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FUNCTIONAL ADAPTATIONS OF OXYGEN-TRANSPORT PROTEINS

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Summary

Oxygen-transport proteins are multisubunit, circulating molecules that provide an efficient supply of oxygen to metabolically active metazoans. Hemoglobins, hemerythrins and hemocyanins have evolved in both structural and functional diversity and exhibit functional repertoires beyond that of simple, monomeric tissue myoglobins. Their phylogenetic distribution is intriguing, especially with respect to those organisms that express more than one type of oxygen-transport protein. An animal can modify the delivery of oxygen to its tissues by varying the rate of synthesis of these proteins or by selective expression of individual subunits and/or molecules. Changes in levels of allosteric modifiers that affect the protein's oxygenation properties will also modify oxygen delivery; some organisms have more ability than others to control concentrations of modulators. Hemoglobins have assumed functions in addition to oxygen transport, while hemocyanins have diversified through multiple gene duplications and functional specializations. Understanding the mechanisms of regulation of expression, synthesis and modulator levels is a key focus of current investigations.

Key words: hemoglobin, hemerythrin, hemocyanin, hypoxia, oxygen transport.

Introduction

Oxygen-binding molecules are ancient proteins. They probably evolved from enzymes that protected the organism against the toxic oxygen molecule. As single-celled organisms developed the capacity to use oxygen as an electron acceptor, capturing oxygen and transferring it to the respiratory chain became increasingly important. Intracellular protoheme or myoglobin-type proteins, and perhaps copper proteins, that could enhance oxygen diffusion and storage began to appear. When multicellular organisms increased in size and complexity, their surface to volume ratios diminished, and simple diffusion of oxygen across the body wall was inadequate to reach all of the cells. The development of vascular and coelomic circulatory systems that could move oxygen away from the inner body wall enhanced the oxygen diffusion rate, but the low solubility of oxygen in body fluids was still limiting. The evolution of simple oxygen-binding proteins into multisubunit, circulating proteins, in combination with the advent of circulatory systems, made possible the transport of oxygen on a significant scale from the periphery of the organism to metabolizing cells in its interior. This discussion will focus on animal hemoglobins, hemerythrins and hemocyanins that function as oxygen-transport molecules and will refer only briefly to monomeric heme proteins, such as myoglobins and myohemerythrins involved in intracellular oxygen transfer and storage. Of course, effective oxygen transport includes delivery, and thus transport molecules have a transfer function as well. So that evolutionary patterns may become more clear, I will describe the phylogenetic distribution and general properties and will present specific examples of how organisms use these proteins to obtain appropriate amounts of oxygen.

Oxygen-transport proteins

There are two major kinds of oxygen-transport proteins, those with iron as the prosthetic group, which reversibly binds to oxygen, and those with copper. As illustrated in Fig. 1, the circulating iron proteins include (1) cellular hemoglobins or red blood cells, (2) giant extracellular hemoglobins and chlorocruorins, and (3) cellular hemerythrins or pink blood cells. In hemerythrins, oxygen binds to iron that is covalently linked to the protein molecule; in hemoglobins and chlorocruorins, the iron is coordinately bound to a protoporphyrin IX or heme group. The heme group is attached to the protein, a globin. Copper-based, blue oxygen-transport proteins include (1) molluscan hemocyanins and (2) arthropodan hemocyanins. While the two hemocyanin proteins are dissimilar in quaternary structure and sequence, the active sites in both hemocyanins are similar although not identical. The active sites include six highly conserved histidines that bind two copper atoms; both coppers together bind one oxygen molecule, reversibly. Hemerythrins and hemocyanins are

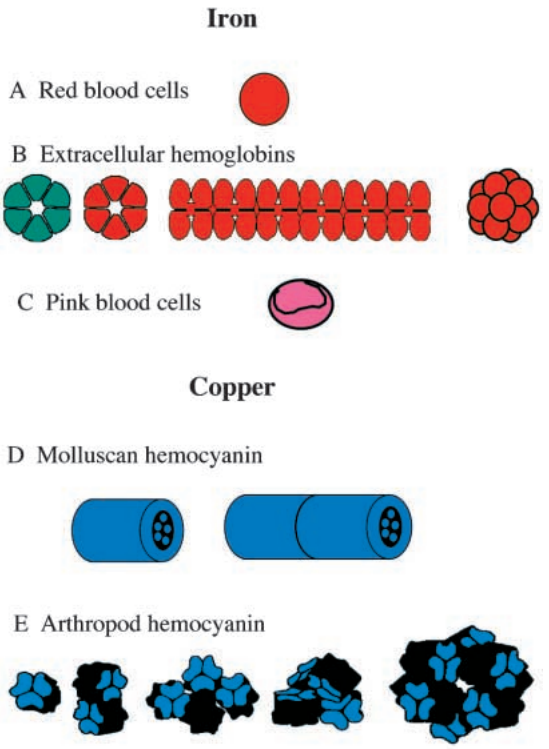

Fig. 1. Oxygen-transport proteins. (A) Cellular hemoglobins or red blood cells; (B) extracellular hemoglobins (annelid chlorocruorin and hemoglobin, mollusc bivalve extracellular hemoglobin, arthropod extracellular hemoglobin); (C) cellular hemerythrins or pink blood cells; (D) molluscan hemocyanin (chiton or cephalopod, gastropod or bivalve); (E) arthropod hemocyanin (one-, two-, six-, four- and eight-hexamers). Models not drawn to scale.

colorless in the deoxy form. Almost all of the iron and copper proteins involved in oxygen transport are multi-subunit proteins. Consequently, they exhibit cooperative oxygen binding and allosteric modulation of oxygen affinity, properties that expand their functional repertoires beyond that of a simple monomeric myoglobin. In the oxygen-transport proteins, then, the evolution of functional diversity parallels that of form. Tracing these patterns requires an overlay of phylogeny with form and function.

Molecular size and structure

Hemoglobins in circulating red blood cells are composed of polypeptide chains or subunits of approximately 17 kDa, each containing a heme group (for a review, see Royer, 1992). Each subunit is a functional unit, or monodomain, that combines with one oxygen molecule. The subunits in red blood cells occur as monomers or aggregate to form dimers, tetramers or, rarely, octomers. The exception to this rule is the hemoglobin of *Barbatia reeveana*, a bivalve mollusc found in the Sea of Cortez. *B. reeveana* red blood cells have a typical tetrameric hemoglobin, plus another large polymeric hemoglobin, of 430 kDa, composed of 34 kDa didomain subunits. Each subunit of the polymeric hemoglobin, the largest intracellular hemoglobin known, contains two oxygen-binding functional units, covalently linked (Grinich and R. Terwilliger, 1980). The DNA coding for this unique hemoglobin is correspondingly long (Riggs *et al.* 1986; Riggs and Riggs, 1990). Since there is one example, there are probably other dogma-confounding large multidomain intracellular hemoglobins; they remain to be discovered.

Extracellular hemoglobins are extraordinarily diverse in both quaternary structure and subunit size, although they share the same myoglobin fold and heme moiety as the red blood cell hemoglobins (for a review, see Terwilliger 1992). Annelid extracellular hemoglobins and chlorocruorins use 17 kDa monodomain subunits, but assemble approximately 200 of them, plus some non-heme-containing linker chains, into beautiful hexagonal bilayers of approximately 3500 kDa (Vinogradov *et al.* 1986; Riggs, 1990, 1998). Arthropod extracellular hemoglobins are composed of mono-, di- or nonadomain subunits, with a range of matching quaternary structures, depending on the species (Moens and Kondo, 1976; Dangott and Terwilliger, 1981; Manning *et al.* 1990). Molluscan extracellular hemoglobins are huge. Each subunit consists of 17 kDa functional units, 10–12 in planorbid snails, 18–20 in the bivalve families Carditidae and Astartidae, that are covalently linked into long polypeptide chains. The 170 kDa snail subunits assemble into 1700 kDa ring-shaped hemoglobins (Wood and Mosby, 1975; Terwilliger *et al.* 1976), while the 340 kDa bivalve hemoglobin subunits form 8000–12 000 kDa rod-shaped assemblages (Waxman, 1975; Terwilliger and Terwilliger, 1978).

Pink blood cell hemerythrin subunits are 13.5 kDa monodomain polypeptides. They are usually assembled into 108 kDa octomers, although trimers, tetramers and dimers have also been described (for a review, see Kurtz, 1992). A 690 kDa hemerythrin aggregate has been reported for the vascular hemerythrocyte of the polychaete annelid *Magelona papillicornis* (Manwell and Baker, 1988); it probably has a low-molecular-mass, monodomain subunit similar to the 14.7 kDa subunit of a closely related species, *Magelona alleni* (N. B. Terwilliger, unpublished observations).

Molluscan hemocyanins, like molluscan extracellular hemoglobins, are composed of large multidomain subunits that self-assemble into elegant symmetries (for a review, see van Holde and Miller, 1995). Seven or eight oxygen-binding units per 350–450 kDa subunit are assembled into decameric cylindrically shaped oligomers of 3500–4500 kDa in chitons and cephalopods. Gastropod and bivalve hemocyanins are didecameric cylinders of 8000–10 000 kDa.

Arthropod hemocyanins are intermediate in size between annelid extracellular hemoglobins and molluscan hemocyanins. Six 75 kDa subunits, each with one oxygen-binding site, assemble into a 450 kDa hexamer. The hexamers assemble further into two-hexamers, four-hexamers, six-hexamers or eight-hexamers depending on the class or species (for a review, see Markl and Decker, 1992).

Most of these oxygen-transport proteins do not consist of a single homogeneous array of subunits, but instead show varying degrees of subunit heterogeneity. Arthropod

hemocyanins are particularly renowned for the number of different kinds of polypeptides within a multisubunit molecule. The heterogeneity expands the functional properties of the respiratory protein, since different subunits may have different oxygen affinities or responses to allosteric modifiers.

Phylogenetic distribution

The expression throughout the animal kingdom of these oxygen-transport proteins and of the tissue protoheme proteins is portrayed in Table 1. Phyla in which no respiratory protein has been identified have not been listed. The extent to which these proteins are expressed within a phylum varies greatly. For example, almost all annelids contain hemoglobins (myoglobin, red blood cells and/or extracellular hemoglobins), while only certain families within two of the six classes of echinoderms contain hemoglobin. Tissue myoglobins are most widely distributed throughout the biosphere and are present in prokaryotes, protists, plants and animals. Red blood cell hemoglobins are represented in almost all protostomes and deuterostomes, triploblasts with the requisite mesodermal potential to form coeloms and vascular systems within which the cells can circulate. A notable exception is the phylum Arthropoda, with no examples of circulating red blood cells. Hemerythrins are expressed in four phyla; however, they are mostly small phyla, except for the Annelida. Here, hemerythrin is known only in one family of worms, the Magelonidae. Extracellular hemoglobins occur frequently in the protostomes but are not expressed in the deuterostome line of evolution. The two kinds of hemocyanins are each found only in a single phylum, and thus at first glance are much more limited in expression than are the heme proteins. The Mollusca and Arthropoda are such huge, diverse phyla, however, that there are a great many species utilizing these copper-based proteins for oxygen transport (Fig. 2). At the same time, many molluscs and some arthropods contain hemoglobins instead of, or in addition to, hemocyanin. What are the selection factors that led to the utilization of a particular oxygen-transport protein? Even more intriguing, why do some organisms express more than one kind, what are the regulatory signals for each protein, and how is the expression of multiple oxygen-transport proteins coordinated?

Adaptations of oxygen-transport molecules

With phylogeny of oxygen-transport proteins as a framework, one can develop a broader understanding of different ways in which organisms use these proteins to facilitate the uptake and delivery of oxygen in a changing environment. How are these molecules adapted to provide adequate oxygen to the tissues under variable external and internal conditions? Detailed information on both the structure and function of oxygen-transport proteins are available in chapters by individual authors in Mangum (1992), as well as in reviews by van Holde and Miller (1995) and Mangum (1997).

Regulation of oxygen-carrying capacity

Changing the rate of synthesis or catabolism of an oxygen-transport protein will obviously affect how much oxygen the blood can transport.

Environmental hypoxia

Environmental hypoxia is a key stimulus to increase the production of an oxygen-transport protein and thereby to increase the oxygen-carrying capacity of the blood. Oxygen sensors and the regulation of hemoglobin expression are topics of current research (Bunn *et al*. 1998; Hand, 1998; Hardison, 1998; Hochachka, 1998; Ratcliffe *et al*. 1998). Several arthropods respond to environmental oxygen levels by increasing or decreasing the rate of synthesis of an extracellular hemoglobin, thus changing the oxygen-carrying capacity to meet the need. The primitive crustaceans *Artemia salina* (Heip

Table 1. *Phylogenetic distribution within the animal kingdom of oxygen-transport proteins and tissue protoheme proteins*

	Tissue protoheme	Red blood cell hemoglobin	Extracellular hemoglobin	Extracellular chlorocruorin	Molluscan hemocyanin	Arthropod hemocyanin	Hemerythrin
Protista	x						
Platyhelminthes	x						
Nematoda	x		x				
Phoronida		x					
Nemertea	x	x	x				
Priapulida							x
Brachiopoda							x
Sipunculida							x
Annelida	x	x	x	x			x
Vestimentifera			x				
Pogonophora			x				
Echiura	x	x					
Mollusca	x	x	x		x		
Arthropoda			x			x	
Echinodermata		x					
Chordata	x	x					

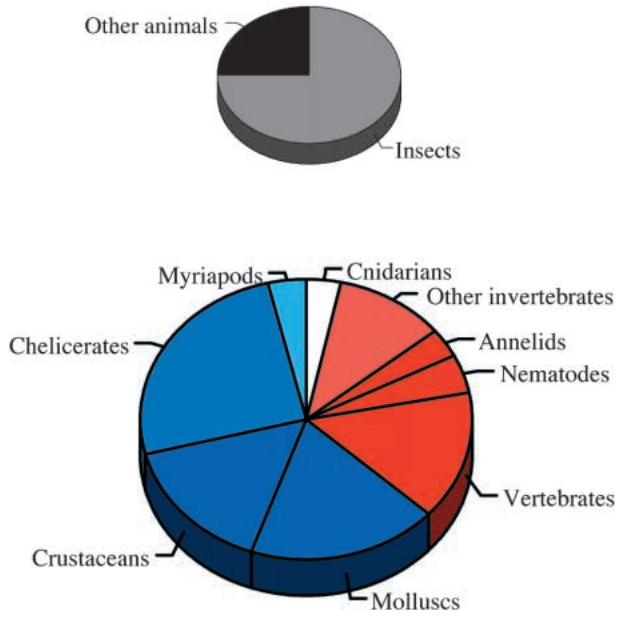

Fig. 2. Relative phylogenetic distribution of hemocyanin and hemoglobin. The lower pie chart shows the relative distribution among all animals except insects. Blue, hemocyanin; dark blue, hemocyanin and/or hemoglobin; red, hemoglobin. Adapted from frontispiece, Ruppert and Barnes (1994).

et al. 1978*b*) and *Daphnia magna* (Kobayashi *et al.* 1988) both respond to hypoxia with an increase in hemoglobin synthesis. Kobayashi and Gonoi (1985) showed that the swimming ability of *Daphnia magna* in hypoxic water was directly proportional to the animal's hemoglobin content. Red *Daphnia* raised under conditions of low oxygen level swam faster and for longer than pale *Daphnia*. Larvae of the phantom midge *Chironomus thummi thummi*, an insect, also produce more hemoglobin under hypoxic conditions (Weber *et al.* 1985). The amphipod *Cyamus scammoni*, an obligate ectosymbiont on the gray whale *Eschrictius robustus*, has an 1800 kDa, multidomain extracellular hemoglobin in its hemolymph (Terwilliger, 1991). *Cyamus scammoni*, along with its host, summers off the coast of Alaska and rides down to Baja, Mexico, for the late winter whale calving season in Scammon's Lagoon. Does *Cyamus scammoni* synthesize more hemoglobin in Baja than in the icy oxygen-rich waters of Alaska?

Hypoxia sensors responsible for the regulation of hemocyanin synthesis are unknown, although oxygen-sensitive receptors in the crustacean gills and central nervous system play roles in the ventilatory and cardiovascular responses to external hypoxia (Wilkens *et al.* 1989; Ishii *et al.* 1989; Massabuau and Meyrand, 1996; Reiber, 1997). Several laboratory studies have indicated that higher hemocyanin levels in crustaceans are induced by hypoxia (Hagerman, 1986; Baden *et al.* 1990). An increase in hemocyanin concentration has been demonstrated in response to hyposalinity and hypoxia in the blue crab *Callinectes sapidus* (Mason *et al.* 1983; deFur *et al.* 1990) and will be discussed below in connection with hypoxia-related changes in hemocyanin subunit composition.

Internal hypoxia or hyperoxia

Internal hypoxia or hyperoxia may be involved in regulating the production of arthropod hemocyanin. Hemocyanin levels are directly related to the nutritional state of a crab (see Dall, 1974; Dumler and Terwilliger, 1996), leading some to suggest that, in times of plenty, hemocyanin serves not only as an oxygen carrier but also as a storage protein. This may occur, although storing excess nutrients as circulating oligomeric proteins seems a bit expensive. An alternative hypothesis is that, with increased nutrition, a potential physiological hypoxia may develop even though external levels of oxygen remain constant. As the well-fed crab's activity levels, metabolic demands and oxygen requirements increase, so might its hemocyanin concentration.

Just as hypoxia may regulate hemocyanin synthesis, incipient hyperoxia may also be involved. It has long been observed that the concentration of hemocyanin plummets when a crab molts (Drach, 1939). This is due both to increased water uptake and to a change in hemocyanin biosynthesis (Mykles, 1980; Mangum *et al.* 1985; Spindler *et al.* 1992; Terwilliger and Otoshi, 1994), but a physiological reason remains unclear. It seems counterproductive that, at such a critical time, late premolt, ecdysis and early postmolt, when the crab is resorbing its old exoskeleton and synthesizing a new one, it would have a lower oxygen-carrying capacity (Fig. 3). Perhaps we should look at the problem from a different perspective. Could the decrease in synthesis of hemocyanin at ecdysis be a defense against too much oxygen related to a temporary increase in oxygen permeability of the new exoskeleton? Might a lower oxygen-carrying capacity help prevent premature cross-linking of internal skeletal structures by slowing the activation cascade of prophenoloxidase? Prophenoloxidase (tyrosinase) is a copper enzyme involved in melanization and arthropod exoskeleton cross-linking (Mason, 1965; Soderhall, 1982; Ashida and Yamazaki, 1990; and see below). These suggestions on the relationship between hypoxia and hemocyanin are obviously speculative but testable. Experimental design will need to take into account the developmental and molt stage of the organism as well as external factors such as oxygen levels, food availability and salinity.

More generally, what are the oxygen response elements in arthropods and other invertebrates and how do they compare with those currently under investigation in bacteria, yeast and mammals? Might oxygen-transport proteins participate in oxygen sensing, as suggested for mitochondrial heme in yeast and *Artemia* (Kwast *et al.* 1998; Hand, 1998)? How do the immediate oxygen sensors translate external or internal hypoxia into regulation of hemocyanin or hemoglobin production? Is there an erythopoietin-like hormonal pathway (Bunn *et al.* 1998; Ratcliffe *et al.* 1998) for regulation of the cells that synthesize extracellular hemoglobin or hemocyanin?

Changes in oxygen affinity

Rather than regulating the rate of expression of a single respiratory protein, many organisms synthesize multiple

Fig. 3. A red king crab *Paralithodes camtschatica* emerging from its old exoskeleton during ecdysis. Carapace width approximately 10 cm. Photograph by Jeff Goddard.

oxygen carriers with different oxygen affinities. These carriers are produced and function either simultaneously, to enhance oxygen transport and transfer to different portions of the body, or sequentially, to meet oxygen demand under changing conditions.

Simultaneous expression

Among the Annelida, some contain red blood cells, while others, especially the oligochaetes and hirudineans, have only extracellular vascular hemoglobin. In contrast, terebellid and opheliid polychaete annelids are outstanding examples of organisms that use multiple proteins in separate body compartments simultaneously to ensure an adequate oxygen supply (Terwilliger, 1974; Mangum *et al.* 1975; Terwilliger *et al.* 1980). *Pista pacifica*, a large terebellid that lives in a vertical leathery tube extending several feet down into the anoxic sediments of a mudflat, contains three different oxygen-binding proteins (Terwilliger, 1974). First, it circulates a giant (3400 kDa) extracellular hemoglobin with a low oxygen affinity (P_{50}=22 mmHg at 20 °C; 1 mmHg=0.1333 kPa) in its vascular system. Blood vessels are present in thin-walled gills at the head of the worm; at high tide, these gills expand into the oxygen-rich sea water above the mudflat. Second, the low-affinity extracellular hemoglobin readily transfers its oxygen to a moderate-affinity hemoglobin (P_{50}=3.8 mmHg at 20 °C) in the red blood cells circulating in the coelomic fluid of the worm. Third, the coelomic cell hemoglobin in turn tranfers oxygen to a high-affinity body wall myoglobin.

Alvinellid worms, also in the order Terebellida, are endemic to the deep-sea hydrothermal vents in the Pacific Ocean (Desbruyeres and Laubier, 1986). In addition to an extracellular hemoglobin in the vascular system (Terwilliger and Terwilliger, 1984; Toulmond *et al.* 1990), alvinellids contain a coelomic cell hemoglobin (Jouin-Toulmond *et al.* 1996). The coelomic cells are concentrated in an internal periesophageal pouch surrounding a plexus of thin-walled blood vessels, suggesting the presence of a complex respiratory gas-transfer system between extracellular and intracellular hemoglobins previously undescribed in polychaetes. The physiological properties of these proteins are still unknown; the authors suggest that the system may also participate in sulfide detoxification.

A similar strategy of using multiple proteins for oxygen transfer is seen in several sipunculids (Manwell, 1960; Mangum and Burnett, 1987; N. B. Terwilliger, unpublished results). *Themiste* sp. produces two different populations of hemerythrin-containing blood cells. Pink blood cells with a low-affinity hemerythrin are present in vessels that extend into the introvert tentacles and back into the coelomic cavity. The tentacles reach into the water column and are probably the prime source of oxygen uptake. Coelomic pink blood cells have a moderate-affinity hemerythrin, and a body wall myohemerythrin has a high oxygen affinity, ensuring oxygen transfer from regions of high to low concentration. The tentacular and coelomic blood cells not only have electrophoretically distinct hemerythrins with different oxygenation properties but differ morphologically as well (Terwilliger *et al.* 1985), reinforcing the conclusion that each hemerythrin is a distinctively different molecule produced by a unique cell population. Other thin-walled, stubby-tentacled sipunculans seem to transfer oxygen in the opposite direction, across the body wall, and there is little difference in oxygen affinity between coelomic and tentacular hemerythrins (Manwell, 1960).

Mollusca show many variations on a theme of multiple oxygen-transporter molecules (Terwilliger and Terwilliger, 1985). Chitons and gastropods have a high-affinity radular muscle myoglobin. This myoglobin ensures a supply of oxygen for the scraping, grazing activities of the radula and odontophore complex. Other body muscles do not contain myoglobin, and their creamy white color is in stark contrast to the red radular muscles. An exception to this is the red-footed

chiton *Lepidochiton rugatus* (Fig. 4). This member of an ancient suborder of chitons, mostly deep-water, has a striking red coloration of its foot and soft tissues, including the mantle, gills and radular muscle, due to a tissue hemoglobin (Eernisse *et al.* 1988). This hemoglobin seems to have a high oxygen affinity. Typical of all other chitons, *L. rugatus* has a circulating hemocyanin. Unlike more advanced, intertidal chitons, it has only a few gills and thus has relatively less surface area for gas exchange. It lives in an oxygen-poor shallow-water habitat. We hypothesize that the presence of the tissue hemoglobin facilitates oxygen transfer from the hemocyanin to the respiring tissues under hypoxic conditions. A note of caution is in order when cataloguing molluscan myoglobins and perhaps other molluscan heme proteins. Suzuki *et al.* (1996) and Suzuki and Imai (1997) have recently reported that the amino acid sequence and gene structure of the radular muscle 'myoglobin' of the abalone *Sulculus diversicolor* is more closely related to the enzyme 2,3-indole dioxygenase than to myoglobins from other sources. These data suggest that we take another look at radular muscle proteins in chitons and gastropods. Perhaps there are others with a didomain structure and sequence like that of *Sulculus.*

Another example of multiple hemoglobin expression in molluscs is found in one group of pulmonate gastropods, the planorbid snails. In addition to a radular muscle myoglobin, they contain a unique high-molecular-mass extracellular hemoglobin in their vascular system, but no hemocyanin. Most other gastropods, including pulmonates, have a high-molecular-mass hemocyanin in their vascular system, along with the radular myoglobin. What evolutionary accident encouraged the expression of hemoglobin in the planorbid snails and hemocyanin in the others?

The expression of oxygen-transport proteins is most varied in the bivalve molluscs. First, primitive bivalves, mostly active foragers, contain a circulating hemocyanin; more advanced bivalves do not (Morse *et al.* 1986; Mangum *et al.* 1987; Terwilliger *et al.* 1988). Second, a tissue myoglobin is often present in the ganglia and sometimes in the adductor, foot and other muscles of bivalves (Kraus and Colacino, 1986). Third, those primitive bivalves such as *Solemya velum*, *S. reidi*, *Myrtea spinifera* and *Lucina pectinata* that incorporate chemoautotrophic bacteria in their gills to utilize sulfide or methane contain several tissue hemoglobins in their gills (Read, 1965; Dando *et al.* 1985; Doeller *et al.* 1988). One or more of these 'branchioglobins' is involved in oxygen uptake, while the others participate in sulfide metabolism (Doeller *et al.* 1988; Kraus, 1995; and see below). Fourth, circulating red blood cells are present in the bivalve family Arcidae (for a review, see Mangum, 1997). The hemoglobins are mostly tetrameric and dimeric, except for the 430 kDa oligomers in *Barbatia reeveana* mentioned above. One bivalve hemoglobin, the cooperative homodimer found in the red blood cells of *Scapharca inaequivalvis*, has an interesting back-to-front assembly of its subunits, the reverse of vertebrate hemoglobins, such that the hemes are in direct contact (Royer *et al.* 1989). Recent crystallography work by Royer *et al.* (1996) suggests that the cooperativity between hemes in *Scapharca* is mediated by ordered water molecules. Two deep-water heterodont bivalves, *Calyptogena magnifica* (Terwilliger *et al.* 1983) and *C. soyoae* (Suzuki *et al.* 1989), also contain red blood cell hemoglobins. A fifth example of oxygen-transport proteins in bivalves is the largest extracellular hemoglobin (12×10^3 kDa) known, found in only two families of bivalves, the Astartidae and the Carditidae (see above). Despite their size and polymeric nature, with approximately 20 functional domains per subunit and many subunits per molecule, the giant hemoglobins have neither the moderate cooperativity of *Scapharca* homodimers nor the supercooperativity of annelid extracellular hemoglobins. Somewhat disappointingly for such dramatically designed oligomers, they show moderate to relatively high oxygen affinities and little or no cooperativity (Terwilliger and Terwilliger, 1978; Yager *et al.* 1982).

With this plethora of hemoglobin and hemocyanin expression among the bivalves, it may soon be possible to correlate the respiratory physiology and molecular phylogeny of bivalve globins with the morphological phylogeny of the bivalve species. This would allow us to determine whether observed functional properties are more closely related to genetics or to habitat and are therefore the result of homology or convergent evolution.

Parasites offer an intriguing view of how two very different respiratory proteins can be utilized for transport and transfer of oxygen – keeping in mind that one protein is synthesized by the host and the other by the parasite. The female rhizocephalan barnacle *Briarosaccus callosus* invades its host, a king crab, and proceeds to grow internally, mature, extrude a brood pouch through the abdomen of the crab and then produce gametes. After fertilization by a male barnacle, the ensuing embryos develop in the barnacle's brood pouch or externa until they are ready to swim away as naupliar larvae. During this bizarre process, the female barnacle synthesizes an extracellular hemoglobin (Fox, 1953; Shirley *et al.* 1986; Terwilliger *et al.* 1986). The hemoglobin circulates through the barnacle's externa and also through thin-walled tissues or

Fig. 4. A red-footed chiton *Lepidochiton rugatus*; ventral view. Length approximately 2 cm. Photograph by Doug Ernisse.

rootlets of the barnacle inside the crab, in close proximity to the crab's hemocyanin-filled hemolymph (N. B. Terwilliger, unpublished observations). Presumably a low-affinity crab hemocyanin and a higher-affinity barnacle hemoglobin provide an oxygen-transfer system to the developing embryos of the barnacle. The interplay between barnacle and crab hormones and oxygen sensors in this system must be a fascinating story. A similar parasitism occurs between the hermit crab *Pagurus samuelis* and rhizocephalans *Peltogaster paguri* and *Peltogasterella gracilis*. Hemocyanin from *P. samuelis*, whether parasitized or not, has a low oxygen affinity (Torchin, 1994).

Sequential expression

Oxygen-transport capabilities can be varied by sequentially expressing multiple oxygen carriers that have different oxygen-binding properties. Developmentally linked changes in hemogobin gene expression are well documented among viviparous vertebrates. Fetal/maternal oxygen affinity differences in red blood cells can be the result of sequential expression of different hemoglobin chains, different concentrations of allosteric effectors such as the organic phosphates 2,3-bisphosphoglycerate or ATP, different sensitivities of the hemoglobins to the effectors, or combinations thereof (for a review, see Ingermann, 1992). The higher affinity of human adult hemoglobin for 2,3-bisphosphoglycerate, a modulator that lowers the oxygen affinity, results in a net higher oxygen affinity in the fetal red blood cell, even though fetal hemoglobin has a slightly lower intrinsic oxygen affinity than adult hemoglobin.

Some arthropod extracellular hemoglobins, such as the two multidomain chains *a* and *b* in the branchiopod crustacean *Artemia*, are sequentially expressed as dimers *aa*, *ab* and *bb* (referred to as Hbs I, II and III) from the naupliar stage onwards (Heip *et al.* 1978*a*,*b*). They differ functionally, but the adaptive response is not clear. The hemoglobin with the lowest oxygen affinity, Hb I, is predominantly expressed in the adult. The phenotype can be altered by hypoxia as well as ontogeny; the highest-affinity Hb III is the most responsive. A more advanced branchiopod, *Daphnia* (Kobayashi *et al.* 1988), and the insect *Chironomus* have also been demonstrated to undergo developmental shifts in hemoglobin synthesis. The chironomid hemoglobins, expressed only in larval hemolymph, have markedly different oxygen affinities and Bohr effects (Weber *et al.* 1985). Because the synthesis of these hemoglobins is under both ontogenetic and environmental control (see above), they would be good candidates in which to identify an arthropod oxygen sensor. I suspect that there are many other examples of ontogenetic changes in hemoglobin expression, especially in benthic annelids and molluscs that have planktotrophic larvae, but they have not yet been investigated.

Arthropod hemocyanin undergoes a developmental shift in expression that is tied in with the development of the renal system. In the Dungeness crab *Cancer magister*, megalopa and juvenile crab hemocyanin differs from adult hemocyanin in both structure and function (Terwilliger and Terwilliger, 1982). The adult crab hemocyanin contains a subunit, C mag 6, that is absent in the young crabs until approximately the sixth juvenile instar. This is the same time at which C mag 6 mRNA first appears in the hepatopancreas (Durstewitz and Terwilliger, 1997*a*,*b*). Two other subunits change in relative abundance during development, while levels of three constitutive subunits are constant. The oxygen affinity of the juvenile hemocyanin is lower by approximately 50 % than that of the adult (Terwilliger and Terwilliger, 1982; Terwilliger and Brown, 1993). At first glance, this seemed to be an environmentally adaptive difference: the megalopa, swimming in well-oxygenated oceanic waters, gets by with a low-affinity oxygen carrier, while the adult, scuttling about and burying itself in the more hypoxic muddy sand floor of the nearshore and estuary, needs a higher-affinity transport protein. In fact, the oxygen affinities of juvenile and adult whole hemolymphs are indistinguishable, in contrast to that of their purified hemocyanins (Brown and Terwilliger, 1998). The growing crab is able to maintain a relatively constant oxygen affinity by coordinated developmental changes in both hemocyanin and ionic regulation (Fig. 5). The juvenile has more magnesium in its hemolymph than the adult (Brown and Terwilliger, 1992). In addition, the juvenile hemocyanin is more sensitive to magnesium, an allosteric effector that causes an increase in hemocyanin oxygen-affinity, than is the adult hemocyanin (Terwilliger and Brown, 1993). With this double jeopardy, more magnesium and a greater hemocyanin sensitivity to it, the juvenile crab would have difficulty unloading oxygen at the tissues if it had an adult-type hemocyanin. Fortunately, juvenile hemocyanin has a lower intrinsic oxygen affinity. As the young crab's ability to excrete more magnesium develops and hemolymph levels of magnesium drop, the synthesis of hemocyanin shifts from the juvenile to the adult form. Changes in the concentration of an internal cofactor rather than environmental oxygen levels seem to be the stimulus for this ontogenetic change in hemocyanin expression and function (Brown and Terwilliger, 1998). It is an interesting case of using different hemocyanins to maintain oxygen affinity while allosteric modulator concentrations vary.

Sometimes the sequential change in oxygen transporter structure and function occurs on a cyclical basis rather than on

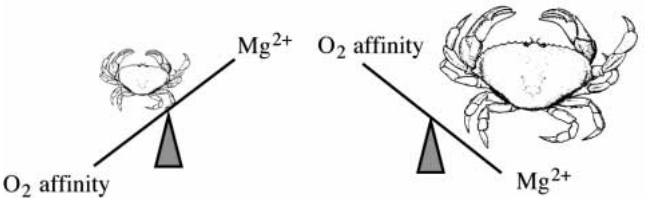

Fig. 5. Ontogeny of hemocyanin expression and ion regulation in the Dungeness crab *Cancer magister*. The low-affinity hemocyanin in the juvenile crab counterbalances high levels of magnesium in the hemolymph (left). As hemolymph magnesium levels decrease during development, the synthesis of hemocyanin shifts from the juvenile form to the higher-affinity adult hemocyanin (right).

a developmental time scale, and the cue is environmental. The blue crab *Callinectes sapidus* migrates seasonally from oceanic waters to dilute coastal marshes and is subjected to major changes in both salinity and oxygen levels due to estuarine stratification. Like *Cancer magister*, *Callinectes sapidus* hemocyanin is composed of six heterogeneous polypeptides that self-assemble into one-hexamers and two-hexamers. Three of the polypeptides are reported to be invariant, the other three are highly variable, from one crab to another and within one adult crab, depending on conditions (Mangum and Rainer, 1988). Normoxic animals have all six hemocyanin chains and low oxygen affinities; hypoxic animals have reduced levels of the three variable chains and progressively higher oxygen affinities. Purification of the oligomers by HPLC revealed that the two-hexamers contain all six chains and have a lower affinity and higher cooperativity, while the one-hexamers are built entirely of the three invariant chains and have a higher oxygen affinity (Mangum *et al.* 1991). Studies on crabs caught in the field show a pattern consistent with the laboratory studies. Crabs caught in the normoxic areas of the York River estuary exhibited the hemocyanin subunit pattern associated with a low oxygen affinity, normoxic conditions and high proportions of two-hexamers, while crabs from hypoxic strata displayed the three-subunit pattern of hemocyanin consistent with high oxygen affinity, hypoxia and one-hexamers (Mangum, 1994). Details at the molecular level have not yet been investigated; it will be interesting to see the level of regulation. Whether undergoing a permanent transition from juvenile to adult or dealing with seasonal hypoxia, functional adaptations in crustacean oxygen-transport proteins utilize sequential changes in protein expression.

Changes in allosteric modifiers

Regulation of the function of oxygen-transport proteins by allosteric modifiers in response to relatively short-term environmental or metabolic changes varies widely depending on the protein. Invertebrate red blood cells are perhaps the least responsive, while annelid extracellular hemoglobins, vertebrate red blood cells and arthropod hemocyanins show a remarkable range of oxygen affinities and cooperativities that are sensitive to pH and inorganic ions. The latter two groups of proteins are modulated by organic metabolites as well (for a review, see Mangum, 1997). Crustacean hemocyanin is particularly responsive to different allosteric modifiers and conditions. In many decapods, increases in pH, CO_2 concentration, divalent cation levels and concentrations of organic molecules such as lactate, urate and dopamine result in an increase in hemocyanin oxygen-affinity, while an increase in temperature causes a decrease in oxygen affinity. Different combinations of these factors come into play, along with ventilatory, cardiovascular and behavioral modifications, to stabilize oxygen uptake during hypoxia or to increase oxygen uptake during exercise (for reviews, see Burnett, 1992; Truchot, 1992; Morris and Bridges, 1994; Morris and Airriess, 1998; Mangum, 1997). In subtidal or intertidal crabs, environmental hypoxia often evokes a hyperventilation response. This promotes CO_2 excretion and results in hemolymph alkalosis and an adaptive increase in oxygen affinity. Since the metabolic demand is not increased, the increased affinity facilitates loading from a low-oxygen environment without adversely affecting oxygen unloading at the tissues. Moderate exercise, in contrast, causes both an increase in oxygen demand and an increase in CO_2 production. The ensuing hemolymph acidosis results in a decrease in hemocyanin oxygen-affinity. External oxygen is not limited during exercise, however. If the crab can keep hyperventilating and perfusing its tissues, oxygen loading at the gills should not be a problem, and the lower affinity will assist at the site of unloading. Prolonged exercise is more of a problem. As a result of lowered blood oxygen levels, urate, an intermediate of purine metabolism, may accumulate (Dykens, 1991), and urate increases hemocyanin oxygen-affinity (Morris *et al.* 1985; Lallier *et al.* 1987). As the exercise continues, insufficient oxygen will be delivered to the tissues and anaerobic metabolism will begin, with the accompanying endproduct, L-lactic acid. Decreased pH will cause a decreased oxygen affinity, but the L-lactate will partially counterbalance the pH-induced reduction in affinity and assist oxygen uptake at the gill (Truchot, 1980). Superimposed on the patterns resulting from hypoxia and exercise are the effects of neurohormones and monoamines; while their primary functions appear to be directed at mechanically tuning the cardiovascular system of the crustacean, they also tend to cause an increase in hemocyanin oxygen-affinity (for a review, see Morris and Airreiss, 1998). Not all hemocyanins are equally responsive to these allosteric effectors. Those from terrestrial crabs seem to be especially insensitive to metabolic modulation. This may be due to the high oxygen content of air *versus* water and the high levels of hemocyanin oxygen saturation at the gas exchange surfaces (Morris, 1991). The Christmas Island red crab *Gecarcoides natalis* shows a decrease in hemocyanin oxygen-affinity with increasing concentrations of lactate. This reverse lactate effect, which may assist in unloading oxygen at the tissues during increased oxygen demand, is unique among the Crustacea (Adamczewska, 1997). Several hemolymph factors have been noted (but not yet identified) that lower oxygen affinity (see Bridges *et al.* 1997; Lallier and Truchot, 1997). These unknown factors, like H^+, act in marked contrast to most of the other allosteric modifiers that cause an increase in oxygen affinity. Collectively, the responses of oxygen-transport proteins to modulators help the organism deal with environmental changes and metabolic demands.

Other functions of oxygen-transport molecules

Besides transporting oxygen, several oxygen carriers have taken on additional roles. One function reported for some invertebrate hemoglobins is transport or detoxification of hydrogen sulfide. Unlike vertebrate hemoglobins, which form sulfhemoglobin in the presence of hydrogen sulfide, the extracellular hemoglobin of the hydrothermal vent vestimentiferan *Riftia pachyptila* binds sulfide reversibly at a site on the molecule different from that binding oxygen (Arp

and Childress, 1983; Arp *et al.* 1987). The hemoglobin thus transports both oxygen and sulfide to the sulfur-oxidizing chemotrophic endosymbionts of *Riftia*. Recent studies by Zal *et al.* (1997*c*) suggest that free cysteine residues on the heme-containing globin chains, and also on the linker chains, of *Riftia* hemoglobin are the principle sulfide-binding sites. Several other extracellular hemoglobin sequences, including those of the mudflat polychaete *Arenicola marina* and the vent polychaete *Alvinella pompejana* from sulfide-rich environments, also have free cysteine groups (Zal *et al.* 1997*a,b*). Sulfide binding may not be a universal property of hemoglobins with free cysteines, however, as no sulfide-binding activity was observed in the vascular blood from either *Alvinella pompejana* or *Alvinella caudata* (Martineau *et al.* 1997).

Bivalves with chemoautotrophic bacteria in their gills contain several hemoglobins in the gill tissue, as mentioned above. These hemoglobins, like the vestimentiferan hemoglobin, are involved in oxygen and/or sulfide uptake and metabolism. Hemoglobin I from the gill of the mangrove swamp clam *Lucina pectinata* is sulfide-reactive, while Hb II and Hb III are oxygen-reactive. Hb I combines with oxygen with high affinity, but at low oxygen concentrations, oxyHb I readily reacts with sulfide to form ferric Hb I sulfide (Kraus and Wittenberg, 1990). The heme-bound sulfide is stabilized by three phenylalanine residues forming a 'cage' around the sulfide (Rizzi *et al.* 1996). Sulfide is believed to be released to the symbiotic bacteria upon the formation of the ferrous protein by electron transfer from an unknown reductant. Thus, this oxygen-binding protein, Hb I, is also implicated in sulfide transport and electron transfer (Navarro *et al.* 1996).

Vertebrate red blood cells have recently been reported to play a significant role in regulating blood pressure through the ability of the oxyhemoglobin molecule to bind nitric oxide as an *S*-nitrosothiol (SNO) (Jia *et al.* 1996). Nitric oxide had been identified as an endothelial relaxing factor (Furchgott and Zawadski, 1980). Its mechanism of action is unclear, because the heme iron of vertebrate hemoglobin is a potent scavenger of nitric oxide, binding it much more tightly than oxygen (Lancaster, 1994). In the red blood cell, however, an equilibrium has been described between nitric oxide bound to the heme iron and to reactive thiol groups of the hemoglobin chain, especially a conserved cysteine residue, CysB193, *versus* nitric oxide bound to glutathione and other small thiols in the erythrocyte. In the lungs, oxygenated hemoglobin is *S*-nitrosylated. During the red blood cell's transit through the body, thiols can transfer nitric oxide from the red blood cell to endothelial receptors as the hemoglobin becomes deoxygenated. The result is vasodilation and a decrease in blood pressure (Jia *et al.* 1996; Kagan *et al.* 1996).

Is this phenomenon restricted to vertebrate red blood cells or does its evolutionary history include invertebrate red blood cells or even extracellular hemoglobins and hemocyanins? Perhaps the thiol groups on the linker chains of polychaete and vestimentiferan extracellular hemoglobins (Zal *et al.* 1997*a,b,c*) can function as transfer agents of SNO from the heme-containing chains of the giant molecules to receptors in cells lining the heart-body or vessels of the worm. Does hemocyanin also form *S*-nitrosothiols and transport them through the hemolymph, and does nitric oxide affect blood pressure in arthropods or molluscs? Our perception of crustacean circulation from a loosely regulated 'open' system, at least in part, to one that is precisely designed and controlled has grown significantly (for reviews, see Airriess and McMahon, 1994; Morris and Airriess, 1998). For many years, it was believed that there was no vasoconstrictive musculature in the blood vessel walls of decapod crustaceans. Recently, striated muscle has been described in the posterior aorta of the prawn *Sicyonia ingentis* (Martin *et al.* 1989) and in the lobster *Homarus americanus* (Wilkens *et al.* 1997). Resistance to blood flow in *Homarus americanus* is thought to be due to cardioarterial valves and to a variety of neurotransmitters and neurohormones (Wilkens, 1997). Perhaps nitric oxide is also involved. Among the molluscs, it would be interesting to look for a nitric oxide effect and an SNO–hemocyanin in the finely tuned respiratory system of cephalopods.

Diversification of function among the hemocyanins seems to have involved gene duplications that resulted not only in multiple hemocyanin subunits with unique oxygen-binding properties but also in new members of the hemocyanin gene family. Phenoloxidases, or tyrosinases, are widespread in the animal kingdom, as well as in plants, fungi and procaryotes; they are able to catalyze the oxidation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones (Mason, 1965). Molluscan hemocyanin has phenoloxidase activity (Salvato *et al.* 1983; Nakahara *et al.* 1983), as does chelicerate hemocyanin (H. Decker, personal communication). Crustacean hemocyanin does not exhibit prophenoloxidase activity under normal circumstances, but it catalyzes the reaction with low efficiency when the hemocyanin molecule is partially unfolded (Zlateva *et al.* 1996). Some chelicerates, crustaceans and insects have a separate protein, a prophenoloxidase, that, when activated, shows strong activity. This enzyme plays a key role in melanization, cuticle hardening and other defense reactions (Ashida and Yamazaki, 1990). It contains two copper-binding sites whose amino acid sequences are nearly identical to those of arthropod hemocyanin, and the entire protein sequence shows some similarity to that of hemocyanin, although the quaternary structures appear unrelated (Aspan *et al.* 1995; Hall *et al.* 1995; Fujimoto *et al.* 1995; Kawabata *et al.* 1995). Thus, it is likely that arthropod hemocyanins and prophenoloxidases evolved from an ancestral arthropod copper protein. Molluscan hemocyanins seem to be more closely related to plant, fungal and vertebrate phenoloxidases, on the basis of sequence comparisons of their copper-binding sites (Drexel *et al.* 1987; Beintema *et al.* 1994; Kawabata *et al.* 1995; Durstewitz and Terwilliger, 1997*b*). They share a similar copper A site that differs from the amino acid sequence of the arthropod copper A site. The copper B sites are similar among the molluscan and arthropod hemocyanins and the phenoloxidases, suggesting that there was a common ancestral copper protein that gave rise

to today's proteins through a series of gene duplications and fusion events (see van Holde and Miller, 1995; Durstewitz and Terwilliger, 1997*b*).

Crustaceans contain another hemolymph protein, cryptocyanin, that is even more similar in sequence and structure to crustacean hemocyanin than is prophenoloxidase – except that it neither contains copper nor binds reversibly with oxygen (Terwilliger and O'Brien, 1992; N. B. Terwilliger, unpublished results). Levels of cryptocyanin increase dramatically during premolt and decrease at ecdysis (Terwilliger and Otoshi, 1994). Insect hemolymph storage proteins, hexamerins, also resemble hemocyanin in quaternary structure and sequence (Telfer and Kunkle, 1991). Like cryptocyanin, they lack copper, and some show molt-cycle-related patterns of biosynthesis and have potential roles in cuticle formation. It is likely that gene duplications in the arthropod hemocyanins allowed the loss of the copper-binding capability in one of the gene products. This then gave rise first to crustacean cryptocyanin and then to insect hexamerins. These proteins have since taken on new functions related to the molt cycle and exoskeleton formation. Thus, there are several members of the arthropod hemocyanin gene family, including crustacean and chelicerate hemocyanins, cryptocyanin, prophenoloxidases and insect hexamerins. Rather than assume additional functions, as some of the hemoglobins have done, hemocyanin has diversified through multiple gene duplications and specializations.

Conclusions

Oxygen-transport proteins are a colorful group of multisubunit molecules with a common function. The observed patterns of biosynthesis of crustacean hemocyanin and related proteins such as cryptocyanin, investigated in individually monitored crabs, indicate that the patterns are closely linked to development and molting and therefore are under some level of hormonal control. If similar monitoring studies were performed on individual polychaetes or molluscs, for example, rather than on pooled samples from many organisms, we would gain further insights into the effects of factors such as develomental stage, nutrition and reproductive cycle on oxygen-transport protein expression.

Changes in oxygen-transport protein synthesis, whether up- or down-regulation or expression of a particular combination of gene products, are often considered to be a long-term response to developmental or environmental change, while allosteric modifiers are thought to be responsible for more immediate, short-term perturbations. This is not always the case, as shown by the rise and fall in levels of modulators such as lactate and urate in the hemolymph. During forced exercise or other stress in crabs, lactate levels rise rapidly within minutes in the hemolymph, but the lactate lingers for hours afterwards until blood levels finally return to normal. Urate is slow to accumulate and is generally felt to be more of a response to prolonged hypoxia. Conversely, oxygen-transport protein synthesis may be much more of a short-term response than previously believed. Studies by Hofmann and Somero (1996) on the mussel *Mytilus trossulus* have shown that two stress proteins were synthesized during the first 2 h of recovery from thermal stress. During the same period of recovery from tidal emersion, damaged proteins were rapidly ubiquitinated and degraded. These results indicate that rapid induction of protein synthesis can occur on a tidal cycle basis. Monitoring the *in vivo* expression of oxygen-transport proteins during a 6–8 h exposure to hypoxia might reveal dynamic changes in synthesis that have not been obvious in longer-duration studies.

Finally, as advances in molecular techniques allow us more easily to obtain gene and protein sequence information, we may discover, as we have for myoglobin and indole amine oxidase, that some of the apparently homologous oxygen-transport proteins are more likely to be the result of the convergent evolution of different proteins. The continuing debate on the relative significance of similarity of structure *versus* amino acid sequence will help sort out molecular phylogenies. Which is more significant, for example, the total number of identical amino acids shared between two proteins or the fact that they have identical active sites? Combining molecular, functional and morphological data should eventually clarify (and probably revise) organismal phylogeny as well. New information may resolve our understanding of some of the paradoxes about the evolution of oxygen-transport proteins pointed out recently by Mangum (1998). Just as new technologies have reintroduced the importance of cell lineage studies to developmental biology, so investigations of respiratory proteins at the level of gene expression and high-resolution three-dimensional structure will provide significant new information about the structure, function and adaptations of oxygen-transport molecules.

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References

ADAMCZEWSKA, A. M. (1997). Eco-physiology of the Christmas Island red crab (*Gecarcoidea natalis*). PhD dissertation, University of Sydney.

AIRRIESS, C. N. AND MCMAHON, B. R. (1994). Cardiovascular adaptations enhance tolerance of environmental hypoxia in the crab *Cancer magister*. *J. exp. Biol.* **190**, 23–41.

ARP, A. E. AND CHILDRESS, J. J. (1983). Sulfide binding by the blood of the hydrothermal vent tube worm *Riftia pachyptila*. *Science* **219**, 295–297.

ARP, A. E., CHILDRESS, J. J. AND VETTER, R. D. (1987). The sulphide-binding protein in the blood of the vestimentiferan tubeworm, *Riftia pachyptila*, is the extracellular hemoglobin. *J. exp. Biol.* **128**, 139–158.

ASHIDA, M. AND YAMAZAKI, Y. H. (1990). Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In *Molting and Metamorphosis* (ed. E. Ohnishi and H. Ishizaki), pp. 239–265. Berlin: Springer.

ASPAN, A., HUANG, T.-S., CERENIUS, L. AND SODERHALL, K. (1995).

cDNA cloning of prophenoloxidase from the fresh water crayfish *Pacifastacus leniusculus* and its activation. *Proc. natn. Acad. Sci. U.S.A.* **92**, 939–943.

BADEN, S. P., PIHL, L. AND ROSENBERG, R. (1990). Effects of oxygen depletion on the ecology, blood physiology and fishery of the Norway lobster *Nephrops norvegicus*. *Mar. Ecol. Prog. Ser.* **67**, 141–155.

BIENTEMA, J. J., STAM, W. T., HAZES, B. AND SMIDT, M. P. (1994). Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Molec. Biol. Evol.* **11**, 493–503.

BRIDGES, C. R., HUPPERTS, V., ESHKY, A. A. AND TAYLOR, A. C. (1997). Haemocyanin oxygen transport in *Ocypode* spp.: modification of oxygen affinity. *J. mar. biol. Ass. U.K.* **77**, 145–158.

BROWN, A. C. AND TERWILLIGER, N. B. (1992). Developmental changes in ionic and osmotic regulation in the Dungeness crab, *Cancer magister*. *Biol. Bull. mar. biol. Lab., Woods Hole* **182**, 270–277.

BROWN, A. C. AND TERWILLIGER, N. B. (1998). Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: hemolymph modulation of hemocyanin oxygen-binding. *J. exp. Biol.* **201** (in press).

BUNN, H. F. GU, J., HUANG, L. E., PARK, J.-W. AND ZHU, H. (1998). Erythropoietin: a model system for studying oxygen-dependent gene regulation. *J. exp. Biol*. **201**, 1197–1201.

BURNETT, L. E. (1992). Integrated function of the respiratory pigment hemocyanin in crabs. *Am. Zool.* **32**, 438–446.

DALL, W. (1974). Indices of nutritional state in the western rock lobster *Panulirus longipes* (Milne-Edwards). I. Blood and tissue constituents and water content. *J. exp. mar. Biol. Ecol.* **16**, 167–180.

DANDO, P. R., SOUTHWARD, A. J., SOUTHWARD, E. C., TERWILLIGER, N. B. AND TERWILLIGER, R. C. (1985). Sulphur-oxidising bacteria and haemoglobin in gills in the bivalve mollusc *Myrtea spinifera*. *Mar. Ecol. Prog. Ser.* **23**, 85–98.

DANGOTT, L. AND TERWILLIGER, R. C. (1981). Arthropod extracellular hemoglobins: structural and functional properties. *Comp. Biochem. Physiol.* **70**B, 549–557.

DEFUR, P. L., MANGUM, C. P. AND REESE, J. E. (1990). Respiratory responses of the blue crab *Calllinectes sapidus* to long-term hypoxia. *Biol. Bull. mar. biol. Lab., Woods Hole* **178**, 46–54.

DESBRUYERES, D. AND LAUBIER, L. (1986). Les Alvinellidae, une famille nouvelle d'annelides infeodées aux sources hydrothermales sous-marines: systematique, biologie et ecologie. *Can. J. Zool.* **64**, 2227–2245.

DOELLER, J. E., KRAUS, D. W., COLACINO, J. M. AND WITTENBERG, J. B. (1988). Gill hemoglobin may deliver sulfide to bacterial symbionts of *Solemya velum* (Bivalvia, Mollusca). *Biol. Bull. mar. biol. Lab., Woods Hole* **175**, 388–396.

DRACH, P. (1939). Mue et cycle d'intermue chez les crustaces decapodes. *Annls Inst. Oceanogr. Monaco* **19**, 103–329.

DREXEL, R., SIEGMUND, S., SCHNEIDER, H. J., LINZEN, B., GIELENS, C., LONTIE, R., KELLERMANN, J. AND LOTTSPEICH, F. (1987). Complete sequence of a functional unit from a molluscan hemocyanin (*Helix pomatia*). *Biol. Chem. Hoppe Seyler* **368**, 617–635.

DUMLER, K. AND TERWILLIGER, N. B. (1996). Effects of food levels and temperature on growth and hemocyanin ontogeny in juvenile *Cancer magister*. *Am. Zool.* **36**, 14A.

DURSTEWITZ, G. AND TERWILLIGER, N. B. (1997*a*). Developmental changes in hemocyanin expression in the Dungeness crab, *Cancer magister*. *J. biol. Chem.* **272**, 4347–4350.

DURSTEWITZ, G. AND TERWILLIGER, N. B. (1997*b*). cDNA cloning of a developmentally regulated hemocyanin subunit in the crustacean *Cancer magister* and phylogenetic analysis of the hemocyanin gene family. *Molec. Biol. Evol.* **14**, 266–276.

DYKENS, J. (1991). Purinolytic capacity and origin of hemolymph urate in *Carcinus maenas* during hypoxia. *Comp. Biochem. Physiol*. **98**B, 579–582.

EERNISSE, D. J., TERWILLIGER, N. B. AND TERWILLIGER, R. C. (1988). The red foot of a lepidopleurid chiton: evidence for tissue hemoglobins. *Veliger* **30**, 244–247.

FOX, H. M. (1953). Hemoglobin and biliverdin in parasitic cirripede Crustacea. *Nature* **171**, 162–163.

FUJIMOTO, K., OKINO, N., KAWABATA, S., IWANAGA, S. AND OHNISHI, E. (1995). Nucleotide sequence of the cDNA encoding the proenzyme of phenol oxidase A_1 of *Drosophila melanogaster*. *Proc. natn. Acad. Sci. U.S.A.* **92**, 7769–7773.

FURCHGOTT, R. F. AND ZAWADSKI, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373–376.

GRINICH, N. AND TERWILLIGER, R. (1980). The quaternary structure of an unusually high molecular weight intracellular hemoglobin from the bivalve mollusc *Barbatia reeveana*. *Biochem. J.* **189**, 1–8.

HAGERMAN, L. (1986). Haemocyanin concentration in the shrimp *Crangon crangon* (L.) after exposure to moderate hypoxia. *Comp. Biochem. Physiol.* **85**A, 721–724.

HALL, M., SCOTT, T., SUGUMARAN, M., SODERHALL, K. AND LAW, J. H. (1995). Proenzyme of *Manduca sexta* phenol oxidase: Purification, activation, substrate specificity of the active enzyme and molecular cloning. *Proc. natn. Acad. Sci. U.S.A.* **92**, 7764–7768.

HAND, S. C. (1998). Quiescence in *Artemia franciscana* embryos: reversible arrest of metabolism and gene expression at low oxygen levels. *J. exp. Biol.* **201**, 1233–1232.

HARDISON, R. (1998). Hemoglobins from bacteria to man: evolution of different patterns of gene expression. *J. exp. Biol.* **201**, 1099–1117.

HEIP, P., MOENS, L., JONIAU, M. AND KONDO, M. (1978*a*). Ontogenetical studies on extracellular hemoglobin of *Artemia salina*. *Dev. Biol*. **64**, 73–81.

HEIP, P., MOENS, L. AND KONDO, M. (1978*b*). Effect of concentrations of salt and oxygen on the synthesis of extracellular hemoglobins during development of *Artemia salina*. *Dev. Biol.* **63**, 247–251.

HOCHACHKA, P. W. (1998). Mechanism and evolution of hypoxia-tolerance in humans. *J. exp. Biol.* **201**, 1243–1254.

HOFMANN, G. E. AND SOMERO, G. N. (1996). Protein ubiquitination and stress protein synthesis in *Mytilus trossulus* occurs during recovery from tidal emersion. *Moec. mar. Biol. Biotech.* **5**, 175–184.

INGERMANN, R. (1992). Structure–function relationships of the ectothermic vertebrate hemoglobins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 411–431. Heidelberg: Springer Verlag.

ISHII, K., MASSABUAU, J. C. AND DEJOURS, P. (1989). Oxygen sensitive chemoreceptors in the branchiocardiac veins of the crayfish, *Astacus leptodactylus*. *Respir. Physiol.* **78**, 73–81.

JIA, L., BONAVENTURA, C., BONAVENTURA, J. AND STAMLER, J. S. (1996). S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* **380**, 221–226.

JOUIN-TOULMOND, C., AUGUSTIN, D., DESBRUYERES, D. AND TOULMOND, A. (1996). The gas transfer system in alvinellids

(Annelida Polychaeta, Terebellida). Anatomy and ultrastructure of the anterior circulatory system and characterisation of a coelomic, intracellular, haemoglobin. *Cah. Biol. Mar.* **37**, 135–151.

KAGAN, V. E., DAY, B. W., ELSAYED, N. M. AND GORBUNOV, N. V. (1996). Dynamics of haemoglobin. *Nature* **383**, 30–31.

KAWABATA, T., YUSAHARA, Y., OCHIAI, M., MATSUURA, S. AND MASAAKI, A. (1995). Molecular cloning of insect pro-phenol oxidase: a copper-containing protein homologous to arthropod hemocyanin. *Proc. natn. Acad. Sci. U.S.A.* **92**, 7774–7778.

KOBAYASHI, M., FUJIKI, M. AND SUZUKI, T. (1988). Variation in and oxygen binding properties of *Daphnia magna* hemoglobin. *Physiol. Zool.* **61**, 415–419.

KOBAYASHI, M. AND GONOI, H. (1985). Horizontal movement of pale and red *Daphnia magna* in low oxygen concentration. *Physiol. Zool.* **58**, 190–196.

KRAUS, D. W. (1995). Heme proteins in sulfide-oxidizing bacteria/mollusc symbioses. *Am. Zool.* **35**, 112–120.

KRAUS, D. W. AND COLACINO, J. M. (1986). Extended oxygen delivery from the nerve hemoglobin of *Tellina alternata* (Bivalvia). *Science* **232**, 90–92.

KRAUS, D. W. AND WITTENBERG, J. B. (1990). Hemoglobins of the *Lucina pectinata*/bacterial symbiosis. I. Molecular properties. Kinetics and equilibria of reactions with ligands. *J. biol. Chem.* **265**, 16043–16053.

KURTZ, D. M., JR (1992). Molecular structure/function relationships of hemerythrins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 151–171. Heidelberg: Springer Verlag.

KWAST, K. E., BURKE, P. V. AND POYTON, R. O. (1998). Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast. *J. exp. Biol.* **201**, 1177–1195.

LALLIER, F., BOITEL, F. AND TRUCHOT, J.-P. (1987). The effect of ambient oxygen and temperature on the haemolymph L-lactate and urate concentrations in the shore crab *Carcinus maenas. Comp. Biochem.Physiol.* **86**A, 255–260.

LALLIER, F. H. AND TRUCHOT, J. P. (1997). Hemocyanin oxygen binding properties of a deep-sea hydrothermal vent shrimp – Evidence for a novel cofactor. *J. exp. Zool*. **277**, 357–364.

LANCASTER, J. R. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc. natn. Acad. Sci. U.S.A.* **91**, 8137–8141.

MANGUM, C. P. (1992). (ed.) *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers*. Heidelberg: Springer Verlag.

MANGUM, C. P. (1994). Subunit composition of hemocyanins from *Callinectes sapidus*: Phenotypes from naturally hypoxic waters and isolated oligomers. *Comp. Biochem. Physiol.* **108**B, 337–341.

MANGUM, C. P. (1997). Invertebrate blood oxygen carriers. In *Handbook of Physiology*, section 13, *Comparative Physiology* (ed. W. H. Dantzler), pp. 1097–1131. New York: Oxford University Press.

MANGUM, C. P. (1998). Major events in the evolution of the oxygen carriers. *Am. Zool*. (in press).

MANGUM, C. P. AND BURNETT, L. E. (1987). Response of sipunculid hemerythrins to inorganic ions and CO_2. *J. exp. Zool.* **244**, 59–65.

MANGUM, C. P., GREAVES, J. AND RAINER, J. S. (1991). Oligomer composition and oxygen binding of the hemocyanin of the blue crab *Callinectes sapidus. Biol. Bull. mar. biol. Lab., Woods Hole* **181**, 453–458.

MANGUM, C. P., MCMAHON, B. R., DEFUR, P. L. AND WHEATLY, M. G. (1985). Gas-exchange, acid–base balance and the oxygen-supply to the tissues during a molt of the blue crab *Callinectes sapidus. J. crust. Res.* **5**, 207–215.

MANGUM, C. P., MILLER, K. I., SCOTT, S. L., VAN HOLDE, K. E. AND MORSE, M. P. (1987). Bivalve hemocyanins – structural, functional and phylogenetic relationships. *Biol. Bull. mar. biol. Lab., Woods Hole* **173**, 205–221.

MANGUM, C. P. AND RAINER, J. S. (1988). The relationship between subunit composition and oxygen binding of blue crab hemocyanin. *Biol. Bull. mar. biol. Lab., Woods Hole* **174**, 77–82.

MANGUM, C. P., WOODIN, B. L., BONAVENTURA, C., SULLIVAN, B. AND BONAVENTURA, J. (1975). The role of coelomic and vascular hemoglobins in the annelid family Terebellidae. *Comp. Biochem. Physiol.* **51**A, 281–294.

MANNING, A., TROTMAN, C. AND TATE, W. (1990). Evolution of a polymeric globin in the brine shrimp *Artemia. Nature* **348**, 653–656.

MANWELL, C. (1960). Histological specificity of respiratory pigments. II. Oxygen transfer systems involving hemerythrins in sipunculid worms of different ecologies. *Comp. Biochem. Physiol.* **1**, 277–285.

MANWELL, C. AND BAKER, C. M. A. (1988). *Magelona papillicornis* haemerythrin: tissue specificity, molecular weights and oxygen equilibria. *Comp. Biochem. Physiol.* **89**B, 453–463.

MARKL, J. AND DECKER, H. (1992). Molecular structure of the arthropod hemocyanins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 325–376. Heidelberg: Springer Verlag.

MARTIN, G. G., HOSE, J. E. AND CORZINE, C. J. (1989). Morphological comparison of major arteries in the ridgeback prawn, *Sicyonia ingentis. J. Morph.* **200**, 175–183.

MARTINEAU, P., JUNIPER, S. K., FISHER, C. R. AND MASSOTH, G. J. (1997). Sulfide binding in the body fluids of hydrothermal vent alvinellid polychaetes. *Physiol. Zool.* **70**, 578–588.

MASON, H. S. (1965). Oxidases. *A. Rev. Biochem.* **34**, 595–634.

MASON, R. P., MANGUM, C. P. AND GODETTE, G. (1983). The influence of inorganic ions and acclimation salinity on hemocyanin oxygen binding in the blue crab *Callinectes sapidus*. *Biol. Bull. mar. biol. Lab., Woods Hole* **164**, 104–123.

MASSABUAU, J. C. AND MEYRAND, P. (1996). Modulation of a neural network by physiological levels of oxygen in lobster stomatogastric ganglion. *J. Neurosci.* **16**, 3950–3959.

MOENS, L. AND KONDO, M. (1976). The structure of *Artemia salina* hemoglobins: a comparative characterisation of four naupliar and adult hemoglobins. *Eur. J. Biochem.* **67**, 397–402.

MORRIS, S. (1991). Respiratory gas exchange and transport in crustaceans: Ecological determinants. *Mem. Queensland Mus*. **31**, 241–261.

MORRIS, S. AND AIRRIESS, C. N. (1998). Integration of physiological responses of crustaceans to environmental challenge. *South Afr. J. Zool.* (in press).

MORRIS, S. AND BRIDGES, C. (1994). Properties of respiratory pigments in bimodal breathing animals: air and water breathing by fish and crustaceans. *Am. Zool*. **34**, 216–288.

MORRIS, S., BRIDGES, C. AND GRIESHABER, M. (1985). A new role for uric acid: Modulator of haemocyanin oxygen affinity in crustaceans. *J. crust. Biol.* **235**, 135–139.

MORSE, M. P., MEYERHOFF, E., OTTO, J. J. AND KUZURIAN, A. M. (1986). Hemocyanin respiratory protein in bivalve mollusks. *Science* **231**, 1302–1304.

MYKLES, D. (1980). The mechanism of fluid absorption at ecdysis in

the American lobster *Homarus americanus*. *J. exp. Biol.* **84**, 89–101.

NAKAHARA, A., SUZUKI, S. AND KINO, J. (1983). Tyrosinase activity of squid hemocyanin. *Life Chem. Reports Suppl.* **I**, 319–322.

NAVARRO, A. M., MALDONADO, M., GONZALEZ-LAGOA, J., LOPEZ-MEJIA LOPEZ-GARRIGA, J. AND COLON, J. L. (1996). Control of carbon monoxide binding states and dynamics in hemoglobin I of *Lucina pectinata* by nearby aromatic residues. *Inorg. chim. Acta* **243**, 161–166.

RATCLIFFE, P. J. O'ROURKE, J. F., MAXWELL, P. H. AND PUGH, C. W. (1998). Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J. exp. Biol.* **201**, 1153–1162.

READ, K. R. H. (1965). The characterization of the hemoglobins of the bivalve mollusc *Phacoides pectinatus* (Gmelin). *Comp. Biochem. Physiol.* **15**, 137–158.

REIBER, C. L. (1997). Oxygen sensitivity in the crayfish *Procambarus clarkii*: peripheral O_2 receptors and their effect on cardiorespiratory functions. *J. crust. Biol.* **17**, 197–206.

RIGGS, A. (1990). Studies of invertebrate hemoglobins: structure, function and evolution. In *Invertebrate Dioxygen Carriers* (ed. G. Preaux), pp. 9–16. Leuven: Leuven University Press.

RIGGS, A. (1998). Self-association, cooperativity and supercooperativity of oxygen binding by hemoglobins. *J. exp. Biol.* **201**, 1073–1084.

RIGGS, A. F., RIGGS, C. K., LIN, R. I. AND DOMDEY, H. (1986). Cloning of the cDNA for the globin from the clam, *Barbatia reeveana*. In *Invertebrate Oxygen Carriers* (ed. B. Linzen), pp. 473–476. Heidelberg, Berlin, New York: Springer Verlag.

RIGGS, C. AND RIGGS, A. (1990). cDNA-derived amino acid sequences of single and two-domain globins from the clam *Barbatia reeveana*. In *Invertebrate Dioxygen Carriers* (ed. G. Preaux), pp. 57–60. Leuven: Leuven University Press.

RIZZI, M., WITTENBERG, J. B., CODA, A. AND ASCENZI, P. (1996). Structural bases for sulfide recognition in *Lucina pectinata* hemoglobin I. *J. molec. Biol.* **258**, 1–5.

ROYER, W. E., JR (1992). Structures of red blood cell hemoglobins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 87–116. Heidelberg: Springer Verlag.

ROYER, W. E., JR, HENDRICKSON, W. A. AND CHIANCONE, E. (1989). The 2.4 Å crystal structure of *Scapharca* dimeric hemoglobin: cooperativity based on directly communicating hemes at a novel subunit interface. *J. biol. Chem.* **264**, 21052–21061.

ROYER, W. E., PARDANANI, A., GIBSON, Q. H., PETERSON, E. S. AND FRIEDMAN, J. M. (1996). Ordered water molecules as key allosteric mediators in a cooperative dimeric hemoglobin. *Proc. natn. Acad. Sci. U.S.A.* **93**, 14526–14531.

RUPPERT, E. E. AND BARNES, R. D. (1994). *Invertebrate Zoology*, sixth edition. Fortworth: Saunders College Publishers.

SALVATO, B., JORI, G., PIAZZESE, A., GHIRETTI, F., BELTRAMINI, M. AND LERCH, K. (1983). Enzymic activities of type-3 copper pair in *Octopus vulgaris* haemocyanin. *Life Chem. Rep. Suppl.* **1**, 313–317.

SHIRLEY, S. M., SHIRLEY, T. M. AND MEYERS, T. R. (1986). Hemolymph responses of Alaskan king crabs to rhizocephalan parasitism. *Can. J. Zool.* **64**, 1774–1781.

SODERHALL, K. (1982). Prophenoloxidase activating system and melanization – a recognition mechanism of arthropods? A review. *Dev. comp. Immunol.* **6**, 601—611.

SPINDLER, K. D., HENNECKE, R. AND GELLISSON, G. (1992). Protein production and the molting cycle in the crayfish *Astacus leptodactylus* (Nordmann, 1842). II. Hemocyanin and protein synthesis in the midgut gland. *Gen. comp. Endocr.* **85**, 248–253.

SUZUKI, T. AND IMAI, K. (1997). Comparative studies of the indoleamine dioxygenase-like myoglobin from the abalone *Sulculus diversicolor*. *Comp. Biochem. Physiol.* **117**B, 599–604.

SUZUKI, T., TAKAGI, T. AND OHTA, S. (1989). Amino acid sequence of the dimeric hemoglobin (HbI) from the deep-sea cold-seep clam *Calyptogena soyoae* and the phylogenetic relationship with other molluscan globins. *Biochim. biophys. Acta* **993**, 254–259.

SUZUKI, T., YUASA, H. AND IMAI, K. (1996). Convergent evolution: The gene structure of *Sulculus* 41 kDa myoglobin is homologous with that of human indoleamine dioxygenase. *Biochim. biophys. Acta* **1308**, 41–48.

TELFER, W. H. AND KUNKEL, J. G. (1991). The function and evolution of insect storage hexamers. *A. Rev. Ent.* **36**, 205–228.

TERWILLIGER, N. B. (1991). Arthropod (*Cyamus scammoni*, Amphipoda) hemoglobin structure and function. In *Structure and Function of Invertebrate Oxygen Carriers* (ed. S. Vinogradov and O. Kapp), pp. 59–63. Berlin, Heidelberg, New York: Springer Verlag.

TERWILLIGER, N. B. (1992). Molecular structure of the extracellular heme proteins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 193–229. Heidelberg: Springer Verlag.

TERWILLIGER, N. B. AND BROWN, A. C. (1993). Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: the interactive effects of developmental stage and divalent cations on hemocyanin oxygenation properties. *J. exp. Biol.* **183**, 1–13.

TERWILLIGER, N. B. AND O'BRIEN, K. (1992). *Cancer magister* 'non-respiratory protein' is a molt-related hemocyanin. *Am. Zool.* **32**, 44A.

TERWILLIGER, N. B. AND OTOSHI, C. (1994). Cryptocyanin and hemocyanin: fluctuations and functions of crab hemolymph proteins during molting. *Physiologist* **37**, A67.

TERWILLIGER, N. B. AND TERWILLIGER, R. C. (1978). Oxygen binding domains of a clam (*Cardita borealis*) extracellular hemoglobin. *Biochim. biophys. Acta* **537**, 77–85.

TERWILLIGER, N. B. AND TERWILLIGER, R. C. (1982). Changes in the subunit structure of *Cancer magister* hemocyanin during larval development. *J. Exp. Zool.* **221**, 181–191.

TERWILLIGER, N. B. AND TERWILLIGER, R. C. (1984). Hemoglobin from the 'Pompeii worm', *Alvinella pompejana*, an annelid from a deep sea hot hydrothermal vent environment. *Mar. biol. Lett.* **5**, 191–201.

TERWILLIGER, N. B., TERWILLIGER, R. C., MEYERHOFF, E. AND MORSE, M. P. (1988). Bivalve hemocyanins – a comparison with other molluscan hemocyanins. *Comp. Biochem. Physiol.* **89**B, 189–195.

TERWILLIGER, N. B., TERWILLIGER, R. C. AND SCHABTACH, E. (1976). The quaternary structure of a molluscan (*Helisoma trivolvis*) extracellular hemoglobin. *Biochim. biophys. Acta* **453**, 101–110.

TERWILLIGER, N. B., TERWILLIGER, R. C. AND SCHABTACH, E. (1985). Intracellular respiratory proteins of Sipuncula, Echiura and Annelida. In *Blood Cells of Marine Invertebrates* (ed. W. Cohen), pp. 193–225. New York: Alan R. Liss

TERWILLIGER, R. C. (1974). Oxygen equilibria of the vascular and coelomic hemoglobins of the terebellid polychaete, *Pista pacifica*. Evidence for an oxygen transfer system. *Comp. Biochem.Physiol.* **48**A, 745–755.

TERWILLIGER, R. C., GARLICK, R. L. AND TERWILLIGER, N. B. (1980). Characterization of the hemoglobins and myoglobin of *Travisia foetida*. *Comp. Biochem. Physiol.* **66**B, 261–266.

TERWILLIGER, R. C. AND TERWILLIGER, N. B. (1985). Molluscan hemoglobins. *Comp. Biochem. Physiol.* **81**B, 255–261.

TERWILLIGER, R. C., TERWILLIGER, N. B. AND ARP, A. (1983). Thermal vent clam (*Calyptogena magnifica*) hemoglobin. *Science* **219**, 981–983.

TERWILLIGER, R. C., TERWILLIGER, N. B. AND SCHABTACH, E. (1986). Hemoglobin from the parasitic barnacle, *Briarosaccus callosus*. In *Invertebrate Oxygen Carriers* (ed. B. Linzen), pp. 125–127. Berlin, Heidelberg: Springer Verlag.

TORCHIN, M. (1994). The effects of parasitism on the hemocyanin of an intertidal hermit crab, *Pagurus samuelis*. MS thesis, University of Oregon.

TOULMOND, A., EL IDRISSI SLITINE, F., DE FRESCHEVILLE, J. AND JOUIN, C. (1990). Extracellular hemoglobins of hydrothermal vent annelids: structural and functional characteristics in three alvinellid species. *Biol. Bull. mar. biol. Lab., Woods Hole* **179**, 366–373.

TRUCHOT, J. P. (1980). Lactate increases the oxygen affinity of crab hemocyanin. *J. exp. Zool.* **214**, 205–208.

TRUCHOT, J. P. (1992). Respiratory function of arthropod hemocyanins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 377–410. Heidelberg: Springer Verlag.

VAN HOLDE, K. V. H. AND MILLER, K. I. (1995). Hemocyanins. In *Advances in Protein Chemistry*, vol. 47, pp. 1–81. New York: Academic Press.

VINOGRADOV, S. N., LUGO, S., MAINWARING, M., KAPP, O. AND CREWE, A. (1986). Bracelet protein: a quaternary structure proposed for the giant extracellular hemoglobin of *Lumbricus terrestris*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8034–8038.

WAXMAN, L. (1975). The structure of annelid and mollusc hemoglobins. *J. biol. Chem.* **250**, 3790–3795.

WEBER, R. E., BRAUNITZER, G. AND KLEINSCHMIDT, T. (1985). Functional multiplicity and structural correlations in the hemoglobin system of larvae of *Chironomus thummi thummi* (Insecta, Diptera): Hb components CTT I, CTT II, CTT III, CTT IV, CTT VI, CTT VIIB, CTT IX and CTT X. *Comp. Biochem. Physiol.* **80**B, 747–753.

WILKENS, J. L. (1997). Possible mechanisms of control of vascular resistance in the lobster *Homarus americanus. J. exp. Biol.* **200**, 487–493.

WILKENS, J. L., DAVIDSON, G. W. AND CAVEY, M. J. (1997). Vascular peripheral resistance and compliance in the lobster *Homarus americanus. J. exp. Biol.* **200**, 477–485.

WILKENS, J. L., YOUNG, R. E. AND DICAPRIO, R. A. (1989). Responses of the isolated crab ventilatory central pattern generators to variations in oxygen tension. *J. comp. Physiol.* B **159**, 29–36.

WOOD, E. AND MOSBY, L. (1975). Physicochemical properties of *Planorbis corneus* erythrocruorin. *Biochem. J.* **149**, 437–445.

YAGER, T., TERWILLIGER, N. B., TERWILLIGER, R. C., SCHABTACH, E. AND VAN HOLDE, K. (1982). Organization and physical properties of the giant extracellular hemoglobin of the clam *Astarte castenea*. *Biochim. biophys. Acta* **709**, 194–203.

ZAL, F., GREEN, B. N., LALLIER, F. H. AND TOULMOND, A. (1997*a*). Investigation by electrospray ionization mass spectrometry of the extracellular hemoglobin from the polychaete annelid *Alvinella pompejana*: an unusual hexagonal bilayer hemoglobin. *Biochemistry* **36**, 11777–11786.

ZAL, F., GREEN, B. N., LALLIER, F. H., VINOGRADOV, S. N. AND TOULMOND, A. (1997*b*). Maximum entropy analysis of electrospray ionisation mass spectra of the heteromultimeric hemoglobin of *Arenicola marina*. *Eur. J. Biochem.* **243**, 85–92.

ZAL, F., SUZUKI, T., KAWASAKI, Y., CHILDRESS, J. J., LALLIER, F. H. AND TOULMOND, A. (1997*c*). Primary structure of the common polypeptide chain *b* from the multi-hemoglobin system of the hydrothermal vent tube worm *Riftia pachyptila*: An insight on the sulphide binding site. *Proteins* (in press).

ZLATEVA, T., DIMURO, P., SALVATO, B. AND BELTRAMINI, M. (1996). The *o*-diphenol oxidase activity of arthropod hemocyanin. *FEBS Lett.* **384**, 251–254.

Note added in proof

The Pogonophora have been reduced from phylum to the rank of family (Siboglinidae) within the Polychaeta [Rouse, G. W. and Fauchald, K. (1997). Cladistics and polychaetes. *Zoologica Scripta* **26**, 139–204], a classification consistent with the structures of the extracellular hemoglobins of pogonophores, vestimentiferans and annelids (Terwilliger, 1992).