ms. Hendrickson is a graduate student in the PI's laboratory and is supported by a Graduate Assistantship funded by this award. She has been enrolled in the Ph.D. program here at the University of Maine during the past year.

Ms. Hendrickson's research project focuses on molecular biological aspects of our studies of parvalbumins in Antarctic fishes. In addition to work in our CONUS laboratory, Ms. Hendrickson participated as a field team member in our recent Antarctic field season at Palmer Station from April - June 2003.

Name: Erickson, Jeffrey

Worked for more than 160 Hours: Yes

Contribution to Project:

Mr. Erickson is a Ph.D. student working under the supervision of Dr. Timothy S. Moerland (see above) at Florida State University. Mr. Erickson participated as a field team member in our recent Antarctic field season at Palmer Station from April -- June 2003.

Mr. Erickson's thesis research focuses on biophysical characterization of parvalbumins from Antarctic fishes.

Name: Borley, Kimberly

Worked for more than 160 Hours: Yes

Contribution to Project:

Kim was a member of our 2005 Antarctic field team and participated in both experiments in the field and analysis of samples in our CONUS laboratory. She received partial Graduate Research Assistantship support from the award.

Name: Wujcik, Jody

Worked for more than 160 Hours: Yes

Contribution to Project:

Jody was a member of our 2005 Antarctic field team and participated in both experiments in the field and analysis of samples in our CONUS laboratory. She received partial Graduate Research Assistantship support from the award.

Undergraduate Student

Technician, Programmer

Name: Babcock, Michael

Worked for more than 160 Hours: Yes

Contribution to Project:

Babcock is a technician who previously worked with Vayda and is now being supervised by Dr. Gregory Mayer. He has assisted Vayda with our collaborative work on myoglobin expression for several years and will now do so under Mayer's supervision. Thus, Babcock's participation contributes important technical continuity in the molecular biological aspects of our project. He will receive 1 calendar month salary during summer 2004, utilizing salary savings from the unfilled Postdoctoral position, which has been partially rebudgeted.

Other Participant

Research Experience for Undergraduates

Organizational Partners

Florida State University

Dr. Timothy S. Moerland, a faculty member at Florida State University, and Mr. Jeffrey Erickson, one of his graduate student advisees both participated as field team members in our recent (April - June 2003) Antarctic Field Season. Moerland and Erickson have also executed studies for the biophysical characterization of parvalbumin from Antarctic icefishes.

Other Collaborators or Contacts

Dr. Stuart Egginton, University of Birmingham Medical School, Birmingham, England, U.K.

We collaborated with Dr. Egginton and his coworkers by combining data that were reported in a coauthored scientific article (Journal of Experimental Biology 206: 411-421, 2003.)

Dr. Kristin M. O'Brien, Institute for Artic Biology, University of Alaska, Fairbanks, AK

Dr. O'Brien also was a contributor and coauthor of the paper referred to above with respect to collaboration with Egginton. She also participated in 2005 field work as a member of our field team.

Dr. Filippo Garofalo, University of Cosenza, Italy. Dr. Garofalo was a member of our 2005 Antarctic field team. We collaborated on isolated, perfused heart experiments using tissues from Antarctic fishes while on-site at Palmer Station. Dr. Garofalo also returned to his home institution with tissue samples that will be used in subsequent histochemical analyses of hearts from Antarctic fish species.

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

Findings:
Major findings are summarized as a unified narrative with the description of research and education activities above.

Training and Development:
The project has supported or partially supported the M.S. thesis research of:

Ms. Jamie Hendrickson, University of Maine (M.S. Degree awarded August 2005)

and the Ph.D. thesis research of

Mr. Jeffrey Erickson, Florida State University (Ph.D. awarded 2005)

Both of these individuals participated in our Antarctic field work as field team members and were actively engaged in activities at Palmer Station and aboard the ARSV L.M. Gould.

Ms. Jody Wujcik (M.S. Student) and Ms. Kimberly Borley (Ph.D. Student), graduate students at the University of Maine (Sidell lab)were field team members during the 2005 field season, actively engaged in activities at Palmer Station and aboard the L.M. Gould and have contributed to the scope of work of the project.

Outreach Activities:
Part of our funded research on variable myoglobin expression in Antarctic icefishes formed the basis for the P.I.'s invited lecture as the 2003 invitee for the C. Ladd Prosser Lecture in Comparative Physiology at the University of Illinois in March 2003. This talk is an endowed lecture that is held annually and draws a broad multidisciplinary audience at the University of Illinois.

P.I. Sidell also has published an invited 'Commentary' article on the evolutionary implications of loss of oxygen-binding proteins in Antarctic icefishes to the Journal of Experimental Biology (JEB) [J. exp. Biol. 209: 1791-1802, 2006]. JEB is a journal that widely read by a broad spectrum of life scientists and the commentary articles tend to reach a very broad audience. In addition, this Commentary was chosen by the JEB Editors to be the first paper as subject of the journal's new online Forum.

Journal Publications

Small, D.J., T. Moylan, M.E. Vayda and B.D. Sidell, "The myoglobin gene of the Antarctic icefish, Chaenocephalus aceratus, contains a duplicated TATAAAA sequence that interferes with transcription.", Journal of Experimental Biology, p. 131, vol. 206, (2003). Published,

O'Brien, K.M., C. Skilbeck, B.D. Sidell and S. Egginton, "Muscle fine stucture may maintain the function of oxidative fibres in haemoglobinless Antarctic fishes.", Journal of Experimental Biology, p. 411, vol. 206, (2003). Published,

Grove, T.J. and B.D. Sidell, "Myoglobin deficiency in the hearts of phylogenetically diverse temperate-zone species", Canadian Journal of Zoology, p. 893, vol. 80, (2002). Published,

Kawall, H.G., J.J. Torres, B.D. Sidell and G.N. Somero, "Metabolic cold adaptation in Antractic fishes: evidence from enzymatic activities of brain.", Marine Biology, p. 279, vol. 140, (2002). Published,

Sidell, B.D. and J.R. Hazel, "Triacylglycerol lipase activities in tissues of Antarctic fishes.", Polar Biology, p. 517, vol. 25, (2002). Published,

Eastman, J.T. and B.D. Sidell, "Measurements of buoyancy for some Antarctic notothenioid fishes from the South Shetland Islands.", Polar Biology, p. 753, vol. 25, (2002). Published,

Winnard, P., B.D. Sidell and M.E. Vayda, "Teleost introns are characterized by a high A+T content.", Comparative Biochemistry and Physiology, p. 155, vol. 133B, (2002). Published,

Dunlap, W.C., A. Fujisawa, Y. Yamamoto, T.J. Moylan and B.D. Sidell, "Notothenioid fish, krill and phytoplankton from Antarctica contain a vitaminE constituent (a-tocomonoenol) functionally associated with cold-water adaptation.", Comparative Biochemistry and Physiology, p. 299, vol. 133B, (2002). Published,

Hazel, J.R. and B.D. Sidell, "The substrate specificity of hormone-sensitive lipase from adipose tissue of the Antarctic fish, Trematomus newnesi.", Journal of Experimental Biology, p. 897, vol. 207, (2004). Published,

Sidell, B.D., "The expression of myoglobin in hemoglobinless Antarctic fish.", The Physiologist, p. 288, vol. 45, (2002). Published,

Moerland, T.S., J.R. Erickson and B.D. Sidell, "Calcium binding of parvalbumin is conserved at normal physiological temperatures in Antarctic and Temperate teleost fishes.", The Physiologist, p. 308, vol. 45, (2002). Published,

Grove, T.J. and B.D. Sidell, "Substrate specificity and structure of fatty acyl CoA synthetase from Notothenioid fishes.", The Physiologist, p. 309, vol. 45, (2002). Published,

Grove, T.J., J.W. Hendrickson and B.D. Sidell, "Two species of antarctic icefishes (genus Champsocephalus) share a common genetic lesion leading to the loss of myoglobin expression.", Polar Biology, p. , vol. , ( ). Accepted,

Grove, T.J. and B.D. Sidell, "Fatty Acyl CoA Synthetase from Antarctic notothenioid fishes may influence substrate specificity of fat oxidation.", Comparative Biochemistry and Physiology, p. , vol. , ( ). Submitted,

Winnard, P., R.E. Cashon, B.D. Sidell and M.E. Vayda, "Isolation, characterization and nucleotide sequence of the muscle isoforms of creatine kinase from the Antarctic teleost, Chaenocephalus aceratus.", Comparative Biochemistry and Physiology, p. 651, vol. 134B, (2003). Published,

Sidell, B.D. and K.M. O'Brien, "When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes.", Journal of Experimental Biology, p. 1791, vol. 209, (2006). Published,

Erickson, J.R., Sidell, B.D. and T.S. Moerland, "Temperature sensitivity of calcium binding for parvalbumins from Antarctic and temperate zone teleost fishes.", Comparative Biochemistry and Physiology, p. 179, vol. 140A, (2005). Published,

Erickson, JR, Wallace, R, Sidell, BD and Moerland, TS, "Thermal adaptation in Mg binding and thermodynamics of parvalbumins in fishes.", Comp. Biochem. Physiol., p. , vol. , ( ). ,

Erickson, JR, Hendrickson, JW, Mayer, G, Moerland, TS and Sidell, BD, "Structural bases for thermal adaptation in ion-binding by fish parvalbumins.", Journal of Experimental Biology, p. , vol. , ( ). ,

Books or Other One-time Publications

Web/Internet Site

Other Specific Products

Contributions

Contributions within Discipline:

Our work on the variable expression of cardiac myoglobin among Antarctic icefishes has further strengthened conclusions about factors driving evolution of Antarctic fish species. In particular, we have presented evidence that multiple independent events have led to the loss of expression of this protein among several species of Antarctic icefish, that loss of the protein resulted in decreased physiological performance of hearts and, that these traits have been retained in populations only because of a combination of unique aspects of the physical environment (very cold temperature, high oxygen availability) and evolutionary history (crash in species diversity, lowering niche competition and the periodic availability of refugia) within the Southern Ocean. Most recently, we have established that the lack of expression of myoglobin in two icefish species, Dacodraco hunteri and Chaenocephalus aceratus, is due to an indentical 15 nucleotide insertion that occurs upstream of the core promoter region of the myoglobin gene. Because the chances of such an identical insertion occuring via 2 independent events are diminishingly small, this finding indicates that the two species have been affected by a single mutational event in shared ancestry and are sister taxa. This conclusion will require a modest modification of the phylogeny of the icefish family.

Results from our work with the intracellular Ca++-binding protein, parvalbumin, is yielding information that indicates ligand-binding characteristics of a non-catalytic (i.e. non-enzyme) protein are subject to the same selective pressures from evolution at cold body temperature as have been observed with enzymes from Antarctic species. In other words, there appears to be a unified set of 'design characteristics' that are necessary for all proteins that must undergo conformational change for appropriate function. It is clear that parvalbumin from Antarctic fishes functions more efficiently at cold temperature than the homologous protein from warmer-bodied animals (see Erickson et al. 2005). The full length sequence that we have obtained for the parvalbumin gene from icefish, further suggests that this adaptive difference in behavior must be attributable to one or more of a limited number of significant amino acid substitutions in the protein. The site-directed mutagenesis experiments that we are completing should permit us definitively to identify the specific amino acid substitions that underlie thermally adaptive parvalbumin function in the polar fishes.

Results of studies with the enzyme fatty acyl CoA synthetase (FACS), which catalyzes an essential step in the catabolism of fatty acids, indicate that this enzyme may play a critical role in determining the types of fatty acids that ultimately are relied upon as metabolic fuel by Antarctic fishes. We have found that the enzyme from nototheniod fishes displays greatest activity and highest substrate affinity for fatty acyl substrates that are unsaturated, a result that is consistent with the behavior of the intact metabolic system of the animal's oxidative muscle tissues (See Grove and Sidell, 2004).

Contributions to Other Disciplines:

Insights about how deleterious traits may have been retained in populations of Antarctic fishes (described for the myoglobin expression studies under Contributions within the Discipline above) are particularly important to fundamental aspects of Evolutionary Biology. In fact, retention of 'disaptive' characters, as evinced by multiple independent losses of myoglobin expression among the Antarctic icefishes, speaks directly to central issues of mechanisms of evolutionary change. Our findings clearly indicate that evolution of Antarctic channichthyid fishes has proceeded via a combination of events of both natural selection and of genetic drift.

Our findings that a duplication of the muscle-specific TATA box promotor element in Chaenocephalus aceratus and Dacodraco hunteri has led to loss of expression of an otherwise functional gene demonstrates that genes can be silenced by very subtle changes in regulatory elements.

Contributions to Human Resource Development:

One component of our project was the focus of M.S. research of Ms. Jamie Hendrickson at the University of Maine (M.S. awarded 2005).

One component of our project was the focus of Ph.D. research of Mr. Jeffrey Erickson at Florida State University (Ph.D. awarded 2005).

Contributions to Resources for Research and Education:

Our research on differential expression of myoglobin in hearts among Antarctic icefishes formed the basis of three different invited lectures to relatively broad scientific audiences:

1. Invited symposium talk at the international intersociety meeting, 'The Power of Comparative Physiology: Evolution, INtegration and Application' held in San Diego, CA during late August 2002.

2. Invited scientific lecture, the C. Ladd Prosser Lecture in Comparative Physiology, an annual named and endowed lecture held at the University of Illinois, Urbana, IL in March 2003.

3. Invited plenary lecture at the International Congress of Fish Biology, St. John's, Newfoundland, CANADA. July 2006.

Additional contributions for research and education:

a. Invited research seminar at Ohio University, October 2004.

b. I presented a talk to students (ca. 10 years of age) at the Junior School in Port Stanley, Falkland Islands on 27 May 2004. This talk was at the invitation of Dr. Paul Brickle of the Falkland Islands Fisheries Office. I spoke to the children about the biology of Antarctic fishes in general and Antarctic Icefishes in particular.

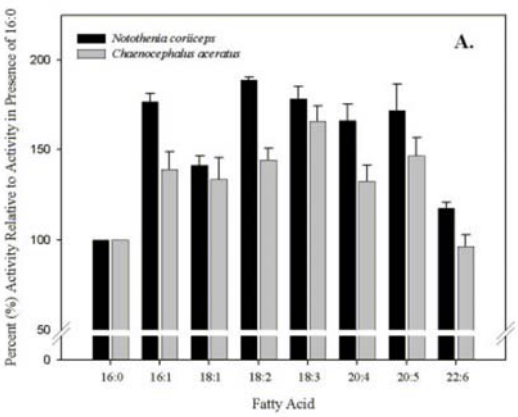
Contributions Beyond Science and Engineering:

Conference Proceedings

Categories for which nothing is reported:

Any Book
Any Web/Internet Site
Any Product
Contributions: To Any Beyond Science and Engineering
Any Conference

a. Objective 1: To identify the amino acid substitutions in the fatty acid-binding pocket of fatty acyl CoA synthetase (FACS) from Antarctic fishes that contribute to its substrate specificity. In earlier work, we successfully cloned and sequenced the gene for FACS from three Antarctic fish species, *Notothenia coriiceps, Gobionotothen gibberifrons* and *Chaenocephalus aceratus.* Specific aims of this component of our project were to purify and characterize fatty acyl CoA synthetase from tissues of Antarctic fishes and then insert the gene into an *E. coli* expression system. Once successful production of enzyme by the expression system was established, we planned to perform site-directed mutagenesis to reverse at least two non-conservative amino acid substitutions found in the critical fatty acid-binding pocket of the Antarctic fish protein, which were good candidates for explaining differences in substrate specificity of the enzyme compared to that seen in warmer-bodied animals. We expended a rather substantial amount of time and effort to purify FACS from Antarctic fishes and found that, as the enzyme was progressively taken to higher degrees of purity, it became more and more unstable. We explored numerous approaches to stabilize the protein in dilute solution but met with little success. Replacement of detergents used in other published purification methods with 1% octyl-β-glucoside and incorporation of SH-protecting reagents did permit sufficient stabilization of the partially purified protein to conduct assessment of substrate specificity, but did not stabilize the protein during additional purification steps. Results of these characterizations of activity do suggest that FACS, in fact, does contribute to establishing the substrate specificity of fatty acid oxidation pathways observed with intact tissues from these fishes (see Sidell *et al.*, 1995). With the exception of $C_{22:6}$, maximal activities of FACS from both *Notothenia coriiceps* and *Chaenocephalus aceratus* were greater with unsaturated substrates that observed with a reference saturated fatty acid, $C_{16:0}$ (Figure 1). At equivalent substrate chain length of 16 carbons, inclusion of a single double bond to form a monoenoic fatty acid ($C_{16:1}$) results in a lower estimated K_m of FACS (1.84 ± 0.85 μM) than seen with the fully saturated ($C_{16:0}$) substrate (3.21 ± 0.44 μM). Thus, <u>FACS from Antarctic fishes displays maximal catalytic activity and highest substrate affinity with unsaturated fatty acyl substrates</u>, a pattern consistent with that displayed by the overall pathway of fatty acid oxidation in these animals. These results were described in Grove and Sidell (2004). Because of persistent stability problems encountered with this relatively large enzyme (697 amino acids), however, we felt that the likelihood of success in producing an expression product that could reliably be used in functional studies was very low. Consequently, abandoned the expression system and site-directed mutagenesis components of this objective in favor of employing this same approach to pursue very promising avenues with parvalbumin, a smaller and more stable protein, subject of the next objective of our project (see below).



b. Objective 2: Biochemical and biophysical characterization of parvalbumin from notothenioid white muscle. The aims of this component of our project are to purify parvalbumins from Antarctic fish species, characterize their Ca^{++}- and Mg^{++}-dissociation constants (K_D), estimate unidirectional rate constants for ion dissociation from the proteins and clone and sequence the gene for the protein from one or more Antarctic fish species.

Parvalbumins are a family of small (11-12 kDa) aqueously soluble Ca^{++}-binding proteins that are found in muscle and in some types of neurons. They are especially abundant in fast-

contracting striated muscles of fish, anurans, and other lower vertebrates (including Antarctic fishes), where their intracellular concentrations can approach several millimolar. The accepted physiological role of parvalbumins is to facilitate relaxation from the active state in rapidly contracting muscles. Because burst-speed swimming of Antarctic notothenioid fishes for prey capture or predator avoidance is dependent upon recruitment of the white axial muscle mass and because these muscle fibers are exceptionally large in size (up to *ca.* 1,000 µm in diameter), we reasoned that parvalbumin's role as in intracellular buffer of [Ca^{++}] would be subject to strong selective pressure.

We have succeeded in purifying parvalbumins from notothenioid fishes, *Gobionotothen gibberifrons* and *Chaenocephalus aceratus*. Two-dimensional electrophoretic analyses indicate that a single form of the protein is expressed in white muscle of each species. Estimates of Ca^{++}-dissociation constants (K_D) for these parvalbumins were initially done by stopped-flow fluorescence methodology during our 2003 field season at Palmer Station. We followed these measurements by more rigorous estimations using isothermal titration microcalorimetry performed with purified protein returned to the CONUS laboratory of collaborator, Dr. Timothy Moerland at *Florida State University*. This technique permits simultaneous estimates of K_D and complete thermodynamic constants for Ca^{++}-binding. Our results indicate that, compared to measurements made with parvalbumin from a warmer-bodied teleost (*Cyrprinus carpio*), parvalbumins from the Antarctic species display an apparent affinity for Ca^{++} at cold temperature that is similar to that of parvalbumin from the warmer-bodied species when measured at 25 ºC (indicating a conservation of Ca^{++}- binding ability at respective physiological temperatures) (Fig. 2). Thermodynamic analyses further show that conservation of Ca^{++}-binding of the Antarctic parvalbumins is characterized by a free energy (ΔG^o) of Ca^{++}-binding that is relatively independent of temperature achieved by: 1) a systematically lower reliance upon enthalpy of Ca^{++}-binding (ΔH^o)

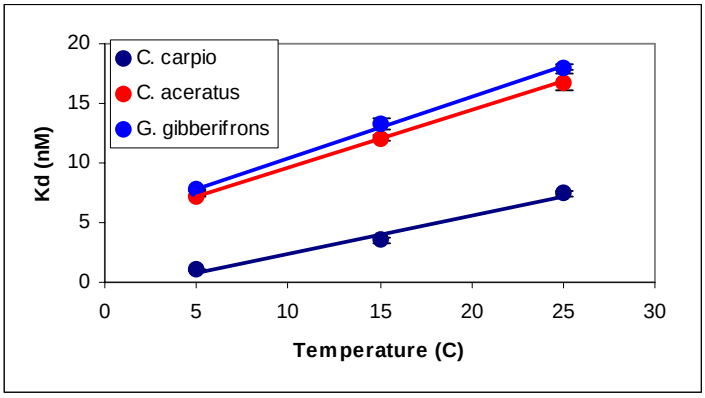


Figure 2. *Dissociation constants (K_D) for Ca^{++} estimated for parvalbumins from Antarctic (G. gibberifrons, C. aceratus) and temperate zone (C. carpio) teleosts by isothermal titration microcalorimetry.*

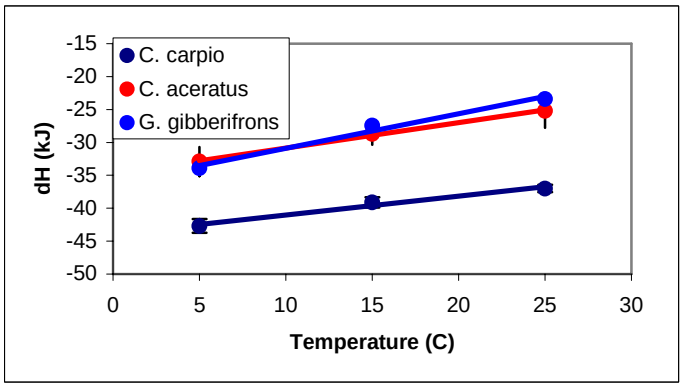


Figure 3. *Enthalpy of Ca^{++}-binding for parvalbumins from Antarctic and temperate zone teleosts.*

(Fig. 3) and, 2) a systematically greater reliance upon the entropic contribution to free energy change during Ca^{++}-binding ($T\Delta S^{o}$) than is characteristic of the warmer-bodied species (Fig. 4).

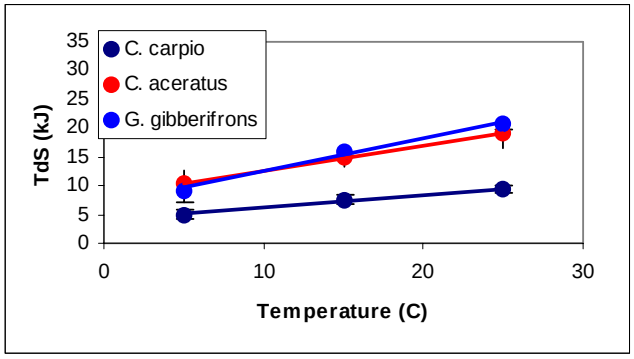


Figure 4. *Entropy of Ca^{++}-binding to parvalbumins from Antarctic and temperate zone teleosts.*

We recently have also collected data estimating the off-constant for Mg^{++}-dissociation from parvalbumin in both Antarctic and temperate zone fishes. This is a particularly important functional step in the overall cycle of Ca^{++}-handling by the protein because, prior to binding Ca^{++}, Mg^{++} is bound to the protein and must be displaced by the calcium. Results from these experiments are consistent with the pattern described above in that they show conservation of the kinetic constant for this process at the respective physiological temperatures of the animals. Thus, the protein from Antarctic species shows characteristics indicative of a more flexible protein structure than observed in warmer-bodied animals, enabling effective function at the colder body temperatures of Antarctic fishes. Results from these experiments formed the basis for Erickson *et al.* (2005).

Graduate student, Jamie Hendrickson used degenerate primers to amplify successfully three apparently distinct parvalbumin gene products from *Chaenocephalus aceratus.* One of these gene products shows nearly complete identity (one amino acid difference) to the directly determined peptide sequence of the single parvalbumin protein purified from the tissue. We obtained full-length coding sequence for this parvalbumin from *Chaenocephalus aceratus* (see Figure 5 at the end of this document). Sequence differences between the protein and those of warmer-bodied animals were then assessed for amino acid substitutions likely to underlie functional differences in the proteins. Antarctic parvalbumin gene was then transfected into an *E. coli* expression system. The expressed parvalbumin from this transfection is functionally identical to the wild type protein (Figure 6).

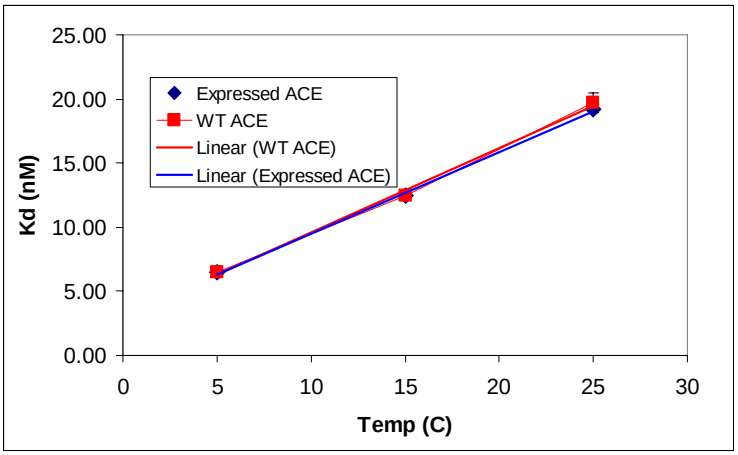


Figure 6. *Parvalbumin produced in the E. coli expression system is functionally identical to the wild-type protein purified from icefish muscle.*

We now have successfully created three different genetic mutants for parvalbumin. Two of these have single amino acid reversions from the icefish sequence to that typically seen in warmer

bodied animals and the third incorporates both mutations. These mutant genes have recently been transfected into the *E. coli* expression systems and we are in the process of harvesting mutant icefish parvalbumin protein. When purified, these mutated proteins will be sent to our collaborators at Florida State University for functional analyses. Results from these experiments will determine whether the candidate amino acid substitutions are, in fact, responsible for adaptive changes in the icefish protein that enable its function at cold temperature.

c. Objective 3: Extend the survey of cardiac myoglobin expression among species of the Suborder Notothenioidei. Expression of the intracellular oxygen-binding protein, myoglobin (Mb) in cardiac muscle is variable among species of Channichthyid icefishes (Sidell *et al.,* 1997; Moylan and Sidell, 2000). Of the sixteen known icefish species, 10 species do express Mb in their heart muscle, while six others do not (Grove *et al.*, 2004). Mapping of the trait of Mb expression on the consensus phylogeny of the family further indicates that ability to produce Mb has been lost *via* 4 independent mutational events and by multiple mechanisms (Moylan and Sidell, 2000; but see additional results below). Despite this pattern, we also have developed convincing data that indicate, when present, Mb is functional in icefishes (Cashon *et al.*, 1997) and enhances mechanical performance of the heart (Acierno *et al.*, 1997). If we assume that: (i) reliance upon myoglobin function is equivalent in icefishes and in their red-blooded notothenioid relatives, (ii) no genetic linkage exists between expression of hemoglobin and myoglobin, and (iii) the normal rate of mutation of the Mb gene has been similar in ancestral Mb(+) icefishes and other notothenioids, we arrive at the following hypothesis. We should expect to encounter a roughly similar rate of loss of cardiac Mb expression in species that belong to the red-blooded notothenioid families compared to that observed in the channichthyids. Testing this hypothesis has been one component of our funded project.

At Palmer Station, we were able to sample hearts from ten species of red-blooded notothenioids representing two families: *Notothenia coriiceps, Notothenia rossii, Gobionotothen gibberifrons, Parachaenichthys charcoti, Lepidonotothen nudifrons, Dissostichus mawsoni, Lepidonotothen larseni, Trematomus hansoni, Trematomus newnesi, Trematomus eulepidotus*. Tissues were extracted and samples subject to SDS-PAGE followed by immunoblotting with Mb-specific antibody. All ten of these species proved to be Mb-expressers in cardiac muscle. Given that the species sampled represent less than 10% of known red-blooded notothenioids, it is entirely premature to conclude that Mb-expression is a trait of all non-Channichthyid notothenioids.

d. Additional results related to myoglobin expression in Channichthyid icefishes: Until very recently, we had identified the mechanisms leading to loss of Mb expression in 3 of the 4 clades of non-expressers identified by mapping the trait on a consensus phylogeny of the Family (Near *et al.*, 2003). The one Mb-lacking species that remained unresolved was *Dacodraco hunteri*. Within the last two weeks, we have obtained sequence of the Mb gene from genomic DNA of *Dacodraco*, which has revealed surprising information. The core promoter, coding sequence and introns (including splice junctions) from the Mb gene of *D. hunteri* reveals no anomalies that would explain lack of expression of Mb in this species. Consequently, we have sequenced 1500 nt of the gene's promoter region upstream of start and found that *D. hunteri* contains the very same 15-nt insertion in the identical position as observed in *Chaenocephalus aceratus*. We have reported convincing evidence from transient expression experiments with promoter constructs linked to a reporter gene that indicates the duplication of the muscle-specific TATAAAA sequence found in this insertion probably leads to lack of Mb expression (Small *et al.*, 2003). Thus, it seems reasonable to conclude that this mechanism also is responsible for loss of Mb expression in *D. hunteri*. The observation also leads to another unavoidable conclusion: because an identical duplication/insertion event occurring two times independently during evolution of the icefishes is extremely unlikely, this must mean that *D. hunteri* and *C. aceratus*

share the same mutational event and are, in fact, sister taxa. This conclusion is rather provocative in light of currently published phylogenies of the icefish family and would require a modest revision of the phylogeny of Family Channichthyidae. Although we are quite confident of this result, we sought to obtain additional samples of *D. hunteri*, where provenance of the samples was very reliable. We have, in fact, recently obtained tissue from an additional specimen of *D. hunteri* from Dr. H. William Detrich of Northeastern University. Sequencing of the myoglobin gene from this sample will be completed shortly. If, as expected, sequence from this specimen also contains the 15-nt insertion observed previously, we will activate a collaboration (already discussed) with an expert in systematics of this group to produce paper describing revision of the phylogeny of the icefish Family.

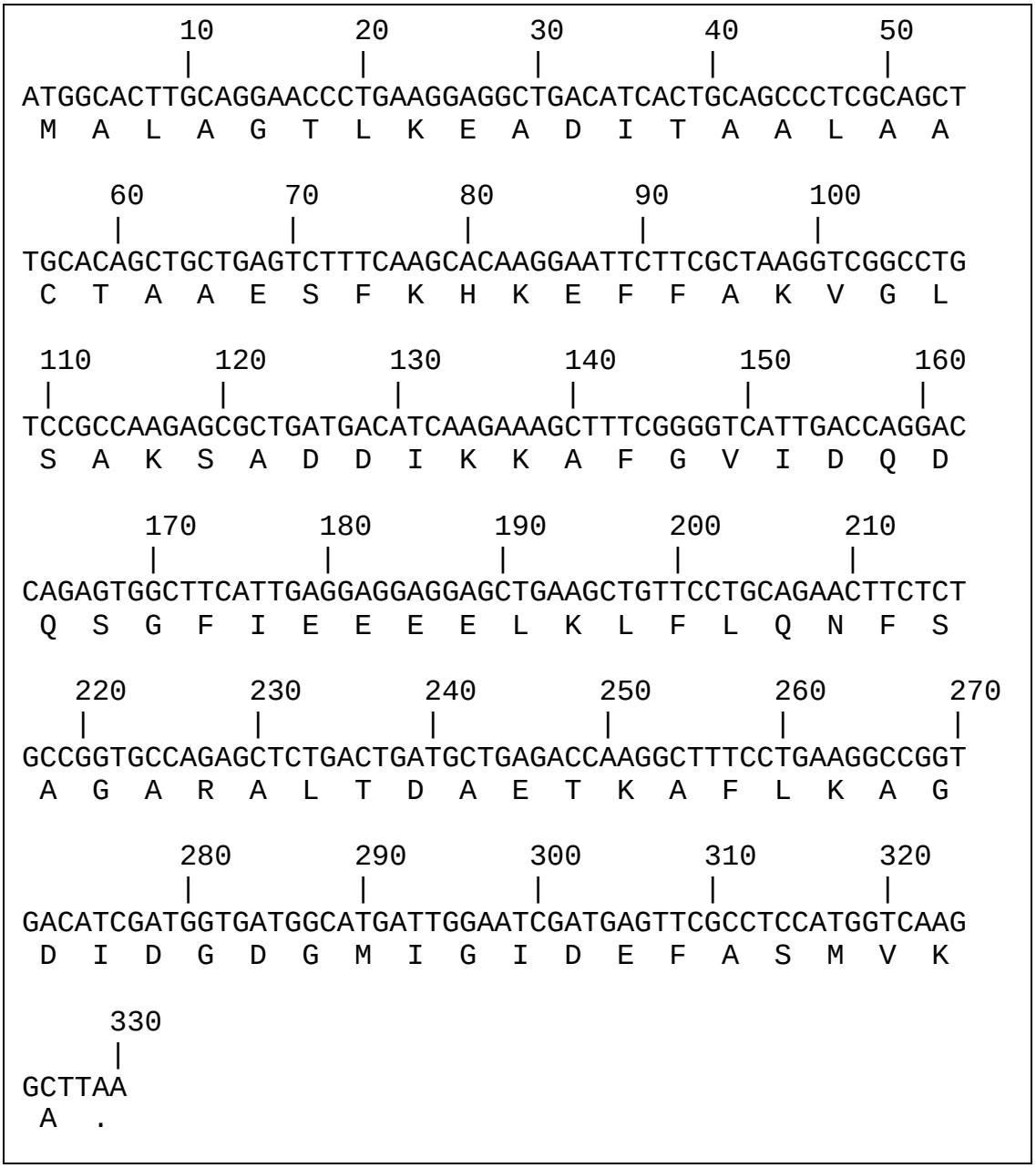


Figure 5. Full-length nucleotide and deduced amino acid sequences for parvalbumin protein from *C. aceratus* white muscle.

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