THE EFFECTS OF TEMPERATURE ON HEMOGLOBIN IN *CAPITELLA TELETA*

by

Alexander M. Barclay

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

Summer 2013

THE EFFECTS OF TEMPERATURE ON HEMOGLOBIN IN *CAPITELLA TELETA*

by

Alexander M. Barclay

Approved: _____
Adam G. Marsh, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Mark A. Moline, Ph.D.
Director of the School of Marine Science and Policy

Approved: _____
Nancy M. Targett, Ph.D.
Dean of the College of Earth, Ocean, and Environment

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I extend my sincere gratitude to the individuals that either contributed to the execution of my thesis project or to my experience here at the University of Delaware. I would like to give special thanks to my adviser, Adam Marsh, who afforded me an opportunity that exceeded all of my prior expectations. Adam will say that I worked very independently and did not require much guidance, but he served as an inspiration and a role model for me during my studies. Adam sincerely cares for each of his students and takes the time and thought to tailor his research program to fit each person's interests. The reason that I initially chose to work in Adam's lab was that he expressed a genuine excitement for science and for his lifes work. His enthusiasm resonated with me and helped me to find my own exciting path in science. Wherever it leads, I was truly fortunate to have had Adam as a graduate adviser, as I am certain, he is one of a kind.

I would also like to express my thanks towards my committee members, Jonathan Cohen and Thomas Hanson. They were supportive of my project ideas from the start and provided valuable guidance and feedback throughout the process. My lab mate, Annamarie, was a good friend and fun to work with in the lab. I also want to thank Mark Warner for so generously sharing much of his lab equipment and Patrick Gaffney, who was a great professor and mentor. Charles Epifanio also provided guidance and mentorship during my first semester. I also want to say thank you to Connie and Janis, who took care of much of the administrative work and were always available to answer questions, as well as Debbie and Ellen, who made the library a welcoming and joyous place to study.

Lastly, I would like to thank my parents, siblings, and Colleen for their continual encouragement, willingness to listen, and unconditional love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Annelid hemoglobin represents an impressive diversity in structure and function that has been highly influential in developing the current understanding of how this large class of protein works. Unique to the annelids are giant extracellular pigments of two main varieties: the 3600 kDa erythrocruorins and the 400 kDa hexagonal-bilayer hemoglobins. The marine polychaete *Capitella teleta* has served as a model organism for numerous biological studies, yet very little work has been dedicated to understanding the types and properties of respiratory pigments utilized by this worm. As a temperate opportunist, this species is ideal for studying the effects laboratory induced changes in temperature and oxygen saturation on the structure and functionality of hemoglobin. The goal of this work was to determine what type of extracellular hemoglobin is normally present in *C. teleta*, and to determine the effects of warm versus cold acclimation on the concentration and subunit composition of this hemoprotein.

It was observed that cold-acclimation induces two major changes in the morphology of *C. teleta*: an increase in body size and a change in red pigmentation. Using the pyridine hemochrome assay in conjunction with a heme activity assay, it was determined that the difference in pigmentation was due to a combination of effects. The 21^{o}C worms had a greater concentration of hemoglobin than the 4^{o}C worms, but less heme b. It has been suggested that this was due to a difference in the functional status of the heme b, as well as a minor difference in hemoglobin stability.

Using size exclusion chromatography and native gel electrophoresis, the major extracellular hemoglobin in both warm- and cold-acclimated *C. teleta* was determined to be a 333kDa hexagonal-bilayer hemoglobin composed of three major and up to three minor polypeptide chains that covalently bind to form disulfide-linked dimers

and trimers. A major difference in the expression of these subunits between the experimental cultures was not detected. This study represents the first attempt at identifying and characterizing extracellular hemoglobins from *C. teleta*. It has established a strong foundation for future work on the purification and analysis of this important respiratory protein in a model annelid.

Chapter 1

INTRODUCTION

1.1 A Historical Perspective

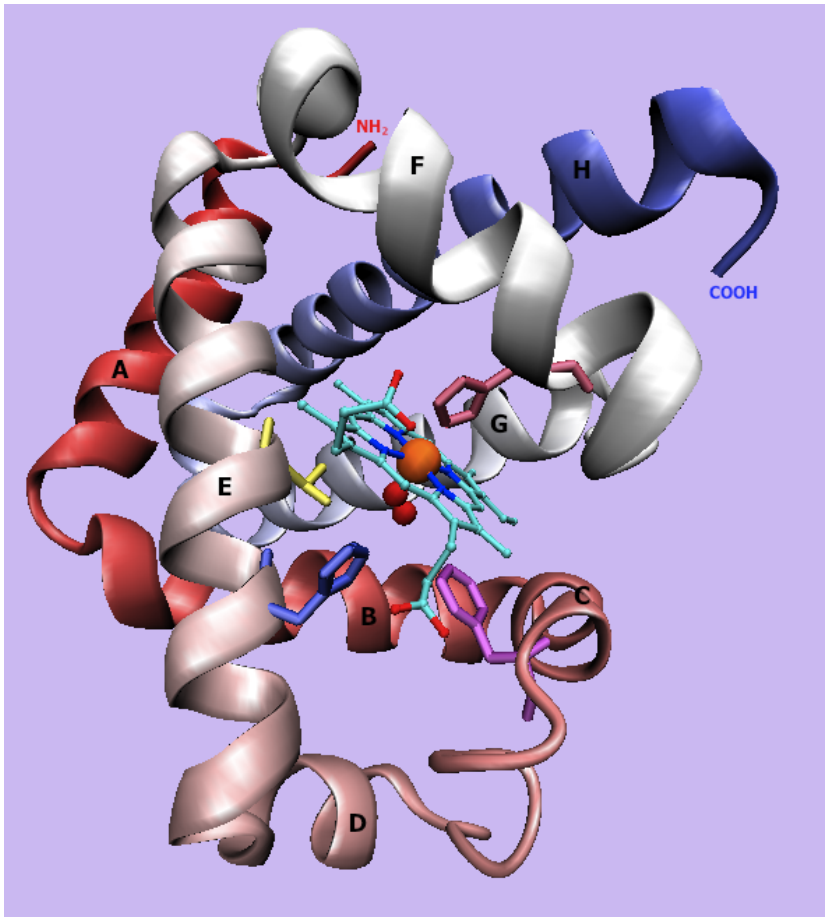
Published literature on invertebrate hemoglobin exists from as early as 1868, when the terms chlorocruorine and red cruorine (now erythrocruorine) were first proposed to describe the green and red "non-corpusculated vascular" pigments of many annelid species (Lankester 1868). The only observable difference between these pigments and those of the vertebrates was their extracellular localization. For many years, it was assumed that since these pigments shared a similar absorbance spectrum to vertebrate hemoglobin, that they were very similar in structure (Barcroft 1924). It was not until the 1930s that ultracentrifugal sedimentation studies determined that these pigments are actually many times larger (Svedberg & Eriksson 1933) and more diverse between species and phyla (Svedberg & Hedenius 1933) than previously thought. Unlike the familiar $\alpha_2\beta_2$ mammalian hemoglobin contained within erythrocytes and myoglobin found in muscle tissues, the invertebrates present a diverse compilation of respiratory pigments that differ structurally and functionally at all levels of organization.

Despite the extraordinary diversity between different hemoglobins, they share numerous commonalities. At the most fundamental level, every hemoprotein, whether vertebrate, invertebrate, bacterial, or even plant, contains a series of five to eight alpha-helices, intricately arranged about and coordinated with the iron center of a heme prosthetic group, forming a three-dimensional pocket. The first protein structures ever determined by x-ray crystallography were sperm whale myoglobin in 1960, followed by horse hemoglobin in 1968 (Royer et al. 2001). In addition to revolutionizing the field of biochemistry, the naming convention originally used to describe the helices

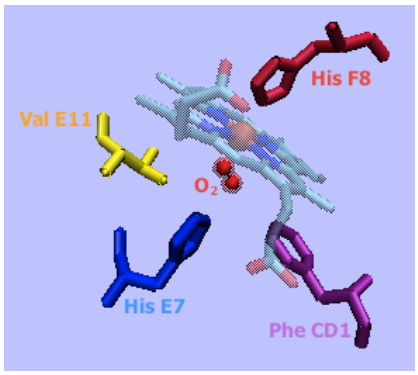
and corners in sperm whale myoglobin have become conventional for the naming of all other globins. Starting at the N-terminus, the helices are labeled A through H, and the corners between them are labeled according to the helices on either side (i.e. C-D is the peptide bend connecting helices C and D). Consequently, all amino acid residues are labeled according to the helix or bend they are located on, followed by the numerical location within the peptide chain making up the helix or bend. To illustrate this, the proximal ligand for the heme-iron, its fifth ligand, is always a histidine residue located in the eighth position on the F-helix (Figure 1.1). For this reason, it is referred to as His F8, and is the most highly conserved feature of all hemoglobins found in nature (Royer et al. 2005).

Regardless, the geometric orientation of His F8 is not conserved between species or even between subunits of the same hemoglobin. The degree to which this residue is staggered or eclipsed heavily impacts the reactivity of the iron center, and consequently, the heme's affinity for oxygen, which binds to the iron as the distal, or sixth ligand, and is typically, but not always, stabilized by a second histidine residue in the E7 position. Carbon dioxide, nitric oxide and sulfide also bind the iron or to free cysteine residues in some species that either take advantage of the prosthetic group's enzymatic properties or harbor microbial symbionts (Vinogradov & Moens 2008). Evolutionarily, these properties most likely precluded hemoglobin as an oxygen transporter.

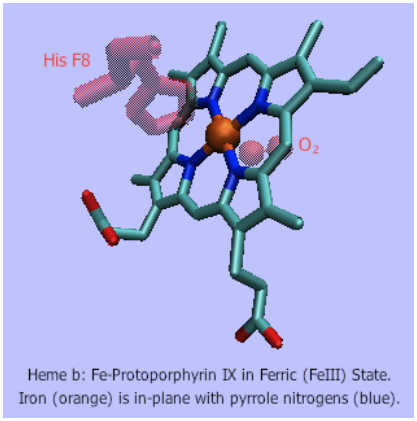
Beyond this modest conservation of structure, hemoglobins have different functions between the domains of life, and are vastly divergent between phyla of the animal kingdom. Within the invertebrate phyla, small, intracellular hemoglobins and myoglobins are found to exist as monomers, dimers, tetramers, as complex aggregates, or not at all (Terwilliger 1980). Giant extracellular pigments of various types are also found in certain groups (Vinogradov 1985). Of all the phyla, it was the annelids and arthropods that showed the greatest range in respiratory pigment size, structure, and function (Bruneaux et al. 2008). Over the past few decades, extensive work has been dedicated to understanding the organization, stability, and function, of the annelid extracellular hemoglobins. These studies have made significant contributions to our



(a) Myoglobin



(b) Key Residues



(c) Heme

Figure 1.1: Cartoon depicting Sperm Whale myoglobin and the proper naming of helices A-H (a). The proximal histidine (His F8) and the distal residues (His E7, Phe CD1, and Val E11), as well as the oxygen ligand (O_2) (b). (c) depicts the heme prosthetic group rotated 90^o about the plane of the page from (b), with the iron atom shown in orange and the proximal histidine shown in translucent red. The red oxygen atoms branching off the heme are part of propionate groups involved in hydrogen bonding.

present understanding of self-assembly of macromolecules (Ebina et al. 1995, Zhu et al. 1996b), association-disassociation kinetics (Rousselot et al. 2006a), mechanisms of cooperativity (Royer et al. 2005), phylogenetic relationships (Lamy et al. 1996, Chabasse et al. 2006), and the development of viable blood-substitutes (Zal et al. 2000, Riess et al. 2001).

1.2 Structural Diversity

Several respiratory pigments dominate the annelid phylum: intracellular myoglobins and hemoglobins contained within the coelomic cells weighing between fifteen and sixteen kilodaltons; dimeric, tetrameric, and octameric hemoglobins, also contained within the coelomic cells; and polymeric extracellular pigments composed of dodecamers of hexagonally arranged subunits, a quaternary structure commonly referred to as a hexagonal bilayer (Aki 2007), floating freely in the closed vascular system and coelomic fluid (Terwilliger 1980, Garlick 1980). The heme content of these extracellular pigments is usually low, unlike the one heme to one globin-polypeptide stoichiometry of vertebrate hemoglobin (Vinogradov 1991). Early hypotheses suggested that annelid globin has a lower affinity for heme (Waxman 1975), but later findings with *Lumbricus* hemoglobin confirmed that twenty-four to thirty-two kilodalton peptide linker chains hold these large pigments together (Vinogradov et al. 1986, Zhu et al. 1996b) in a supramolecular structure that has been well characterized at the atomic level (Royer et al. 2000, Royer et al. 2006). Although giant extracellular hemoglobins have been identified in many annelid species (Bruneaux et al. 2008), four species have served as the primary research subjects since the late 1980s: the earthworm *Lumbricus terretris*, the Capitellid polychaete *Arenicola marina*, the deep sea hydrothermal vent tubeworm *Riftia pachyptila*, and the beard worm *Oligobrachia mashikoi*.

The annelid giant extracellular hemoglobins have been divided into four groups based on common structural features. The first type, chlorocruorin, is a green pigment found in some polychaete species that differs primary by its modified heme group (Weber & Vinogradov 2001, Pallavicini et al. 2001). The discovery of this pigment

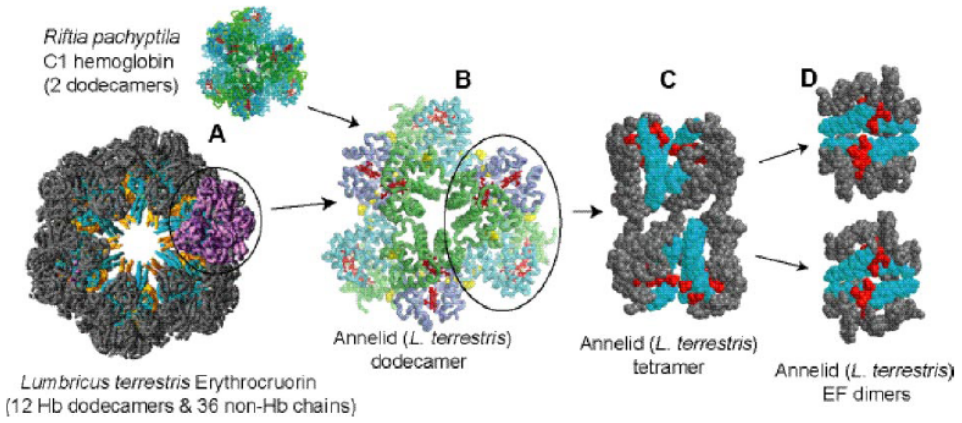


Figure 1.2: Crystal structures showing the hierarchical arrangement of subunits in *L. terrestris* erythrocruorin and *R. pachyptila* HBL Hb. Figure taken from Royer et al. 2005.

contributed to many early findings on invertebrate respiratory pigments (Roche 1933), but will not be discussed here extensively. The next two types are both erythrocruorins with molecular weights of approximately 3.6 x 10^6 kDa. Both types of erythrocruorins are hexagonal bilayer hemoglobins (HBL-Hbs) that are held together by a scaffold of 36 non-globin linker subunits. The native complex is composed of 144 globin peptides symmetrically arranged in twelve dodecameric units, each composed of a trimer of heterotetramers. The subunit composition of the heterotetramers is variable between species. Each dodecamer associates with three linker chains in a one-twelfth submultiple called a protomer (Figure 1.2).

The difference between the two erythrocruorins is that Type I, discovered in *L. terrestris*, have a staggered arrangement in which one half of the bilayer is rotated 16 degrees about the other half, and the linker subunits hold the complete molecule more compactly. Type II erythrocruorins, discovered in *A. marina*, have an eclipsed arrangement and have more space between halves of the bilayer and between neighboring submultiples (Jouan et al. 2001, Royer et al. 2007). The final group of giant extracellular hemoglobins is specifically called hexagonal bilayer hemoglobin (HBL-Hb) and has a molecular weight of approximately 400kDa. HBL-Hb will now be used exclusively to describe this type of giant extracellular hemoglobin. HBL-Hb, discovered in

O. mashikoi, is also arranged in a bilayer, but it is not held together by linker subunits. Each half of the bilayer is composed of twelve globins, analogous to a one-twelfth submultiple erythrocruorin (Numoto et al. 2005). Interestingly, *R. pachyptila* has three different giant extracellular hemoglobins: a vascular erythrocruorin, a vascular HBL-Hb, and a coelomic HBL-HB, all of which also bind sulfide reversibly (Zal et al. 1996a, Zal et al. 1996b). The types and native molecular masses for a selection of known anneid hemolgobins, including those mentioned already, are shown in Table 1.1a and b.

Table 1.1: Selection of some annelid extracellular hemoglobins. Tables describe the masses and subunit stoichiometry of the native proteins. G: gobin subunits, L: linker subunits.

(a) Some Native Hemoglobins

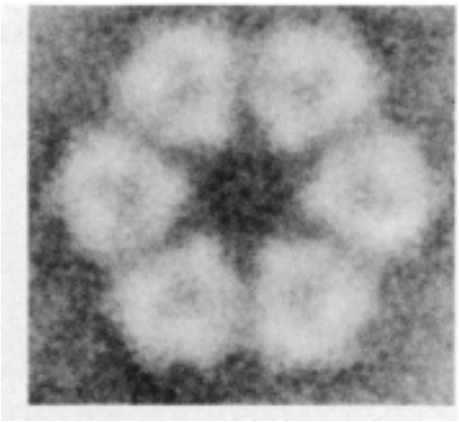
	A. marina	*L. terrestris*	*R. pachyptila*		
Habitat	sandy intertidal	soil	black smokers		
Native Hb	Type II Ec	Type I Ec	Type I Ec	HBL-Hb	HBL-Hb
Mass (kDa)	3648±24	3410-4100	3503±13	433±8	380±4
Stoichiometry	$G_{144}L_{36}$	$G_{144}L_{36}$	$G_{144}L_{36}$	G_{24}	G_{24}
Reference	Zal et al. 1997	Zhu et al. 1996	Zal et al. 1996		

(b) More Native Hemoglobins

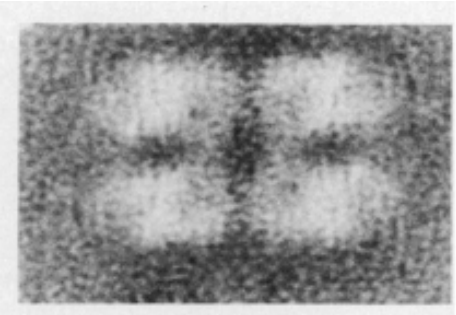
	O. machikoi	*O. webbi*	*A. pompejana*	*S. contortum*	
Habitat	sulfide-rich sediments	cold seeps	hydrothermal vents	cold seeps	
Native Hb	HBL-Hb	HBL-Hb	Type II Ec	Ec	HBL-Hb
Mass (kDa)	440	409±4	3833±14	3190±50	461±46
Stoichiometry	G_{24}	G_{24}	$G_{144}L_{36}$	G_{24}	$G_{144}L_{36}$
Reference	Numoto et al. 2005	Meunier et al. 2010	Zal et al. 1997b	Meunier et al. 2010	

1.3 Self-Assembly and Hierarchal Organization

Determining the hierarchal organization of subunits and their arrangements in the different native proteins was a challenging feat that required the combined efforts of many researchers and several revisions of the models over a few decades. New



(a) Top View



(b) Edge View

Figure 1.3: Electron Micrographs of *P. pacifica* erythrocruorin. Figure taken from Garlick 1980.

technologies, namely electrospray ionization mass spectrometry (ESI-MS), multi-angle laser light scattering (MALLS), and better crystallization methods allowed ambiguities between earlier models to be resolved with unprecedented confidence (Zal et al. 1997, Bruneaux et al. 2008). The common features that have persisted throughout all of the models are the high molecular weights of the native proteins, the functionality of sub-multiples, and the overall hexagonal bilayer geometries that were first observed in the chlorocruorins and hemoglobins of various annelid species by electron microscopy (Figure 1.3) (Terwilliger et al. 1976a). The fact that the giant extracellular hemoglobins were all symmetrical, despite their varying sizes, forced mathematical limits on the possible arrangement and total number of subunits possible (Hanin & Vinogradov 2000). Without symmetry, no one would have understood how nature took 192 polypeptide chains and put them together in exactly the same arrangement every time; a remarkable process known as self-assembly (Ebina et al. 1995).

What makes self-assembled molecules unique, albeit not uncommon, is that independently synthesized units are brought together by non-covalent electrostatic interactions and hydrogen bonds without any molecular machinery. The annelid giant extracellular hemoglobins have been influential in the study of this process. Although

the mode of assembly varies between hemoglobin types, and the specific contacts between globin and linker (if present) subunits differ markedly, dissociation-reassembly studies have shown that upon partial to complete dissociation of native protein into protomers, dodecamers, or smaller subunits, the quaternary structure is spontaneously restored, at least for a fraction of the protein in solution, following return to native conditions (Zhu et al. 1996b, Lamy et al. 1996, Rousselot et al. 2006a). The fact that this can be achieved *in vitro* implies that no molecular machinery is required to assemble these complexes once stable secondary structures are formed. It was also found that upon denaturation, the globin subunits release their hemes, which then act to oxidatively degrade the linker subunits. Reassembly was not observed here, demonstrating the importance of the linker chains in supporting quaternary structure of erythrocruorins and the heme's role in stabilizing local secondary structure.

Similar studies were also done to examine the subunit composition and hierarchal organization of the various annelid extracellular hemoglobins. Early studies relied on sedimentation equilibrium, size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the masses and multiplicity of subunits or larger substructures (Terwilliger et al. 1980, Garlick 1980, Vinogradov 1986, Zhu et al. 1996a, Nakagawa 2005). Later studies, like those mentioned previously, used high performance chromatography methods in conjunction with mass spectrometry to determine more precisely the masses of the different constituents. All of these studies relied on crystallographic data and information on the symmetry observed by electron microscopy to match mass measurements with stoichiometries. The known stoichiometries and masses for the largest submultiple hemoglobins in have been added to the species listed in Table 1.1 (Table 1.2).

1.4 Protein Function and Stability

Annelids provide an ideal system for studying the effects of environmental impacts on hemoglobin structure and function. Like all invertebrates, annelids are ectotherms and exchange matter and energy freely with their surroundings. Factors

Table 1.2: Largest submultiples of the annelid extracellular hemoglobins from Table 1.1. Tables describe stoichiometry and experimental masses of the submultiple proteins. Masses calculated based on stoichiometry rarely agree with experimental mass, and are therefore omitted, but are available in the original papers. All data were reported by the authors listed in Table 1.1. M: monomers, D: dimers, T: trimers, L: linker subunits, NA: not applicable.

(a) Some Submultiple Hemoglobins

	A. marina	*L. terrestris*	*R. pachyptila*		
Submultiple	1/12	1/12	1/12	1/2	1/2
Protomer	$(M_9T)L_{3.5}$	$(M_3T_3)L_3$	$(M_4D_4)L_3$	NA	NA
Mass (kDa)			274		
Dodecamer	M_9T	M_3T_3	M_4D_4	M_4D_4	M_4D_4
Mass (kDa)	204	214	199		

(b) More Submultiple Hemoglobins

	O. machikoi	*O. webbi*	*A. pompejana*	*S. contortum*	
Submultiple	1/2	1/2	1/12	1/12	1/2
Protomer	NA	NA			NA
Mass (kDa)					
Dodecamer			M_3T_3		
Mass (kDa)			212		

including temperature, salinity and ionic composition, carbon dioxide concentration and pH, and oxygen concentration all strongly affect protein structure, stability, and affinity for oxygen and other ligands. In the face of environmental change, there are a few physiological responses that can take place at the molecular level in order to maintain or alter hemoglobin function. The most obvious response is to alter the rate of hemoglobin synthesis or breakdown, which can rapidly change the oxygen carrying capacity of an organism. While this does increase the amount of oxygen that can be carried throughout the organism, it can be metabolically expensive and is only effective at dealing with low oxygen levels.

Other factors mentioned, like temperature or ionic composition, may negatively affect the affinity of the existing hemoglobin for oxygen by disrupting the stability or conformation of the protein. In these cases, synthesizing more hemoglobin will not

help the organism. Allosteric modulators can greatly increase or decrease the affinity of hemoglobin for oxygen by stabilizing a desired protein conformation or by helping to expose the heme pocket. In humans, the organic phosphate 2,3-bisphosphoglycerate binds adult hemoglobin, lowering its affinity for oxygen during pregnancy so that the fetal hemoglobin can readily extract oxygen from the adult supply. Many invertebrates use similar mechanisms, except that inorganic ions like magnesium and calcium are used since they are easier to concentrate in salt-water environments (Terwilliger 1998, Numoto et al. 2008).

A third molecular level adaptation that occurs frequently in many invertebrate phyla is the simultaneous expression of different respiratory proteins at the same time. Terwilliger (1998) illustrated several examples of this, notably, the expression of three different types of respiratory protein in the polychaete *Pista pacifica*. This marine worm simultaneously utilizes an extracellular erythrocruorin with a low oxygen affinity, a circulating red blood cell hemoglobin with a moderate affinity for oxygen, and a body wall myoglobin with a high affinity for oxygen. By using this cascade of respiratory pigments, the worm can efficiently extract oxygen from its environment by facilitated diffusion, transport this oxygen throughout its body, and distribute it to individual tissue cells. Due to their lack of cooperativity, the high affinity myoglobins also function as intracellular stores of oxygen during short bouts of hypoxia. A very similar mechanism was observed in the peanut worm, *Themiste sp.*, which uses an octameric and a monomeric hemerythrin, a hemoglobin that binds iron without the heme group (Loehr 1978), to transfer oxygen from the tentacles that extend into the water to the deep tissues that have no direct contact with the environment (Terwilliger et al. 1983).

A fourth, similar adaptation is the sequential expression of subunits during times of stress or change, also termed heterogeneous expression. Like the examples with simultaneous expression, this mechanism takes advantage of the differing oxygen affinity properties of different hemoglobin molecules, except that it does not necessarily occur constitutively and the process can be intramolecular, in which a single hemoglobin

molecule can have variable subunit composition depending on the conditions. This has been identified as an adaptive mechanism in fish (Ariaeenejad et al. 2012, Methling et al. 2010, Rasmussen et al. 2009), a brachiopod crustacean (Guadagnoli et al. 2005), the blue crab (Defur et al. 1990), a few gastropod species (Medeiros et al. 1998), and myoglobin variability in a polychaete (Kleinschmidt & Weber 1998). This differential expression of subunits at the protein level helps explain how certain organisms are able to live aerobically in variable oxygen, pH, and temperature environments. The distinctive subunits have differing affinities for oxygen, allowing optimization to various oxygen conditions. Methling et al. (2010) found that Atlantic cod from the same species, *Gadus morhua*, had different preferences in temperature and oxygen saturation depending which of two hemoglobin isoforms were being expressed. Kleinschmidt & Weber (1998) identified five sequence-based isoforms of myoglobins in a marine polychaete, which each function at a characteristic oxygen tension, broadening the range of oxygen conditions that the worms can inhabit.

Differential subunit expression in response to environmental change has been observed extensively in the high molecular weight hemocyanins of many crustaceans (Bruneaux et al. 2008). Although this effect has not been observed as a response to environmental change in annelid extracellular hemoglobins, the presence of multiple low-abundance subunits of both globin and linker chains in the erythrocruorins of *A. marina* and *L. terrestris* is highly suggestive and worth investigating (Zal et al. 1997, Fushitani et al. 1996, Maier et al. 1997). The specific subunit stoichiometries for globin and linker chains have been added to the table (Table 1.3). It is at this level of organization that variability is expected to exist.

1.5 The Cooperative Binding of Oxygen

In 1970, Max Perutz used the two state model of allosteric theory to describe the binding of oxygen to mammalian hemoglobin at the lungs and its subsequent unloading in the tissues (Perutz 1970). Although there have been revisions and disputes to this day, the basic model describes binding molecular oxygen to the iron of a free

Table 1.3: Subunit Stoichiometry of largest submultiples of the annelid extracellular hemoglobins from Table 1.2. Tables describe stoichiometry in terms of globin chains and linker chains. Specific peptide isoforms are ommited. All data were reported by the authors listed in Table 1.1. Subunit nomenclature is that used by the authors. M: monomers, D: dimers, T: trimers, L: linker subunits, NA: not applicable, +: intratetramer disulfide bond, $\oplus$: intertetramer disulfide bond.

(a) Some Hemoglobin Subunits

	A. marina	*L. terrestris*	*R. pachyptila*		
Submultiple	$(M_9T)L_{3.5}$	$(M_3T_3)L_3$	$(M_4D_4)L_3$	M_4D_4	M_4D_4
Globins	a,b,c,d; T=b+c+d; M=a	a,b,c,d; T=a+b$\oplus$c; M=d	b,c,d,e; D1=d+e	a,b,c,d,e,f; D1=d+e; D2=e+f	a,b,c,e,f; D2=e+f
Linkers	L1,L2; D1=L1+L1; D2=L1+L2	L1,L2,L3,L4	L1,L2,L3,L4	NA	NA

(b) More Hemoglobin Subunits

	O. machikoi	*O. webbi*	*A. pompejana*	*S. contortum*	
Submultiple	M_3T_3		M_3T_3		
Globins	A1,A2,B1,B2; T=A1+B2$\oplus$ B1; M=A2	a,b,c	a1,a2,a3,a4, b,c,d; T=b+d+c; M=a1,a2,a3,a4	a,b,c,d,e,f,g; D1=b+c, D2=b+d, D3=b+e; M=a,b,c,d,e,f,g	a,b,c,d,e,f,g; D1=b+c, D2=b+d, D3=b+e; M=a,c,d,e,f,g
Linkers	NA	NA	L1,L2,L3,L4	L1,L2,L3,L4	NA

heme group on an alpha subunit, coordinating a conformational change in the tertiary structure of the protein between each set of alpha-beta dimers. This conformational change enhances the binding of oxygen to the second iron atom on the beta subunit, a cooperative process that Perutz remarked upon. It was also determined that while the allosteric effect seemed to occur at the dimer level, disulfide bridges holding together the complete tetramer were necessary to stabilize the low-affinity state, which is required for oxygen unloading.

While this model of cooperativity as a type of allostery appears in every textbook that contains the topic, researchers have recently observed that mammalian hemoglobin

actually exists in more than two affinity states (T- and R-), in which multiple high affinity (R-) states actually exist (Kern et al. 2003, Bellelli & Brunori 2011). This idea was theorized in the earlier stages of research, but was rejected temporarily and practically as a result of technological limitations (Edelstein 1971). The transition between the two states is a dynamic process that takes about 20 microseconds (Cui & Karplus 2007). Since the original mechanism was based solely on crystal structures, the states that exist intermediately were not seen, but as a model of allosteric regulation, it is important to understand in greater detail what actually occurs during the transition stage.

Cooperative binding of oxygen to hemoglobin can be observed by the shape of the oxygen binding curve, and is measured spectrophotometrically. These curves, recorded either during association or dissociation, describe the proportion of total available ligand binding sites that are in complex with oxygen over a range of partial pressures. Once generated, a lot of information can be extracted from these curves, incuding the effects of temperature, pH, and ions on hemoglobin-oxygen affinity, which is determined by the stereochemical interactions within the heme pocket (Figure 1.1). The key piece of information to describe the oxygen binding affinity is known as the half-saturation constant, denoted P_{50}, which is a measure of the partial pressure of oxygen at which 50% of the total available binding sites of hemoglobin in solution are occupied. For reference, sperm whale myoglobin has a relatively high affinity for oxygen, with a P_{50} equal to 0.5 Torr. The hemoglobin of the nematode parasite *Ascaris suum* has one of the highest known oxygen affinities with a P_{50} equal to 0.004 Torr. *L. terrestris* erythrocruorin is known to cover a wide range of P_{50}s, from 1.6 to 17.3 Torr, depending on the experimental conditions (Royer et al. 2001).

The other useful piece of information that can be inferred from oxygen binding curves is the degree of cooperativity that the hemoglobin exhibits. Myoglobin, which only has one oxygen binding site per molecule, produces a hyperbolic oxygen binding curve, which is signature of no cooperativity. Multisubunit hemoglobins, which can potentially exhibit cooperativity, produce sigmoidal oxygen binding curves. The degree

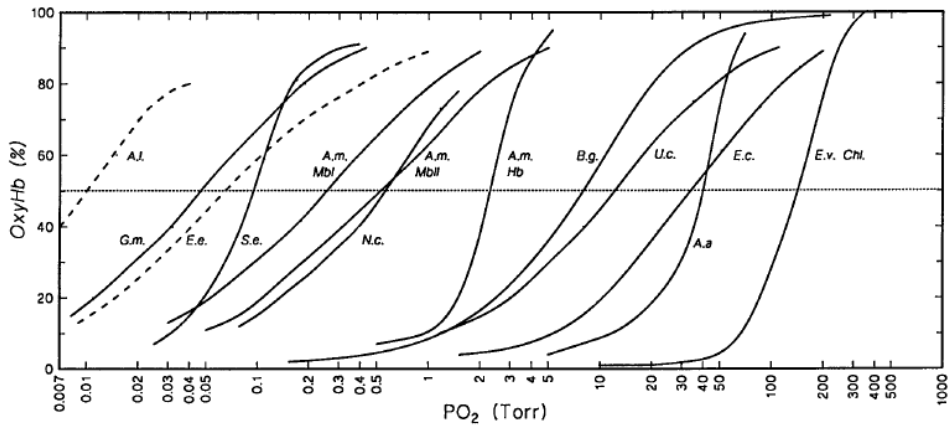


Figure 1.4: Plot depicts the range of oxygen affinities observed in several model invertebrates. Figure taken from Weber & Vinogradov 2001.

of interaction between binding sites is quantified using the Hill equation, $\mathrm{Log}(\frac{\Theta}{1-\Theta}) = \mathrm{nLog}(\mathrm{pO_2})$ - $\mathrm{nLog}(\mathrm{P_{50}})$, which is derived from general allosteric theory in which a ligand binds to a multisubunit protein. Here, Θ is the fraction of occupied binding sites at a particular partial pressure of oxygen ($\mathrm{pO_2}$), and $\mathrm{P_{50}}$ is the half-saturation constant determined from the oxygen binding curve. The Hill coefficient (n), which has an upper limit equal to the total number of oxygen binding sites, is conventionally reported to describe the level of cooperativity. When n equals 1, as is the case for myoglobin, no cooperativity is observed. n-values greater than 1 correspond to increasing degrees of cooperativity. n-values can theoretically be less than 1, but are not observed in this special case of allostery. For reference, human hemoglobin A, with four binding sites, has an n ranging from 2.5 to 3.0. *L. terrestris* erythrocruorin, with twelve binding sites per dodecameric submultiple, has an n ranging from 2.5 (modest cooperativity) to 7.9 (high cooperativity) (Royer et al. 2001). A wide range of oxygen affinities and degrees of cooperativity are observed throughout the invertebrate phyla (Figure 1.4), and can usually be correlated with the organism's habitat. The known half-saturation constants, Hill coefficients, and total number of oxygen binding sites have been added to the table (Table 1.4).

Studying cooperative binding of oxygen to annelid extracellular hemoglobins

Table 1.4: Some available data for oxygen affinity and cooperativity for the annelid extracellular hemoglobins from Table 1.1 are recorded here.

(a) Some P_{50} values and Hill Coefficients

	A. marina	*L. terrestris*	*R. pachyptila*		
Habitat	sandy intertidal	soil	pelagic		
P_{50} (Torr)	1.0-5.7	1.6-17.3	0.5	0.3	
n	2.5-4.0	2.5-7.9	2.4	1.6	
Maximum O_2 binding sites	144	144	144	24	24

(b) More P_{50} values and Hill Coefficients

	O. machikoi	*O. webbi*	*A. pompejana*	*S. contortum*	
Habitat	sulfide-rich sediments	cold seeps	hydrothermal vents	cold seeps	
P_{50} (Torr)	0.82		0.3		
n	1.1-2.5		3.0		
Maximum O_2 binding sites	24	24	144	144	24

has provided much insight into other mechanisms of cooperativity that occur in nature, and could be highly influential in revising the current understanding of allosteric modulation. First of all, multiplicity of subunits does not imply cooperativity. Rather, the presence of what is called an EF dimer, a motif in which the E helix of one subunit comes into contact with the F helix of a neighboring subunit, has been found to exist in three invertebrate phyla (annelids, molusks, echinoderms) with hemoglobins that exhibit cooperativity (Royer et al 2005). One notable exception is the twenty-four subunit HBL Hb of *O. mashikoi*, which has an EF dimer motif, but virtually no cooperativity with a Hill coefficient equal to 1.1. It was determined that a single amino acid substitution from a histidine to an arginine on the F-helix that interacts with the heme propionate group of the neighboring subunit may restrict movement that is necessary for tertiary rearrangement upon ligand binding (Numoto et al. 2005). Similarly, the submultiples of *L. terrestris* have the same affinity for oxygen as the native protein,

but have a reduced cooperativity (Lamy et al. 1996). This may imply that the linker subunits have a role in cooperativity. A major difference that the annelid extracellular hemoglobins introduce is that during cooperative binding of oxygen, conformational changes occur at the tertiary level between subunits sharing an EF dimer, but hardly any quaternary level changes occur. This is in stark contrast to the mammalian model, in which quaternary rearrangement is a necessary component of the allosteric mechanism (Royer et al. 2005).

1.6 Summary

Capitella teleta, formerly identified as *Capitella sp. I* (Blake et al. 2009), is a small, highly opportunistic benthic polychaete of the phylum Annelida with a wide geographic distribution and a high tolerance for stress. It has long been used as the primary invertebrate indicator of pollution in marine settings and is frequently observed to be the first colonizer of recently contaminated marine sediment habitats (Blake et al. 2009, Li et al. 2004, Sanders et al. 1980, Grassle et al. 1976). *C. teleta* has been utilized as a model organism in studies on invertebrate development (Holbrook et al. 1984, Biggers 1992, Boyle 2008). Its proteome and phosphoproteome are beginning to be studied (Chandramouli et al. 2011), which has lead to investigations of post-translational modifications in other benthic invertebrates (Chandramouli et al. 2012).

The first laboratory culture of *C. teleta* was started in the mid-1970s, and is currently maintained in laboratories around the world (Blake et al. 2009). While much attention has been given to *C. teleta* as a model of invertebrate development and its role in biodegradation in marine sediments, very little work has been done on the composition and functional properties of its respiratory pigments. Two research papers exist on oxygen affinity in *C. teleta* (Wells & Warren 1975, Mangum et al. 1992), however, the type of hemoglobin present in this polychaete has not been identified. Mangum et al. (1992), who made the assumption that measurements were being made on a homogenous mixture of red blood cell (RBC) hemoglobins, commented "the nature of the apparent cooperativity exhibited by capitellid RBCs will remain unclear

until their Hbs have been investigated in more detail." The apparent discrepancy in their results, in which monomeric cellular hemoglobins exhibited cooperativity, suggests that larger, extracellular hemoglobins went undetected and were influencing the RBC oxygen affinity measurements.

The goal of this study is to characterize hemoglobin in normal and cold-acclimated *C. teleta* and to find if variability of subunits exists between cultures. Temperature may affect the electrochemical stability of the heme pockets, or the free energy required for oxygen binding. To compensate, the two cultures may synthesize subunit isoforms that are functionally more stable at their respective acclimation temperatures. I hypothesize that if variability exists in the hemoglobin structures between the two cultures, it would be manifested at the subunit level, whereas tertiary and quaternary structures would be conserved. Alternatively, the quantity of hemoglobin or its functional status may be altered upon cold-acclimation. The previously determined structural information from the marine polychaetes *A. marina* and *O. machikoi* will be used to postulate a potential stoichiometry for the *C. teleta* hemoglobin.

Chapter 2

INHIBITION OF PROTEASE ACTIVITY AND SAMPLE PREPARATION

2.1 Introduction

The overall goal of this study was to quantify hemoglobin extracted from *Capitella teleta* cultured at two temperatures and to visualize the protein on polyacrylamide gels for size analysis. Foremost of the many challenges involved was the need to successfully produce a stable extraction containing sufficient quantity of intact protein from the worms. The fact that the worms are small necessitated that whole individuals were homogenized and many individuals were pooled and processed together in order to have enough protein to work with. However, *C. teleta* is known to exhibit exceptionally high protease activity (Ito et al. 2011). Thus, the goal of this section was to develop a sample preparation technique that would halt protease activity and result in crude, but stable protein extracts that could be used for subsequent isolation and characterization.

2.2 Methods

2.2.1 *Capitella* Cultures

A laboratory culture of *Capitella teleta* was maintained at room temperature ($\sim$21^{o}C) in plastic trays containing 32-35ppt filtered seawater that was continuously aerated with small bubbling stones. Each tray had a thin layer of 500μm filtered sand enriched with a small quantity of organic sediment. Cultures were fed weekly with Omega One brand fish flakes and water was changed on a bimonthly basis to prevent buildup of organic material and the onset of anaerobiosis. Cultures were tended to daily to check salinity, temperature, and to aerate the sediment.

A second culture was set up in an environmental chamber set at 10^oC. This chamber served primarily as a transition space before being moved to a second environmental chamber set at 4^oC. *C. teleta* were left to acclimate to 10^oC for five to eight weeks before transfer to 4^oC, where they were left for an additional five weeks before samples were taken.

Six culture trays were maintained at 21^oC for the duration of this study to assure that worms could be sampled at any time. Six culture trays were also maintained at 4^oC for the duration of this study. Two trays were kept in the 10^oC transition chamber in case the 4^oC cultures did not survive.

2.2.2 Sample Preparation

For the first method, adult worms were randomly and individually collected and transferred directly to pre-chilled microfuge tubes containing $500\mu L$ thiourea lysis buffer (Mok et al. 2009). Briefly, approximately eighteen individuals were selected and pooled from the 21^oC culture trays, and approximately twelve individuals were selected and pooled from the 4^oC culture trays. More worms were required from the 21^oC cultures because individuals were inherently smaller. The lysis buffer contained 8M urea and 2M thiourea as chaotropic denaturants, 4% CHAPS detergent as a solubilizing agent, 10mM dithiothreitol as a reducing agent, and 1mg/mL Pefabloc SC serine protease inhibitor (Sigma), all prepared in deionized water (dH_2O) and passed through a $2\mu m$ syringe filter before use. Following collection, worms were homogenized in lysis buffer using a mechanical pestle homogenizer, and sonicated on ice. Sonicates were then centrifuged twice at 14,000g, 3^oC for 25 minutes to clarify the extracts and remove cellular debris. Samples brought to this stage of preparation are referred to as homogenates for the remainder of this study.

While the first method is sufficient for most protein sampling protocols, it was ineffective against gut proteases in *C. teleta*. The goal of the second method was to neutralize these gut proteases prior to rupturing of the digestive tract. To accomplish this, adult worms were colleced in twelve-well culture plates containing filtered seawater

with Roche cOmplete ULTRA protease inhibitor cocktail. Briefly, one tablet was dissolved in 10mL filtered seawater at culture temperature and aliquoted 1mL/well in a plate. Six to ten worms were placed directly into each well and left for one hour to consume and absorb the cocktail. Following this pretreatment, worms were collected and sampled in thiourea lysis buffer without protease inhibitor and prepared as described in the first method.

2.2.3 TCA Precipitation

A 75% (w/v) stock solution of trichloroacetic acid (TCA) was prepared in dH_2O and stored in an amber glass vial. 40μL of TCA stock was mixed with 220μL of sample homogenate for a final concentration of 15%. This mixture was incubated on ice for 60 minutes and then centrifuged at 13,000g for 15 minutes. The precipitated protein, which appeared as a soft white pellet, was washed twice with 200μL ice-cold acetone. Briefly, the pellet was resuspended in acetone by vortexing, incubated on ice for 10 minutes, and centrifuged at 13,000g for 5 minutes. Samples precipitated with TCA were resuspended in (1X) urea sample loading buffer, and required readjustment of the pH by adding 0.5-1μL of 1M Tris-HCl pH 8.0 (the tracking dye turned from yellow back to blue).

2.2.4 SDS-PAGE

Denaturing, sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed either with Pierce Precise Tris-HEPES-SDS precast polyacrylamide mini-gels (10 x 8.5cm, 12%T), or with the Tricine-SDS polyacrylamide gel system (Schaegger et al. 1987). Briefly, the precast gels are pH 7.0 without SDS and utilize a Tris-HEPES-SDS running buffer at pH 8.0. The Tricine-SDS-PAGE system utilizes a resolving gel and a 3.5% stacking gel prepared in Tris-HCl buffer at pH 8.45 with 0.3% SDS. A Tris-HCl anode buffer was prepared at pH 8.9, and a SDS-Tricine cathode buffer was prepared at pH 8.25. Electrophoresis was carried out at constant voltages: 40V for 30 minutes, 100V for 90 minutes, and 150V for 45 minutes or until

the tracking dye reached the end of the gel. Samples were mixed with SDS-PAGE (2X) sample loading buffer (100mM Tris-HCl pH 6.8, 8% SDS, 24% Glycerol, 0.2% bromophenol blue), reduced with 2% β-mercaptoethanol, heat-denatured at 95^{o}C for 5 minutes, and cooled to 4^{o}C prior to loading onto gels.

The primary staining method utilized Pierce Imperial Blue protein stain, a preformulated solution of coomassie brilliant blue R-250 that combines the fixing and staining steps into one 60 minute incubation. Gels stained with this were destained in dH_2O overnight with several water changes. A second staining method utilized Amido Black 10B, a versatile dye that amongst other uses, is used in forensic science (Laberke et al. 2011), as a histological stain (Puchtler & Sweat, 1962), for western blots (Yonan et al. 2005), for polyacrylamide disc gels (Kruski & Narayan), but also for polyacrylamide slab gels (Achilanu & Goldring, 2010). It is known to be especially sensitive to proteins in the blood, namely hemoglobin, but also stains other proteins (Cucchi & Basaglia 1998). Briefly, gels are fixed in methanol, stained with 1% amido black 10B in 7.5%(w/v) acetic acid for 20 minutes and destained in 7.5% acetic acid overnight with several changes.

2.3 Results

2.3.1 *Capitella* Cultures

The goal of the experimental design used in all parts of this study was to establish cultures of *Capitella teleta*: one that is maintained at a near constant 21^{o}C water temperature, and one that is maintained at a near constant 4^{o}C water temperature, while keeping salinity at 35ppt and bubbling stones to keep all cultures well aerated. Assuming that all cultures were 100% saturated with oxygen, at 35ppt salinity, seawater has a concentration of 7.2mg O_2/L (250μM) at 21^{o}C and a concentration of 10.4mg O_2/L (350μM) at 4^{o}C (Benson et al. 1980). In order to make the transition from 21^{o}C to 4^{o}C, the 21^{o}C worms were subcultured and moved to 10^{o}C for a minimum of five weeks to allow the specimens to acclimate and to prevent shock before transition to 4^{o}C.

It was observed early that upon acclimation from 21^{o}C to 10^{o}C, the worms became much larger in size and more abundant in the culture trays. For this reason, the 10^{o}C worms were selected during the initial testing of sample preparation techniques (Figure 2.1B). Once a stable 4^{o}C culture was established, use of the 10^{o}C worms was discontinued.

2.3.2 Protease Inhibitor Treatment

Two strategies of protease inhibition were tested here. The first strategy was to include a potent protease inhibitor cocktail in the sample lysis and solubilization buffer prior to loading the sample on a denaturing polyacrylamide gel. This method resulted in complete (Figure 2.1A) to near (Figure 2.1B) degradation of all protein present in the sample. To confirm that the major issue was protein degradation and not protein quantity, samples were precipitated with TCA to concentrate them prior to electrophoresis (Figure 2.1B). Although peptide bands did become visible following TCA precipitation, degradation was still observed. The addition of 8M urea to the sample loading buffer as a substitute for glycerol helped with peptide solubilization after TCA precipitation, but also did not prevent early cleavage of peptides by proteases.

To demonstrate that the problem was a result of sample preparation and not an artifact of electrophoresis, samples were run on a gel with bovine serum albumin (BSA, MW$\sim$67kDa), which served as a control (Figure 2.2). Here, the *C. teleta* 10^{o}C samples were prepared in thiourea lysis buffer and precipitated with TCA as before, and loaded on the gel with urea sample loading buffer. The BSA standards were not precipitated with TCA, but the lyophilized stock was dissolved in urea sample loading buffer. It is shown that peptides were resolved in the BSA control, but not in the *C. teleta* samples.

A similar experiment was performed, this time using protein extracts from two algal species as controls, and a different electrophoresis buffer system (Figure 2.3). It is evident from this gel that protein degradation in the *C. teleta* samples occurred

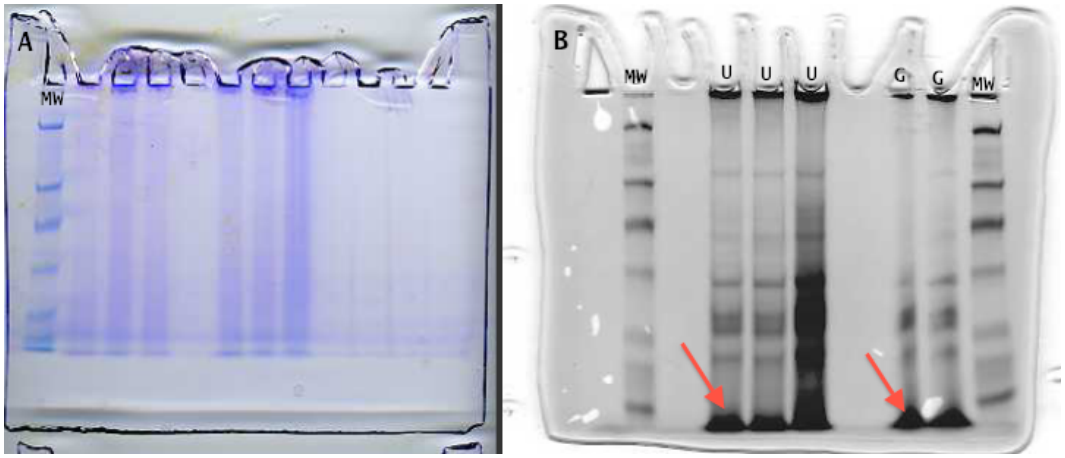


Figure 2.1: (A) *C. teleta* 21^{o}C samples prepared in thiourea lysis buffer containing protease inhibitor cocktail and run on a 12% Pierce Precise Tris-HEPES-SDS polyacrylamide gel stained with Pierce Imperial Blue protein stain. (B) *C. teleta* 10^{o}C samples prepared in thiourea lysis buffer containing protease inhibitor cocktail, precipitated with TCA, and run on a 12% Pierce Precise Tris-HEPES-SDS polyacrylamide gel stained with Pierce Imperial Blue protein stain. MW: Molecular weight marker, U: urea sample loading buffer, G: glycerol sample loading buffer. Red arrows indicate degraded peptides

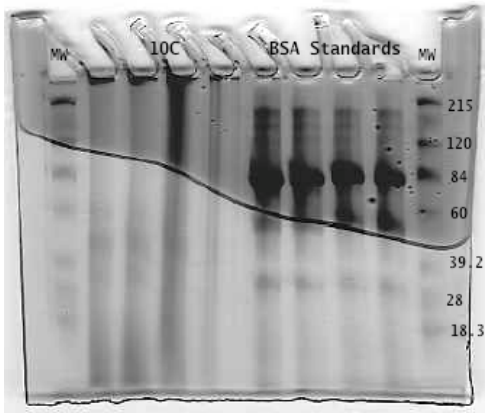


Figure 2.2: *C. teleta* 10^{o}C samples prepared in thiourea lysis buffer containing protease inhibitor cocktail, precipitated with TCA, and run on a 12% Pierce Precise Tris-HEPES-SDS polyacrylamide gel stained with Pierce Imperial Blue protein stain. MW: molecular weight marker, 10C: four lanes contain the *C. teleta* samples, BSA Standards: four lanes contain bovine serum albumin (BSA) standards.

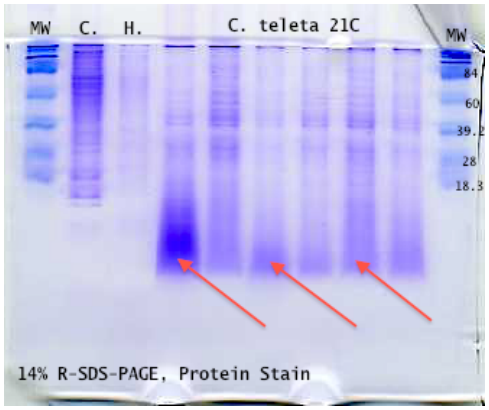


Figure 2.3: *C. teleta* 21^{o}C samples prepared in thiourea lysis buffer containing protease inhibitor cocktail, precipitated with TCA, and run on a 14% Tricine-SDS polyacrylamide gel stained with Pierce Imperial Blue protein stain. MW: molecular weight marker, C.: *Chattonella subsalsa*, an algal species, H.: *Heterosigma akashiwo*, an algal species, *C. teleta* 21^{o}C occupies the remaining 6 lanes. Red arrows indicate degraded peptides.

during sample preparation and not during electrophoresis. While the Tricine-SDS polyacrylamide gel system improved band resolution over the Tris-HEPES-SDS precast gel system, significant degradation product was still observed.

The second strategy of protease inhibition was to treat the worms with a potent protease inhibitor cocktail prior to sampling. The theory is that if the worms were moved from the culture trays to a small well plate containing filtered seawater with protease inhibitor, the worms would take up the drug by ingestion and absorption, causing inhibition of gut and tissue proteases before sampling in lysis and solubilization buffer. When compared to worms that were not pretreated with protease inhibitor and were exposed to it only during sample preparation, the pretreated worms exhibit no noticeable degradation products on a polyacrylamide gel (Figure 2.4). With minor modifications, this sample pretreatment was employed for the remainder of this study.

2.3.3 Comparing Acclimation Temperatures

To confirm that the protease inhibitor pretreatment was successful, *C. teleta* 21^{o}C and 4^{o}C were placed in 35ppt filtered seawater with protease inhibitor cocktail at their respective culture temperatures for 45 minutes prior to sampling in thiourea

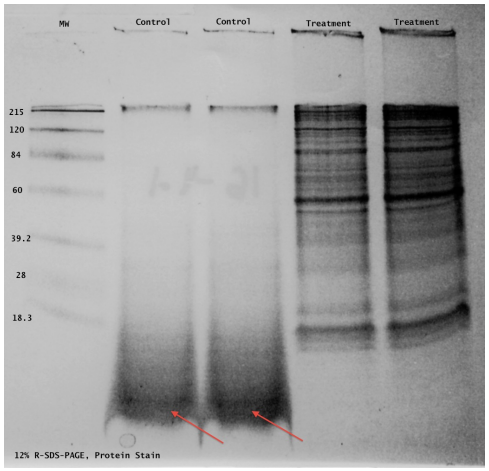


Figure 2.4: 12% Tricine-SDS polyacrylamide gel stained with Pierce Imperial Blue protein stain. MW: molecular weight marker, Treatment: *C. teleta* 21^{o}C worms treated with Roche Complete Ultra protease inhibitor cocktail dissolved in 35ppt filtered seawater for 60 minutes at room temperature prior to sample preparation, Control: directly sampled in lysis buffer containing protease inhibitor cocktail. Red arrows indicate degraded peptides.

lysis buffer without protease inhibitor. These samples were run on a SDS-PAGE gel and stained with Amido Black 10B (Figure 2.5). Although minor degradation was observed, band resolution was good. Human hemoglobin A (MW$\sim$16kDa) was also run on this gel for comparison and to test the Amido Black stain. Although Amido Black does appear to be more sensitive with less background than Imperial Blue, its use was discontinued due to the length of time required to destain ($\sim$48hours) and the quantity of acetic acid required to do so (at least 1L of 7% solution in dH_2O).

2.4 Conclusion

Two cultures of *C. teleta* were successfully established at 21^{o}C and 4^{o}C, and proved to be very simple to take care of. Traditional sample preparation methods utilize either a protease inhibitor or a denaturant such as urea in the sample lysis buffer to slow or prevent degradation of peptides before gel analysis. This technique was not sufficient when working with whole-organism homogenates of *C. teleta*. Although precipitation with TCA and resuspension in urea sample loading buffer did improve band visibility by concentrating the peptides and denaturing any remaining proteases,

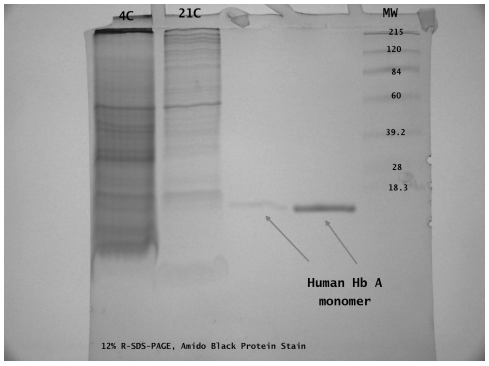


Figure 2.5: 12% Tricine-SDS polyacrylamide gel stained with Amido Black 10B. *C. teleta* cultured at either 21^{o}C or 4^{o}C were treated with protease inhibitor cocktail at their respective acclimation temperatures for 45 minutes prior to homogenization in thiourea lysis buffer without protease inhibitor. Two lanes were loaded with standard human hemoglobin A following reduction and denaturation at two concentrations for comparison.

peptide degradation was still observed on polyacrylamide gels. The fact that the BSA standard and the two algal protein extracts showed no signs of degradation or smearing on the gel confirmed that the issue was not the electrophoresis methodology, but the sample preparation technique. Pretreating the worms with protease inhibitor while they were still alive solved the degradation problem. This was validated by running *C. teleta* 21^{o}C and 4^{o}C samples that were pretreated with protease inhibitor and not treated during homogenization. This gel was stained with Amido Black 10B, which proved to be more sensitive than Imperial Blue, but less convenient to work with. Although Amido Black 10B is highly sensitive to hemoglobins, it also stains other proteins. For this reason, it was determined that a different in-gel hemoglobin detection method would be needed (see Chapter 4).

Chapter 3

TEMPERATURE ACCLIMATION

3.1 Introduction

Preliminary observations of adult *Capitella teleta* revealed visually distinct differences in size and color when acclimated to 4^{o}C compared to the original 21^{o}C cultures. The worms acclimated to 4^{o}C appeared larger and to be darker in color. These morphological differences raised several important questions concerning the underlying mechanisms that permit individuals of the same species to change so drastically over such a short time period (5-8 weeks), and whether these changes are also observed in the natural environment during seasonal fluctuations in temperature. The goal of this section was to quantify these differences in size, measured as cylindrical volume, and color, measured in terms of heme b (Fe-protoporphyrin IX) concentration. Heme b is the non-covalently bound prosthetic group of many hemoproteins, including the respiratory proteins that are the subject of this study, and is responsible for the observed production of color.

3.2 Methods

3.2.1 Size Measurement

Previous work has shown that there exists a strong correlation (r=0.9072, n=60) between the volume of the fifth setiger and dry weight of juvenile *C. teleta* (Marsh et al. 1989). In this study, the volume of the cylinder formed by the third, fourth, and fifth setiger was used as a measure of *C. teleta* body size. Randomly selected individuals from 21^{o}C and 4^{o}C cultures were sampled in Petri dishes and relaxed with a solution of 2% $MgCl_2$ in filtered seawater. Under a dissecting microscope, the length of the third to fifth setiger and the width of the fifth setiger (Figure 3.1) of individual worms were

measured with an ocular micrometer calibrated with a cell counter (mm) and used to calculate worm volume, using the relationship volume (mm^3) = $\pi \times (\frac{width}{2})^2 \times (length)$, assuming that volume of these anterior segments is independent of total length and total number of segments.

3.2.2 Sample Preparation

Protein extracts were prepared as in Chapter 2, except the thiourea lysis buffer was replaced with a biological buffer, Tris-HCl pH 7.2 with 10mM $MgCl_2$. It became evident throughout this study that the harsh denaturants, detergent, and reducing agent were not necessary or were detrimental to most of the experimental procedures that were performed, and that they could be added later if required for electrophoretic steps. The resulting homogenates were concentrated and fractionated using Amicon Ultra centrifugal filter devices. Samples were first passed through a membrane with a 100kDa size cutoff. The concentrate was diluted in biological buffer and passed through the membrane a second time to maximize size fractionation. The first filtrate was then passed through a second filter with a 3kDa size cutoff. The concentrate from this step was saved for analysis. All sample preparation was carried out at 3^oC and samples were kept on ice.

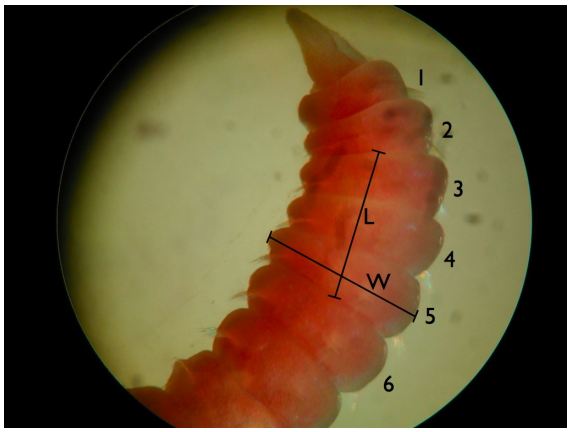


Figure 3.1: Diagram of the two anterior measurements made to determine volume. (Photograph by S. Guida 2010)

3.2.3 Protein Determination

Protein concentration was determined using ProStain Protein Quantification Kit (Active Motif). This assay has a very narrow, but highly sensitive range of detection

(0.15-10.0μg/mL). As a result, samples required large dilutions (500-2500X) before protein quantification. Because heme concentration was normalized to the protein concentrations determined by this assay, minor modifications were made to minimize experimental error. Briefly, two sets of six BSA standards and a 0μg/mL standard were prepared separately. Each assay was run using both sets of standards. Standard curves were only accepted with linear regression coefficients greater than 0.95, with both sets of standards plotted together. To minimize pipetting errors associated with small volumes (<5μL), samples were prepared in larger volumes. Standards and samples were incubated with a fluorescent dye reagent for 35 minutes at room temperature in the dark. Reactions were left to proceed before aliquoting samples in triplicate on a well plate to minimize error between sample replicates. Fluorescence was measured with a FLUOstar Omega microplate reader (BMG Labtech) equipped with 488/635 excitation/emission filters.

3.2.4 Pyridine Hemochrome Assay

Heme b was quantified using the pyridine hemochrome assay (Berry & Trumpower 1987), scaled to a microplate format (Sinclair et al. 1999), with some modifications for this study. The assay uses dual-wavelength extinction coefficients of reduced minus oxidized difference spectra to accurately quantify different heme species (a, b, and c) in samples containing a variety of absorbing molecules that otherwise cause interference with single wavelength determinations. Under alkali denaturing conditions, the non-covalently bound heme b dissociates from its protein pocket and becomes free heme. The nitrogenous base, pyridine, binds free heme in a coordination complex with the iron center, in what is known as a hemochrome. Because the heme is no longer bound to the protein, oxidation or reduction of the iron center of the hemochrome yields characteristic absorbance spectra. For heme b, the reduced minus oxidized difference spectrum has characteristic absorbance maxima at 419nm, 525nm, and 557nm.

Fractionated samples (>100kDa and 3-100kDa) and unfractionated homogenates were diluted in biological buffer at room temperature to a final volume of 420μL. It was

found that diluting samples from a deep red color to a light yellow, usually five times for 4^oC samples and two times for 21^oC samples, produced results with minimum background absorbance. Diluted samples were then vortexed with 120μL pyridine and 60μL 1M NaOH. 92μL of each sample was then pipetted onto a 96 well plate. Meanwhile, a saturated solution of sodium dithionite ($Na_2S_2O_4$) in dH_2O was prepared immediately before use. A 10mM solution of potassium ferricyanide ($K_3(Fe(CN)_6)$) was prepared from a previously made stock solution (1M, stored in the dark). One set of each sample was reduced by addition of 20μL sodium dithionite. The second set of each sample was oxidized by addition of 20μL potassium ferricyanide. After incubating at room temperature for approximately 10 minutes, absorbance spectra were taken from 400 to 600nm with 1nm resolution using the FLUOstar Omega microplate reader.

To calculate the concentration of heme b, measured as pyridine hemochrome b, the oxidized spectra were subtracted from the reduced specra. Using these difference spectra, the peak minus trough (557-541nm) absorbance difference (ΔA) was used to determine heme b concentration according to the Beer-Lambert Law, (mmol heme b) = $0.100\times\left(\Delta A_{\overline{\varepsilon\times L}}\right)$, using a millimolar extinction coefficient (ε) of 20.7 $mM^{-1}cm^{-1}$. The microplate reader software automatically corrected for path length (L) given a volume of 112μL. Both heme b and protein concentration were normalized to a sample volume of 0.100mL. Heme b concentration was then normalized to protein concentration for each sample.

3.2.5 Statistics

Size Measurement

The null hypothesis that there is no difference between the size of 21^oC and 4^oC acclimated *C. teleta* was tested using a Kruskal-Wallis non-parametric ANOVA in R because the assumptions of normality and homogeneity of variances were not supported (Shapiro-Wilk test for 21^oC data, $p < 0.01$, Bartletts chi-squared = 355.4, $p < 0.01$). Each observation was made independently.

Normalized Heme b Concentration

To test if there was a significant difference in heme b concentration between homogenates from the two temperature treatments, a one-way ANOVA was done in R. A logarithmic transformation of the raw data was necessary to support the assumptions of normality and homogeneity of variance. Each observation was made independently.

A randomized complete block design was used to test the significance between temperature treatments after fractionation, in which the two size fractions (>100kDa and 3-100kDa) were blocking factors and triplicate biological replicates within each temperature treatment and block were subsamples. Boxplots as well as a residual plot and normal q-q plot were used to determine if the assumptions of normality and homogeneity of variances were met. Although the data set is small, there were minor deviations from normality and variances were much larger for the 4^oC data set. A logarithmic transformation of the raw data improved normality and homogeneity of variance (Figure 3.2). A plot of the interaction between temperature treatment and size block was also done (Figure 3.3).

3.3 Results

3.3.1 Morphology

A total of 230 measurements were made on *C. teleta* 21^oC (A. Pasqualone), and a total of 36 measurements were made on *C. teleta* 4^oC. The 21^oC culture has existed for much longer than the 4^oC culture, allowing more measurements to be made. The 4^oC cultures were given at least five weeks before any measurements were taken. All size measurements were made within a three-week period following this initial acclimation time. The 21^oC worms had a size of 0.085 ± 0.003 mm^3 and the 4^oC worms had a size of 0.53 ± 0.04 mm^3 (mean $\pm$ SEM), a volume 6.2 times greater than the 21^oC worms (Kruskal-Wallis Chi-squared = 91.6, p $<2.2e^{-16}$). This highly significant difference in size can be seen clearly under a dissecting mocrscope (Figure 3.4).

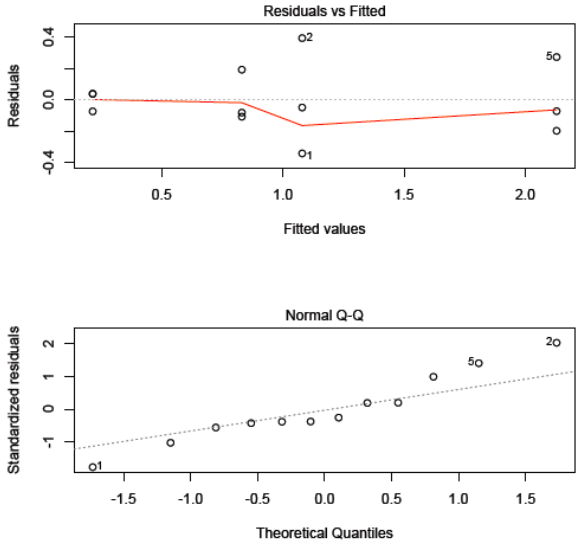
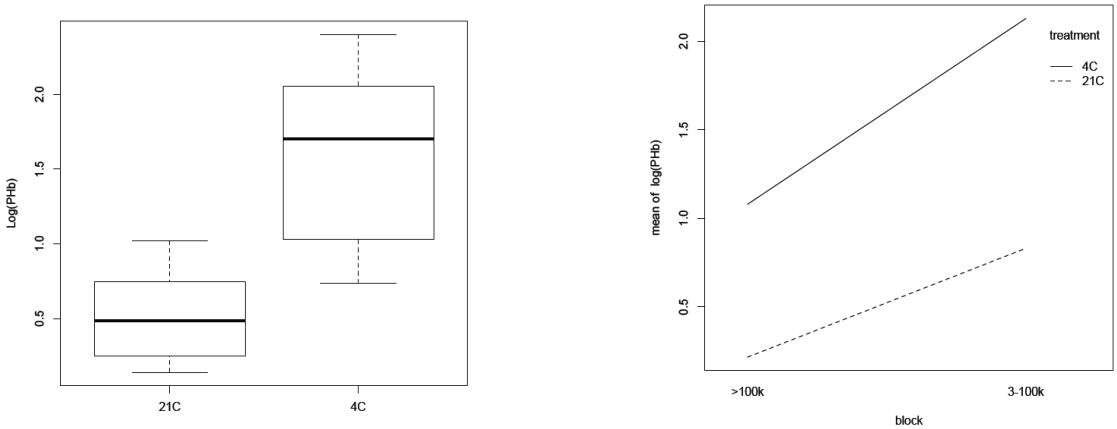


Figure 3.2: Residual plot and normal Q-Q plot for RCBD Diagnostics of Log transformed data. No obvious wedges appear in the residual plot and there are no strong deviation from normal in the Q-Q plot.



(a) Box Plot

(b) Interaction Plot

Figure 3.3: Boxplot and interaction plot for the Log transformed data. Since the slopes of the two lines in the interaction plot are nearly equal, it is assumed that temperature treatment is independent of the blocking factor.

3.3.2 Pyridine Hemochrome Assay

Raw homogenates, as well as two size fractions were prepared to compare heme b content between the two acclimation temperatures. The third size fraction (<3kDa) was not used because it appeared as a clear solution to the naked eye. The other two fractions resulted in an intense production of color (Figure 3.5). The hemoglobin protein that is the subject of this study has a molecular weight much greater than 100kDa, a measurement that will be discussed in Chapter 6. The other major hemoproteins that also associate with heme b are not part of this larger size class. It has been demonstrated that *C. teleta* constitutively express two cytochrome P450 enzymes, a fact that is not surprising considering the worm's central role in marine detoxification processes. The molecular weights of these two important enzymes are 62.66kDa and 56.42kDa (Li et al. 2004). For this reason, it has been assumed that heme associated with these other abundant proteins are in the 3-100kDa size fraction and did not interfere with the quantification of heme b associated with >100kDa hemoglobins.

Figure 3.4: Photograph taken through the lense of a dissecting microscope. A representative worm from each acclimation temperature is shown for comparison.

Following treatment with pyridine under alkaline conditions, each sample was split so that an oxidized and reduced spectrum could be recorded at the same time and under identical spectrophotometric conditions. Representative difference spectra were acquired for *C. teleta* 21^{o}C and 4^{o}C homogenates, and normalized to the absorbance maxima at 421nm (Figure 3.6). Even prior to fractionation, these spectra show peaks near 419nm and at 525nm and 557nm, the characteristic maxima for pyridine hemochrome b. Pyridine hemochrome a and c have absorbance maxima at 588nm

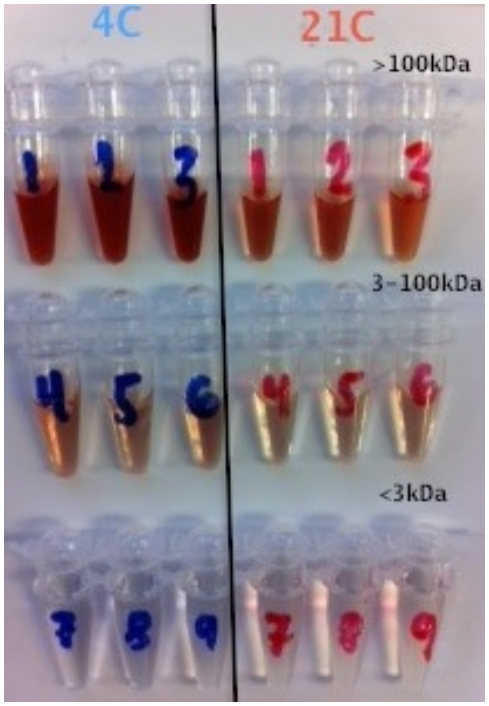


Figure 3.5: Photograph showing the color of three size fractions obtained from each culture prior to performing pyridine hemochrome assay. Protein concentrations are not normalized between the tubes shown, resulting in the differences in color intensity.

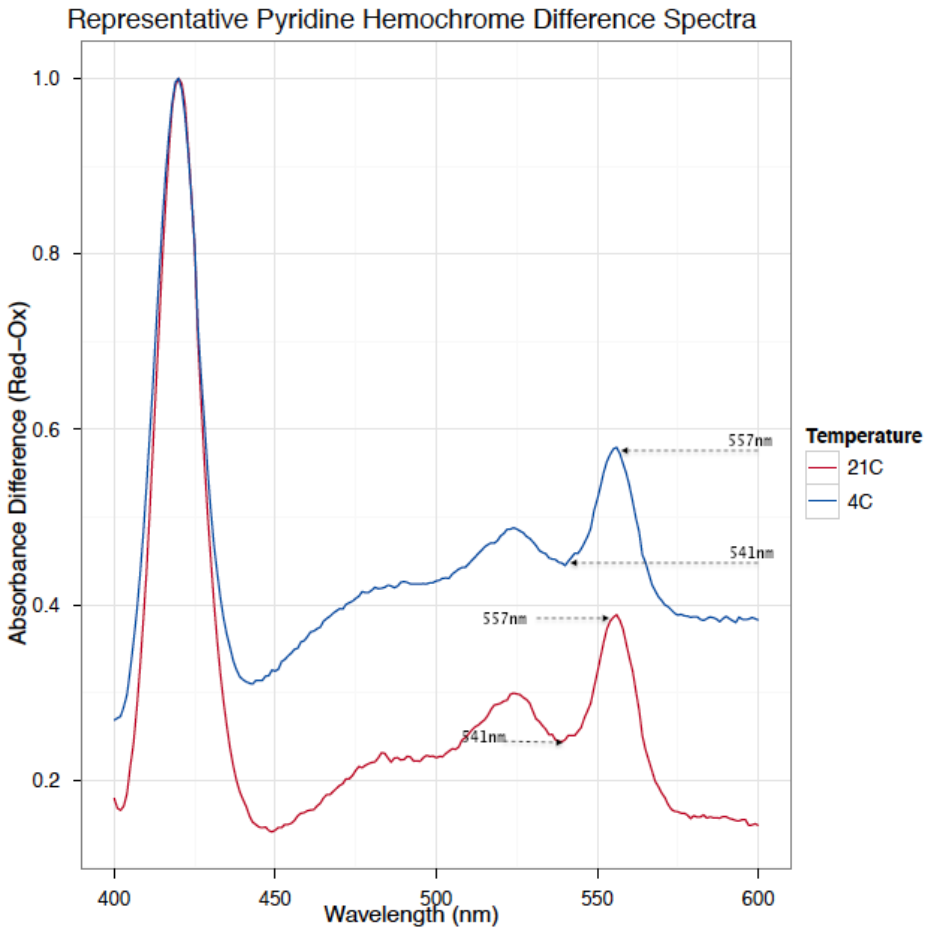


Figure 3.6: Plot of representative pyridine hemochrome difference spectra for the two cultures. Spectra for each temperature are normalized to their respective peak maxima at 421nm.

and 550nm, respectively. In addition to the color observation, this is the first compelling piece of evidence that hemoglobin is one of the most abundant hemoproteins present in the worms from both cultures. The peak and trough are labeled on the plot to illustrate that although the total protein concentrations or populations may be highly varied between the two cultures, the difference in absorbance between these two wavelengths provides an accurate measure to calculate heme b concentration in the samples.

Heme b, quantified as picomoles of pyridine hemochrome b per microgram protein, has been used a proxy for hemoglobin concentration (Figure 3.7). Measurements

were made on homogenates, proteins greater than 100kDa, and proteins between 3 and 100kDa, but it was assumed that the giant extracellular hemoglobin would be in the >100kDa fractions. It was found that *C. teleta* 4^{o}C had significantly more heme b per unit protein in homogenates than *C. teleta* 21^{o}C (One-Way ANOVA on log transformed data, p = 0.0330) (Figure 3.7a). This data set was obtained separately from the measurements on fractionated protein, and therefore was treated separately for statistical analysis. When the variance associated with protein size fraction was subtracted from the total error, this relationship was even more significant (p=0.0001) (Figure 3.7b, asterisks). The effect of size fraction as a blocking factor was determined to be highly significant (p=0.0007) (Figure 3.7b, plus signs). The analysis of variance table for the complete linear model is shown in Table 3.1.

Table 3.1: Analysis of Variance Table

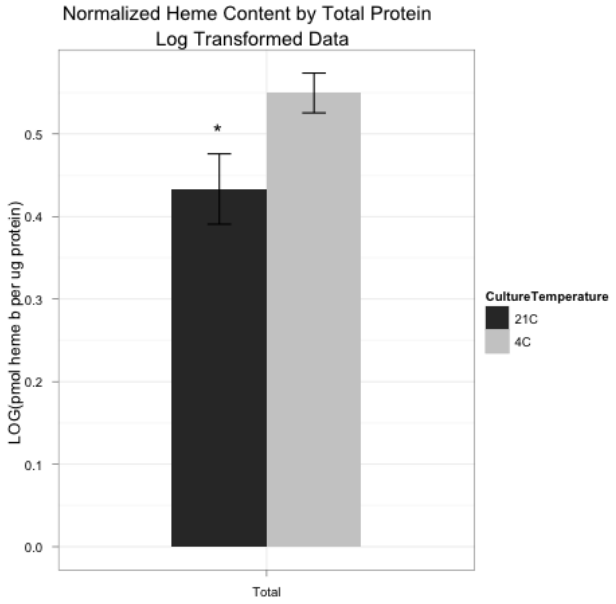
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Block	1	2.08	2.08	33.29	0.0007
Treatment	1	3.51	3.51	56.20	0.0001
Replicates	2	0.16	0.08	1.29	0.3346
Residuals	7	0.44	0.06		

After determining that the 4^{o}C worms had a greater concentration of heme b than the 21^{o}C worms, the distribution of heme b between the two size fractions was analyzed (Figure 3.8). It is shown that heme b is distributed 35% to 65% in the larger to smaller protein size fractions for the 21^{o}C worms, and 26% to 74% in the larger to smaller protein size fractions for the 4^{o}C worms. Though heme b in the 4^{o}C worms may be skewed slightly towards the smaller protein size fraction, the difference in distributions between the two cultures in not large.

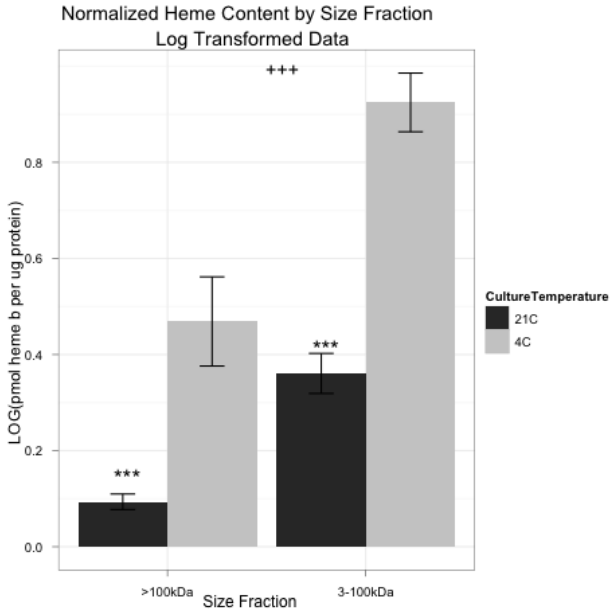
3.4 Conclusion

It has been determined that *C. teleta* maintain a significantly larger body size when acclimated at 4^{o}C compared to their usual culture temperature of 21^{o}C, and that the primary heme species present in total homogenates is heme b, the prosthetic group

of hemoglobin as well as several other important hemoproteins. The findings show that *C. teleta* 4^{o}C have significantly more heme b per unit total protein, and the distribution of this heme between large (>100kDa) and small (3-100kDa) proteins is only slightly different between the two temperatures. Though it is interesting that the heme b distribution is similar while the heme b concentration is very different between the two cultures, the pyridine hemochrome assay does not indicate the functional importance of these findings.



(a) By Total Protein



(b) By Size Fraction

Figure 3.7: Plots show the amount of pyridine hemochrome b (PH b) measured for each acclimation temperature using log transformed data. Quantities are sample means ± SEM (n=3 for both size fractions, n=25 for 21^{o}C homogenates and n=108 for 4^{o}C homogenates), which is represented by the black error bars. The single asterisk indicates a significant difference between temperature treatments ($p<0.05$) and a triple asterisk indicates a highly significant difference between temperature treatments ($p<0.001$). The triple plus indicates a highly significant difference between size fractions ($p<0.001$)

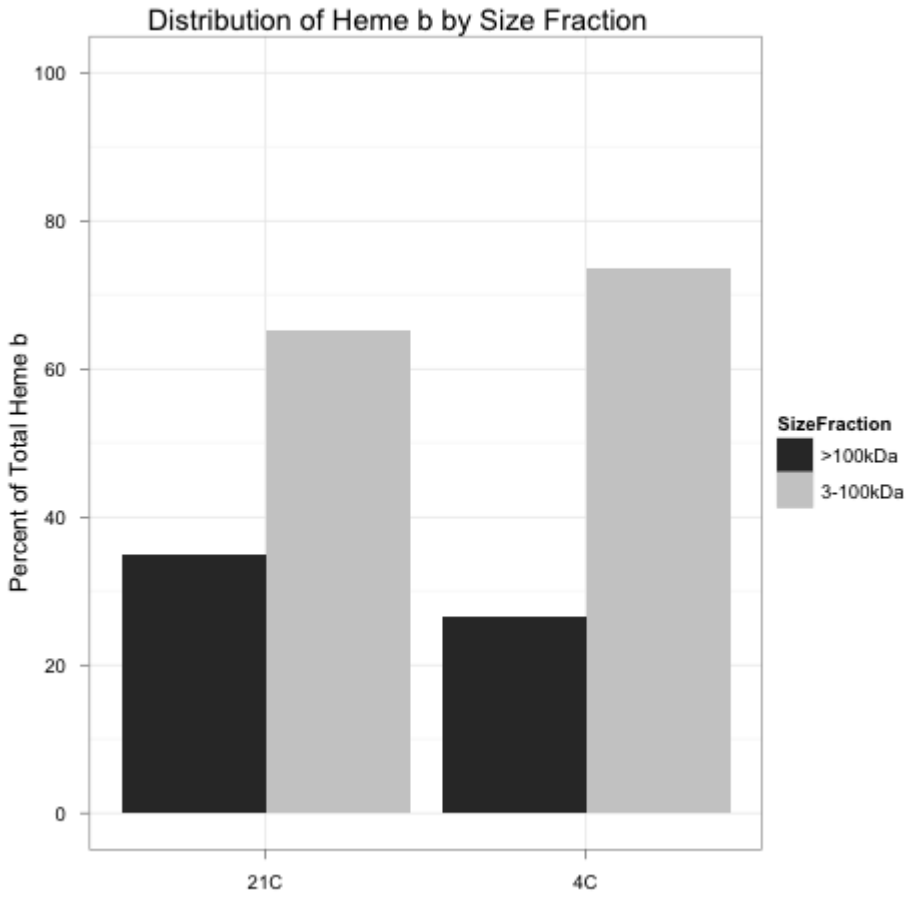


Figure 3.8: Plot shows the proportion of heme b as a percent of the total in each size fraction for the two *C. teleta* cultures.

Chapter 4

DETECTION OF HEMOGLOBIN IN POLYACRYLAMIDE GELS

4.1 Introduction

A necessary component of a gel-based protein study, regardless of the type of gel or the purity of the protein, is the ability to identify the protein or peptide of interest and its location on the gel relative to standards or other sample components. While the separation properties of the gel provide useful information (i.e. molecular mass or isoelectric point), initial identification requires an assay that can be performed in the gel or on a solid support during or following electrophoresis (Gabriel & Gersten 1992). One way to obtain a positive identification is to excise bands of interest and sequence them. However, this procedure is time consuming, uneconomical, and requires very high-resolution separation of peptides on the gel. The advantage of utilizing an in-gel assay is that they can be rapid, sensitive, reproducible, and inexpensive.

Though hemoglobin is not usually classified as an enzyme, it does possess intrinsic peroxidase activity as a function of its redox-active prosthetic group. A frequently encountered difficulty with in-gel detection of hemoglobin is that many different proteins besides hemoglobin also bind heme prosthetic groups, and those that contain heme c are covalently bound, whereas heme b is not. One advantage here is that the hemoglobin that is the subject of this study was found to be much larger than most other known hemoproteins, as mentioned in Chapter 3. For this reason, the combination of size and peroxidase activity were used to identified hemoglobin on native polyacrylamide gels.

In-gel assays for heme-associated peroxidase activity have been used for a wide range of studies, including identification of hemoglobin (Broyles et al. 1979), identification of cytochrome P450 variants (Welton & Aust 1974, Thomas et al. 1976), studying

c-type cytochromes in general (Goodhew et al. 1986), haptoglobin phenotyping (Hasan et al. 2012), and many others. The only issue with most of these studies is that the substrate, benzidine, is highly carcinogenic and its use is now tightly regulated. Although benzidine is still used today, great efforts have been put into finding a safe alternative, considering the value and versatility of a heme-stain. Amongst the many alternatives tested, the compound N,N,N'N'-tetramethyl-p-phenylenediamine (TMPD) proved to be safe, sensitive, and particularly effective in this study (Butler et al. 1987). A major difference between TMPD staining and benzidine staining is that upon oxidation, benzidine forms a colored product that polymerizes in the gel, while it is apparent that TMPD does not polymerize (Broyels et al 1979). This means that gels stained with benzidine remain stable for long periods of time and can be saved for future reference. TMPD forms a small colored product that diffuses out of the gel within a few minutes of destaining. The advantage of this effect is that gels stained with TMPD can subsequently be fixed and stained for total protein, allowing direct comparison between heme-stained bands and protein-stained bands without question. In this section, the use of TMPD-H_2O_2 staining in native polyacrylamide gels is presented, it is used to quantifying relative heme associated peroxidase activity, and its versatility through the analysis of hemoglobin subunit composition on denaturing polyacrylamide gels is demonstrated.

4.2 Methods

4.2.1 Sample Preparation

Samples were prepared using biological buffer as in Chapter 3. Following protease inhibitor pre-treatment, *C. teleta* were homogenized and sonicated on ice in Tris-HCl buffer pH 7.2 containing 10mM $MgCl_2$. Homogenates were centrifuged to clarify the extracts as previously described. Total protein was quantified using ProStain Protein Quantification Kit and mixed with 2X native or dissociating sample loading buffer prior to electrophoresis. Native sample loading buffer had a final concentration and composition of 100mM Tris-HCl pH 6.8, 24% glycerol, 5mM sodium chloride, and 0.2%

bromophenol blue. Dissociating sample loading buffer had a final concentration and composition of 100mM Tris-HCl pH 6.8, 8% SDS, 24% glycerol, and 0.2% bromophenol blue. Samples were not reduced or boiled.

4.2.2 Native PAGE

Native polyacrylamide gel electrophoresis (Native PAGE) was performed under non-dissociating, non-denaturing, and non-reducing conditions, in order to preserve the extracellular hemoglobin in its native quaternary structure as much as possible. Auto-dissociation of the native complex into presumed one-twelfth submultiples was observed during every run and could not be avoided, as was the case in previous studies. A small quantity of sodium chloride was added to the native sample loading buffer to help stabilize quaternary structure for as long as possible.

Native PAGE was run using the discontinuous Tris-Glycine buffer system (Laemmli 1970), without SDS. A 3% stacking gel was prepared in Tris-HCl pH 6.8, and a 7% resolving gel was prepared in Tris-HCl pH 8.8. The running buffer contained glycine as the leading ion for stacking and was pH 8.3. Native-PAGE was run with an ice block in the anode buffer to keep the gel cold. Electrophoresis was carried out at constant voltages: 40V for 60 minutes and 100V for three hours. The gel was pre-electrophoresed at 40V for about 60 minutes to remove some of the ammonium persulfate from the gel, which is typically done to preserve the structural integrity of the native proteins prior to doing enzyme activity assays (Gabriel & Gersten 1992).

4.2.3 SDS-PAGE

Dissociating, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed either with Pierce Precise Tris-HEPES-SDS precast polyacrylamide mini-gels, or with the Tricine-SDS polyacrylamide gel system as described in Chapter 2, except that samples were not reduced or boiled to prevent denaturation of the dissociated subunits. This was to prevent loss of heme b from at least a fraction of the protein. Samples were loaded onto dissociating gels in either native (N-SLB)

or dissociating sample loading buffer (D-SLB). Samples loaded in N-SLB were only exposed to SDS in the running buffer, where as samples loaded in D-SLB had SDS in both the sample buffer and the running buffer.

4.2.4 In-Gel Heme Assay

Following electrophoresis, gels were stained for heme using the in-gel activity assay described by Hasan et al. (2012). Briefly, gels were rinsed in dH_2O for a few minutes while the staining reagent was prepared. Gels containing SDS were rinsed longer than native gels. The reagent was made by mixing about 10μg of solid TMPD and 300μL of H_2O_2 in 50mL of 100mM Tris-HCl pH 7.5. According to Hasan et al. (2012), the exact quantity of TMPD added is not important. After making an initial measurement on a spatula, the amount of TMPD was estimated for all subsequent assays. Due to the lower amount of heme in the samples relative to Hasan et al. (2012), the 75μL of H_2O_2 was increased to 300μL. This was determined during the initial trial experiments of this study.

The reagent was poured over the gel and left to incubate at room temperature without shaking for five to ten minutes. The reagent was then decanted, and the gel was rinsed briefly in dH_2O to remove background staining. The gels were photographed as soon as possible (within about 10 minutes) because the colored bands in the gel diffused out rapidly. Restaining in attempt to improve resolution or stability of the bands was found to have no effect (results not shown), which implies that the heme groups were left in the met- (oxidized) state following the first exposure to the substrate.

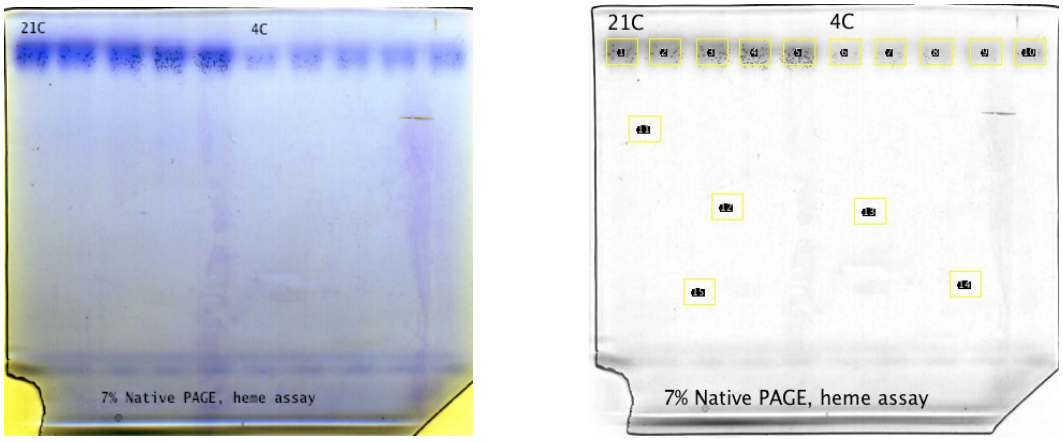
4.2.5 ImageJ Analysis

According to Hasan et al., TMPD-H_2O_2 staining of polyacrylamide gels is not quantitative. There are a few reasons for this. An important factor is that the peroxidase activity associated with hemoglobins is due to their heme moieties, which can easily dissociate from the protein, even under native conditions. A second reason is that the colored product is small and highly soluble in water, and therefore diffuses

from the place of activity and out of the gel quickly. While other reasons exist, comparing relative amounts of hemoglobin based on the intensity of the stain between bands on the same gel and at the same time (i.e. in a photograph) can be informative for comparing between the two *C. teleta* cultures. It is also of interest to compare these results to the results obtained by the pyridine hemochrome assay in Chapter 3.

ImageJ is an open-source image processing and analysis software developed by a scientist at the NIH in 1987 (Schneider et al. 2012). Amongst its many uses in biological science today, it can be used to compare the intensity of bands on a gel. A gel image is first digitized in 8-bit grayscale format, so that each pixel is associated with an integer value, between 1 and 256. Given a selected area, the program calculates the intensity as the sum of all of the integer values of the pixels. This value is termed integrated density, and is used here to compare the intensity of bands stained with TMPD-H_2O_2 on the same gel. Because the bands are on the same gel, no calibration was done, and the results are presented as integrated density per unit pixel area, normalized to gel background intensity.

To analyze gels, scanned images were converted into 8-bit tiff format, and the process Light background was selected. The rectangular selector was used to choose the first band or region of activity on the gel. The data for this region was sent to the ROI Manager. The yellow box was then dragged over a second band, with the horizontal coordinate remaining constant, and the data was sent to the ROI manager. This process was repeated for all bands of interest. Next, reference areas were selected in the same sample size as the bands, in order to obtain a background measurement. Values for integrated density and pixel area were obtained from the ROI manager and used for analysis. The ratio of heme-associated peroxidase activity to hemoglobin quantity from this assay was compared to concentration of heme b from the pyridine hemochrome assay.



(a) (b)

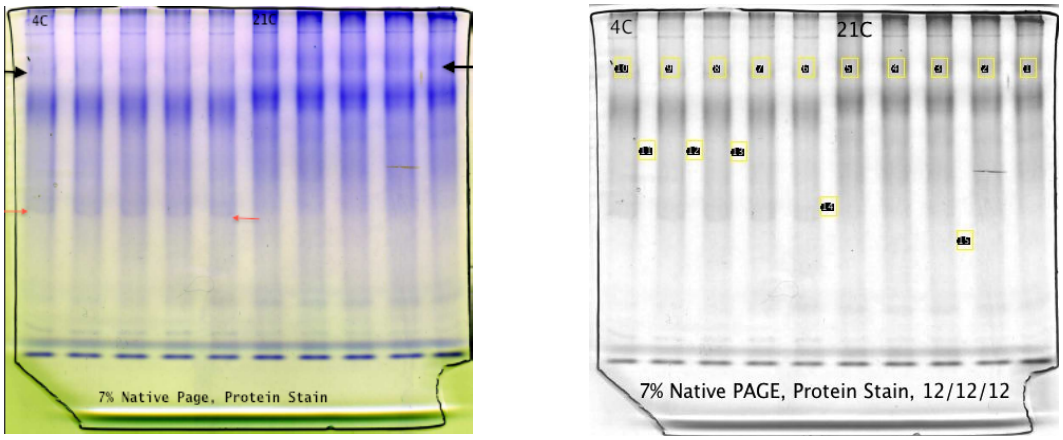
Figure 4.1: Native polyacrylamide gel stained with TMPD-H_2O_2 (a) and digitized to 8-bit format for ImageJ analysis (b). In (b), the yellow boxes encompass regions used for quantification of heme activity for the 21^{o}C and 4^{o}C protein extracts, as well as for the five, randomly selected, background regions.

4.3 Results

4.3.1 Native PAGE

C. teleta cultured at either 21^{o}C or 4^{o}C were pretreated with protease inhibitor at their respective acclimation temperatures for 45 minutes prior to sampling in ice-cold Tris-HCl pH 7.2 containing 10mM $MgCl_2$. Unfractionated homogenates were adjusted to 15μg total protein and run on a 7% native polyacrylamide gel. Following electrophoresis, the gel was incubated at room temperature for about five minutes in the presence of TMPD-H_2O_2 to detect heme-associated peroxidase activity (Figure 4.1a). The bubbles that appear over the blue colored product in the gel are oxygen gas, a byproduct of the reaction. This gel image was digitized for ImageJ quantification of band intensity (Figure 4.1b). Each of the five lanes corresponding to 21^{o}C (boxes 1-5) and 4^{o}C (boxes 6-10) were treated as biological replicates for the analysis. Five randomly selected rectangular regions were used to quantify background intensity (boxes 11-15).

After a scan was obtained, the native polyacrylamide gel from Figure 4.1 was rinsed with dH_2O until all TMPD eluted out of the gel, which took fifteen to twenty



(a) (b)

Figure 4.2: Native polyacrylamide gel from Figure 4.1 was rinsed with dH_2O and subsequently fixed and stained with Pierce Imperial Blue protein stain, and used for ImageJ analysis.

minutes. The gel was then fixed and stained with Pierce Imperial Blue protein stain (Figure 4.2a). The black arrows indicate the protein band across all lanes that correspond to the heme-associated peroxidase activity observed in Figure 4.1, and is presumed to be hemoglobin. This gel image was then digitized and analyzed by ImageJ as in the heme-stained gel (Figure 4.2b). The numbering of the boxes is identical. Note that this image is a mirror image of the corresponding heme-stained gel, which did not interfere with the analysis. The five background boxes are clearly selected in regions between lanes so that no protein would interfere with the analysis.

The results of the ImageJ analysis on heme-stained and protein-stained gels revealed that when the total amount of protein loaded was controlled (15μg), the 21^{o}C culture had more hemoglobin and a higher level of heme-peroxidase activity than the 4^{o}C culture (Figure 4.3, Gel Quantification). While this simple relationship makes sense and is expected, both within and between temperature treatments, the ratio of heme-peroxidase activity to hemoglobin quantity is not the same between the two temperature treatments (Figure 4.3, ratio).

To analyze this further, this ratio was compared to the concentration of pyridine hemochrome b in the >100kDa size fraction from Figure 3.7a in Chapter 3. Here, the

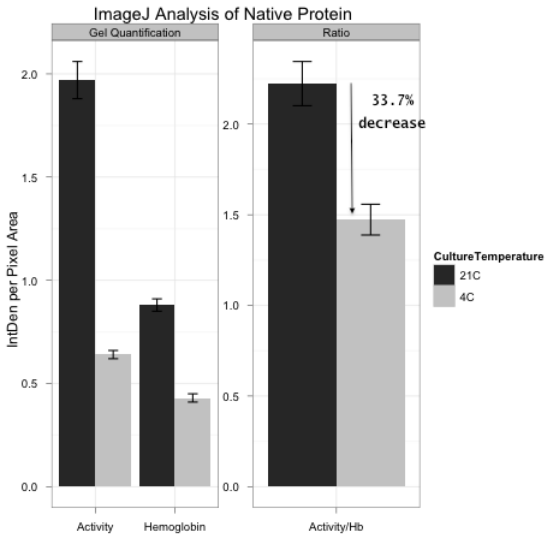


Figure 4.3: Gel Quantification: Plot shows pixel intensity for heme-associated peroxidase activity and protein staining from Figure 4.1 and 4.2 as Integrated Density per unit Area. Error bars represent ± 1 SEM (n=5). Ratio: Plot shows the mean ratio of activity per hemoglobin protein. Error bars represent ± 1 SEM.

raw data were used, not the log transformed data as in Figure 3.7a. As discussed, the >100kDa size fraction is assumed to be primarily hemoglobin, and will be treated as such here (Figure 4.4, Assay). Since both of these quantities are normalized to hemoglobin concentration, a second ratio can be made that assesses the level of heme-peroxidase activity to heme b concentration (Figure 4.4, ratio). Note that for this ratio, error bars are not included since the two values used to generate the ratio come from two different assays.

While the numerical values of the two ratios are not comparable, the relative difference between temperature treatments is. When comparing activity per unit hemoglobin protein, there is a 33.7% decrease between the 21^{o}C and 4^{o}C worms (Figure 4.3, ratio). When comparing activity per unit heme b, there is a 73.3% decrease between the 21C and 4C worms (Figure 4.4, ratio). This apparent disagreement is highly suggestive that there is a more complex mechanism at work here than a difference in hemoglobin concentration between the two *C. teleta* cultures.

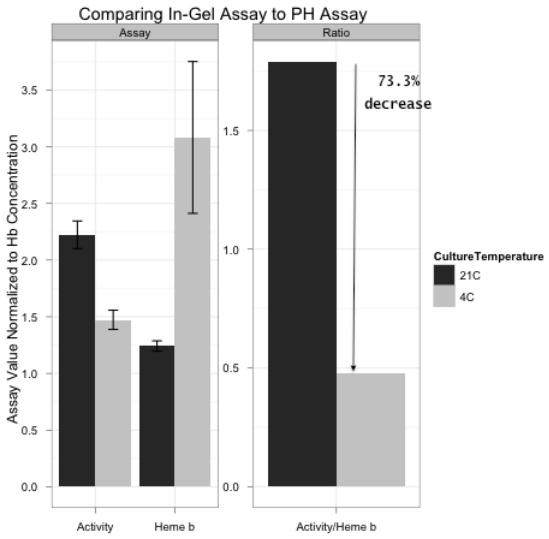
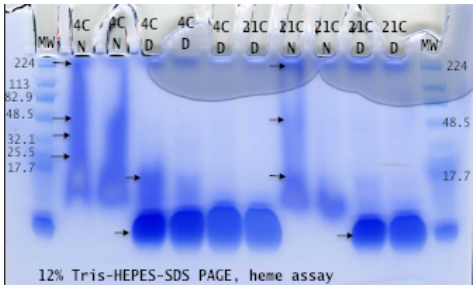


Figure 4.4: Assay: Plot shows the assay values for activity per unit hemoglobin from Figure 4.3, ratio, and heme b per unit hemoglobin from Figure 3.7a, >100kDa. Ratio: Plot shows the ratio of activity per heme b.

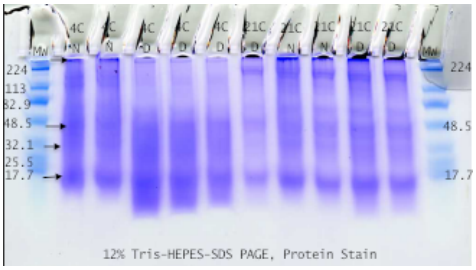
4.3.2 SDS-PAGE

C. teleta 21^{o}C and 4^{o}C were sampled as described and loaded onto a Pierce 12% Tris-HEPES-SDS polyacrylamide gel in either N-SLB or D-SLB, without quantifying protein. Under these mild conditions, it is expected that if *C. teleta* has an erythrocruorin, the dissociated globin subunits would be detected by heme staining of the gel. Using the examples given in Chapter 1, the major substructures would be one-twelfth protomers (with linkers) or dodecamers (no linkers), heterotetramers, dimers, and individual monomeric subunits. It is also expected that linker chains would be seen on the protein stained gel but not the heme stained gel, but positive identifications of linkers cannot be made without first purifying the native protein. It is also expected that samples loaded in N-SLB would retain more of the larger substructures, where as samples loaded in D-SLB dissociate further, but would retain a good portion of heme.

Upon treatment with TMPD-H_2O_2, a few areas of heme-peroxidase activity were identified on the SDS-PAGE gel, and were matched to proteins and peptides when stained with Imperial Blue protein stain (Figure 4.5). As expected, the samples



(a) Heme Stain

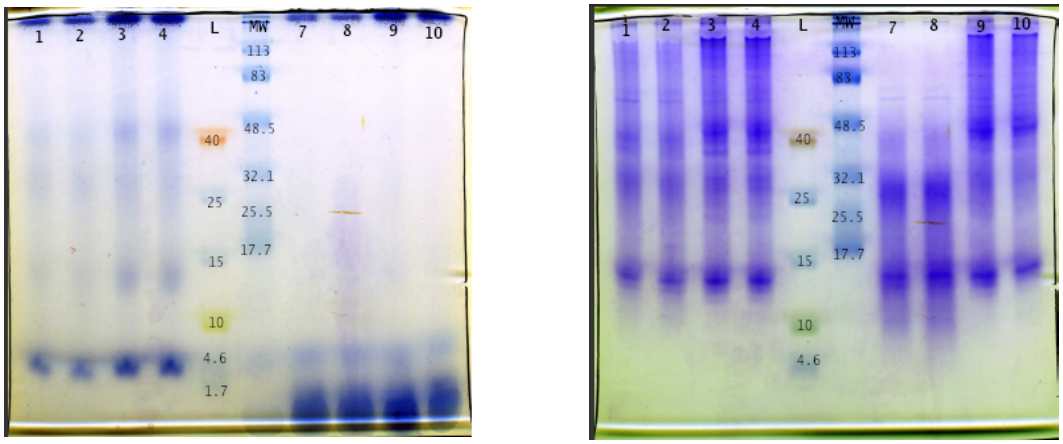


(b) Protein Stain

Figure 4.5: A 12% Tris-HEPES-SDS polyacrylamide gel stained for heme and then for protein. The arrows indicate all detected hemoglobin substructures and free heme.

loaded in N-SLB showed activity throughout the lanes, whereas samples loaded in D-SLB showed most activity in the smaller size region of the gel. Interestingly, the heaviest stained band (MW $\approx$ 224kDa) appeared in all lanes. This band is likely a one-twelfth protomer or dodecamer substructure. The lanes loaded in N-SLB for both cultures show two or three smeared bands between 17.7 and 48.5 kilodaltons. These likely correspond to tetramers, dimers, and monomer subunits. The monomers are also seen in the lanes loaded in D-SLB. The large area of activity in the D-SLB lanes that did not stain for protein is free heme that dissociated from its protein pocket.

Following the analysis of the Tris-HEPES-SDS polyacrylamide gels, the same experiment was done using a 14% Tricine-SDS polyacrylamide gel, which can produce slightly higher resolution bands, and therefore, better molecular weight estimation that allows assignment of bands to erythrocruorin substructures more accurately. Samples were prepared as for the previous gel, except that each lane was loaded with 15μg total protein. This allows better comparison between the two cultures. Upon treatment with TMPD-H_2O_2, the same pattern of substructures was identified, except that regions of heme-peroxidase activity were more distinct (Figure 4.6a). Two features stand out most in this gel: the one-twelfth submultiples show high activity in all lanes, yet all other substructures are very faint in the samples loaded in D-SLB (lanes 7, 8, 9, and 10). As expected, a large quantity of free heme was detected in the D-SLB lanes, as it did not stain for protein (compare to Figure 4.6b). In addition, a series of very small



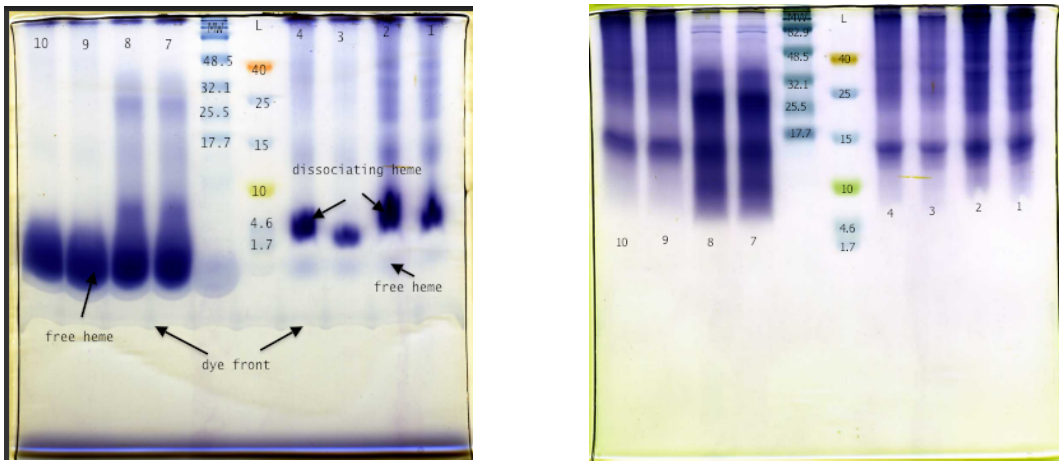
(a) Heme Stain

(b) Protein Stain

Figure 4.6: A 14% Tricine-SDS polyacrylamide gel stained for heme and then for protein. 1 and 2: 4^{o}C Loaded in N-SLB, 3 and 4: 21^{o}C Loaded in N-SLB, L: Ladder Marker, MW: Molecular Weight Marker, 7 and 8: 4^{o}C Loaded in D-SLB, 9 and 10: 21^{o}C Loaded in D-SLB.

peptides appear in all lanes in line with the 4.6 and 1.7 kilodalton Ladder markers. These are too large to be free heme (MW $\approx$ 600Da), and much smaller than would be expected for a hemoglobin monomer, yet they do not stain for protein. These small bands appear more intensely in the N-SLB lanes than the D-SLB lanes, and may be interpreted as heme-loss during electrophoresis. It seems unlikely that these bands are due to the bromophenol blue tracking dye since it diffuses from the gel rapidly and should travel ahead of the smallest peptides. This is seen more clearly in a heme-stained gel image in which the run time was not as long and the dye front did not reach the end of the gel (Figure 4.7).

Some interesting features also become apparent in the Tricine-SDS protein-stained gel that could not be resolved in the Tris-HEPES-SDS protein-stained gel. Although the monomeric subunits were not resolved and therefore could not be compared between the cultures, yet, it is apparent that the one-twelfth submultiple is more abundant in the 21^{o}C worms than the 4^{o}C worms (Figure 4.6b). This is in agreement with the native polyacrylamide gels. It also appears that the one-twelfth bands in the 21^{o}C lanes do not loose intensity when loaded in D-SLB, where as the 4^{o}C bands do.



(a) Heme Stain

(b) Protein Stain

Figure 4.7: 14% Tricine-SDS polyacrylamide gel. Lane identities are identical to Figure 4.6. Dissociating heme, free heme, and the bromophenol blue dye front are labeled in the heme-stained gel. Note that by the time the gel was scanned, much of the bromophenol blue already diffused from the gel. These areas of activity were not seen on the corresponding protein-stained gel.

4.4 Conclusion

These simple experiments produced a lot of interesting information concerning the concentration, composition, and stability of what is believed to be *C. teleta* erythrocruorin. Three important pieces of information were drawn from the Native PAGE experiment after analysis with ImageJ. Considering the first ratio only (Figure 4.3, Ratio), it was determined that heme-peroxidase activity per hemoglobin protein was not equal between the two cultures. There are three logical conclusions that can be made. The first will be termed the kinetic explanation, in which the activity assay is not linear over the hemoglobin concentration range within which the two samples fell. If the hemoglobin is treated as an enzyme, a greater concentration of enzyme will lead to a faster rate of conversion of substrate to product, and thus an exponential increase in the amount of product produced relative to the amount of protein, per unit time. This means that a greater level of activity per unit hemoglobin implies a greater concentration of hemoglobin in the gel. The second conclusion will be termed the structural explanation, in which the heme groups are less accessible on the 4^{o}C

acclimated hemoglobin than on the 21^{o}C acclimated hemoglobin. The third possible conclusion will be termed the thermodynamic explanation (or experimental explanation) in which the heme from the 4^{o}C hemoglobin dissociates from the protein pocket more easily than the heme from the 21^{o}C protein pocket during electrophoresis. Based on this first ratio, the first explanation seems the most likely, but further analysis sheds light on other possibilities.

Considering the second ratio only (Figure 4.4, Ratio), it was determined that heme-peroxidase activity per unit heme b was not equal between the two cultures. According to the pyridine hemochrome assay in Chapter 3, the 4^{o}C cultures had a greater concentration of heme b than the 21^{o}C cultures, yet here, the 21^{o}C cultures showed a greater level of activity per unit heme b. This observation seems to negate the kinetic explanation and support the thermodynamic explanation. However, it seemingly confuses the question about hemoglobin concentration. In Chapter 3, it was concluded that since the 4^{o}C culture had a higher concentration of heme b, it had a higher concentration of hemoglobin.

When looked at together, a third piece of information comes into view. The fact that the magnitude of the percent decrease is greater when looking at activity per heme b than activity per hemoglobin leads to the conclusion that a combination of effects are taking place. At first glance, there is clearly more hemoglobin in the 21^{o}C worms than the 4^{o}C worms when comparing protein stain. There is also clearly more heme b per unit protein in the 4^{o}C worms than the 21^{o}C worms, from the pyridine hemochrome assay. These two values alone contradict each other. The difference in activity per unit hemoglobin is in agreement with the kinetic explanation, in which the 21^{o}C worms do have more Hb than the 4^{o}C worms. The even greater difference in activity per unit heme b, the actual enzymatic constituent, is in agreement with the structural explanation and the thermodynamic explanation that the greater quantity of heme b in the 4^{o}C hemoglobin is less accessible, and also the possibility that the 4^{o}C hemoglobin is less stable and dissociates at a faster rate than the 21^{o}C hemoglobin, given the electrophoretic conditions. This would cause both a loss of heme and a loss

of native protein. The horizontal set of bands between the red arrows on the 4^oC half of the native gel (Figure 4.2a) mark a possible hemoglobin dissociation product that does not appear as intensely on the 21^oC half of the gel. This band did not show heme-peroxidase activity, but to take this hypothesis further is beyond the scope of the data presented so far and would be purely speculative.

TMPD-H_2O_2 activity staining of SDS polyacrylamide gels also provided valuable structural information. The fact that samples loaded in N-SLB stained heme better than samples loaded in D-SLB, and that the staining is smeared, not in clear bands, is very strong evidence that the hemoproteins being stained contained heme b and not heme c, in agreement with the pyridine hemochrome assay. Using the stoichiometry models for erythrocruorin presented in Chapter 1, five substructures were plausibly identified on the dissociating gels: high molecular weight one-twelfth submultiples or protomers (indistinguishable), tetramers with molecular weight of about forty-five to fifty kilodaltons, dimers of about twenty-five kilodaltons, unresolved monomers between ten and fifteen kilodaltons, and free heme that dissociated either before or during electrophoresis. The small, non-peptide bands that exhibited heme-peroxidase activity in the Tricine-SDS polyacrylamide gel may be free heme that dissociated during electrophoresis as SDS entered the gel. This can be explained by the higher stability of proteins in N-SLB than D-SLB. A final piece of evidence that was drawn from the SDS-PAGE experiments is that the one-twelfth bands stained strongly for 21^oC worms in both N-SLB and D-SLB, but only stained strongly in N-SLB for 4^oC worms. This is in agreement with the thermodynamic explanation drawn from the native gels in which the eryrthrocruorin from the 4^oC worms is less stable than the protein from the 21^oC worms given the electrophoretic conditions.

These experiments compared heme-activity staining and protein staining on polyacrylamide gels under native and dissociating conditions between protein extracts from warm and cold-acclimated *C. teleta*. In contrast to Chapter 3, the data presented here provides a bridge between the functional and structural differences that may be occurring in the hemoglobin between *C. teleta* cultures. The fact that at 4^oC, *C. teleta*

synthesizes or stores more heme b per unit protein than at 21^{o}C does not imply that these heme units are functionally active within the live organisms. One possibility is that the non-functional heme b is in the oxidized met-state, in which it cannot bind oxygen, and also would not react with the TMPD in the heme-assay, but the ratiometric differences in activity per unit heme and activity per unit hemoglobin observed between the two cultures strongly suggests that structural differences in the proteins also exist. The major difference in banding patterns between the two cultures was in the intensity, and not in the molecular weight distributions. This means that any differences that are present are likely manifested in the primary and secondary structures of the erythrocruorin subunits. A higher-resolution analysis of subunit composition will be necessary to determine if this hypothesis is true.

Chapter 5

PROTEIN FRACTIONATION

5.1 Introduction

The previous three chapters described the successful development of a sampling protocol resulting in the isolation of total soluble protein from whole organism homogenates, the effect of temperature acclimation on the size and heme pigmentation of *C. teleta*, and the use of a quick and easy assay for detecting heme-peroxidase activity in polyacrylamide gels. Based on the data presented so far, it was concluded that *C. teleta* has a high molecular weight erythrocruorin, and that this hemoglobin protein automatically dissociates into smaller substructures, even under *in vitro* native conditions. After the major hemoglobin protein in *C. teleta* was identified (Figure 4.1) and a temperature effect detected (Figure 4.3 and 4.4), it became necessary to develop a purification protocol so further structural and functional analyses could be performed on the pure protein. This chapter details the attempts, challenges, and successes in developing a purification protocol. Unfortunately, total purification was not achieved, but three individual steps led to fractionation of hemoglobin from the greater protein pool.

The overall strategy for the isolation work is as follows. First, the goal of the purification was to obtain homogenous, native hemoglobin in sufficient quantity to perform downstream structural and functional analysis. In order to achieve this goal, a series of steps based on different physical and chemical properties of the hemoglobin needed to be selected. An initial selection was based on the purification of mammalian hemoglobin by affinity chromatography (Hsia 1990). This procedure is based on the binding of the organic phosphate ATP to the allosteric site of the mammalian

tetramer. This presented the first challenge, since invertebrate hemoglobins do not bind organic phosphates, as mentioned in Chapter 1. Recall, invertebrate hemoglobins are structurally diverse, which makes generalization of properties difficult. One property, however, and the basis of this study, is size.

Two methods can be used to fractionate proteins based on size. The first method, ultracentrifugation, separates macromolecules based on their differential sedimentation velocities in a centrifugal field (Lebowitz et al. 2002). Although this method was used by Svedberg to make the first molecular weight measurements of annelid extracellular hemogobins in 1933, it requires at least a few milliliters of sample to perform. The second method, size exclusion chromatography, can be performed with very small sample sizes and has previously been used as a reliable fractionation step in the purification of annelid extracellular hemoglobins (Terwilliger et al. 1976, Garlick & Terwilliger 1977, Terwilliger et al. 1980, Garlick & Riggs 1981). What makes *C. teleta* hemoglobin so difficult to work with, even amongst the annelids, is the small size of the individual worms, as discussed in Chapter 2. In addition to the increased risk of protease activity when homogenizing an entire worm, the complexity of the protein pool is also a problem. The researchers cited above were able to take blood samples from their specimens by careful excision of the vascular system without rupturing the digestive tract and with minimal contamination from the body wall. *C. teleta* is a challenging organism to purify protein from because of the small volume and complexity of the starting samples. For this reason, multiple fractionation steps (Ammonium Sulfate Precipitation, Size Exclusion Chromatography, and Anion Exchange Chromatography) were tested individually. Coupling of steps led to very low yield of protein thwarting the ability to make functional measurements like oxygen affinity and cooperative binding by the hemoglobin complex.

5.2 Methods

5.2.1 Ammonium Sulfate Precipitation

Salting-out with ammonium sulfate ($(NH_4)_2SO_4$, or AS) was selected as an initial fractionation step in which proteins in a complex mixture differentially precipitate out of solution as the ionic strength of the solution increases upon addition of this highly soluble salt. Unlike TCA precipitation, this procedure does not significantly denature proteins. Trial experiments were done to determine the saturation range at which *C. teleta* erythrocruorin precipitates.

Precipitation was done on samples prepared in biological buffer as in Chapter 3. Following clarification of the cellular extract by centrifugation, 540μL of homogenate was brought to nearly 50% saturation by adding 440μL of a ice-cold 100% saturated solution of ammonium sulfate drop-wise with a Pasteur pipette. Typically, precipitation is done while stirring the sample and adding solid ammonium sulfate using tables that provide information on the amount of solid to add. Here, the ammonium sulfate was added as an aqueous saturated solution because the small sample volume made weighing out the solid inaccurate and difficult. The solution was added drop-wise while shaking to prevent localized areas of high salt concentration and undesired precipitation of proteins.

Once at 45% saturation, the samples were incubated on ice for 30-40 minutes, with occasional mixing. After incubation, the samples were centrifuged at 8000g, 3^{o}C for 20 minutes to pellet the precipitated proteins. The pellet was resuspended in biological buffer and saved for gel analysis. 900μL of supernatant was brought to 75% saturation by adding 300μL of ice-cold 100% saturated ammonium sulfate. These samples were then incubated and centrifuged as before. The second pellet was resuspended and the supernatant was saved for gel analysis. Before mixing with SLB, all three fractions (pellet 1, pellet 2, supernatant 2) were diluted in biological buffer, desalted, and concentrated using Amicon Ultra centrifugal filter devices with a 100kDa size cutoff. The concentrates from the desalting process were used for gel analysis and the flow-through was discarded.

5.2.2 Anion Exchange Chromatography

C. teleta 4^{o}C samples were prepared in biological buffer as in Chapter 3, concentrated using Amicon Ultra centrifugal filter devices with 100kDa size cutoff, and brought back to a volume of about 750μL in anion exchange buffer A (100mM Tris-HCl pH 8.59) for immediate use. Ion exchange chromatography was done using a Bio-Rad Bio-Sclae Mini UNOsphere Q Cartridge with a 1mL bed volume, packed with a strong anion exchange resin. Chromatography was done at 4^{o}C using a Bio-Rad BioLogic LP system equipped with a pre-column conductivity flow meter, a post-column UV flow meter, a BioFrac Fraction Collector, and LP Data View Software (Bio-Rad). The sample loop was set to a volume capacity of 500μL and chromatography was carried out at a constant flow rate of 0.5mL/min. Protein was eluted from the column with a positive step gradient of buffer B (100mM Tris-HCl pH 8.57, 1.0M NaCl) and collected every 3 minutes in fraction collection tubes, corresponding to 1.5mL volumes. Care was taken to ensure that buffer B was slightly more acidic than buffer A. The buffers were refrigerated and pH adjusted prior to use to avoid the increase in pH that would occur at 4^{o}C relative to room temperature. Optimal buffer concentration and pH were determined experimentally (data not shown). The volumetric distance between the UV flow meter outlet and the fraction collection drip outlet was measure to be 0.75mL.

Following chromatography, 100μL of each fraction was pipetted into a 96 well plate and absorbance at 415nm was recorded using a FLUOstar Omega microplate reader (BMG Labtech). Fractions that absorbed strongly at 415nm were selected, concentrated, diluted in N-SLB, and run on a 7% native polyacrylamide gel. Gels were stained for heme-peroxidase activity and total protein as in Chapter 4. Two anion exchange elution programs were tested and are described in the results. A protein stained gel for the second program is not available.

5.2.3 Size Exclusion Chromatography

Two size exclusion chromatography (SEC) resins were tried during this study: Sepharose 4B and Sepharose CL-6B (Sigma). Sepharose 4B has been successfully used

as an early purification step of annelid extracellular hemoglobins in many older studies because it has a wide molecular weight separation range ($70x10^3$-$20x10^6$). Though durable and inexpensive, this wide separation range requires a larger bed volume to achieve high enough resolution. For this reason, Sepharose CL-6B was selected for this study because the narrower separation range ($10x10^3$-$4x10^6$) is better suited for the smaller column that was available for this study (300mm x 9mm), and it was expected that a high molecular weight erythrocruorin would still be well within this size range. In addition, the cross-linking (CL) of the agarose beads allows chromatography to be run at higher flow rates, a broader range of temperature and pHs, and is easier to clean and sterilize between uses. In general, the Sepharose brand chromatography media are suited for early stage fractionation of crude homogenates to reduce the complexity of the protein solutions and to remove other molecular contaminants.

Sepharose CL-6B was packed into a 300mm K9 low-pressure column equipped with a flow-adaptor and 80μm mesh filter (Amersham) according to the manufacturers instructions, except a packing reservoir was not used. Care was taken to ensure that there was no air space between the surface of the column and the filter of the flow-adaptor. The column was packed at room temperature and atmospheric pressure in 20% ethanol before hooking up to the LP system at 4^{o}C. The actual bed height was about 280mm, corresponding to a volume of about 25mL. Before use, the column was equilibrated with three bed volumes of elution buffer (0.1M Tris-HCl pH 7.2 with 10mM $MgCl_2$), which was the same as the biological buffer used for sample preparation. Performance testing was done with a solution of 2% acetone in dH_2O according to the manufacturers instructions. It was necessary to empty and repack the column several times before performance criteria were adequately met.

Samples were prepared for SEC as in Chapter 3, concentrated using Amicon Ultra centrifugal filter devices with 100kDa size cutoff, and brought back to a volume of about 750μL in elution buffer immediately before use. Lyophilized standards (Sigma kit MWGF1000) were dissolved in elution buffer with 10% glycerol and used to calibrate

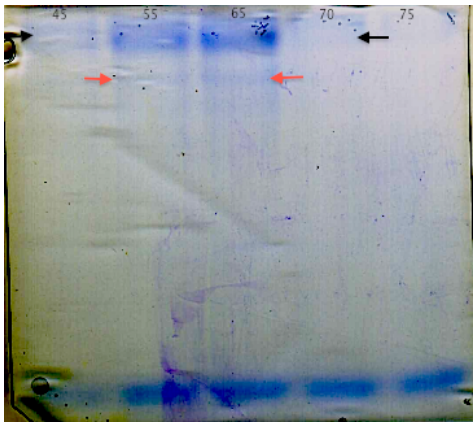
the column. The standards included thyroglobulin (669kDa), apoferritin (443kDa), β-amylase (200kDa), alcohol dehydrogenase (150kDa), bovine serum albumin (66kDa), and carbonic anhydrase (29kDa). The representative *C. teleta* 4^oC elution profile shown in the results was done at a flow rate of 0.1mL/min, whereas the 21^oC elution profile was done at a flow rate of 0.39mL/min. Standards were tested at both flow rates, but there was no difference in elution volume, just elution time. Blue dextran (2000kDa) was also run as a standard, but the peak could not be resolved, even with multiple attempts, and therefore was not included in the generation of the calibration curve.

Following chromatography, fraction absorbance was measured at 415nm, as with the anion exchange fractions. Because the standards were only detected at the UV flow meter, sample elution profiles at 415nm were adjusted to account for the volumetric distance separating the UV flow meter from the fraction collector outlet. To generate the calibration curve, the elution volume (V_e) was measured as the volumetric distance from the start of the chromatographic run to the center of the absorbance peak for each standard. Since blue dextran did not resolve, the next highest molecular weight standard, thyroglobulin (MW 669kDa) was used to determine the void volume (V_o), measured in the same manner as V_e. The ratio of V_e/V_o was plotted as a function of the molecular weight of each standard, and a linear regression was fitted to the points (r^2=0.9874, $p<0.001$). The equation for the linear regression was used to plot a calibration curve directly on the elution profiles for each of the two cultures using the V_e/V_o relationship in which V_e was the elution volume. Curves were only plotted to encompass the reliable molecular weight separation range of Sepharose CL-6B (10-4000kDa).

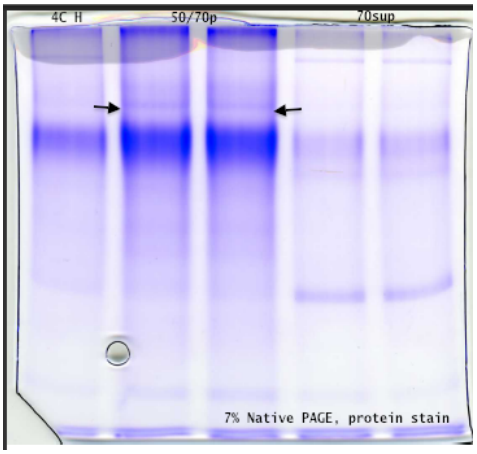
5.3 Results

5.3.1 Ammonium Sulfate Precipitation

It was determined that a saturation cutoff range of 45-75% produced the highest yield based on the in-gel activity assay (Figure 5.1a). Although using a narrower range



(a) Trial 1: Heme Stain

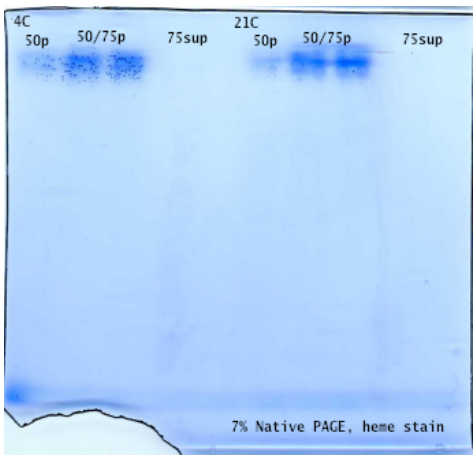


(b) Trial 2: Protein Stain

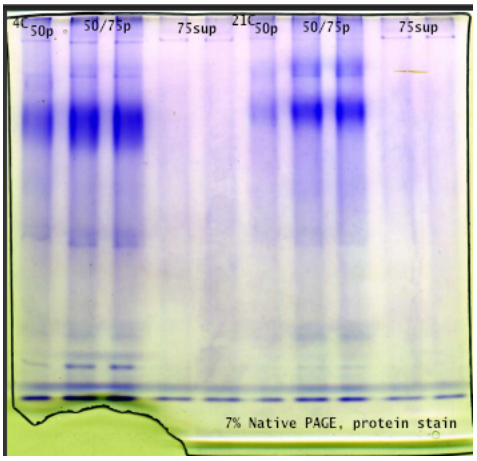
Figure 5.1: Native polyacrylamide gels from two separate AS precipitation trial experiments using *C. teleta* 4^{o}C. In (a) 45: 45% AS pellet, 55: 45-55% AS pellet, 65: 55-65% AS pellet, 70: 65-70% AS pellet, 75: 70-75% AS pellet. In (b) 4C H: 4^{o}C homogenate, 50/70p: 50-70% AS pellet, 70sup: 70% AS supernatant.

of 55-65% saturation improved purity, yield was not as high. An example protein-stained gel for AS precipitation is shown with the hemoglobin band marked by arrows (Figure 5.1b). Since this was to be used as an initial fractionation step, high-yield was chosen over high-purity, and subsequent experiments utilized a saturation cutoff range of 50-75%. It has also been shown that heme-peroxidase activity was maintained during precipitation, concentration, and electrophoresis, meaning the erythrocruorin was not denatured during the process. A minor band appears in the 55% and 65% saturation lanes on the native gel, as indicated by the red arrows (Figure 5.1a). This may be a dissociated substructure.

After determining an AS saturation range using 4^{o}C worms, *C. teleta* 4^{o}C and 21^{o}C were brought to 50% and then to 75% AS saturation, desalted, and quantified. 20μg of protein from homogenate, 50-75% pellet, and 75% supernatant were run together on a 7% native polyacrlyamide gel for comparison (Figure 5.2). No heme-peroxidase activity was detected in the 75% supernatant from either culture (Figure 5.2a) but a small quantity of protein was detected (Figure 5.2b). The 50-75% pellet appears to have stained more intensely in the 21^{o}C lanes than the 4^{o}C lanes, as expected.



(a) Heme Stain



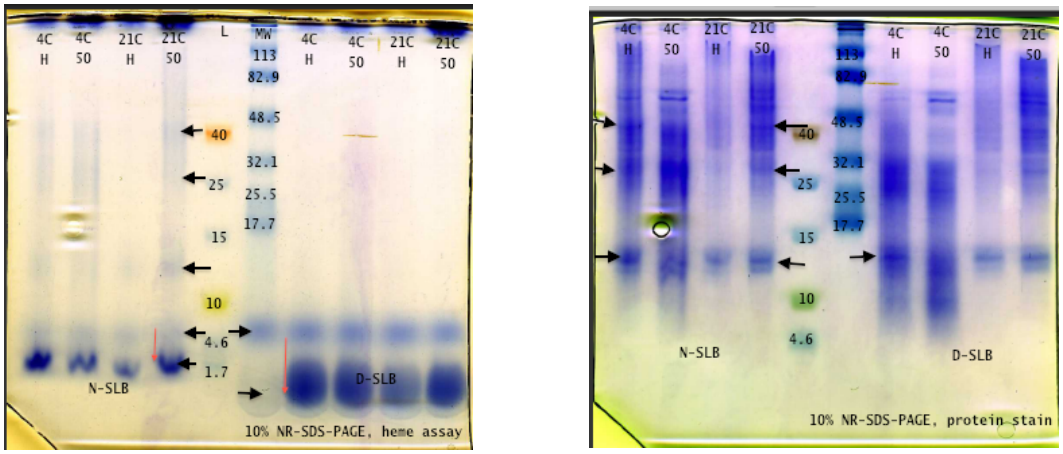
(b) Protein Stain

Figure 5.2: *C. teleta* 21^{o}C and 4^{o}C homogenates and AS fractions were run on a native polyacrylamide gel and stained for heme and protein. 50p: 50% AS pellet, 50/75p: 50-75% AS pellet, 75sup: 75% AS supernatant.

It should also be noted that the 50-75% pellet fractions have a similar composition to the homogenates but stain more intensely, which leads to the possibility that this served more as a concentration step than a fractionation step.

The 50-75% AS fractions and the homogenates from the two cultures were also run on a non-reducing, non-denaturing, 10% Tricine-SDS polyacrylamide gel, in which 20μg protein of each sample was loaded in either N-SLB or D-SLB, as in Chapter 4. The samples loaded in D-SLB only show heme-peroxidase activity in the 4.6 to 10 kilodalton molecular weight range and in the form of free heme at the base of the gel, whereas the samples loaded in N-SLB also show activity corresponding to tetramers, dimers, and monomers (Figure 5.3a), similar to Figure 4.6a. One-twelfth submultiples also stained for heme at the top of all lanes for both cultures, and is most intense in the 21^{o}C 50-75% AS fractions.

It is difficult to verify if the activity in the 4.6 to 10-kilodalton range is mitochondrial cytochromes, but since they do not stain for protein, it is deduced that these regions are free heme that dissociated during the process of electrophoresis. The presence of this band in the molecular weight lane is likely due to cross contamination from the neighboring sample lane during loading. The process of heme dissociation can



(a) Heme Stain (b) Protein Stain

Figure 5.3: *C. teleta* 21^{o}C and 4^{o}C homogenates and AS fractions were run on a dissociating polyacrylamide gel in either N-SLB or D-SLB and stained for heme and protein. H: homogenates, 50: 50-75% AS fractions.

be seen by comparing the D-SLB lanes to the N-SLB lanes, in which the heme appears to have dissociated during electrophoresis in the N-SLB and was still in progress by the end of the run (red arrows). In the protein-stained gel (Figure 5.3b), it becomes evident that the monomeric band encompasses two or three separate peptides, and the relative expression of these peptides may be different between the two cultures.

To improve the quality of the protein staining, the 4^{o}C and 21^{o}C homogenates and 50-75% AS fractions were fully dissociated in D-SLB, reduced in the presence of β-mercaptoethanol, denatured by heating at 95^{o}C for 5 minutes, and run on a 10% Tricine-SDS polyacrylamide gel (Figure 5.4). It is expected that if any intermolecular disulfide bonds exist, they would not be seen under reducing conditions. This gel provided strong evidence that further fractionation was required before a complete stoichiometry could be determined.

5.3.2 Anion Exchange Chromatography

Without prior knowledge of the isoelectric point of a protein, developing an ion exchange protocol requires a lot of trial and error. Initially during this study, a strong cation exchange resin (UNOsphere S, Bio-Rad) equilibrated with phosphate

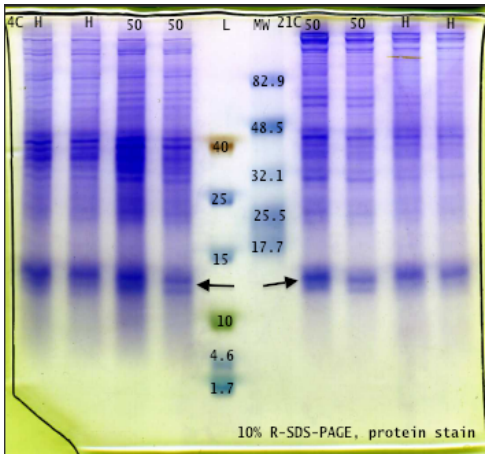
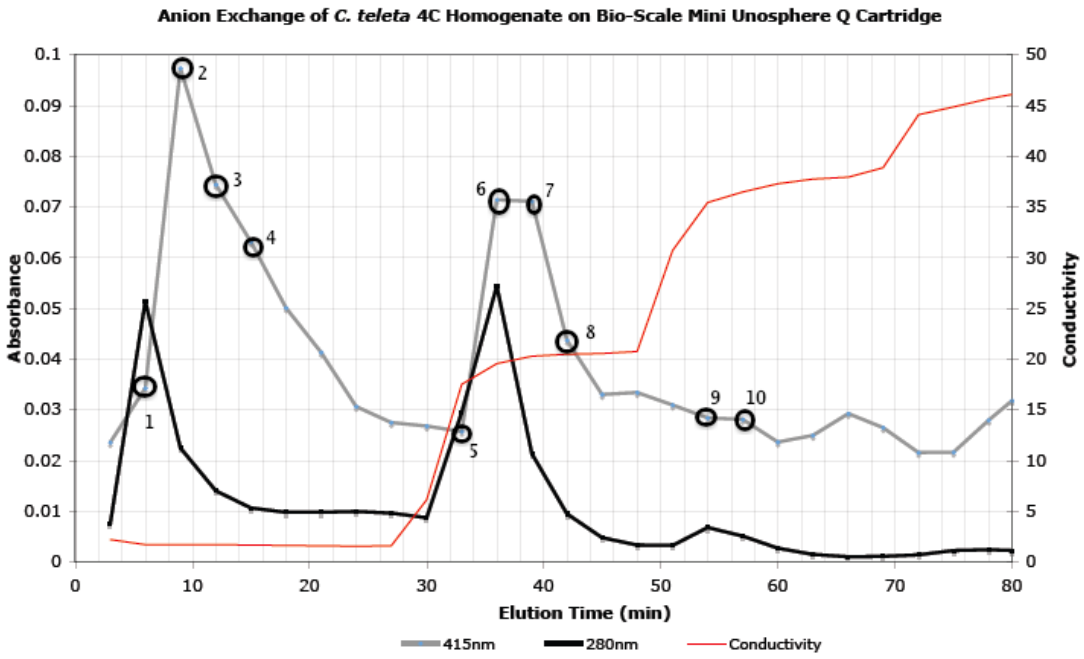


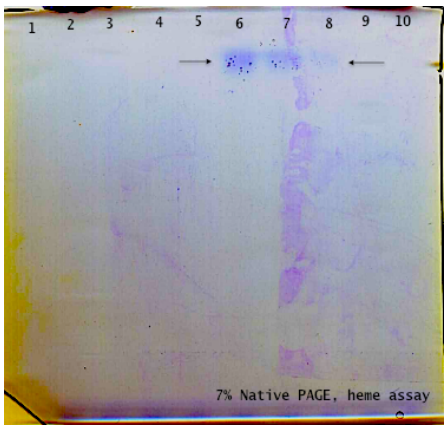
Figure 5.4: *C. teleta* 21^{o}C and 4^{o}C homogenates and AS fractions were run on a denaturing polyacrylamide gel and stained for protein. H: homogenates, 50: 50-75% AS fractions.

buffer using three different pH regimes (pH 7.6, pH 6.7, and pH 5.8) was tested in the fractionation of erythrocruorin from *C. teleta* homogenates (elution profiles not shown). Based on the absorbance at 415nm, it was found that the erythrocruorin did not bind to the column under any of these conditions. This should have been expected since the annelid extracellular hemoglobins known to date, including all those mentioned in Chapter 1, have characteristically acidic isoelectric points (Bruneaux et al. 2008). While the fractionation did not prove successful, it was determined that the hemoglobin of interest has a pI less than 5.8, which is in good agreement with those of other annelid species.

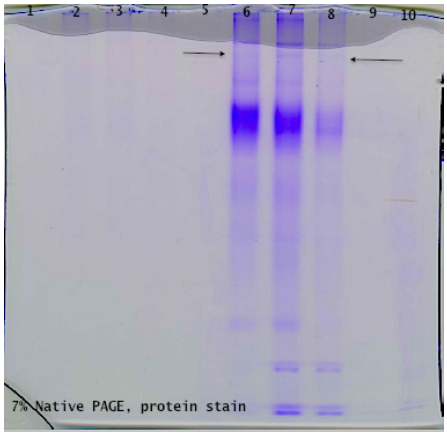
Rather than dropping the pH of the cation exchange buffer further, and risk denaturing the proteins, a strong anion exchange resin was used. With a pI less than 5.8, a anion exchange buffer at pH 6.8 would have sufficed to bind the protein of interest. To ensure that as much hemoglobin protein as possible bound to the resin, a Tris-HCl buffer was prepared at pH 8.59. At this pH, any protein with a pI less than 8.59 would bind to the resin. Two chromatography elution programs were tested using *C. teleta* 4^{o}C homogenates. The first program was as follows: 20min buffer A at 0.5mL/min, 20min 40% buffer B at 0.5mL/min, 20min 80% buffer B at 0.5mL/min, and 20min 100% buffer B at 0.5mL/min (Figure 5.5a). Selected fractions were concentrated and



(a) Elution Profile



(b) Heme Stain



(c) Protein Stain

Figure 5.5: Anion exchange elution profile showing protein absorbance at 280nm, hemoglobin absorbance at 415nm, and the change in conductivity, corresponding to an increase in NaCl concentration in the elution buffer with time. Fractions selected for gel analysis are circled and numbered.

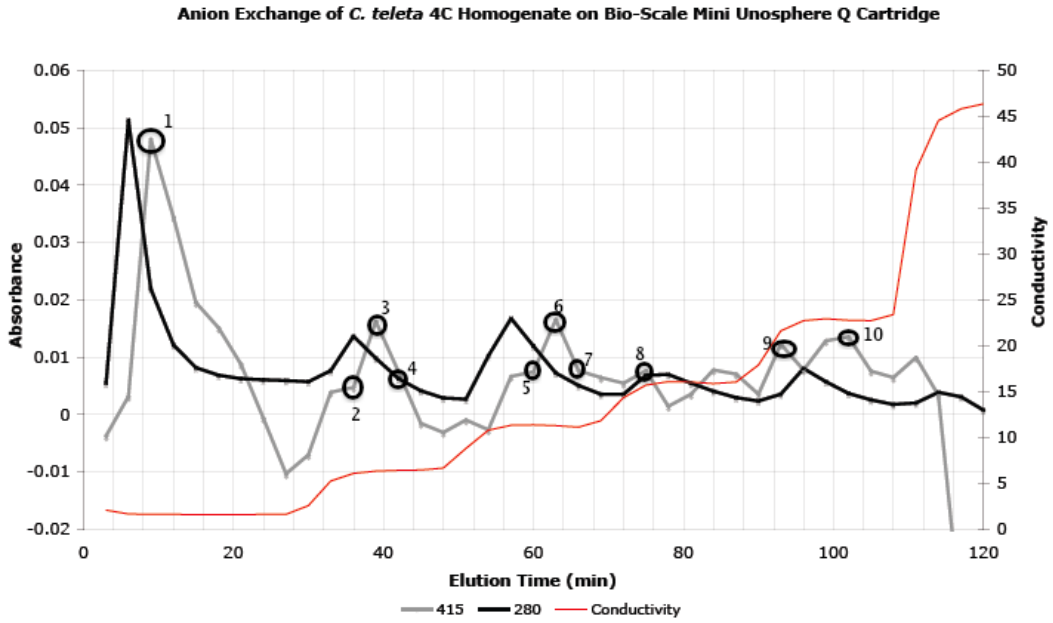
run on a 7% native polyacrylamide gel and stained for heme (Figure 5.5b) and total protein (Figure 5.5c).

It was determined that *C. teleta* erythrocruorin bound to the ion exchange resin and eluted during the first step (40% buffer B = 0.0-0.4M NaCl). The sample applied to the column was highly concentrated, so it is not known if the large amount of protein that did not bind was due to the pI of the proteins in those fractions or the binding capacity of the cartridge. It is likely a combination of these two factors. It was intriguing that the first four fractions absorbed strongly at 415nm, but did not stain for heme and only faintly for protein. The proteins that did stain in the native gel for these fractions were very large and barely entered the gel.

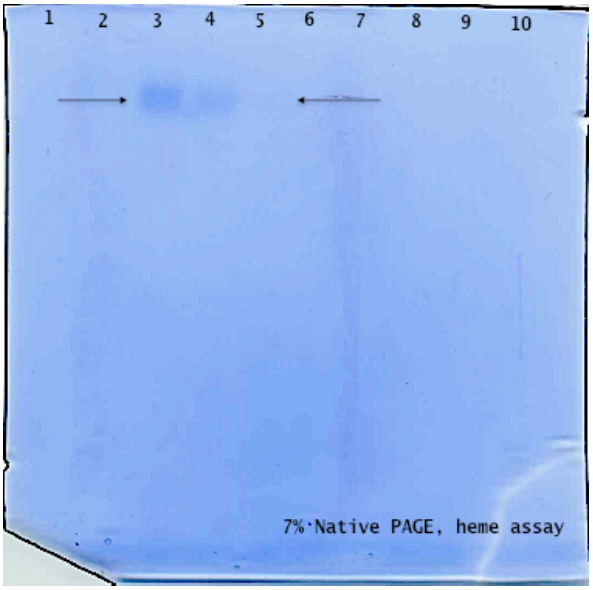
The second ion exchange elution program was as follows: 20min buffer A at 0.5mL/min, 20min 10% buffer B at 0.5mL/min, 20min 20% buffer B at 0.5mL/min, 20min 30% buffer B at 0.5mL/min, 20min 40% buffer B at 0.5mL/min, and 20min 100% buffer B at 0.5mL/min (Figure 5.6a). To increase the binding capacity of the resin, two 1mL UNOsphere Q cartridges were attached in series. In this case, the *C. teleta* erythrocruorin bound to the ion exchange resin and eluted with a step gradient of 10% buffer B (0.0-0.1M NaCl), and stained for heme on a 7% native polyacrylamide gel (Figure 5.6b). This gel was not stained for protein. Follow-up test programs increased the length of the elution steps, but no improvement in separation was observed.

5.3.3 Coupling AS Precipitation with AE Chromatography

It has been shown that *C. teleta* erythrocruorin could be fractionated using a 50-75% AS precipitation and separately by eluting from a anion exchange resin at pH 8.6 with 0.1M NaCl. Though each of these steps requires refinements, greater progress could be obtained by coupling the two steps. Briefly, *C. teleta* 4^oC samples were prepared as in Chapter 3 and brought first to 45% AS saturation and then to 75% AS saturation. The pellet was resuspended in anion exchange buffer A, desalted using an Amicon Ultra centrifugal filter device with a 100kDa size cutoff, and resuspended again in anion exchange buffer A. This sample was run on the anion exchange column

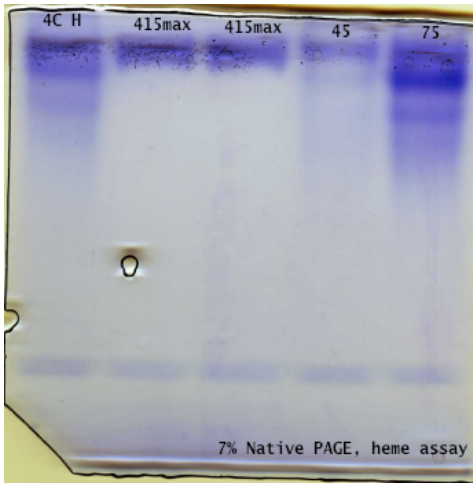


(a) Elution Profile

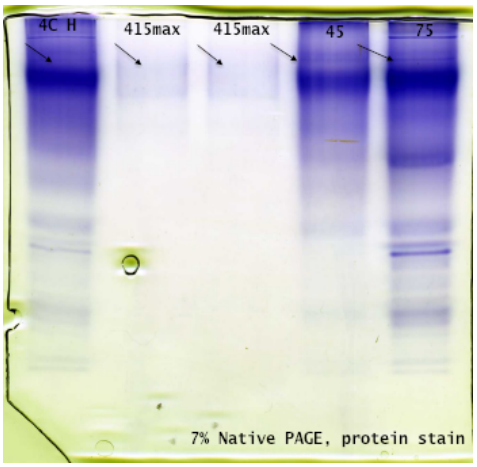


(b) Heme Stain

Figure 5.6: Anion exchange elution profile showing protein absorbance at 280nm, hemoglobin absorbance at 415nm, and the change in conductivity, corresponding to an increase in NaCl concentration in the elution buffer with time. Fractions selected for gel analysis are circled and numbered.



(a) Heme Stain



(b) Protein Stain

Figure 5.7: *C. teleta* 4^{o}C was fractionated by AS precipitation and then by anion exchange and run on a native polyacrylamide gel. H: homogenates, 45: 45% AS pellet, 75: 45-75% AS pellet, 415max: AE fractions.

according to the second elution program. Fractions 3 and 4 were pooled, concentrated, and desalted using a centrifugal filter device. The starting homogenate, the 45% AS pellet fraction, the 45-75% AS pellet fraction, and the pooled anion exchange fractions were run on a 7% native polyacrylamide gel and stained for heme and protein (Figure 5.7). It becomes clear here that the erythrocruorin one-twelfth submultiple is a relatively low-abundance protein, but was detected in all fractions. The coupling of the two steps greatly improved purity but yield was significantly reduced.

5.3.4 Size Exclusion Chromatography

Size exclusion chromatography on Sepharose CL-6B was tested independently of AS precipitation and anion exchange, and used as a fractionation technique as well as to determine the approximate molecular weights of the native erythrocruorin. The elution profile for *C. teleta* 21^{o}C shows four major absorbance peaks at 415nm (Figure 5.8). Using the calibration curve, the first peak is thought to be the native erythrocruorin. It is lower in abundance, due to dissociation into substructures, and has a molecular weight of about 4MDa. This is in the expected range for an annelid erythrocruorin.

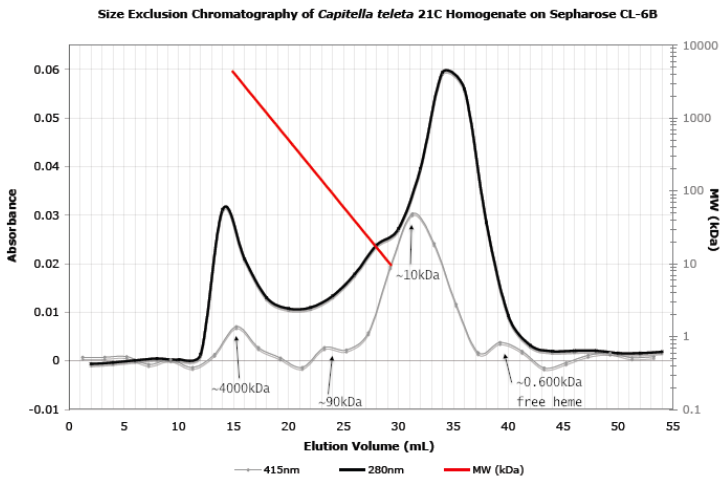


Figure 5.8: A representative size exclusion chromatography elution profile for *C. teleta* 21^{o}C showing protein absorbance at 280nm and hemoglobin absorbance at 415nm as a function of buffer elution volume. Calibration curve is shown in red. Major hemoglobin absorbance peaks are labeled with their approximate molecular weights as determined by the calibration curve.

The second peak at about 90kDa could be a one twelfth submultiple, in which case the molecular weight is an underestimation. Including blue dextran in the calibration curve greatly influences this region. The third peak, which is the largest, may contain a mixture of tetramers, dimers, and monomers. The fourth peak is outside of the reliable size estimation range of the resin, but is most likely free heme.

A very similar elution profile was produced when running a 4^{o}C sample, except that a higher level of resolution was obtained because of the reduction in flow rate (Figure 5.9). Fractions from this chromatography elution were pooled, precipitated with TCA, and run on a 12% reducing Tricine-SDS polyacrylamide gel (Figure 5.10). This gel was run before native PAGE or the TMPD-H_2O_2 assay were used in this study. There is still insightful information that can be taken from this gel. The two black arrows point to very large proteins that stained but barely entered the stacking gel. It is not understood how the protein could remain intact following precipitation in a strong acid, heating, and reduction, but for the purpose of this analysis, and considering the remarkable self-assembly mechanics of known erythrocruorins, this possibility will not

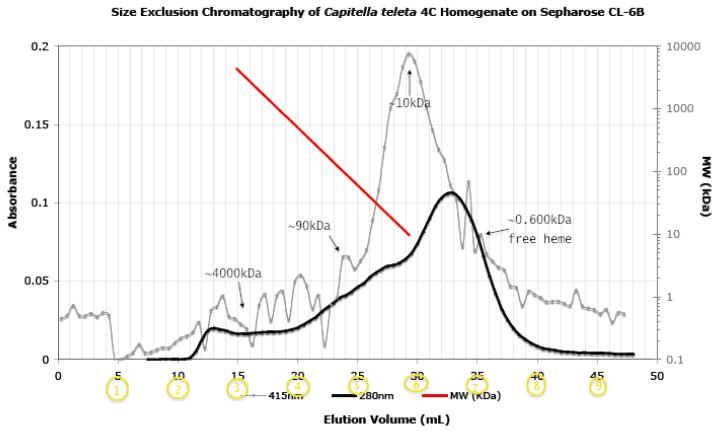


Figure 5.9: A representative size exclusion chromatography elution profile for *C. teleta* 4^{o}C showing protein absorbance at 280nm and hemoglobin absorbance at 415nm as a function of buffer elution volume. Calibration curve is the same as in Figure 5.8. Pooled fractions are labeled 1-9 in yellow.

be dismissed. Pooled fractions used in lanes 3 and 4 are exactly where it was expected that the native erythrocruorin would elute. The small peptides in lanes 6, 7, and 8 are hemoglobin monomers. The presence of peptides or proteins between 113 and 224kDa in lane 8 provide evidence that the calibration of the column was inaccurate or that reassembly of larger protein complexes can take place after denaturation.

5.4 Conclusion

It was demonstrated that the erythrocruorin from *C. teleta* could be fractionated using ammonium sulfate precipitation with a saturation cutoff range of 50-75% based on the heme-peroxidase activity in native polyacrylamide gels. The erythrocruorin has an acidic isoelectric point, and is retained on an anion exchange resin at pH 8.6. The protein then elutes from the resin in the presence of 0.1M NaCl as determined by measuring absorbance of fractions at 415nm. Coupling of these steps improved purity, but yield was very low.

Size exclusion chromatography on a 280mm bed of Sepharose CL-6B was also used to fractionate erythrocruorin and to estimate the molecular weight of the native

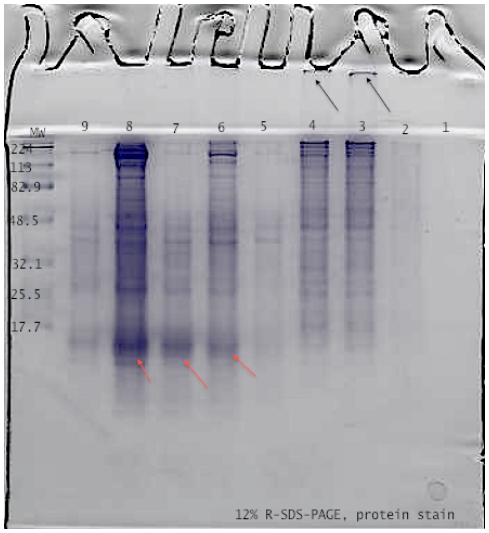


Figure 5.10: A representative denaturing polyacrylamide gel stained with Imperial Blue protein stain. Pooled fractions 1-9 correspond to the lane numbering here. The red arrows indicate hemoglobin monomers. The black arrows indicate possible reassembled native hemoglobin or large substructure.

protein. As a fractionation step, the protein eluted at different points depending on the level of dissociation. While some native protein eluted at about fifteen milliliters, the majority of protein eluted between twenty-five and thirty-five milliliters, based on the absorbance at 415nm and the relative quantity of protein observed by R-SDS-PAGE.

While it seems unlikely, two high molecular weight proteins are seen in lanes three and four (Figure 5.10), and are hypothesized to be native erythrocruorin or hemoglobin aggregates. Using the calibration curve, the native protein has a molecular weight of about four megadaltons. By comparison to previous gels, the hemoglobin monomers are identified in lanes six, seven, and eight. The calibration curve was not used to make size estimations for these regions of the elution profile (>30mL), but for comparison, carbonic anhydrase (MW=29kDa), a standard near the lower size exclusion limit for the resin, eluted at a volume of 26.7 milliliters. The elution profile for the two cultures was very similar, inferring similar protein populations. Heterogeneity most likely occurs at the level of hemoglobin monomers, which cannot be detected by chromatography with Sepharose media.

Chapter 6

PROTEIN PURIFICATION AND SUBUNIT COMPOSITION

6.1 Introduction

Traditional protein purification techniques have been demonstrated in the fractionation of a giant extracellular hemoglobin, erythrocruorin, from the marine polychaete *Capitella teleta*. The types of hemoglobin present in this species of annelid have never before been determined, but the results of this study have so far shown that at least this giant extracellular protein is expressed. It was hypothesized that if long-term temperature induced heterogeneity exists between the erythrocruorin from 21^{o}C and 4^{o}C acclimated *C. teleta* as a result of molecular-level physiological alterations, it would occur in the distribution of monomeric subunits of varying primary and secondary structures. As stated in Chapter 5, developing a protein purification scheme requires some knowledge of the chemical and physical properties of the protein of interest. Purification is necessary if accurate stoichiometric determinations are to be made. In addition to its large size, erythrocruorin produces color. The purification technique presented in this chapter takes advantage of the fact that as a hemoprotein, erythrocruorin can be seen as it migrates through a gel matrix, as long as the heme group remains associated with the protein and as long as enough protein is loaded onto the gel.

The ability to see hemoglobin as red bands in a native polyacrylamide gel before staining has been demonstrated in at least one previous study (Rousselot et al. 2006). The researchers were analyzing the molecular mass of native extracellular hemoglobin and its subunits from a brachiopod crustacean, *Triops cancriformis*. They saw two hemoglobin isoforms near the 669-kilodalton molecular weight marker as red bands,

which then stained with Coomassie-blue. Two major differences exist between that study and this study. First, Rousselot et al. had already purified the protein before gel analysis, which meant that the concentration of protein loaded onto the gel was equal to the concentration of hemoglobin. In this study, the hemoglobin was not purified, and thus the concentration of hemoglobin was an unknown fraction of the total protein concentration. This meant that the unpurified hemoglobin precipitated out of solution at a lower concentration than would have been possible with pure protein. This dilution in hemoglobin concentration resulted in a dilution of color production, so that the red bands actually appeared yellow in the native gels.

The second major difference was that the hemoglobin of *T. cancriformis* has a molecular mass close to 669-kilodaltons. The mass of *C. teleta* erythrocruorin was estimated to be about 4000-kilodaltons by size exclusion chromatography, making it difficult to resolve by standard native PAGE methodologies. For this reason, agarose, a polyacrylamide gradient, and an agarose-polyacrylamide hybrid were used as matrices in the electrophoresis techniques described in this chapter, all of which have previously been used to separate high molecular weight proteins (Oh-ishi et al. 2000, Schaegger et al. 1994, Tatsumi & Hattori 1995). The advantage of agarose is that since it does not form a polymer, protein can easily be recovered following electrophoresis by melting the agarose. A polyacrylamide gradient can be used to accurately estimate the molecular weight of native proteins, and is less fragile than a continuous low-percentage gel. The agarose-polyacrylamide hybrid has the resolving power of a low-percentage polyacrylamide gel (as low as 2%T), but added tensile strength provided by the solidified agarose. All three methods were valuable here in learning more about this novel erythrocruorin.

6.2 Methods

6.2.1 Native Agarose Electrophoresis

Agarose electrophoresis was done using a Mini-Horizontal electrophoresis unit (Fisher Biotech) and an EPS 301 power supply (Amersham Pharmacia). Native gels

were cast using a 1% solution of SeaPlaque GTG low melting temperature (<65^{o}C) agarose in gel buffer, and left to solidify at room temperature for 30 minutes, and were chilled in a refrigerator for an additional 60 minutes before use. To keep the gel cool during electrophoresis, the entire apparatus was placed in an ice-water bath and the running buffer was kept in the refrigerator until use. *C. teleta* 21^{o}C and 4^{o}C homogenates were prepared as in Chapter 3, and loaded into wells with N-SLB of the same composition as that used for vertical native PAGE. Samples were concentrated using Amicon Ultra centrifugal filter devices until they took a deep red color, and were loaded onto gels in this highly concentrated form. Electrophoresis was run at a constant 125V for about 90-120 minutes.

Two buffer systems were tried here. The first was the Tris-glycine system that was used for the vertical native gels described previously, except that no stacking gel was prepared. The second system was a discontinuous Tris-borate buffer, which supports higher voltages and longer run times without getting as hot as the Tris-glycine buffer. The gel buffer consisted of 500mM Tris base, 160mM boric acid, pH 8.6, and the running buffer was 90mM Tris base, 90mM boric acid, pH 8.5. Following each run, the gel was carefully removed from the apparatus and the colored bands were excised and placed in 2.0mL microfuge tubes. Agarose was melted in a water bath set to 85^{o}C and diluted in 3X D-SLB.

The diluted agarose band was prepared for second-dimension SDS-PAGE in one of two ways. In the first method, diluted samples were centrifuged at 10,000g, room temperature, until the agarose cooled and formed a gelatinous matrix. The supernatant was saved and run on a 12% Tris-HEPES-SDS polyacrylamide gel under reducing or non-reducing conditions without quantifying protein. In the second method, diluted samples were melted and poured over a 12% Tricine-SDS polyacrylamide gel under reducing conditions and left to solidify in the refrigerator for about 45 minutes before running. Here, no stacking gel was used since the agarose itself acted as a pseudo-stacking gel.

6.2.2 Colorless-Native PAGE (CN-PAGE)

CN-PAGE was run using the NativePAGE Novex 4-16% Bis-Tris Gel System (Invitrogen) according to the method of Schaegger et al. 1994, but omitting the use of Coomassie Blue G-250 as a charge-shift molecule. This way, erythrocruorin and its one-twelfth substructures could be identified by their intrinsic production of color and the heme-peroxidase assay before making molecular weight measurements by BN-PAGE. Total protein (300μg) from each sample was loaded with 4X N-SLB provided by the manufacturer (50mM BisTris, 6N HCl, 50mM NaCl, 10% w/v glycerol, 0.001% Ponceau S, pH 7.2). An unstained protein standard (NativeMARK, Invitrogen) was used for comparative purposes, even though CN-PAGE cannot be used for accurate molecular weight determinations. The 4-16% gradient gels have a resolving range of 15-1000kDa, which means that any color remaining in the wells after electrophoresis would be native erythrocruorin or hemoglobin aggregates that were too large to enter the gel. The gel and the running buffer are prepared at pH 7.5 and electrophoresis was run at a constant 125V for up to two hours. The gel was then stained for heme and for protein as in Chapter 4.

6.2.3 Blue-Native PAGE (BN-PAGE)

BN-PAGE was done in the same way as CN-PAGE, except that 0.02% G-250 was added to the cathode buffer. This dye acts as a charge-shift molecule, similar to SDS in dissociating PAGE. The G-250 binds to the surface of native proteins, without causing them to unfold or dissociate. This means that proteins migrate through the gradient gel according to their mass to charge ratio, not the intrinsic charge of the protein. This has two advantages in native PAGE: one is that the protein bands can be seen during electrophoresis because of the dye, and second, accurate estimations of molecular weight can be made. To do this, the native gradient gel was fixed and stained with Imperial Blue protein stain, and a standard curve was generated by measuring the relative mobility of the standards of known molecular weight as the distance traveled on the gel. The standard curve produced by BN-PAGE was compared to the curve

produced by CN-PAGE, and also to estimate the molecular weight of the erythrocruorin one-twelfth submultiple, and consequently, the native protein.

6.2.4 Tris-Borate Native PAGE

Due to the success of the horizontal slab gels in purifying erythrocruorin by excision of the colored bands, the same buffer system was used to prepare native polyacrylamide gels reinforced with low melting temperature agarose. The vertical gels were much thinner and provided sharper band resolution than the horizontal gels. The resolving gel buffer and the running buffer were the same as those used for the horizontal gels. A stacking gel buffer composed of 125mM Tris-HCl, pH 8.0 was used for the vertical gels. Initially, 3% polyacrylamide with 0.5% agarose was used in the resolving gel, but it was found that the native erythrocruorin dissociated into one-twelfth submultiples during electrophoresis, as in Chapter 4. When a 6% polyacrylamide gel with 0.25% agarose was used for the resolving gel and a 3% polyacrylamide gel with 0.5% agaorse was used for the stacking gel, the native protein entered the stacking gel and halted at the interface with the resolving gel, whereas the one-twelfth submultiples moved ahead into the resolving gel. Though counterintuitive, this approach provided interesting results.

A 6% resolving gel was prepared as follows: 1.5mL of 40% acrylamide/bis-acrylamide solution was mixed with 975μL of 87% glycerol in 5.0mL of 2X Tris-borate buffer, pH 8.6. Meanwhile, a 1% mixture of low melting temperature agarose in dH_2O was prepared in a flask and melted in a microwave until fully dissolved. Once cool to touch, 2.5mL of 1% agarose was mixed with the acrylamide solution. 30μL of 10% APS and 10μL of TEMED were added and a vertical mini-gel (10 x 10.5 x 0.075 cm) was cast between glass plates. Following polymerization, the gel was cooled in the refrigerator to allow the agarose to solidify. A 3% polyacrylamide stacking gel reinforced with 0.5% agarose was then poured on top of the stacking gel with a five-well comb and left to polymerize and solidify.

C. teleta 21^{o}C and 4^{o}C homogenates were concentrated and loaded into wells with 300μg total protein. Since it was difficult and impractical to quantify protein after excision of bands from the gel, this initial quantification was considered adequate to assume equal loading of hemoglobin on the second dimension gel relative to the original amount of total protein for each sample. Electrophoresis was run as follows: 75V for 30 minutes, 150V for 45 minutes, and 250V for 90 minutes. The anode buffer was kept cold with an ice block. One gel with a 4^{o}C sample was stained with Imperial Blue protein stain. A separate gel was run with samples from both cultures so that the colored bands could be excised and used for SDS-PAGE.

6.2.5 Reducing SDS-PAGE (R-SDS-PAGE) and Subunit Analysis

Once excised from the Tris-Borate native gel, the colored bands were mixed with about 4 volumes of 4X D-SLB and heated in an 85^{o}C water bath with frequent mixing for 60 minutes to initiate protein dissociation and diffusion out of the gel. These samples were either reduced and used for second-dimension R-SDS-PAGE immediately, or left to incubate at 4^{o}C overnight prior to running the second dimension. Monomeric subunit distribution was compared between the two cultures using ImageJ analysis. Since only one sample for each culture was run, statistical analysis could not be done.

6.3 Results

6.3.1 Native Agarose Electrophoresis

Electrophoresis of native protein on a horizontal agarose gel allowed for the visualization of *C. teleta* hemoglobin as a colored band, which looked yellow in the gel. A horizontal gel was chosen because a 1% low melting temperature agarose gel was too fragile to prepare in a vertical format. Every attempt to utilize the vertical apparatus resulted in the crumbling of the agarose upon removal of the comb. Of the two buffer systems used for horizontal electrophoresis, Tris-borate resulted in tighter band formation than Tris-glycine (Figure 6.1a and b). A representative Tris-borate agarose gel stained with Imperial Blue protein stain is shown (Figure 6.1c). What is

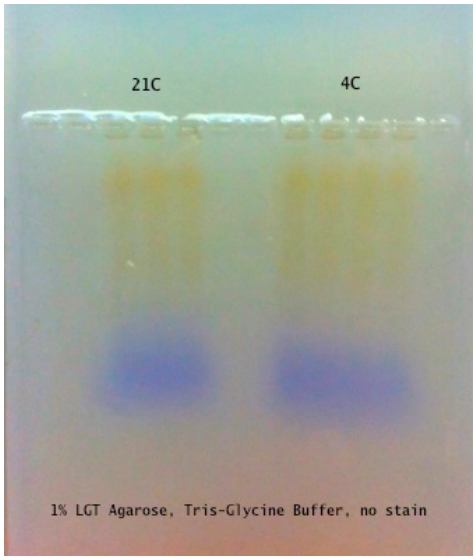
believed to be native erythrocruorin and the dissociated submultiples are indicated with black arrows. The colored bands were visible in these two locations. The one-twelfth submultiples were carefully excised and used for SDS-PAGE.

It was determined that the one-twelfth submultiples from the native agarose electrophoresis were nearly pure. Following dissociation of the *C. teleta* 4^{o}C bands in D-SLB, five peptides were visualized on a Tris-HEPES-SDS polyacrylamide gel (Figure 6.2a). The red arrow on top indicates the position of two bands between the 113kDa and the 224kDa molecular weight markers, the red arrow in the middle indicates the position of two bands between the 32.1kDa and 48.5kDa molecular weight markers, and the red arrow at the bottom indicates the position of a single band below the 17.7kDa marker. The submultiple from the native agarose gel was also fully denatured by heating at 95^{o}C for five minute in the presence of 10mM EDTA and reducing agent. The resulting Tris-HEPES-SDS polyacrylamide gel did not have good band resolution, but a series of small peptides believed to be hemoglobin monomers were seen near the 15kDa ladder and the 17.7kDa molecular weight markers (Figure 6.2b).

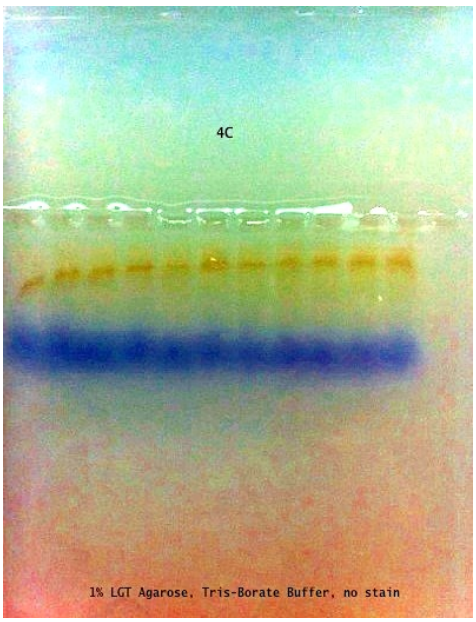
Due to the poor quality of resolution on the Tris-HEPES-SDS polyacrylamide gels, the one-twelfth submultiple was also run on a 12% Tricine-SDS polyacrylamide gel under reducing and denaturing conditions (Figure 6.3). Although the resolution on this gel was equally poor, possibly due to the absence of a polyacrylamide stacking gel, two regions stained for protein very clearly. It was not surprising to see the lower molecular weight bands, which are likely hemoglobin monomers, but the doublet bands near the 32.1kDa molecular weight marker are interesting. Though some background staining of bands did take place, the intensity of the two regions indicated by the red arrows is strong evidence that the protein excised from the native agarose gel was nearly pure, suggesting that the doublet bands are two non-globin linker chains.

6.3.2 CN-PAGE

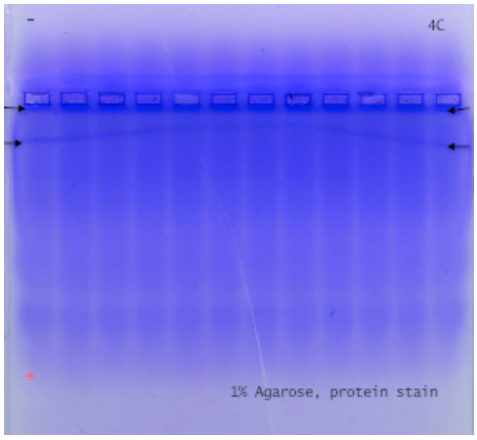
The purpose of running CN-PAGE was to determine how *C. teleta* erythrocruorin acts in the gradient gels before running BN-PAGE to determine the molecular



(a) Trs-Glycine

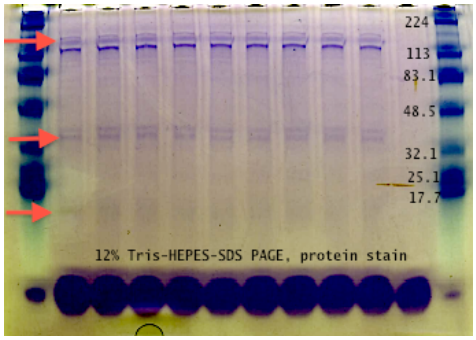


(b) Tris-Borate

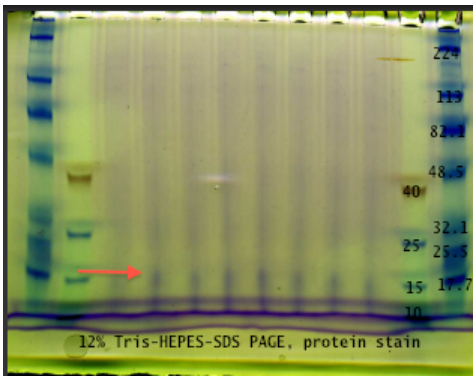


(c) Tris-Borate, protein stain

Figure 6.1: Representative native agarose gels using two different buffer systems. The Tris-Borate buffer system provided greater resolution than the Tris-Glycine buffer system. (b) and (c) are the same buffer but separate electrophoretic runs. (b) was used for band excision and SDS-PAGE. The arrows in (c) indicate possible native erythrocruorins and dissociated submultiples.



(a) Dissociating PAGE



(b) R-SDS-PAGE

Figure 6.2: Hemoglobin band from the Tris-Borate native agarose gel was excised, heated gently in D-SLB, and run on two Tris-HEPES-SDS polyacrylamide gels and stained for protein.

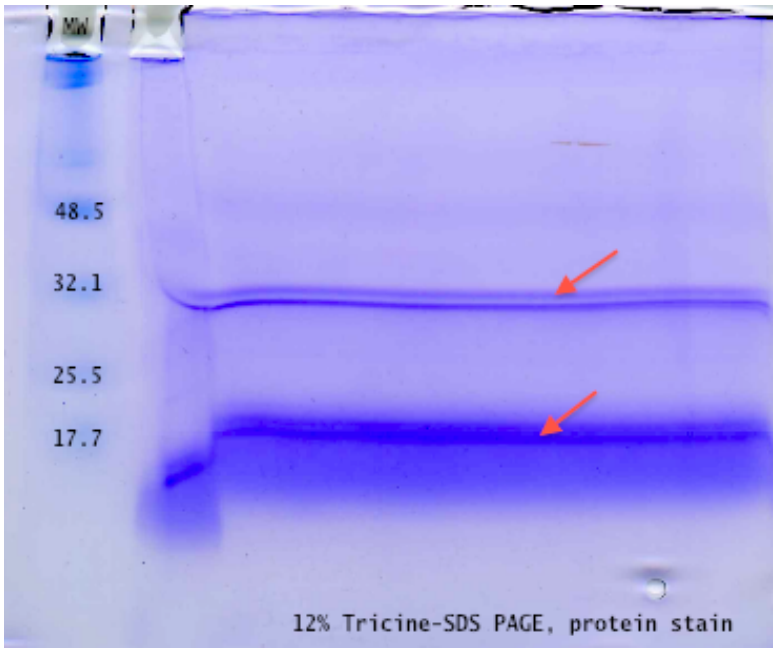
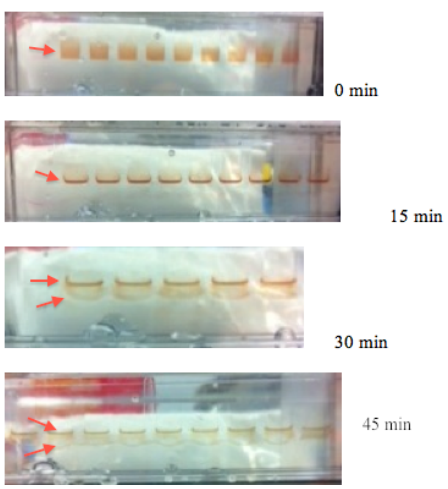


Figure 6.3: Hemoglobin band from the Tris-Borate native agarose gel was excised, heated gently in D-SLB, reduced, and run on a Tricine-SDS polyacrylamide gel and stained for protein.

weight of the one-twelfth submultiple. On the CN-polyacrylamide gels, the protein standard β-phycoerythrin is seen as a pink band prior to staining. For this reason, the hemoglobin of interest could be identified in relation to this pink band before and after staining. The results turned out to be more interesting than expected. Rather than simply locating which band to watch for on the BN-PAGE, further insights were gained into the dissociation mechanics of this giant extracellular protein as it migrates in an electric field.

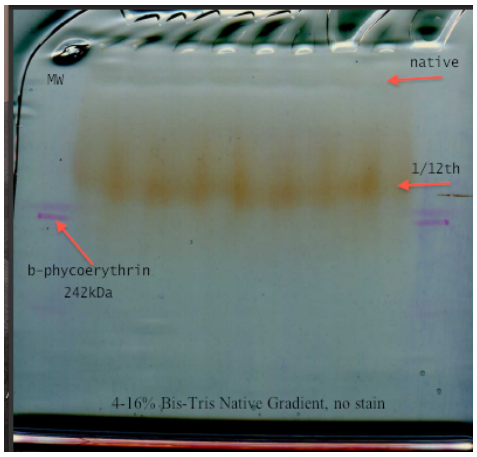
To start, 300μg total protein from *C. teleta* 4^{o}C was loaded onto a 4-16% gradient gel to confirm that erythrocruorin or the one-twelfth submultiple migrates as a colored band down the gel. What became immediately apparent, and was more difficult to see on the horizontal agarose gels, was that during the electrophoresis process, two events occur simultaneously. The first is that when the sample is loaded, it has a deep red color, but as electrophoresis progresses, this color shifts from red to yellow, to nearly brown. While this change in color is happening, the sample divides into three independent bands (Figure 6.4). One band remains in the well, a second band appears as a diffuse region of color just entering the gel, and a third band migrates into the gel far ahead of the other two. It is hypothesized that the first band is native erythrocruorin, which is too large to enter the gel. The diffuse region is the result of dissociating of the native protein into smaller submultiples and subunits as electrophoresis progresses. The third band is the one-twelfth submultiples that dissociated prior to or early on during electrophoresis, possibly the result of an equilibrium that occurs between the native structure and its subunits. On the final gel, only the one-twelfth submultiples could be seen in the gel (Figure 6.5a). Upon staining for total protein, the one-twelfth submultiples form a heavy band near the middle of the gel (Figure 6.5b). The colored bands near the top also become visible. Three regions are labeled native, dissociating, and denaturing. Although the protein may not be unfolding, the loss of color during electrophoresis leads to the possible conclusion that heme is being released as a result of protein unfolding.



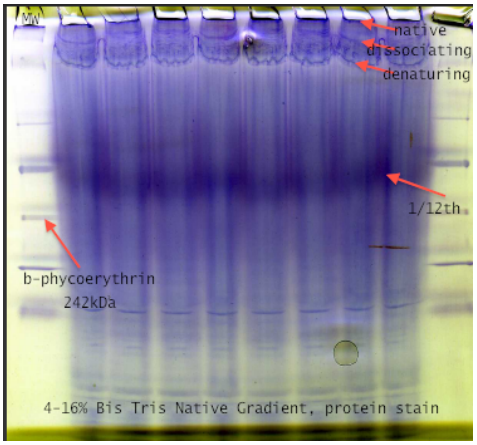
(a) Two Bands Visible

(b) Three Bands Visible

Figure 6.4: Photographs were taken while running *C. teleta* 4°C samples on a polyacrylamide gradient gel to illustrate the rapid loss of color and dissociating of the red protein during electrophoresis.



(a) No Stain



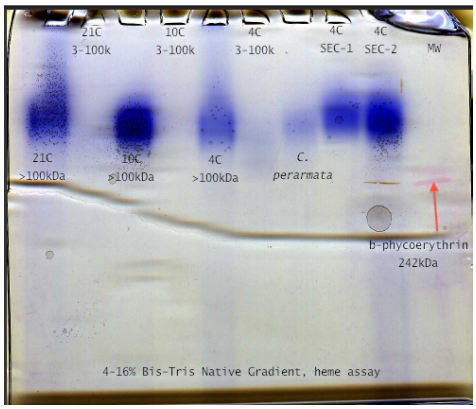
(b) Protein Stain

Figure 6.5: The gel from Figure 6.4 was run to completion and stained for total protein. The native erythrocruorin and the one-twelfth submultiples are labeled.

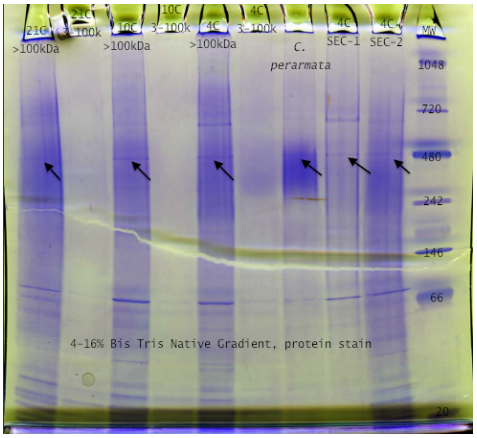
In order to compare *C. teleta* cultures, 21^{o}C, 4^{o}C, and the 10^{o}C transition culture were run together on a CN-polyacrylamide gel (Figure 6.6). Each culture was run as a >100kDa fraction and a 3-100kDa fraction, as in the pyridine hemochrome assay. Each of these samples contained 30μg total protein, except for the 10^{o}C 3-100kDa fraction, which only had 15μg total protein. In addition to these samples, a single worm homogenate (15μg total protein) from the Antarctic polychaete *C. perarmata* was also run, but is not part of this study. Two sets of pooled and concentrated size exclusion fractions from *C. teleta* 4^{o}C were also run. SEC-1 (10μg total protein) comes from the first hemoglobin peak on the elution profile (14-20mL), and SEC-2 (15μg total protein) comes from the third peak (28-34mL). This gel was stained for heme and for protein. Since far less protein was loaded onto this gel, hemoglobin could not be seen as red bands. However, the location of the one-twelfth submultiple was in agreement with the gel in Figure 6.5. It should also be noted that both SEC fractions stained for the one-twelfth submultiple. This implies that the native erythrocruorin in SEC-1 dissociated into submultiples, while the various subunits in SEC-2 reassociated into submultiples. This is further evidence that the one-twelfth submultiple is a highly stabile thermodynamic state.

6.3.3 BN-PAGE

Once the location of *C. teleta* erythrocruorin was identified relative to the other proteins in the sample and to the β-phycoerythrin standard, BN-PAGE was used to determine the molecular weight of the one-twelfth submultiple with good accuracy. A BN-polyacrylamide gel was run with the same samples and quantities as CN-PAGE and stained with Imperial Blue protein stain (Figure 6.7). It can be seen that the banding pattern is very similar to that observed by CN-PAGE, so the band corresponding to a one-twelfth erythrocruorin submultiple was identified and is indicate with black arrows where present. A standard curve was generated by measuring the migration distance of each protein standard, and plotting them against molecular weight (Figure 6.8). Measurements were made directly on the gel and not on a scanned image of the gel.



(a) Heme Stain



(b) Protein Stain

Figure 6.6: *C. teleta* samples from the three culture temperatures and two size fractions, pooled SEC fractions from *C. teleta* 4^{o}C, and a sample from *C. perarmata* were run on a CN-PAGE and stained for heme and total protein. The black arrows in (b) correspond to the heme-peroxidase activity observed in (a)

A curve was also made for the CN-polyacrylamide gel for comparison. It was found that a second-order polynomial fit the points best, which was used to estimate the molecular weight of the erythrocruorin submultiple. It was determined that the mass of the one-twelfth submultiple, across all three temperature treatments, was about 333kDa. Multiplying this value by twelve gives a mass of 3996kDa, which is in close agreement with the native mass determined by size exclusion chromatography. This conversion assumes that the 333kDa protein is a one-twelfth protomer, which includes both globin and linker chains.

6.3.4 Tris-Borate Native PAGE

Once the mass of the one-twelfth submultiple was identified by BN-PAGE, the next step was to identify the subunit composition, and if this differed between the two culture temperatures. While the horizontal agarose gels worked well for purifying one-twelfth submultiples from protein samples, using a thinner vertical gel has several advantages: it permits the use of a stacking gel, runs can be longer because it is easier to keep the gel cool, and excised bands come with less gel material. These three factors improve both yield and purity. It was mentioned above that vertical agarose gels are

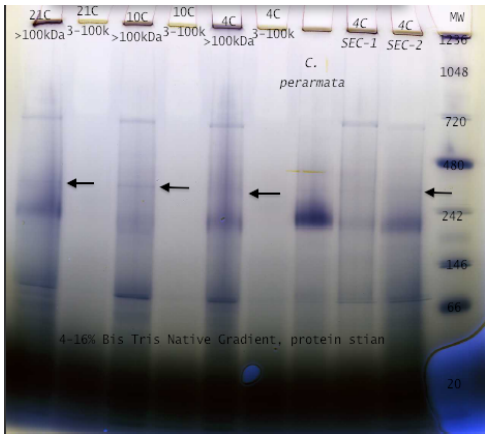


Figure 6.7: *C. teleta* samples from the three culture temperatures and two size fractions, pooled SEC fractions from *C. teleta* 4^{o}C, and a sample from *C. perarmata* were run on a BN-PAGE and stained for total protein. The black arrows correspond to the one-twelfth submultiples identified in Figure 6.6.

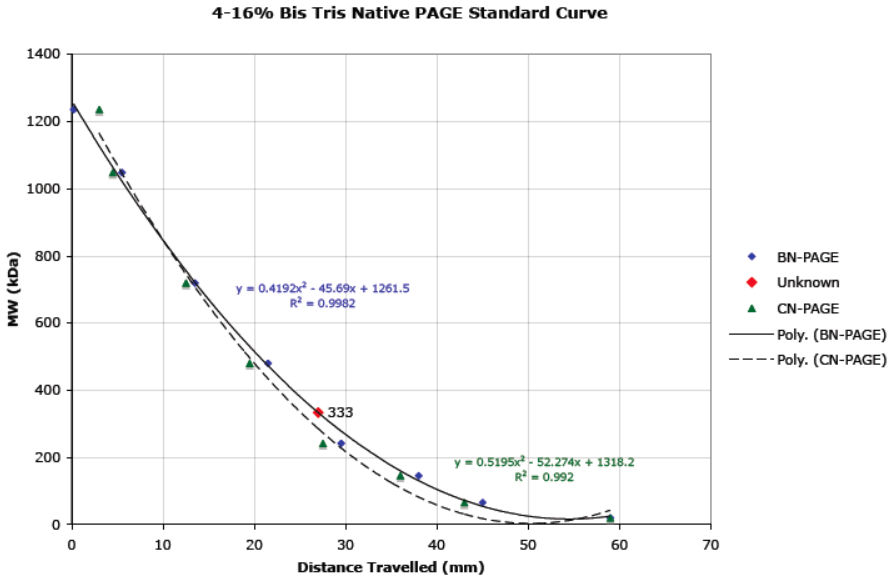


Figure 6.8: A second order polynomial was fit to standard curves generated from CN and BN-PAGE. The mass of the one-twelfth submutiple was determined to be 333kDa using the BN standard curve.

fragile and challenging to work with. Here, a polyacrylamide gel strengthened with agarose was used to purify hemoglobin from *C. teleta* samples with greater resolution and yield than the horizontal agarose gels.

The original reason for using agarose was that the native erythrocruorin is too large to separate on typical polyacrylamide gels. From the CN-PAGE, it was determined that the native protein dissociates into one-twelfth submultiples during electrophoresis. For this reason, the objective was changed slightly so that the one-twelfth submultiples, and not the native protein, would be purified by electrophoresis. In terms of subunit analysis, both of these protein complexes should yield the same results if the native protein is in fact an annelid erythrocruorin. The reason that the CN-polyacrylamide gels could not be used to purify the hemoglobin is that on gradient gels, large proteins will migrate until the porosity of the gel is too small to allow further movement. At this point, the proteins stop and become trapped in the gel. From the BN-PAGE, the percent total acrylamide at which the one-twelfth submultiples stopped moving was approximately 8.5%T, which corresponds to a migration distance of about 39% of the total length of the gel. For this reason, it was decided that a 6%T polyacrylamide gel reinforced with 0.25% agarose would be dense enough to resolve the one-twelfth submultiple from the rest of the proteins in the sample, but porous enough that the protein would not become stuck in the gel, and could therefore be recovered.

Similar results were obtained for the Tris-borate native PAGE as for the CN-PAGE, in which the native protein entered the stacking gel, but became immobilized at the interface with the resolving gel, while the one-twelfth submultiple dissociated and continued to migrate (Figure 6.9a). Note that the stacking gel was 3%T and was porous enough for the native protein to enter, where as the gradient gels started with 4%T, which did not permit the native protein to enter. Schaegger et al. (1994) used 3-12% gradient gels to resolve protein complexes of nearly 10MDa, which demonstrates that this seemingly minute difference results in nearly an order of magnitude increase in the resolving range of a polyacrylamide gel. It will also be noted that the tensile strength of the stacking gel and the resolving gel were noticeably and significantly

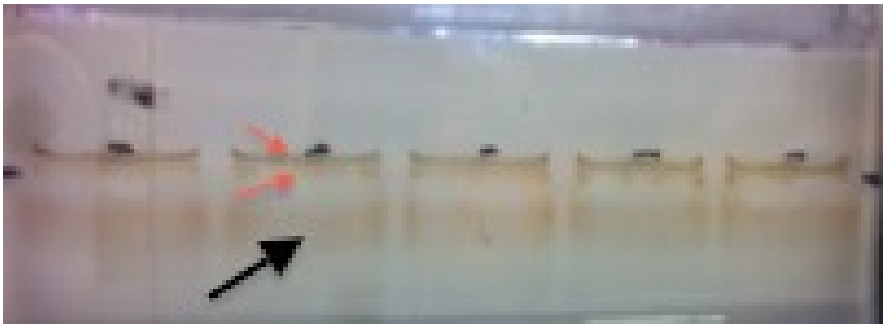
greater when agarose was added to the matrix.

Four lanes of the native gel were used to excise the colored band (Figure 6.9b), while the fifth lane was isolated and stained for total protein (Figure 6.9c). This identification and purification process was also done for a 21^{o}C sample, but a protein stained gel is not shown. Two bands are labeled, a "top" band and a "bottom" band. 6%T corresponds to the migration distance of the 720kDa marker on the gradient gel, which means that the top band, which appeared yellow on the unstained gel, is most likely an aggregate of two or three one-twelfth submultiples, or a partial dissociation product of the native protein. The bottom band is the one-twelfth submultiple of interest, but it may be migrating near a different, highly abundant protein (thick band). The top and bottom bands were excised as tightly as possible and used for SDS-PAGE. Whether this other band is hemoglobin or not has been a conflicting question throughout this study, but all attempts were made to avoid cutting into this region of the gel.

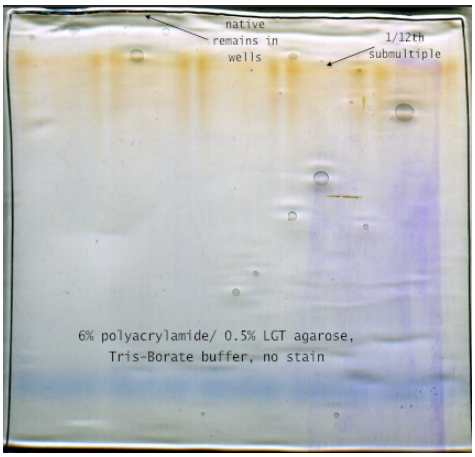
6.3.5 R-SDS-PAGE and Subunit Analysis

Comparing the gel results between samples incubated in D-SLB for 60 minutes (Figure 6.10a) and overnight (Figure 6.10b) show clearly that 60 minutes was not a sufficient time for the protein to fully diffuse from the polyacrylamide slice. While it can be said with certainty that the top band did include hemoglobin because of its color on the unstained native gel, the high abundance of protein on denaturing gel leads to the possibility that this band contained many aggregated proteins and peptides, which is not unusual on native gels. For this reason, just the bottom band was used for hemoglobin subunit analysis.

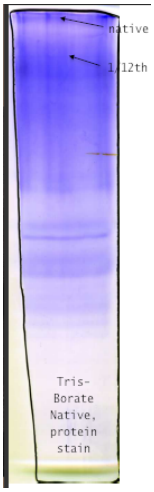
It was assumed that banding patterns on the reducing-denaturing-polyacrylamide gel was representative of the subunit composition for *C. teleta* erythrocruorin acclimated to 21^{o}C and 4^{o}C. Previous gels comparing the subunit composition of gel-purified one-twelfth submultiples from 4^{o}C protein extracts under reducing and non-reducing conditions revealed the presence of up to six hemoglobin monomers (Figure 6.11 black



(a) Three bands visible

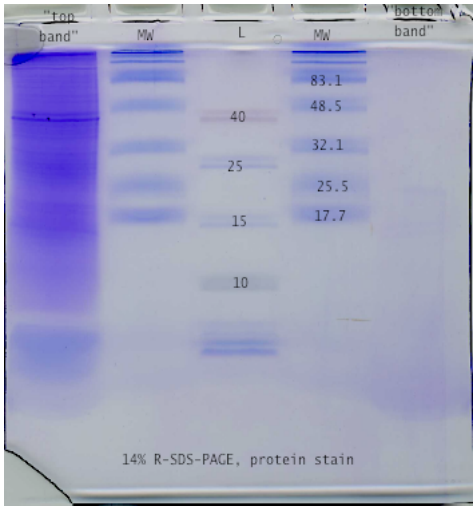


(b) No Stain

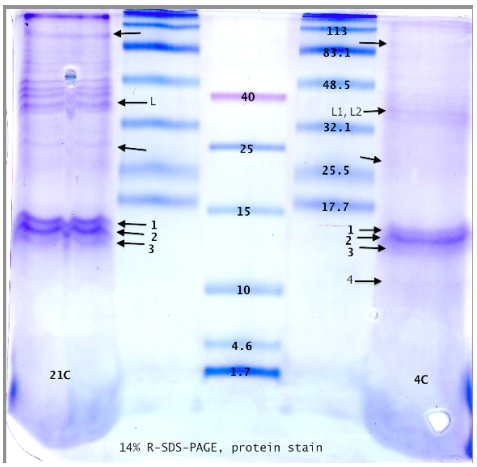


(c) Protein Stain

Figure 6.9: *C. teleta* 4^{o}C samples were prepared as described previously and loaded with N-SLB and run on a 6% polyacrylamide/ 0.5% agarose, Tris-Borate native gel on ice. Each well contains 300μg total protein. (a) Photograph taken during electrophoresis showing three distict bands and a loss of color. (b) Gel after electrophoresis showing the 1/12th submultiple that is visible and was excised for R-SDS-PAGE analysis. (c) Gel slice from (b) stained with Imperial Blue protein stain.



(a) 60 minute incubation



(b) Overnight Incubation

Figure 6.10: (a) *C. teleta* 4^{o}C "top" and "bottom" hemoglobin bands purified by Tris-Borate Native PAGE were excised and run on a 14% R-Tricine-SDS polyacrylamide gel following a 60 minute incubation period. (b) "bottom" hemoglobin bands purified by Tris-Borate Native PAGE from both cultures were run on a 14% R-Tricine-SDS polyacrylamide gel following an overnight incubation period.

arrows), and two disulfide linked dimers (Figure 6.11 red arrows). The gel shown in Figure 6.10b was the only one in which the same quantity of starting protein was used between the two cultures, and will therefore be used to compare subunits. There are three primary bands for both cultures, but a fourth, less abundant band can be seen in the 4^{o}C lane. The presence of at least two high molecular weight linkers between 32.1 and 48.5kDa was also observed. Multiple isomeric forms of the hemoglobin monomers have been observed in every other annelid erythrocruorin that has been studied to date. These minor subunits usually make up a small percentage of the total, and are not used to describe the subunit stoichiometry of the proteins. Upon digitization of the image, this fourth band became even more difficult to see, so the composition of the three primary bands was compared using ImageJ (Figure 6.12). The expression level of each band, measured as integrated density per unit pixel area, was normalized to the band of lowest expression, which happened to be the 21^{o}C band 3. Though statistics could not be performed here, it does look like there is a difference in the subunit composition

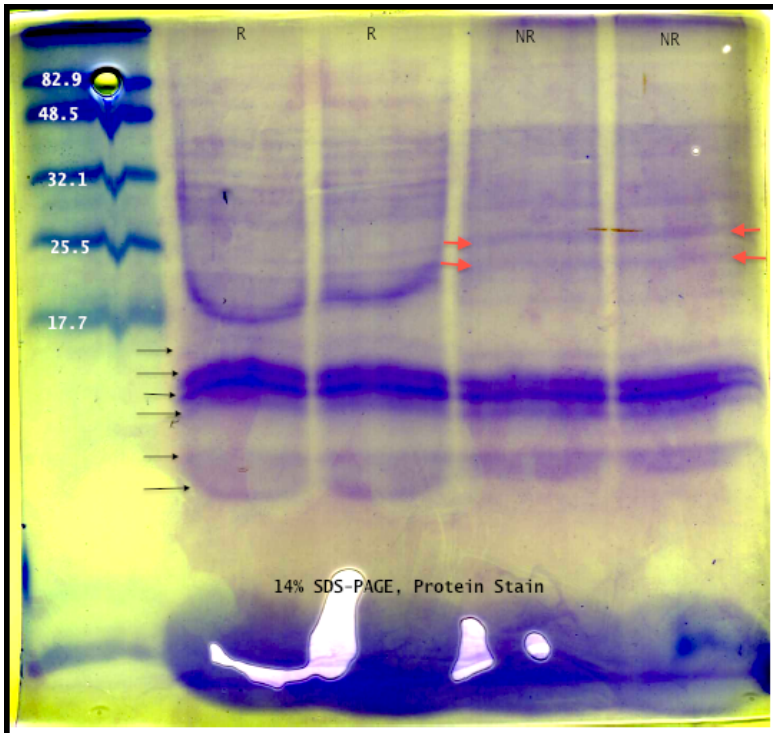
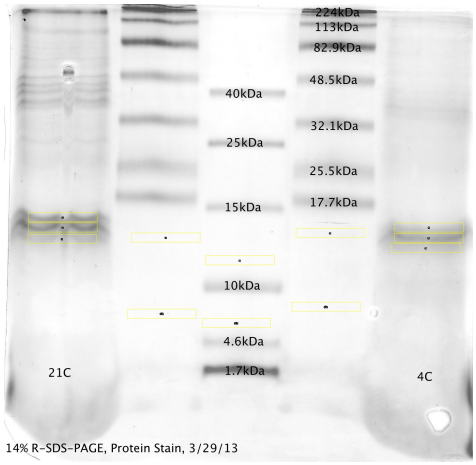


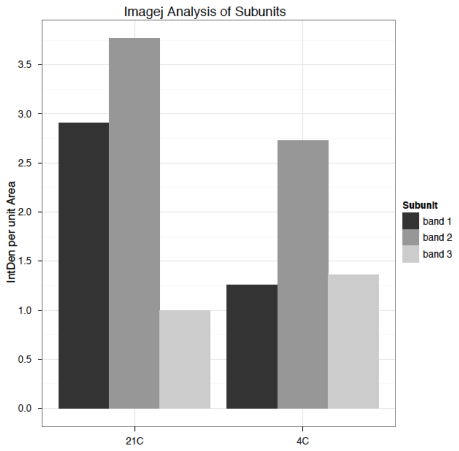
Figure 6.11: *C. teleta* 4^{o}C erythrocruorin purified by Tris-Borate Native PAGE were excised and run on a 14% Tricine-SDS polyacrylamide gel under reducing (R) or non-reducing (NR) cconditions. The six visibile subunits are labeled with black arrows.

between the two temperature treatments.

Using the models for annelid erythrocruorin in Chapter 1, the monomer subunits present should non-covalently interact to form tetrameric subunits, three of which interact together with three linker chains to form a one-twelfth submultiple. From the data available here, *C. teleta* 21^{o}C has a possible tetrameric subunit composed of a homodimer of heterodimers, most of which are peptide bands 1 and 2, while a smaller proportion are peptides 2 and 3 (Figure 6.12). *C. teleta* 4^{o}C also makes primary use of peptide band 2, but has an even distribution of heterodimers composed of peptide bands 1 and 2 and peptide bands 2 and 3. From other gels (Figure 6.11), it is evident that other subunit combinations likely occur, but it is also evident that three subunits are involved in the majority of tetramers. Three other areas are also marked with black arrows on the reducing-denaturing polyacrylamide gel (Figure 6.10b). While some of these are probably non-globin linker chains (L, L1, and L2), there is not enough data



(a) Digitized Gel Image



(b) Subunit Composition

Figure 6.12: The gel from Figure 6.10b was digitized and used for ImageJ analysis of subunit composition. The yellow boxes are drawn to show the areas used by the program to determine pixel density for subunits from each temperare treatment and for obtaining baseline pixel density. The results are plotted to show relative subunit expression or each culture normalized to the raw pixel density of 21^{o}C band 3.

to make proper assignments. Logically, the stoichiometry models presented in Chapter 1 have been used here at the subunit level, but the possibility that *C. teleta* has a giant extracellular hemoglobin with a novel, alternative stoichiometrty, never seen before, is always a possibility that may be revealed with future research.

6.4 Conclusion

The results presented in this chapter exhibit an alternative method for the purification and subunit characterization of *C. teleta* hemoglobin. Provided the challenges of working with these small worms, the ability to use color to physically see the protein of interest, even if it is relatively low abundance, is a great advantage. The horizontal agarose gels were easy to prepare and were ideal for protein recovery since the agarose could simply be melted away. While this may be useful for a final purification step to be used after the steps outlined in Chapter 5, it did not provide the desired level of resolution. The native gradient polyacrylamide gels provided greater resolution of

the hemoglobin protein and were used to analyze the molecular weight of the one-twelfth submultiple, a measurement that cannot be made by traditional native PAGE. The molecular mass of 333kDa is in very good agreement with the mass of the native protein, estimated to be about 4000kDa by size exclusion chromatography. This conversion was made under the assumption that the "top" band seen in all of the native gels was an erythrocruorin similar to those described in Chapter 1. The disconnect arises when trying to fit the 333kDa mass with the subunit stoichiometries associated with the known erythrocruorins, of which many models exist (See Table 1.3). The data presented here is not sufficient to make any conclusions on this matter.

The analysis of subunits did reveal that the distribution shifts from a three-to-one relationship between bands 1 and 3 for the 21^{o}C worms, to a one-to-one relationship between bands 1 and 3 for the 4^{o}C worm. This result suggests that hemoglobin band 3 provides some functional advantage for cold-acclimated *C. teleta*, and may also be involved in the differences in structural stability observed by electrophoresis throughout this study. This difference in stability may also be attributed to the presence of several other minor peptide bands (Figure 6.11), but the resolution on this gel was not sufficient to make accurate determinations. The presence of at least three major bands below the 17.7 kDa marker does support the hypothesis that a larger hemoglobin structure was observed. Whether this structure follows the stoichiometry of known erythrocruorins, HBL-Hbs, or whether it is a novel structure, is left as a topic for future work.

Chapter 7

DISCUSSION

The objectives of this study were to purify hemoglobin from the opportunistic benthic polychaete, *Capitella teleta*, to characterize the native protein based on mass and color, and to determined if the increased body size and change in pigmentation observed in cold-acclimated laboratory cultures was accompanied by a measurable change in hemoglobin concentration and or an adjustment in hemoglobin subunit composition. A principal challenge of working with this polychaete was its relatively small size compared to other annelids that have been studied for the same purpose. The need to pool many worms and homogenize whole-individuals resulted in complex protein extracts with high protease activity. Treating the worms with a protease inhibitor dissolved in filtered seawater prior to sample collection and processing neutralized gut proteases and successfully mitigated this problem (Figure 2.4). It was also determined that the harsh detergents and denaturants, like CHAPS, urea, and thiourea, typically used in cell lysis and protein extraction buffers were not necessary to obtain a stable solution containing the hemoglobin of interest. If the hemoglobin is extracellularly located in vascular or coelomic fluid, then it must be soluble in water and only needed certain cations to remain stable (Royer et al. 2000), which is why $MgCl_2$ was added to the sample buffer.

This research was based on the observation that cold-acclimated individuals looked morphologically different from individuals reared at 21^{o}C, in both size and pigmentation. Size was quantified by measuring the volume of the cylinder formed by the third, fourth, and fifth setiger for individuals from randomly selected sample populations for each culture. It was determined that the 4^{o}C acclimated worms were on average 6.2 times larger than the 21^{o}C acclimated worms (Figure 3.4). Color was more

difficult to qualify and quantify, but it was hypothesized that the red pigmentation was a result of hemoglobin in the body fluids of the worms. This observation was used to drive the secondary hypothesis that the difference in color was due to a difference in hemoglobin concentration and or composition between the worm cultures. Using the pyridine hemochrome assay, it was determined that *C. teleta* 4^{o}C had a significantly greater quantity of heme b in the >100kDa protein size fraction than the 21^{o}C worms (Figure 3.7). This difference in heme pigmentation did explain the observed difference in color between the two cultures, but the results of the pyridine hemochrome assay do not speak to the functional significance of these differences.

These findings were further elucidated by the in-gel heme-peroxidase assay, in which the oxidation of the substrate TMPD by heme in the presence of hydrogen peroxide produced a soluble purple colored product. The results of the in-gel heme assay showed that *C. teleta* 21^{o}C had a greater level of heme b associated peroxidase activity in the large protein fraction. It was also determined that the 21^{o}C worms exhibited 33.7% greater peroxidase activity per unit hemoglobin protein than the 4^{o}C worms (Figure 4.3). However, it was shown that when activity is normalized to heme b content, the difference between the two cultures increased from 33.7% to 73.3%. If the only difference between the hemoglobin from the two cultures was concentration, one would expect the difference in activity per unit hemoglobin and activity per unit heme to be about the same between them. The observed variation points towards two possible explanations. The first explanation has to do with the elevated oxygen affinity at 4^{o}C, and the second has to do with temperature induced structural differences between the proteins from the two cultures that cause heme dissociation from the 4^{o}C worms to a greater extent than from the 21^{o}C worms.

As mentioned in Chapter 2, a direct effect of temperature on a marine organisms habitat is that oxygen solubility increases as temperature decreases. When the temperature of 35ppt aerated seawater is reduced from 21^{o}C to 4^{o}C, the concentration of oxygen increases nearly one and a half times, from 250μM to 350μM, or about 40%.

Biologically, this effect cannot be ignored when analyzing the consequences of temperature reduction on hemoglobin since its primary function *is* oxygen transport. Although the experimental design did not allow the effects of temperature and oxygen availability on hemoglobin concentration or structure to be determined separately, it can be assumed that both factors played a critical role in the observed differences between *C. teleta* hemoglobin from the two experimental temperatures. Functionally, the effect of oxygen availability can be inferred from a generic oxygen-binding curve. An increase in oxygen saturation would favor the bound, high-affinity state of hemoglobin, causing a shift in the total equilibrium of the hemoglobin pool so that a greater fraction of hemoglobins are saturated with oxygen. This greater saturation may contribute to the ability of *C. teleta* to support a larger body size by forcing oxygen-unloading to deeper tissues that are further removed from the higher O_2 external environment and surface tissues. This interpretation may also be inferred by the greater quantity of oxidized, potentially non-functional, heme in the 4^oC worms compared to the 21^oC worms.

To begin to understand the structural differences, the in-gel heme assay was used on dissociating polyacrylamide gels to compare the distribution and staining intensity of subunits between the two cultures. The first feature shared by all of these gels was that samples loaded in the presence of SDS did not stain as well as samples exposed to SDS only after electrophoresis began (Figure 4.6), proving that heme b is the primary heme species in *C. teleta* from both experimental temperatures (Goodhew et al. 1986). Additionally, a high molecular weight protein exhibiting heme-peroxidase activity was seen in all samples, regardless of the sample preparation conditions. It was hypothesized that these large proteins are one-twelfth submultiples of a native erythrocruorin, but later findings suggested the added possibility that these bands were native HBL-Hbs or dodecameric submultiples (Figure 6.2 and 6.9a). Though this cannot be known for sure, the evidence presented in the different gels is very compelling. Four other protein size ranges stained on all of the gels, and were hypothesized to be further dissociation products of the giant hemoglobin. Based on the molecular weights, they are thought to be trimers or tetramers, dimers, monomers, and free heme, although

other minor, less stabile substructures likely exist.

From the heme staining, it was determined that *C. teleta* has a high molecular weight hemoglobin, and that the mass of the native protein does not vary between temperature treatments (Figure 4.1). It was expected that if differences do occur, they would be seen at the subunit level, in which amino acid differences or post-translational modifications imparting alterations to secondary structures would be seen as peptides with slightly different molecular weights on a denaturing polyacrylamide gel. Since the heme b fully dissociates from proteins under the reducing-denaturing conditions necessary to look at the distribution of globin monomers in detail, it became evident that hemoglobin would need to be purified in its native state.

Although the three protein fractionation techniques tried in this study did not lead to the complete purification of hemoglobin from *C. teleta*, some valuable observations were made. Hemoglobin was detected in gels following a 50-75% AS precipitation, but there seemed to be a low level of actual fractionation. This can be seen most clearly in Figure 5.4, in which the precipitated fractions do not look much different than the raw homogenates. One possibility is that the homogenates were too concentrated prior to adding any ammonium sulfate. This would cause nearly all proteins to co-precipitate upon addition of the salt, even if they have different solubilities. Diluting the samples back to their original volume in buffer after passing through a 100kDa Amicon Ultra filter would allow the filtration step to act as a crude fractionation prior to AS precipitation, rather than precipitating nearly all proteins at once.

Information can even be taken from the failed cation exchange step. It was determined that the pI of *C. teleta* hemoglobin is below 5.8. The isoelectric point for *A. marina* erythrocruorin is 4.7. It was suggested that this high negative charge density is why annelid hemoglobins require divalent cations for support and function (Pionetti & Pouyet 1980). The *C. teleta* hemoglobin was successfully retained on an anion exchange resin (Figure 5.5 and 5.6), but the protein was not purified. Although the high pH of the elution buffer promoted a greater protein yield, annelid extracellular hemoglobins tend to dissociate at alkaline pH, and the dissociation products do not

necessarily have the same pI as the native protein (Rousselot et al. 2006). This may explain why the hemoglobin always eluted with the first salt step. The fact that the hemoglobin did not bind to the cation exchange column at pH 5.8 meant that at that same pH, it should have bound to the anion exchange column, while most other proteins would not. Dropping the 100% concentration of sodium chloride from 1.0M to 0.4M would have allowed a greater level of resolution once the salt gradient began.

From the size exclusion chromatography, the molecular weight of the native hemoglobin was estimated to be about 4000 kilodaltons (Figure 5.8 and 5.9). This suggested that the large *C. teleta* hemoglobin was an erythrocruorin. It was evident that size exclusion chromatography could have been used as a fractionation step, but the length of the column was a major limitation. This is apparent by the steep slope of the calibration curve. The column eluted proteins spanning three orders of magnitude over a relatively short interval of about fifteen milliliters. For the purpose of this study, the molecular weight estimation of 4000 kilodaltons will be accepted, but using a 600-900mm column of the same type would have increased the bed volume from 28 milliliters to 50-75 milliliters. The addition of 10mM sodium chloride and 10mM calcium chloride, as well as the magnesium chloride, may also have helped by stabilizing the protein in its native state and increasing yield.

It became obvious that a few changes in the three fractionation steps tried in this study could potentially increase the purity, yield, and stability of *C. teleta* hemoglobin, leading the way to studies on the oxygen binding properties of the pigment, but success was found in an alternative purification method that proved to be easy to manipulate and faster to perform than the fractionation techniques that were tried. The purification by electrophoresis led to findings on the subunit composition, as well as the dissociation mechanics, of *C. teleta* hemoglobin. The ability to see the protein as it migrates through the gel was advantageous over other techniques because no assay was required to determine where the protein was located. Even at pH 7.5, with the native gradient gels, dissociation of native hemoglobin into submultiples was observed (Figure 6.4). Rather than try to stabilize the native protein, the objective was changed

so that the target became the one-twelfth submultiple, which was estimated to have a mass of 333 kilodaltons (Figure 6.8), or one-twelfth the mass of the 4000 kilodalton erythrocruorin. A comparison between the mass and possible stoichiometry of *C. teleta* hemoglobin, *A. marina* erythrocruorin, and *O. machikoi* HBL-Hb is summarized in Table 7.1.

Table 7.1: Table summarizes and compares the experimental and theoretical masses of *C. teleta* extracellular hemoglobins to those of two previously studied annelid species. Since the type of hemoglobin is not known for certain, models are proposed for both erythrocruorin and HBL-Hb. Experimental values are bold and theoretical values are not. G: globin, L: linkers, M: monomer, D: dimer, T: trimer, NA: not applicable.

	A. marina	*O. machikoi*	*C. teleta*	
Habitat	sandy intertidal	sulfide-rich	disturbed sediment	
Native Hb	Type II Ec	HBL-Hb	Ec	HBL-Hb
Mass (kDa)	3648±24	440	**4000**	**333**
Stoichiometry	$G_{144}L_{36}$	G_{24}	$G_{144}L_{36}$	G_{24}
Submultiple	1/12	1/2	1/12	1/2
Protomer	$(M_9T)L_{3.5}$	NA	$(M_4D_4)L_3$	NA
Mass (kDa)		NA	**333**	NA
Dodecamer	M_9T		M_4D_4	M_6T_2
Mass (kDa)	204		228	167
Reference	Zal et al. 1997	Numoto et al. 2005	This Study	This Study

From the start of this project, the search was for a giant extracellular hemoglobin, with a mass close to that of known annelid erythrocruorin (≈3600 kDa), or HBL-Hb (≈400kDa). The fact that the one-twelfth submultiple had a mass, as measured by BN-PAGE, almost exactly one-twelfth that of the native protein, as measured by size exclusion chromatography, was persuasive, but not conclusive. When the "top" band on the native gels was excised and run on a denaturing gel, there was little evidence that the protein had been purified (Figure 6.9 (a)), but the "bottom" band looked pure (Figure 6.9 (b)), especially for the 4^{o}C samples. Using the model for *A. marina* erythrocruorin, a trimer of tetrameric subunits is held together by a trimer of linker chains. In total, 144 globin chains are held together in a hexagonal bilayer by 36 non-gobin linker subunits (Zal et al. 1997). Here, two linker chains were estimated to have a mass of about 35kDa (Figure 6.10b), and the monomers were estimated to have an

average mass of about 13.5 kDa (Figure 6.10b). Using this stoichiometry, *C. teleta* erythrocruorin should have a one-twelfth protomer mass of about $(3 \times (13.5 \times 4)) + (35 \times 3)$, which equals 267 kilodaltons, a one-twelfth dodecamer mass of $(3 \times (13.5 \times 4))$, equal to 162 kilodaltons, and a native mass of $(144 \times 13.5) + (36 \times 35)$, equal to 3204 kilodaltons. However, the "top" band always remained in or barely entered the stacking gel, leading to the possible conclusion that it could actually be a massive aggregate of hemoglobins and other proteins. In this case, the model of *O. machikoi* HBL-Hb makes more sense.

The first piece of evidence that the type of hemoglobin present in *C. teleta* is a HBL-Hb, and not an erythrocruorin, is that 333 divided into twenty-four equal parts gives a subunit mass of 13.88 kilodaltons, in great agreement with the subunit mass measured for the "bottom" bands for both cultures (Figure 6.9 (b)). The model for *O. machikoi* HBL-Hb states that two dodecamers of globin subunits combine to form a native hemoglobin composed of twenty-four globins without any linker chains (Numoto et al. 2005). Applying this model to *C. teleta* results in a theoretical native mass of (24×13.5), equal to 324 kilodaltons, and a one-half dodecamer mass of (12×13.5), equal to 162 kilodaltons. This theoretical mass for the native protein is very close to the mass of 333 kilodaltons measured by BN-PAGE. Nakagawa et al. (2005) only observed native HBL-Hb, and not dodecamers, when determining the mass of *O. machikoi* hemoglobin by SEC, which supports the fact that 162 kilodalton bands were not observed on the gels for *C. teleta*, with the exception of the mildly denaturing gel in Figure 6.2a. A comparison between the masses and possible subunits of *C. teleta* hemoglobin, *A. marina* erythrocruorin, and *O. machikoi* HBL-Hb are summarized in Table 7.2.

The final analysis done in this study was to determine if subunit composition is different between the two *C. teleta* cultures (Figure 6.11). While the presence of multiple low-abundance subunits has been observed in *L. terrestris* (Maier et al. 1997), the relative expression of these subunits in response to temperature or any other property has not been studied. It is, however, reasonable to hypothesize that the presence

Table 7.2: Table summarizes and compares the theoretical subunit stoichiometries for *C. teleta* extracellular hemoglobins to those of two previously studied annelid species. Bold values are from Mangum et al. (1992). +: intratetramer disulfide bond; ⊕: intertetramer disulfide bond; b: band from Figures 6.10 and 6.11; M: monomer, mass from Figure 6.10b; D: dimer, mass from Figures 4.6a and 6.11; T: trimer, mass from Figures 4.6a and 6.2a; L: linker, mass from Figure 6.10b

	A. marina	*O. machikoi*	*C. teleta*	
Submultiple	$(M_9T)L_{3.5}$	M_3T_3	$(M_4D_4)L_3$	M_6T_2
Globins	a,b,c,d; T=b+c+d; M=a	A1,A2,B1,B2; T=A1+B2⊕ B1; M=A2	b1,b2,b3, b4,b5,b6; D=2b	b1,b2,b3, b4,b5,b6; T=3b
Linkers	L1,L2; D1=L1+L1; D2=L1+L2	NA	L1,L2	NA
Mass (kDa)	M=15.9-17.6; T≈49.6; L1≈25.2; L2≈26.8		M≈13.5; D≈25; L≈35	T≈40
P_{50} (Torr)	1.0-5.7	0.82	**6.9**	
n	2.5-4.0	1.1-2.5	**7**	
Maximum O_2 binding sites	144	24	144	24

of multiple hemoglobin monomer isoforms are due to environmental selection. It was found that while up to six subunits are expressed in the 4^oC worms (Figure 6.10), both cultures show high expression levels for only three primary subunits. It has been hypothesized that the 4^oC worms show equal expression of dimers of bands 1 and 2 and bands 2 and 3, where as the 21^oC worms show preference for the band 1 and 2 dimers. It is not known here if this is related to a functional advantage, however, since band 3 is smaller than the other two, it may be metabolically less expensive to produce.

Twenty-one years ago, Mangum et al. (1992) proposed that hemoglobin be studied in *C. teleta* in more detail. Though long overdue, this study provides the first insight into understanding the type of hemoglobin present and how it is affected by warm and cold temperature acclimation. As a model organism, it is important to understand the biochemical and physiological processes that allow *C. teleta* to inhabit such a diverse array of environments. Given the high level of cooperativity observed by

Mangum et al. (1992), it comes as no surprise that *C. teleta* has a giant extracellular hemoglobin. The results of this study more strongly support the possibility that this hemoglobin is a 333kDa HBL-Hb than a 4000kDa erythrocruorin, but the evidence is not conclusive. It was also determined that the functional status of this protein may be compromised upon cold-acclimation, and that if structural changes do occur at the monomer level, they are minor and would require higher-resolution techniques to detect. Future studies are necessary to fully characterize this hemoglobin, but *C. teleta* can now be added to the growing list of annelid species that exhibit this remarkable and universally essential molecule.

REFERENCES

Achilonu, Ikechukwu and J. P. Dean Goldring. 2010. "Direct Red 81 and Amido Black Stain Proteins in Polyacrylamide Electrophoresis Gels within 10 Min." Analytical Biochemistry 400 (1): 139-141.

Aki, Yayoi, Taro Nakagawa, Masako Nagai, Yuichi Sasayama, Yoshihiro Fukumori, and Kiyohiro Imai. 2007. "Oxygenation Properties of Extracellular Giant Hemoglobin from Oligobrachia Mashikoi." Biochemical and Biophysical Research Communications 360 (3): 673-678.

Ariaeenejad, Shohreh, Mehran Habibi-Rezaei, Shahla Jamili, Mohammad Reza Fatemi, Najmeh Poursasan, Faizan Ahmad, Nader Sheibani, Kaveh Kavousi, and Ali A. Moosavi-Movahedi. 2012. "Biochemical Characterization of Hemoglobins from Caspian Sea Sturgeons (Acipenser Persicus and Acipenser Stellatus)." Cell Biochemistry and Biophysics 62 (1): 73-81.

Barcroft, J. and H. Barcroft. 1924. "The Blood Pigment of Arenicola." Proceedings of the Royal Society of London.Series B, Containing Papers of a Biological Character 96 (672): 28-42.

Bellelli, Andrea and Maurizio Brunori. 2011. "Hemoglobin Allostery: Variations on the Theme." Biochimica Et Biophysica Acta-Bioenergetics 1807 (10): 1262-1272.

Berry, E. A. and B. L. Trumpower. 1987. "Simultaneous Determination of Hemes-A, Hemes-B, and Hemes-C from Pyridine Hemochrome Spectra." Analytical Biochemistry 161 (1): 1-15.

Blake, J. A., J. P. Grassle, and K. J. Eckelbarger. 2009. "Capitella Teleta, a New Species Designation for the Opportunistic and Experimental Capitella Sp. I, with a Review of the Literature for Confirmed Records." Zoosymposia 2: 25-53.

Broyles, R. H., B. M. Pack, S. Berger, and A. R. Dorn. 1979. "Quantification of Small Amounts of Hemoglobin in Polyacrylamide Gels with Benzidine." Analytical Biochemistry 94 (1): 211-219.

Bruneaux, Matthieu, Morgane Rousselot, Emmanuelle Leize, Francois H. Lallier, and Franck Zal. 2008. "The Structural Analysis of Large Noncovalent Oxygen Binding Proteins by MALLS and ESI-MS: A Review on Annelid Hexagonal Bilayer Hemoglobin and Crustacean Hemocyanin." Current Protein Peptide Science 9 (2): 150-180.

Butler, M. J. and M. A. Lachance. 1987. "The use of N,n,n',n'-Tetramethylphenylenediamine to Detect Peroxidase-Activity on Polyacrylamide Electrophoresis Gels." Analytical Biochemistry 162 (2): 443-445.

Chabasse, Christine, Xavier Bailly, Sophie Sanchez, Morgane Rousselot, and Franck Zal. 2006. "Gene Structure and Molecular Phylogeny of the Linker Chains from the Giant Annelid Hexagonal Bilayer Hemoglobins." Journal of Molecular Evolution 63 (3): 365-374.

Chandramouli, Kondethimmanahalli H., Lisa Soo, and Pei-Yuan Qian. 2011. "Differential Expression of Proteins and Phosphoproteins during Larval Metamorphosis of the Polychaete Capitella Sp I." Proteome Science 9: 51.

Chandramouli, Kondethimmanahalli H., Yu Zhang, Yue Him Wong, and Pei-Yuan Qian. 2012. "Comparative Glycoproteome Analysis: Dynamics of Protein Glycosylation during Metamorphic Transition from Pelagic to Benthic Life Stages in Three Invertebrates." Journal of Proteome Research 11 (2): 1330-1340.

Cucchi, C. and F. Basaglia. 1998. "Tetramethylbenzidine Staining Procedure After Starch Gel Electrophoresis of Human Haemoglobin." Comparative Haematology International 8 (3): 178-181.

Cui, Qiang and Martin Karplus. 2008. "Allostery and Cooperativity Revisited." Protein Science 17 (8): 1295-1307.

Defur, P. L., C. P. Mangum, and J. E. Reese. 1990. "Respiratory Responses of the Blue-Crab Callinectes-Sapidus to Long-Term Hypoxia." Biological Bulletin 178 (1): 46-54.

Ebina, S., K. Matsubara, K. Nagayama, M. Yamaki, and T. Gotoh. 1995. "Carbohydrate Gluing, an Architectural Mechanism in the Supramolecular Structure of an Annelid Giant Hemoglobin." Proceedings of the National Academy of Sciences of the United States of America 92 (16): 7367-7371.

Edelstein, S. J. 1971. "Extensions of the Allosteric Model for Haemoglobin." Nature 230: 224-227.

Fushitani, K., K. Higashiyama, M. Asao, and K. Hosokawa. 1996. "Characterization of the Constituent Polypeptides of the Extracellular Hemoglobin from Lumbricus Terrestris: Heterogeneity and Discovery of a New Linker Chain L4." Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1292 (2): 273-280.

Gabriel, O. and D. M. Gersten. 1992. "Staining for Enzymatic-Activity After Gel-Electrophoresis .1." Analytical Biochemistry 203 (1): 1-21.

Garlick, R. L. 1980. "Structure of Annelid High Molecular-Weight Hemoglobins (Erythrocruorins)." American Zoologist 20 (1): 69-77.

Garlick, R. L. and A. Riggs. 1981. "Purification and Structure of the Polypeptide-Chains of Earthworm Hemoglobin." Archives of Biochemistry and Biophysics 208 (2): 563-575.

Garlick, R. L. and R. C. Terwilliger. 1977. "Structure and Oxygen Equilibrium of Hemoglobin and Myoglobin from Pacific Lugworm Abarenicola-Pacifica." Comparative Biochemistry and Physiology B-Biochemistry Molecular Biology 57 (3): 177-184.

Goodhew, C. F., K. R. Brown, and G. W. Pettigrew. 1986. "Heme Staining in Gels, a Useful Tool in the Study of Bacterial C-Type Cytochromes." Biochimica Et Biophysica Acta 852 (2-3): 288-294. Grassle, J. F. and J. P. Grassle. 1974. "Sibling Species in the Marine Pollution Indicator Capitella (Polychaeta)." Science 192: 567-569.

Guadagnoli, JA, AA Braun, SP Roberts, and CL Reiber. 2005. "Environmental Hypoxia Influences Hemoglobin Subunit Composition in the Branchiopod Crustacean Triops Longicaudatus." Journal of Experimental Biology 208 (18): 3543-3551.

Hanin, LG and SN Vinogradov. 2000. "Combinatorics of Giant Hexagonal Bilayer Hemoglobins." Mathematical Biosciences 163 (1): 59-73.

Ito, Katsutoshi, Mana Nozaki, Takashi Ohta, Chiemi Miura, Yuzuru Tozawa, and Takeshi Miura. 2011. "Differences of Two Polychaete Species Reflected in Enzyme Activities." Marine Biology 158 (6): 1211-1221.

Jouan, L., JC Taveau, S. Marco, FH Lallier, and JN Lamy. 2001. "Occurrence of Two Architectural Types of Hexagonal Bilayer Hemoglobin in Annelids: Comparison of 3D Reconstruction Volumes of Arenicola Marina and Lumbricus Terrestris Hemoglobins." Journal of Molecular Biology 305 (4): 757-771.

Kern, D. and ERP Zuiderweg. 2003. "The Role of Dynamics in Allosteric Regulation." Current Opinion in Structural Biology 13 (6): 748-757.

Kleinschmidt, T. and RE Weber. 1998. "Primary Structures of Arenicola Marina Isomyoglobins: Molecular Basis for Functional Heterogeneity." Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1383 (1): 55-62.

Kruski, A. W. and K. A. Narayan. 1974. "Some Quantitative Aspects of the Disc Electrophoresis of Ovalbumin using Amido Black 10B Stain." Analytical Biochemistry 60: 431-440.

Laberke, P. J., S. Ilg, H-P Bieri, R. Hausmann, and B. Balitzki. 2011. "Amido Black 10B in Forensic Stain Analysis. Comparative Investigations on Forensic Stain Material." Rechtsmedizin 21 (5): 452-456.

Laemmli, U. K. 1970. "Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4." Nature 227 (5259): 680-.

Lamy, JN, BN Green, A. Toulmond, JS Wall, RE Weber, and SN Vinogradov. 1996. "Giant Hexagonal Bilayer Hemoglobins." Chemical Reviews 96 (8): 3113-3124.

Lankester, E. Ray. 1868. "Preliminary Notice of some Observations with the Spectroscope on Animal Substances." Journal of Anatomy and Physiology 2 (1): 114-116.

Lebowitz, J., MS Lewis, and P. Schuck. 2002. "Modern Analytical Ultracentrifugation in Protein Science: A Tutorial Review." Protein Science 11 (9): 2067-2079.

Li, B., HC Bisgaard, and VE Forbes. 2004. "Identification and Expression of Two Novel Cytochrome P450 Genes, Belonging to CYP4 and a New CYP331 Family, in the Polychaete Capitella Capitata Sp.I." Biochemical and Biophysical Research Communications 325 (2): 510-517.

Loehr, J. S., P. J. Lammers, B. Brimhall, and M. A. Hermodson. 1978. "Amino-Acid Sequence of Hemerythrin from Themiste-Dyscritum." Journal of Biological Chemistry 253 (16): 5726-5731.

Maier, CS, B. Arbogast, U. Hahn, ML Deinzer, AR Kuchumov, SN Vinogradov, and DA Walz. 1997. "A Mass Spectrometric Study of the Heterogeneity of the Monomer Subunit of Lumbricus Terrestris Hemoglobin." Journal of the American Society for Mass Spectrometry 8 (4): 352-364.

Mangum, C. P., J. M. Colacino, and J. P. Grassle. 1992. "Red-Blood-Cell Oxygen Binding in Capitellid Polychaetes." Biological Bulletin 182 (1): 129-134.

Marsh, A. G., A. Gremare, and K. R. Tenore. 1989. "Effect of Food Type and Ration on Growth of Juvenile Capitella Sp-i (Annelida, Polychaeta) - Macronutrients and Micronutrients." Marine Biology 102 (4): 519-527.

Medeiros, R., L. Serpa, C. Brito, H. De Wolf, K. Jordaens, B. Winnepenninckx, and T. Backeljau. 1998. "Radular Myoglobin and Protein Variation within and among some Littorinid Species (Mollusca : Gastropoda)." Hydrobiologia 378: 43-51.

Methling, Caroline, Neelakanteswar Aluru, Mathilakath M. Vijayan, and John F. Steffensen. 2010. "Effect of Moderate Hypoxia at Three Acclimation Temperatures on Stress Responses in Atlantic Cod with Different Haemoglobin Types." Comparative Biochemistry and Physiology A-Molecular Integrative Physiology 156 (4): 485-490.

Meunier, Cedric, Ann C. Andersen, Matthieu Bruneaux, Dominique Le Guen, Peran Terrier, Emmanuelle Leize-Wagner, and Franck Zal. 2010. "Structural Characterization of Hemoglobins from Monilifera and Frenulata Tubeworms (Siboglinids): First Discovery of Giant Hexagonal-Bilayer Hemoglobin in the Former "Pogonophora" Group." Comparative Biochemistry and Physiology A-Molecular Integrative Physiology 155 (1): 41-48.

Mok, Flora S. Y., Vengatesen Thiyagarajan, and Pei-Yuan Qian. 2009. "Proteomic Analysis during Larval Development and Metamorphosis of the Spionid Polychaete Pseudopolydora Vexillosa." Proteome Science 7: 44.

Nakagawa, T., S. Onoda, M. Kanemori, Y. Sasayama, and Y. Fukumori. 2005. "Purification, Characterization and Sequence Analyses of the Extracellular Giant Hemoglobin from Oligobrachia Mashikoi." Zoological Science 22 (3): 283-291.

Numoto, N., T. Nakagawa, A. Kita, Y. Sasayama, Y. Fukumori, and K. Miki. 2005. "Structure of an Extracellular Giant Hemoglobin of the Gutless Beard Worm Oligobrachia Mashikoi." Proceedings of the National Academy of Sciences of the United States of America 102 (41): 14521-14526.

Numoto, Nobutaka, Taro Nakagawa, Akiko Kita, Yuichi Sasayama, Yoshihiro Fukumori, and Kunio Miki. 2008. "Structural Basis for the Heterotropic and Homotropic Interactions of Invertebrate Giant Hemoglobin." Biochemistry 47 (43): 11231-11238.

Numoto, Nobutaka, Taro Nakagawa, Akiko Kita, Yuichi Sasayama, Yoshihiro Fukumori, and Kunio Miki. 2008. "Structure of the Partially Unliganded Met State of 400 kDa Hemoglobin: Insights into Ligand-Induced Structural Changes of Giant Hemoglobins." Proteins-Structure Function and Bioinformatics 73 (1): 113-125.

Oh-Ishi, M., M. Satoh, and T. Maeda. 2000. "Preparative Two-Dimensional Gel Electrophoresis with Agarose Gels in the First Dimension for High Molecular Mass Proteins." Electrophoresis 21 (9): 1653-1669.

Pallavicini, A., E. Negrisolo, R. Barbato, S. Dewilde, A. Ghiretti-Magaldi, L. Moens, and G. Lanfranchi. 2001. "The Primary Structure of Globin and Linker Chains from the Chlorocruorin of the Polychaete Sabella Spallanzanii." Journal of Biological Chemistry 276 (28): 26384-26390.

Pionetti, Jean-Michel and Jean Pouyet. 1980. "Molecular Architecture of Annelid Erythrocruorins." European Journal of Biochemistry 105 (1): 131-138.

Perutz, M. F. 1970. "Stereochemistry of Cooperative Effects in Haemoglobin." Nature 228 (5273): 726-.

Putchler, H. and F. Sweat. 1962. "Amidoblack as a Stain for Hemoglobin." Archives of Pathology 73 (3): 245-.

Rasmussen, Jonas R., Rufus M. G. Wells, Kristen Henty, Timothy D. Clark, and Thomas Brittain. 2009. "Characterization of the Hemoglobins of the Australian Lungfish Neoceratodus Forsteri (Krefft)." Comparative Biochemistry and Physiology A-Molecular Integrative Physiology 152 (2): 162-167.

Riess, JG. 2001. "Oxygen Carriers ("Blood Substitutes") - Raison d'Etre, Chemistry, and some Physiology." Chemical Reviews 101 (9): 2797-2919.

Roche, J. and HM Fox. 1933. "Crystalline Chlorocruorin." Nature 132: 516-516.

Rousselot, M., D. Le Guen, C. Chabasse, and F. Zal. 2006. "Novel Dissociation Mechanism of a Polychaetous Annelid Extracellular Haemoglobin." Febs Journal 273 (7): 1582-1596.

Rousselot, Morgane, Elmar Jaenicke, Tobias Lamkemeyer, J. Robin Harris, and Ralph Pirow. 2006. "Native and Subunit Molecular Mass and Quarternary Structure of the Hemoglobin from the Primitive Branchiopod Crustacean Triops Cancriformis." Febs Journal 273 (17): 4055-4071.

Royer, W. E., H. Zhu, T. A. Gorr, J. F. Flores, and J. E. Knapp. 2005. "Allosteric Hemoglobin Assembly: Diversity and Similarity." Journal of Biological Chemistry 280 (30): 27477-27480.

Royer, WE, JE Knapp, K. Strand, and HA Heaslet. 2001. "Cooperative Hemoglobins: Conserved Fold, Diverse Quaternary Assemblies and Allosteric Mechanisms." Trends in Biochemical Sciences 26 (5): 297-304.

Royer, WE, K. Strand, M. van Heel, and WA Hendrickson. 2000. "Structural Hierarchy in Erythrocruorin, the Giant Respiratory Assemblage of Annelids." Proceedings of the National Academy of Sciences of the United States of America 97 (13): 7107-7111.

Royer, William E., Jr., Michael N. Omartian, and James E. Knapp. 2007. "Low Resolution Crystal Structure of Arenicola Erythrocruorin: Influence of Coiled Coils on the Architecture of a Megadalton Respiratory Protein." Journal of Molecular Biology 365 (1): 226-236.

Royer, William E., Jr., Hitesh Sharma, Kristen Strand, James E. Knapp, and Balaji Bhyravbhatla. 2006. "Lumbricus Erythrocruorin at 3.5 Angstrom Resolution: Architecture of a Megadalton Respiratory Complex." Structure 14 (7): 1167-1177.

Sanders, H. L., J. F. Grassle, G. R. Hampson, L. S. Morse, S. Garnerprice, and C. C. Jones. 1980. "Anatomy of an Oil-Spill - Long-Term Effects from the Grounding of the Barge Florida Off West Falmouth, Massachusetts." Journal of Marine Research 38 (2): 265-380.

Schagger, H., W. A. Cramer, and G. Vonjagow. 1994. "Analysis of Molecular Masses and Oligomeric States of Protein Complexes by Blue Native Electrophoresis and Isolation of Membrane-Protein Complexes by 2-Dimensional Native Electrophoresis." Analytical Biochemistry 217 (2): 220-230.

Schagger, H. and G. Vonjagow. 1987. "Tricine Sodium Dodecyl-Sulfate Polyacrylamide-Gel Electrophoresis for the Separation of Proteins in the Range from 1-Kda to 100-Kda." Analytical Biochemistry 166 (2): 368-379.

Schneider, Caroline A., Wayne S. Rasband, and Kevin W. Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." Nature Methods 9 (7): 671-675.

Svedberg, T. and IB Eriksson. 1933. "The Molecular Weight of Erythrocruorin." Journal of the American Chemical Society 55: 2834-2841.

Svedberg, T. and A. Hedenius. 1933. "Molecular Weights of the Blood Pigments of the Invertebrates." Nature 131: 325-325.

Tatsumi, R. and A. Hattori. 1995. "Detection of Giant Myofibrillar Proteins Connectin and Nebulin by Electrophoresis in 2-Percent Polyacrylamide Slab Gels Strengthened with Agarose." Analytical Biochemistry 224 (1): 28-31.

Terwilliger, NB. 1998. "Functional Adaptations of Oxygen-Transport Proteins." Journal of Experimental Biology 201 (8): 1085-1098.

Terwilliger, N. B., R. C. Terwilliger, and E. Schabtach. 1983. "2 Populations of Hemerythrin-Containing Cells in the Sipunculan Themiste-Dyscritum." American Zoologist 23 (4): 1025-1025.

Terwilliger, R. C. 1980. "Structures of Invertebrate Hemoglobins." American Zoologist 20 (1): 53-67.

Terwilliger, R. C, R. L. Garick, and N. B. Terwilliger. 1980. "Characterization of the Hemoglobins and Myoglobin of Travisia-Foetida." Comparative Biochemistry and Physiology B-Biochemistry Molecular Biology 66 (2): 261-266.

Terwilliger, R. C., N. B. Terwilliger, and E. Schabtach. 1976. "Comparison of Chlorocruorin and Annelid Hemoglobin Quaternary Structures." Comparative Biochemistry and Physiology A-Physiology 55 (1): 51-.

Thomas, P. E., D. Ryan, and W. Levin. 1976. "Improved Staining Procedure for Detection of Peroxidase-Activity of Cytochrome-P-450 on Sodium Dodecyl-Sulfate Polyacrylamide Gels." Analytical Biochemistry 75 (1).

Vinogradov, Serge N. and Luc Moens. 2008. "Diversity of Globin Function: Enzymatic, Transport, Storage, and Sensing." Journal of Biological Chemistry 283 (14): 8773-8777.

Vinogradov, S. N. 1985. "The Structure of Invertebrate Extracellular Hemoglobins (Erythrocruorins and Chlorocruorins)." Comparative Biochemistry and Physiology B-Biochemistry Molecular Biology 82 (1): 1-15.

Vinogradov, S. N, S. D. Lugo, M. G. Mainwaring, O. H. Kapp, and A. V. Crewe. 1986. "Bracelet Protein - a Quaternary Structure Proposed for the Giant Extracellular Hemoglobin of Lumbricus-Terrestris." Proceedings of the National Academy of Sciences of the United States of America 83 (21): 8034-8038.

Vinogradov, S. N., P. K. Sharma, and D. A. Walz. 1991. "Iron and Heme Contents of the Extracellular Hemoglobins and Chlorocruorins of Annelids." Comparative Biochemistry and Physiology B-Biochemistry Molecular Biology 98 (2-3): 187-194.

Waxman, L. 1975. "Structure of Annelid and Mollusk Hemoglobins." Journal of Biological Chemistry 250 (10): 3790-3795.

Weber, RE and SN Vinogradov. 2001. "Nonvertebrate Hemoglobins: Functions and Molecular Adaptations." Physiological Reviews 81 (2): 569-628.

Wells, R. M. G. and L. M. Warren. 1975. "Function of Cellular Hemoglobins in Capitella-Capitata (Fabricius) and Notomastus-Latericeus Sars (Capitellidae-Polychaeta)." Comparative Biochemistry and Physiology A-Physiology 51 (4): 737-740.

Welton, A. F. and S. D. Aust. 1974. "Multiplicity of Cytochrome P450 Hemoproteins in Rat-Liver Microsomes." Biochemical and Biophysical Research Communications 56 (4): 898-906.

Yonan, CR, PT Duong, and FN Chang. 2005. "High-Efficiency Staining of Proteins on Different Blot Membranes." Analytical Biochemistry 338 (1): 159-161.

Zal, F., B. N. Green, P. Martineu, F. H. Lallier, A. Toulmond, S. N. Vinogradov, and J. J. Childress. 2000. "Polypeptide Chain Composition Diversity of Hexagonal-Bilayer Haemoglobins within a Single Family of Annelids, the Alvinellidae." European Journal of Biochemistry 267 (16): 5227-5236.

Zal, F., BN Green, FH Lallier, and A. Toulmond. 1997. "Investigation by Electrospray Ionization Mass Spectrometry of the Extracellular Hemoglobin from the Polychaete Annelid Alvinella Pompejana: An Unusual Hexagonal Bilayer Hemoglobin." Biochemistry 36 (39): 11777-11786.

Zal, F., BN Green, FH Lallier, SN Vinogradov, and A. Toulmond. 1997. "Quaternary Structure of the Extracellular Haemoglobin of the Lugworm Arenicola Marina - A Multi-Angle-Laser-Light-Scattering and Electrospray-Ionisation-Mass-Spectrometry Analysis." European Journal of Biochemistry 243 (1-2): 85-92.

Zal, F., F. H. Lallier, B. N. Green, S. N. Vinogradov, and A. Toulmond. 1996. "The Multi-Hemoglobin System of the Hydrothermal Vent Tube Worm Riftia Pachyptila .2. Complete Polypeptide Chain Composition Investigated by Maximum Entropy Analysis of Mass Spectra." Journal of Biological Chemistry 271 (15): 8875-8881.

Zal, F., F. H. Lallier, J. S. Wall, S. N. Vinogradov, and A. Toulmond. 1996. "The Multi-Hemoglobin System of the Hydrothermal Vent Tube Worm Riftia Pachyptila .1. Reexamination of the Number and Masses of its Constituents." Journal of Biological Chemistry 271 (15): 8869-8874.

Zhu, H., M. Hargrove, Q. Xie, Y. Nozaki, K. Linse, SS Smith, JS Olson, and AF Riggs. 1996. "Stoichiometry of Subunits and Heme Content of Hemoglobin from the Earthworm Lumbricus Terrestris." Journal of Biological Chemistry 271 (47): 29999-30006.

Zhu, H., DW Ownby, CK Riggs, NJ Nolasco, JK Stoops, and AF Riggs. 1996. "Assembly of the Gigantic Hemoglobin of the Earthworm Lumbricus Terrestris - Roles of Subunit Equilibria, Non-Globin Linker Chains, and Valence of the Heme Iron." Journal of Biological Chemistry 271 (47): 30007-30021.