Recent Structural Work on the Oxygen Transport Protein Hemocyanin

Karen A. Magnus,* Hoa Ton-That, and Joan E. Carpenter

Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio, 44106–4935

Received November 2, 1993 (Revised Manuscript Received February 2, 1994)

Contents

I. Introduction

The ability to transport oxygen is a key biological process in multicellular organisms. Three general classes of respiratory proteins have evolved: hemoglobins, hemerythrins, and hemocyanins. The hemocyanins (Greek for "blue bloods") are large, multisubunit proteins capable of transporting oxygen and are found in various arthropods and mollusks. Unlike the other two general classes of respiratory proteins that contain iron, hemocyanins bind oxygen at a coupled dinuclear active site containing two copper atoms directly ligated by protein side chains. Hemocyanins circulate extracellularly in the hemolymph as multisubunit complexes rather than in specialized cells. Some hemocyanins have also been shown to demonstrate a high degree of cooperativity in oxygen binding.[1] Recent advances have been made in the crystal structures of the spiny lobster, *Panulirus interruptus*[2] and horseshoe crab, *Limulus polyphemus*, hemocyanins.[3] Many primary sequences have also been recently determined, which has led to a greater understanding of hemocyanins, their structures, and their functions as molecules under regulatory control that bind oxygen reversibly and cooperatively. This article will concentrate primarily upon developments in the past five years that have advanced our knowledge of primary, tertiary, and quaternary structures of the hemocyanins and their cooperative properties.

Major differences exist between the architectures of hemocyanins of arthropod and molluskan origin. Extensive discussion of spectral features of hemocyanins is outside the scope of this review. In brief, the two types of hemocyanins have similar spectral properties, including absorption maxima at about 340 and 580 nm when oxygenated and similar circular dichroic and fluorescence spectra. Also they both normally do not exhibit electron paramagnetic resonance, again suggesting similarity in their active sites. Electron microscopic studies show that the quaternary structures of the two types of hemocyanins are quite distinct,[4,5] with the principal difference being that each arthropod hemocyanin molecule is composed of hexamers or multiple hexamers of subunits of ~75 kDa while each molluskan hemocyanin has subunits in the 350–450-kDa range assembled into cylinders with apparent 5- and 10-fold symmetry (see Figure 1). In the arthropod hemocyanins, each subunit contains a single oxygen binding site, whereas molluskan hemocyanin subunits have multiple oxygen binding sites, each in ~50-kDa domains termed "functional units". A representative example of arthropod hemocyanin is that of the horseshoe crab, the largest of the arthropod hemocyanins. It is made up of 48 subunits, each containing a dinuclear copper active site (see Figure 2). In contrast, molluskan hemocyanins usually contain 10 or 20 subunits, with seven or eight functional units in each subunit.[6] Primary sequence results from assorted arthropod subunits and molluskan domains show some similarities in the region containing the three histidines binding the so-called "copper B" atom (CuB), but similarities in the area of the residues that ligate the other copper atom "copper A" (CuA) are not obvious.

X-ray diffraction studies of single crystals provide three-dimensional information about the entire protein structure at a detailed level not usually accessible with other techniques for proteins of such large size. Three important structures have recently been determined including those of subunits from arthropod hemocyanin from spiny lobster[2] and the horseshoe crab in two different ligation states.[3,7] Establishment of the geometry of oxygen binding in the active site of hemocyanin occurred concomitantly with the synthesis and characterization of an interesting series of bioinorganic copper compounds with properties similar to hemocyanins,[8] which will be covered in a separate article.

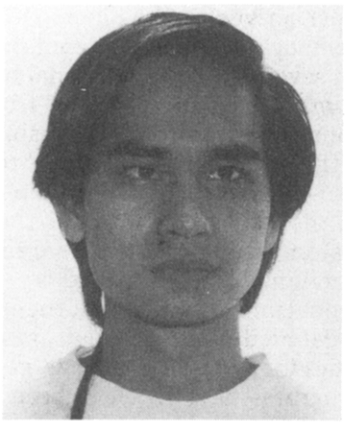
The functional control of hemocyanins and the cooperative binding of ligands has also been extensively studied in the last several years. Because of their large sizes and multiplicities of subunits or functional units, there are a number of cases where relatively simple models of cooperativity fail to describe cooperative oxygen binding accurately, leading to more complex modeling efforts. The ability to label specific subunits and to disassemble and reassemble multisubunit hemocyanin complexes[9] greatly enhances our ability to understand the cooperative ligand binding.

II. Primary Structures

Many of the latest advances in the study of hemocyanin are dependent on knowledge of the primary structure. The crystal structures of *P. interruptus* hemocyanin[2] and *L. polyphemus* hemocyanin[3,7] could not have been determined at high resolution without first obtaining the amino acid sequence. Molecular biological studies on the active site or on the binding of subunits have the same prerequisite: a thorough

0009-2665/94/0794-0727$14.00/0

Karen A. Magnus is currently Assistant Professor of Biochemistry at Case Western Reserve University School of Medicine, where she has been since 1989. Dr. Magnus received her Ph.D. at Johns Hopkins University under the direction of Professor Warner E. Love. She carried out postdoctoral work first in the laboratory of Professor Paul B. Sigler and then with Professor Eaton E. Lattman at the Johns Hopkins University School of Medicine. Her present research efforts are concentrated in the areas of oxygen transport and motility.



Hoa Ton-That received a B.A. in mathematics and microbiology from Miami University in 1989. He is completing his doctoral work in the Department of Biochemistry at Case Western Reserve University. Under the supervision of Professor Karen A. Magnus, he is determining the high-resolution structure of the oxygenated form of *Limulus polyphemus* subunit II hemocyanin.

Joan E. Carpenter received her B.A. in chemistry from Hiram College in 1985. She then obtained a masters in biochemical research at Case Western Reserve University under the supervision of Professor Harland G. Wood, where she worked on the enzymatic hydrolysis of polyphosphates. She is currently following her interests in biochemistry in the laboratory of Professor Karen A. Magnus.

understanding of the primary structure. The currently available sequences are listed in Table 1.

P. interruptus hemocyanin consists of three major subunits *a*, *b*, and *c* and a minor subunit *b'*.[10] The differences between subunits *a* and *b* are few, 18 amino acids or 2.7% of the total sequence. No deletions or additions were found, suggesting that subunits *a* and *b* are gene duplication products. Earlier, the molecular weights for these subunits were reported to be 94, 90, and 80 kDa for subunits *a*, *b*, and *c* respectively on the basis of SDS/PAGE.[11] This generated a certain amount of controversy since the theoretical value was approximately 77 kDa per subunit.[10] Further, only in the case of subunit *c* were the experimental and theoretical values in agreement. The difference between calculated and apparent molecular masses from polyacrylamide gel electrophoresis experiments has been explained by the presence of negative charges on the carboxy terminal end of subunits *a* and *b*. Moreover, it appears to be the distribution of the charges rather than the exact number which is important, since subunit *c* actually contains more acidic residues than either *a* or *b*. Heterogeneities were observed while analyzing peptide maps of subunit *b* and determined to be due to the presence a minor subunit component labeled *b'*.

Panulirus subunit *c* does not closely resemble either subunit *a* or *b*.[12,13] The sequence identity between subunits *a* and *c* is only about 59%. On the basis of the crystal structure of the spiny lobster hemocyanin described more fully in section V, arthropod hemocyanin subunits can be divided into three structural domains. The primary sequence in structural domain II of subunit *a* has a greater identity with subunit *c*, ~75%. This is not surprising since the oxygen-binding site is buried in the middle of domain II.[2] The other two domains show less identity, 60% and 45%, for domains I and III, respectively, and on the basis of sequence comparisons with other arthropod hemocyanins, subunits *a* and *c* are also thought to be products of gene duplication.[12] This duplication must have occurred before the divergence of the crayfish, *Astacus leptodactylus*, and *Panulirus*.

Another intriguing difference between subunits *a* and *c* of *Panulirus* hemocyanin is the attachment of their carbohydrate groups. In subunit *a*, the carbohydrate attachment occurs in domain I, while in subunit *c* the carbohydrate is bound in domain III. Since the exact function of the carbohydrate moiety is unclear, the effect of this difference is unknown.[12]

Sequence determination is also being undertaken on other species of arthropod hemocyanins. Schartau *et al.*[14] report the amino acid sequence for subunit *a* of the North American tarantula, *Eurypelma californicum*. The cDNA encoding its subunit *e* has been determined, suggesting that *Eurypelma* hemocyanin is very similar to other arthropod hemocyanins.[15-17]

Comparisons of the molluskan hemocyanin sequences with those of arthropod hemocyanins indicate that there are potentially different ligands coordinating the copper atoms in the oxygen binding sites. For example, Drexel *et al.*[18] compared the amino acid sequences of hemocyanins from the vineyard snail, *Helix pomatia* β_c, and *Panulirus*. The only protein sequence homology they found was a region corresponding to the CuB site in arthropod hemocyanins.[18] No regions corresponding to the sequences providing the histidine ligands to CuA

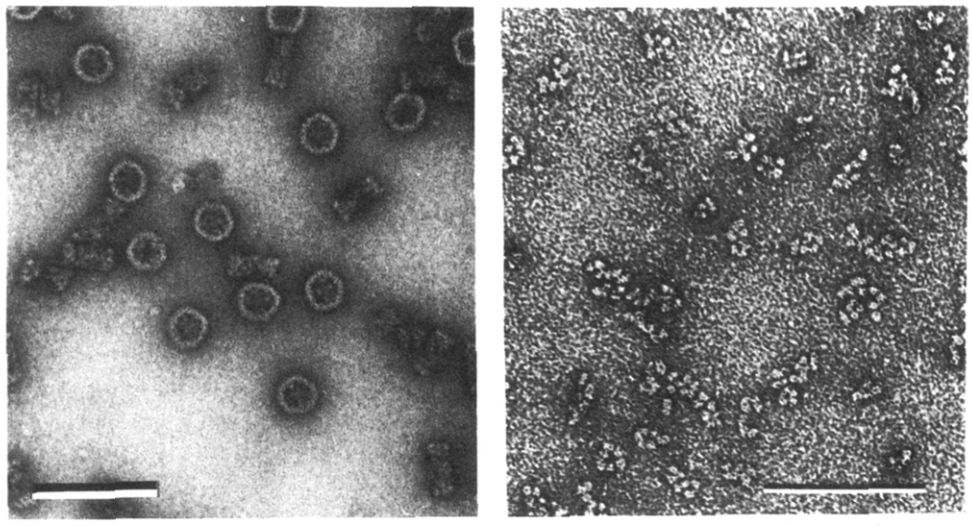


Figure 1. Electron micrographs of *O. dofleini* hemocyanin: (a, left) whole-molecule *Octopus* hemocyanin and (b, right) disassociated subunits. In both views, the bar is equivalent to 0.1 μm. The micrographs were kindly provided by Dr. K. I. Miller.

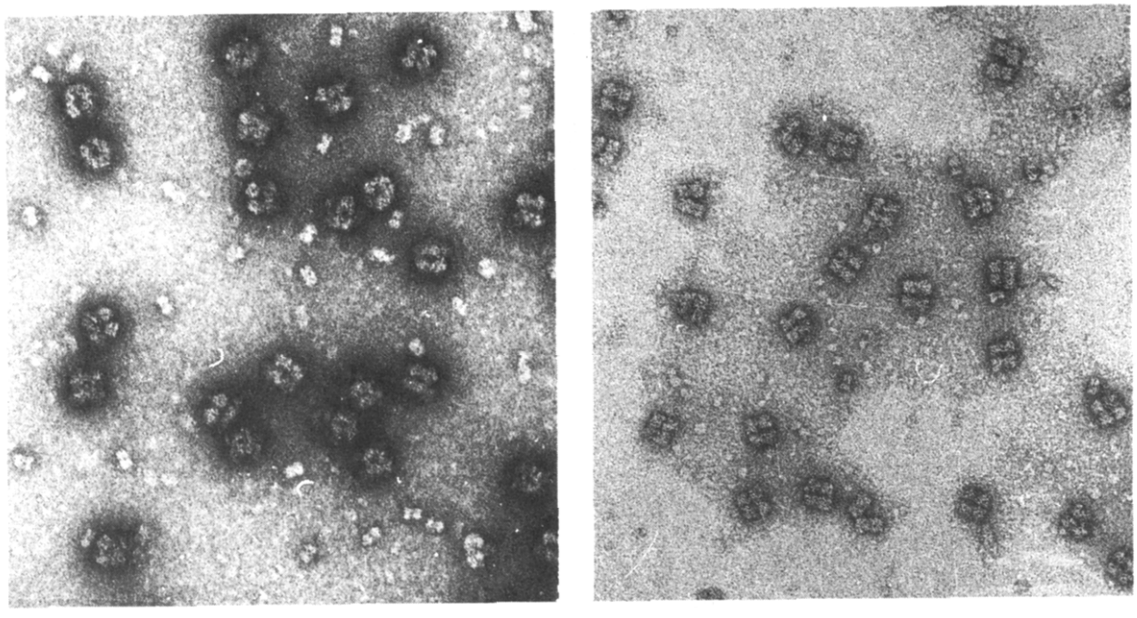


Figure 2. Electron micrographs of the arthropod hemocyanin *L. polyphemus* courtesy of Drs. E. F. J. van Bruggen and C. Bonaventura: (a, left) the native 48-mer of *Limulus* hemocyanin, (the molecule has an approximate diameter of 250 Å) and (b, right) reconstituted 24-mers of *Limulus* hemocyanin.

Table 1. Primary Structures of Hemocyanins[a]

organism	common name	subunit	method of sequencing	ref(s)
Astacus leptodactylus	crayfish	*b*	NA[b]	28
E. californicum	North American tarantula	*a,e*	cDNA	14–16
E. californicum	North American tarantula	*d*	proteolytic digestion	17
H. pomatica	vineyard snail	*βc*-HC*d*[c]	proteolytic digestion	18
L. polyphemus	horseshoe crab	II	proteolytic digestion	56
O. dofleini	Giant Pacific octopus	ode, odf, odg[c]	cDNA	19,20
P. interruptus	spiny lobster	*a,c,b*	proteolytic digestion	10,12
Tachypleus tridentatus	horseshoe crab	*α*	NA[b]	28

[a] Note: Only complete sequences are listed. Other partial sequences have been reported, most notably for *H. pomatia* (Xin, X-Q.; Gielen, C.; Witters, R.; Préaux, G. In *Invertebrate Dioxygen Carriers*; Preaux, G., Lontie, R., Eds. Leuven University Press: Louvain, 1990; pp 113–117) and *Sepia officinalis* (Préaux, G.; Declercq, L.; Tielemans, K.; Vanderzande, M.; Witters, R.; Gielens, C. In *International Congress on Invertebrate Dioxygen Carriers*; Reprodienst Chemische Laboratoria: Rijksuniversiteit Groningen, The Netherlands, 1992; II-P03). [b] Not available. [c] The sequenced mollusk hemocyanin functional domains are shown.

were obvious. A similar situation was observed with the protein sequence data from the giant Pacific octopus, *Octopus dofleini* hemocyanin. Potential CuB ligands were apparent but corresponding ligands for CuA were not clearly visible.[19,20]

A possible site for ligating CuA atoms has been proposed on the basis of conserved residues in molluskan hemocyanins and tyrosinases. Tyrosinases are proteins that catalyze the addition of an oxygen atom to a phenol group and are involved in the synthesis of

tyrosine.[21] Their active sites are believed to be similar but not identical to hemocyanins on the basis of their spectral properties and primary structures. Lang and Van Holde[20] suggested two of the copper ligands might be histidines, specifically, residues His 74 and His 46 in *Octopus dofleini*. These residues are conserved in known molluskan hemocyanins and tyrosinases, and their mutations can cause both loss of enzymatic activity and loss of a copper atom.[22] The third ligand is even more difficult to identify, and three possibilities have been suggested. First, that the ligand may be a nonconserved histidine. This would imply either different tertiary structures or different ligation geometries. Although neither of these possibilities is unprecedented they are relatively uncommon. Next, the ligand could be something other than histidine, perhaps a cysteine or a methionine. Both of these amino acids have been shown to coordinate copper. A third possibility suggested by Lang and Van Holde is that no third ligand exists so that the copper atom is coordinated by two protein ligands instead of three. These possibilities are all consistent with the observations that it is possible to remove one of the copper atoms with less effort than the other.[23,20] The answer to this question should be apparent when a crystal structure has been determined for a functional domain of a molluskan hemocyanin.[24]

Several proteins found in insect larvae have significant (25–30%) primary sequence identity to those of arthropod hemocyanins[25,26] with ~32% sequence identity among the hemocyanin proteins themselves.[26] Although the exact function of these proteins is unknown, they are not oxygen transport proteins. The insect proteins do not contain copper and analyses of their protein sequences indicate that there are no histidine residues at the locations corresponding to those that ligate either copper atom in arthropod hemocyanins.[27] The similarity in primary structure suggests the possibility that the larval proteins may have the same tertiary structures as arthropod hemocyanins described later in section V. The sequence identity between hemocyanins and larval proteins exists throughout all three structural domains, in contrast to the identity between arthropod hemocyanin subunits, which are high in their second domains, but less so in the other two.[28] Biophysical studies of the insect storage proteins indicate that they exist as hexamers with 32 symmetry or multihexamers in the native state, raising the possibility that the association of subunits is similar to that described for arthropod hemocyanins in the next section.[26,27]

III. Quaternary Structures

Arthropod and molluskan hemocyanin polypeptide subunits associate into distinct and complex structures. The two types of hemocyanin quaternary structures, those found in arthropods and those in mollusks, have been intensively studied using electron microscopic techniques. A single molluskan subunit resembles beads on a string. Multiples of five or 10 subunits can associate to form whole molluskan hemocyanin molecules that resemble hollow tubes or cylinders.[6] In the last few years, molecular weights of many different species of molluskan hemocyanin have become available.[29–33] Dimers, and to a lesser extent, trimers of the 10-subunit molluskan hemocyanin complexes have also been reported; however, there is no detailed outline of the role these multiple complexes play in the function of molluskan hemocyanin.[29,30,33] Although the stoichiometry of molluskan hemocyanins association is fairly clear, more needs to be learned about the specific interactions involved. Electron microscopic investigations show that the polypeptide subunits associate in a "head-to-tail" formation, generating the cylindrical polymers.[6] A kinetic study of a typical molluskan hemocyanin from *O. dofleini* indicates that assembly of its 10-subunit complex is nucleated by subunit dimerization.[34]

Arthropod hemocyanin subunits are globular in shape and organize into quaternary structures that are multiples of six subunits (Figure 2). They contain one, two, four, six, or eight hexamers, depending upon the species of animal and environmental factors.[5] The patterns of inter-hexamer contacts can be very complex and require specific subunit types for the interactions.[35] Careful analysis of hemocyanin quaternary structures requires accurate measurement of molecular weights of individual subunits and subunit complexes. Recent advances in mass spectroscopic techniques make this a much more feasible enterprise.

Studies of the scorpion, *Androctonus australis* hemocyanin, a 24-mer, use electron microscopy to localize immunological epitopes, and reveal information at or better than 20 Å.[36] These studies show that hemocyanin subunits are found at specific locations within the hemocyanin complexes.[36] A model of the complete quatrohexamer *Androctonus* hemocyanin has been reconstructed by this method.[37] The *Limulus* hemocyanin 48-mer bears a striking resemblance to two *Androctonus* 24-mers stacked up to form the larger molecule,[4] suggesting that the multihexamer motif was developed before the *Androctonus* and *Limulus* hemocyanins diverged.[4]

IV. Cooperative Ligand Binding

Accurate data on ligand binding by hemocyanins has led to the development of several methods to handle the problems in modeling their oxygen-binding behavior. No single model seems to be appropriate to all the known hemocyanin experimental data. Four general classes of models appear in the recent liturature. Three types of models have been employed to explain the behavior of arthropod hemocanins and one to describe molluskan hemocyanin data.

The first model type is found in a recent example of the complexity of arthropod hemocyanin ligand binding in the study of the hexameric hemocyanin from the spiny lobster, *P. japonicus*.[38] This work demonstrates that even in such a simplified system as that made by reassembly of the individual monomeric subunits to form homohexamers, a two-state Monod, Wyman, and Changeux (MWC) type model[39] for cooperativity is not sufficient and a three-state concerted model is required. A similar result that necessitates extended or hybrid three-state MWC modeling is found for the allosteric properties of six- or 12-subunit hemocyanin from the blue crab *Callinectes sapidus*.[40]

The second general approach to theoretical treatment of ligand binding is encompassed in the idea of "Nested Allostery" (Figure 3). The concept was first applied to

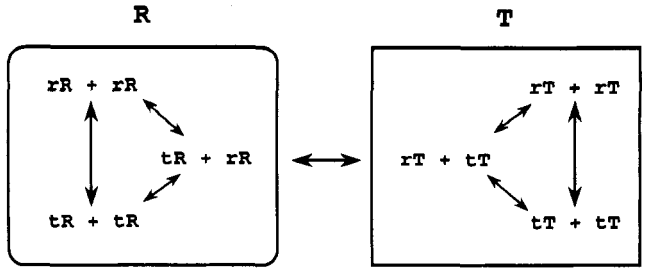


Figure 3. Schematic showing the allosteric states used in a nested allostery type model of cooperative ligand binding. The lower case letters indicate the oxygen affinity states within for example 12-mers, while upper case letters show the state of a higher order structure such as a 24-mer.

hemocyanins by Robert *et al.*[41] and was necessary to explain the cooperative oxygen-binding properties of the 24-subunit tarantula hemocyanin. The hemocyanin of the tarantula is composed of four hexameric units. The structural repeating unit of this hemocyanin is a dodecamer made of seven types of subunits termed *a*, *b*, *c*, *d*, *e*, *f*, and *g* present respectively in 2:1:1:2:2:2:2 copies. By studying intermediate structures of six, seven, 12, or 19 subunits Savel-Niemann *et al.*[42] showed that the cooperative behavior requires all of the subunits. Therefore the minimum structural repeating unit of the hemocyanin is not the minimum unit necessary to establish cooperative functional behavior.

The nesting concept, originally suggested by Wyman,[43,44] as applied to tarantula hemocyanin[41] is that there are four allosteric states: rR, tR, rT, and tT for each of the 12-mers as shown in Figure 3. In this case, there are allosteric interactions within each of the two 12-mers, but there are also interactions between the two 12-mers that make up the 24-mers. Among the possible combinations of these sets of allosteric units, only the rRrR, rRtR and tTtT, rTtT states are allowed for the 24-mer and equilibra exist among all of them.

The four states predicted by the nesting model for allosteric behavior as applied to the tarantula hemocyanin can be detected experimentally. A fluorescent probe, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole,[45] binds specifically to a lysine residue on only one of the seven types of subunits, *d*. The four different fluorescence emission maxima representing the different states reflect the differences in the environments of the probe and are dependent upon the pH and relative oxygenation of the hemocyanin molecules.

In a slightly later paper, Decker *et al.*[9] reported the necessary conditions to dissociate the hemocyanin of tarantula into its component parts. More importantly, the subunits can then be reassembled into 24-mers and the correctness of the reassembly verified using crossed immunoelectrophoresis. Because the different types of subunits may be modified so that they are in apo (copper free) or met (oxidized copper) forms before being reassembled into whole molecules, this paper also demonstrated that all the types of subunits contribute to cooperative oxygen binding.

Both the 12-subunit hemocyanin from the lobster, *Homarus americanus*, and the 24-subunit hemocyanin from *Eurypelma* described above, have had their complex oxygen-binding behavior modeled using nested hierarchies of allosteric units and in both cases protons are allosteric effectors.[46] Data obtained by binding oxygen and carbon monoxide competitively to tarantula hemocyanin is also satisfactorily fit using this type of model.[47] Properties of the 24-subunit hemocyanin from another scorpion, *Pandinus imperator* are also nicely described using a nesting model.[48] Interestingly, the oxygen affinities for the various allowed states in both *Eurypelma* and *Pandinus* are essentially the same, suggesting conservation of affinities among arachnids with similar subunit assemblies.

The third model system was deduced using molluskan, not arthropod, hemocyanin. The binding properties of oxygen and carbon monoxide were also used to study the molluskan hemocyanin from *O. dofleini*.[49] In this system, which comprises 70 oxygen binding sites with seven per polypeptide chain, the simple MWC model with allosteric unit size of seven and two types of sites *is* sufficient to describe the binding states. An automated procedure for determining models appropriate to analyze data to provide a best fit for this type system is given by Zhou *et al.*[50]

The fourth model type occurs in the arthropod hemocyanins, the 48-subunit complex (8 hexamers) from *L. polyphemus*. Once again a simple MWC treatment is insufficient to model accurately ligand binding data. Brouwer and Serigstad[51] propose an "interacting cooperative unit" model in which allosteric entities include not only hexameric T6 and R6 states but also dodecameric T12 and R12 states in equilibrium with each other. In the case of *Limulus* hemocyanin at neutral pH, the hexamers and dodecamers are demonstrated experimentally to have the same oxygen affinities in their R states, but the affinity of the dodecamers is higher than that of the hexamers in the T states. This model, like the others discussed above, incorporates the known physical subassemblies of hemocyanins into a formalism that is useful in modeling more complicated biological structures.

V. X-ray Crystallographic Studies

Single-crystal X-ray crystallographic studies have greatly increased the understanding of hemocyanins. Two types of arthropod hemocyanin structures have been determined by this method, that of the *P. interruptus* hexamer[2] and those of the *L. polyphemus* subunit II in the oxygenated[7] and deoxygenated[3] forms. Crystals of subunit *Aa*6 hemocyanin from *A. australis* are also reported,[52] but a high-resolution structure of this crystal form is not currently available. As yet, no crystal structure has been determined of any of the molluskan hemocyanins, although work on crystals of the carboxyl terminal domain, one "functional unit", of *O. dofleini* is in progress.[24] These crystals are of a 47-kDa proteolytic fragment that can reversibly bind one oxygen molecule.[20] Table 2 lists more detailed information on each of the hemocyanin crystal forms that have been reported. As the only high-resolution hemocyanin structures available are those of the arthropods *Panulirus* and *Limulus*, this section will discuss them in detail.

P. interruptus hemocyanin exists *in vivo* as a hexamer that consists of three different subunit types. Each subunit is approximately 76 kDa in molecular weight so that the hexamer is about 460 000 molecular weight.[10] Only two of the subunit types, *a* and *b*, were found in crystals of the *Panulirus* hemocyanin; they associate

Table 2. The Current Status of Crystal Structures of Hemocyanins

	color of crystals	maximum diffraction resolution, Å	structure available	Cu–Cu distance, Å	bound oxygen	ref(s)
P. interruptus hexamer	none	3.2	yes	2.9–3.8[a]	no	2
Oxygenated *L. polyphemus* subunit II	blue	1.9	yes	3.6 ± 0.2	yes	7,58
Deoxygenated *L. polyphemus* subunit II	none	2.1	yes	4.6 ± 0.2	no	3
A. australis subunit *Aa*6	blue	2.8	no	*b*	*b*	52
H. pomatia subunit *βc*	none/pale blue	10.0	no	*b*	*b*	*c*
carbonyl terminal fragment from *O. dofleini*	pale blue	1.9	no	*b*	*b*	24

[a] The range given is from the six subunit active sites within the hexamer. [b] Not yet determined. [c] Hazes, B, Ph.D. Dissertation, Rijksuniversiteit Groningen, The Netherlands, 1993.

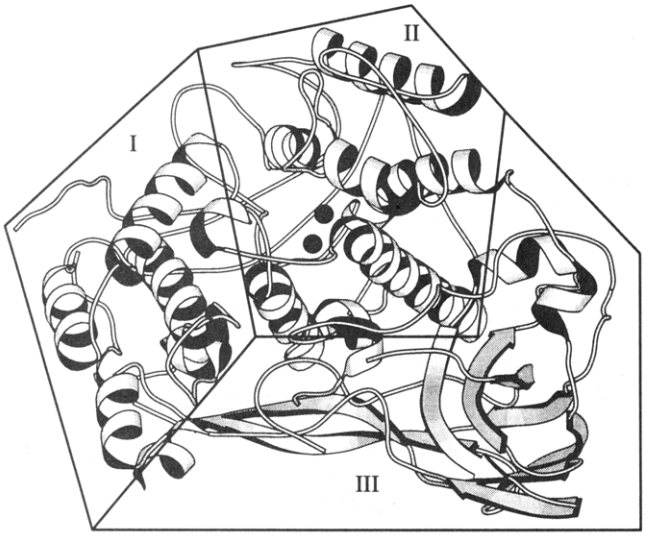


Figure 4. A ribbon diagram of the oxygenated *Limulus* subunit II hemocyanin crystal structure.[58] The α-helical secondary structural elements are represented by coils and the β-strand secondary structural elements are represented by sheets. The two copper atoms are shown as dark spheres. The approximate locations of the three folding domains found in this subunit are delineated in dark lines and labeled I, II, and III.

to form a hexamer similar in appearance and molecular weight to the native hemocyanin that contains all three subunits.[2] Subunits *a* and *b* are 97% identical in their primary structures and indistinguishable in the *Panulirus* crystal structure at 3.2 Å.[11] At this resolution, it is usually difficult to observe slight differences in amino acid sequence.[2] X-ray absorbance measurements have shown that these crystals are in a deoxygenated state.[53]

L. polyphemus hemocyanin consists of 48 subunits[54] of eight subunit types.[55] Of these, subunit II is the best characterized, with a molecular weight of about 73 kDa.[56] Purified subunit II forms hexamers similar to those of other arthropods including *Panulirus* and can bind oxygen cooperatively under certain conditions.[57] Both oxygenated and deoxygenated forms of the *Limulus* subunit II structure have been determined independently.[3,7]

The topologies of the *Panulirus* and *Limulus* hemocyanin subunits are the same (Figure 4).[58] As was apparent from the similarity of amino acid sequences, it is likely that all other arthropod hemocyanin subunits will also have similar tertiary structures to those presently known.[28] The *Panulirus* subunit structure was determined first and was shown to consist of three structural domains.[2] Domain I consists of the first 175 amino acid residues of the protein and has largely α-helical secondary structure. Domain II, also mostly helical in nature, consists of residues 176–400 and includes the two coppers that are the site of oxygen binding and the histidines that coordinate them. The remaining 257 residues (from 401–658) make up domain III. The third domain has a similar folding pattern to other proteins, most notably antibodies[59] and superoxide dismutase.[60] This so-called Greek key β-barrel folding motif seems to be an example of a non-functionally related folding pattern.[61] The same three domains are also found in the *Limulus* II structures, even though there are 29 fewer amino acid residues in the *Limulus* subunit II sequence.[3,7,28] Proteolytic cleavage of both *Panulirus* and *Limulus* hemocyanin subunits produces three separate fragments; in each case one of which appears to be the second domain.[62]

The three known crystal structures all reveal oxygen binding sites that are centered around two copper atoms and six histidine residues.[2,3,7] Each copper atom is ligated to the protein by N^{ϵ} atoms of three histidine imidazole rings. Both copper atoms are required to bind one molecule of oxygen.[5] As was discussed more fully in section II, these six histidines have equivalents in all known arthropod sequences.[3,28] Close examination of the three-dimensional arrangement of the arthropod hemocyanin dicopper active sites shows they possess a high degree of pseudo-2-fold symmetry.[63] This suggests that the dicopper active site may be a result of gene duplication and may thus have evolved from a protein that bound one metal ion.

There are other interesting structural features of the arthropod hemocyanin active sites. For example, the Cu–Cu distance seems to depend on the oxygen affinity state of the hemocyanin subunit. In the oxygenated form of *Limulus* subunit II, the Cu–Cu distance is 3.6 ± 0.2 Å.[7] On the other hand, the deoxygenated *Limulus* subunit II structure has a Cu–Cu distance of 4.6 ± 0.2 Å.[3] The closer Cu–Cu distance is presumably required to coordinate the oxygen molecule in the fashion described in the next section. There are also significant differences in the six Cu–N^{ϵ} distances between the oxygenated and deoxygenated forms of *Limulus* subunit II hemocyanin.[58] The *Panulirus* active sites, which have been shown to be deoxygenated,[53] have Cu–N^{ϵ} distances that more closely resemble the oxygenated *Limulus* subunit II site and may be the result of crystallization at low pH.[53,58] Further discussion of the active site is presented in section V.

Many amino acids that surround the dinuclear copper site are found in all the sequenced arthropod subunits including those from different parts of the primary structure.[3] These amino acids are mostly aromatic in nature and, like the six copper ligating histidines, may be required to form a functional oxygen binding site.

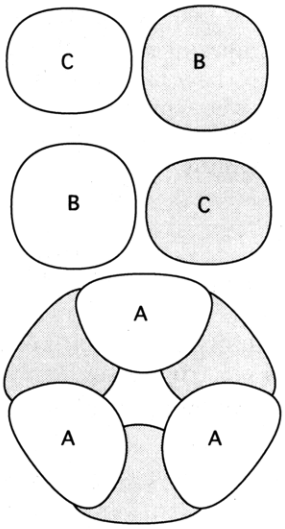


Figure 5. A schematic of an arthropod hemocyanin dodecamer. Positions of the subunits were taken from Lamy *et al.*[36] The lower hexamer is shown parallel to the 3-fold axis with the subunits labeled A being the ones involved in trimer interactions. The upper hexamer is viewed ~90° to the lower hexamer. This view shows the 2-fold interactions: that between the different-colored B and C subunits and that between the C and C subunits (The B and B subunit interactions are equivalent to the C and C subunit interactions).

The active sites are located at the core of the second domain of each hemocyanin subunit. No obvious path to the active site is accessible to the solvent without at least some readjustment of the structures although multiple potential channels through the protein that may connect the active site to the solvent exposed surface have been suggested.[2,3]

The known arthropod hemocyanin crystal structures all have some form of hexameric quaternary arrangement[2,3,64] like that seen in electron micrographs.[4] Three types of contacts are found in the arthropod hemocyanin hexamers. Two types of contacts make up dimers, and one type can be considered to form trimers of subunits. In the *Panulirus* crystal structure, the hexamer has most of its contacts between one of the types of dimers, so that the *Panulirus* hexamer can be thought of as constructed from a trimer of dimers.[2] On the other hand, the *Limulus* subunit II hexamers have more interactions in the trimer contacts than the dimers, consequently, this form is referred to as a dimer of trimers[3] (Figure 5). Analyses of the crystal structures have shown many of the amino acids responsible for the hexamer contacts.[65] The specific hexamer interactions are slightly different between the *Limulus* subunit II homohexamers and the *Panulirus* hexamer.[3] These interactions are mostly hydrophobic in nature and are located throughout the hemocyanin subunit's three domains.[2,3]

The most obvious contrast between the *Panulirus* and *Limulus* hemocyanin hexameric structures can be attributed to different relative positions of domain I with respect to domains II and III in the two species.[3,58] The *Panulirus* and *Limulus* hemocyanin structures can best be superimposed if domain I in one of the subunits is rotated by about 8° relative to domains II and III. The disagreement in contacts and quaternary structure may be solely a result of differences in their primary structures. A more interesting hypothesis is that the two species' structures are in different oxidation states as a result of their crystallization conditions. In this hypothesis, the *Limulus* subunit II structures would be in a low oxygen affinity form with allosteric effectors binding between domain I and the rest of the hemocyanin subunit. By determining more high-resolution structures of arthropod hemocyanins, especially in assorted cooperative oxygen affinity states, a clearer picture of cooperative oxygen binding should emerge.

VI. Chemical Properties of the Active Site

In a formal way, oxygenation of the arthropod hemocyanins may be thought of as changing the copper atoms from the Cu(I) to the Cu(II) state and the oxygen is bound as O_2^{2-}.[66] The ligation and binding-site geometries in models derived from X-ray crystallographic data of the oxygenated and deoxygenated forms of *Limulus* subunit II hemocyanin are consistent with such an interpretation[58] (Figure 6). In the oxygenated form of *Limulus* subunit II hemocyanin, the copper atoms are coordinated in approximately a square planar geometry by the two oxygen atoms and N^{ϵ} atoms of the four closest of the six histidine residues that coordinate the two copper atoms in the deoxygenated state.[7,58] This is a favorable coordination for the Cu(II) state of the oxygenated structure. This $\eta^2:\eta^2$ geometry of oxygen binding of oxygenated hemocyanin was found earlier in small, copper-containing compounds,[8] but was largely unexpected in hemocyanins

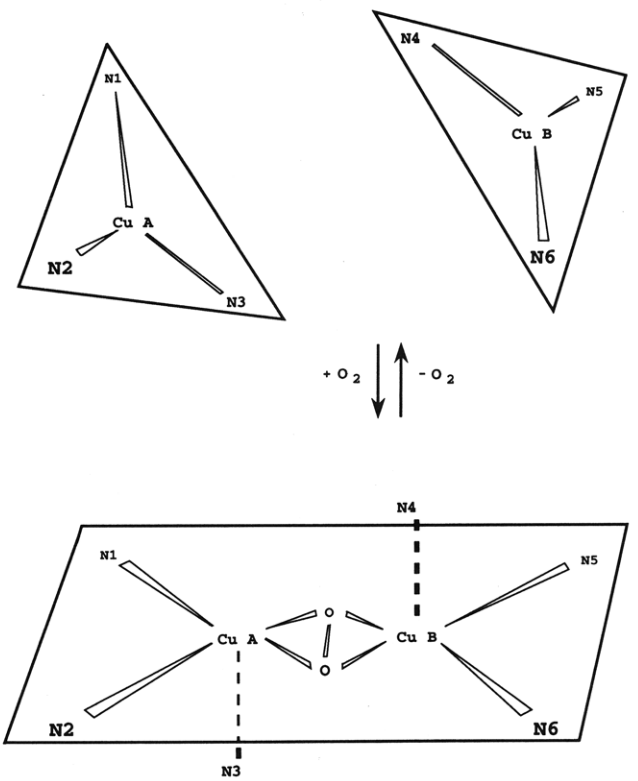


Figure 6. Schematic diagrams of the deoxygenated (top) and oxygenated (bottom) *Limulus* subunit II hemocyanin dicopper sites. In the deoxygenated state, each copper atom and its three ligating nitrogen are nearly coplanar (top). Each copper atom in the oxygenated *Limulus* II hemocyanin is strongly coordinated by the two closest nitrogen atoms of histidine residues and the oxygen atoms of the bound oxygen molecule (bottom). Cu A to N1 = 2.1 Å, Cu A to N2 = 2.1 Å, Cu A to N3 = 2.5 Å, Cu A to O = 2.0 Å, Cu B to N3 = 2.2 Å, Cu B to N4 = 2.4 Å, Cu B to O = 2.1 Å.

before the crystal structure of oxygenated *Limulus* II hemocyanin was determined.[58] In the deoxygenated *Limulus* subunit II form, the coppers are now ~4.6 Å apart so that they have relative little interaction. Each copper atom has essentially trigonal coordination by three of the six histidine residues,[3] consistent with the Cu(I) oxidation state.

The hemocyanin dicopper site is known to coordinate other compounds besides oxygen. Recently, particular interest has been paid to the binding of nitrous oxide[67-69] and hydrogen peroxide[70] to hemocyanins. NO reversibly binds to hemocyanin, producing nitrosylhemocyanin whose characteristics differ depending on the species of hemocyanin.[67-69] Nitrosylhemocyanin from *Astacus leptodactylus*[68] readily reverts to the unliganded state, whereas the *Helix pomatia*[69] and *Octopus vulgaris*[71] nitrosylhemocyanins are more stable. A possible mode of NO binding would be for it to displace one of the six histidine imidazole rings coordinating the copper atoms in arthropod hemocyanin.[68,69] If, in fact, the molluskan hemocyanins have five histidine ligands and one non-histidine ligand, one of the possibilities discussed in section II, their behavior toward ligands requiring displacement to bind might be as observed.

The same sort of heterogeneous reaction behavior by different hemocyanins is observed in their interactions with hydrogen peroxide.[5] Mollusk and some arthropod deoxygenated hemocyanins, including *Panulirus*, are able to convert H_2O_2 to molecular oxygen and water.[70,72-74] Interestingly, the catalase activity has also been correlated to the production of ATP from ADP in the *A. australis*.[75]

VII. Future Directions

The most recent knowledge about hemocyanins has led to a better understanding of these molecules structures, functions, and regulation. Future developments in this field are likely to be concentrated in expansions of the work considered here. Because of their large sizes and their compositions of multiple subunits or functional units, molluskan hemocyanins represent an as yet poorly understood level of organizational complexity for biological macromolecules. Thus work on modeling cooperative ligand binding behavior and its modulation by allosteric effectors will serve as a prototype for other systems of equivalent size and heterogeneity. Electron microscopic, small angle X-ray scattering, mass spectroscopic and X-ray crystallographic methods provide largely complementary types of information about hemocyanins. The synthesis of data from these techniques makes the study of larger, more complex structures a feasible enterprise.

The ability to dissociate whole hemocyanin molecules into components and reassemble them into specific structures is a particularly powerful technique for testing ideas about hemocyanins. In conjunction with molecular biological methods that allow engineered variants of hemocyanins to be designed and constructed, reassembly procedures open an overwhelming range of possibilities for testing ideas about the roles in structural, functional, and regulatory properties of hemocyanins. Designed primary structure variants of arthropod hemocyanins may be employed to test ideas about the chemical behavior of hemocyanins. The same methodology will be useful in attempts to create variants that are designed to have affinities for ligands other than oxygen. As more of the molluskan hemocyanin primary structures become available and with the determination of the three-dimensional structural information these approaches will also be extended to molluskan hemocyanins.

VIII. Abbreviations

CuA	copper A
CuB	copper B
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
cDNA	complementary deoxyribonucleic acid
His	histidine
MWC	Monod–Wyman–Changeux
N^{ϵ}	epsilon nitrogen
ATP	adenosine triphosphate
ADP	adenosine diphosphate

IX. References

(1) Volbeda, A.; Hol, W. G. J. In *Oxidases and Related Redox Systems*; King, T. E., Mason, H. S., Morrison, M., Eds.; Alan R. Liss. Inc.; New York, 1988; pp 291–307.
(2) Volbeda, A.; Hol, W. G. J. *J. Mol. Biol.* **1989**, *209*, 249–279.
(3) Hazes, B.; Magnus, K. A.; Bonaventura, C.; Bonaventura, J.; Dauter, Z.; Kalk, K. H.; Hol, W. G. J. *Protein Sci.* **1993**, *2*, 576–619.
(4) Lamy, J.; Compin, S.; Lamy, J. N. *Arch. Biochem. Biophys.* **1983**, *223*, 584–603.
(5) Van Holde, K. E.; Miller, K. I. *Quart. Rev. Biophys.* **1982**, *15*, 1–129.
(6) Miller, K. I.; Schabtach, E.; Van Holde, K. E. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1496–1500.
(7) Ton-That, H., Unpublished data, 1993.
(8) Kitajima, N.; Fujisawa, K.; Fujimoto, C.; Moro-oka, Y.; Hashimoto, S.; Kitagawa, T.; Toriumo, K.; Tatsumi, K.; Nakamura, A. *J. Am. Chem. Soc.* **1992**, *114*, 1277–1291.
(9) Decker, H.; Savel-Niemann, A.; Korschenhausen, D.; Eckerskorn, E.; Markl, J. *Biol. Chem. Hoppe-Seyler* **1989**, *370*, 511–523.
(10) Jekel, P. A.; Bak, H. J.; Soeter, N. M.; Vereijken, J. M.; Beintema, J. J. *Eur. J. Biochem.* **1988**, *15*, 403–412.
(11) Gaykema, W. P. J.; Hol, W. G. J.; Vereijken, J. M.; Soeter, N. M.; Bak, H. J.; Beintema, J. J. *Nature* **1984**, *309*, 23–29.
(12) Neuteboom, B.; Jekel, P. A.; Beintema, J. J. *Eur. J. Biochem.* **1992**, *206*, 243–249.
(13) Soeter, N. M.; Beintema, J. J.; Jekel, P. A.; Bak, H. J.; Vereijken, J. J.; Neuteboom, B. In *Invertebrate Oxygen Carriers*; Linzen, B., Ed.; Springer-Verlag: Berlin 1986; pp 153–163.
(14) Schartau, W.; Metzger, W.; Sonner, P.; Geisert, H.; Storz, H. In *Hemocyanin in Spiders, XXIII*; Hoppe-Seyler, B. C., Ed.; Walter de Gruyter & Co.: Berlin, 1990.
(15) Voll, W.; Voit, R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5312–5316.
(16) Voit, R.; Feldmair-Fuchs, G. *J. Biol. Chem.* **1990**, *265*, 19447–19452.
(17) Schartau, W.; Eyerle, F.; Reisinger, P.; Geisert, H.; Storz, H.; Linzen, B. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 1383–1409.
(18) Drexel, R.; Siegmund, S.; Schneider, H.-J.; Linzen, B.; Gielens, C.; Preaux, G.; Lontie, R.; Kellermann, J.; Lottspeich, F. *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 617–635.
(19) Lang, W. H. *Biochemistry* **1988**, *27*, 7276–7282.
(20) Lang, W. H.; Van Holde, K. E. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 244–248.
(21) Jolley, R. L.; Evans, L. H.; Makino, N.; Mason, H. S. *J. Biol. Chem.* **1973**, *249*, 335–345.
(22) Huber, M.; Lerch, K. *Biochemistry* **1988**, *27*, 5610–5615.
(23) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 243–247.
(24) Cuff, M. E.; Hendrickson, W. A.; Lamy, J.; Lamy, J. N.; Miller, K. I.; Van Holde, K. E. *J. Mol. Biol.* **1990**, *213*, 11–15.
(25) Naumann, U.; Scheller, K. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 963–972.
(26) Markl, J.; Burmester, T.; Decker, H.; Savel-Niemann, A.; Harris, J. R.; Suling, M. *J. Comp. Physiol.* **1992**, *162B*, 1284–1291.
(27) Telfer, H. W.; Kunkel, J. G. *Annu. Rev. Entomol.* **1991**, *36*, 205–228.
(28) Linzen, B.; Soeter, N. M.; Riggs, A. F.; Schneider, H.-J.; Schartau, W.; Moore, M. D.; Yokata, E.; Behrens, P. Q.; Nakashima, H.; Takagi, T.; Nemoto, T.; Vereijken, J. M.; Bak, H. J.; Beintema, J. J.; Volbeda, A.; Gaykema, W. P. J.; Hol, W. G. J. *Science* **1985**, *229*, 519–524.

(29) Herskovits, T. T.; Blake, P. A.; Gonzalez, J. A.; Hamilton, M. G.; Wall, J. S. *Comp. Biochem. Physiol.* **1989**, *94B*, 415–421.
(30) Herskovits, T. T.; Guzman, A. E.; Hamilton, M. G. *Comp. Biochem. Physiol.* **1989**, *92B*, 181–187.
(31) Herskovits, T. T.; Zou, J.; Hamilton, M. G. *Comp. Biochem. Physiol.* **1992**, *103B*, 447–453.
(32) Herskovits, T. T.; Blake, P. A.; Hamilton, M. G. *Comp. Biochem. Physiol.* **1988**, *90B*, 869–874.
(33) Herskovitz, T. T.; Gonzalez, J. A.; Hamilton, M. G. *Comp. Biochem. Physiol.* **1992**, *98B*(*2*/*3*), 271–278.
(34) Van Holde, K. E.; Miller, K.; Schabtach, E.; Libertini, L. *J. Mol. Biol.* **1991**, *217*, 307–321.
(35) Markl, J. *J. Comp. Physiol.* **1980**, *140*, 199–207.
(36) Lamy, J.; Billiald, P.; Taveau, J. C.; Boisset, N.; Motta, G.; Lamy, J. *J. Struct. Biol.* **1990**, *103*, 64–74.
(37) Boisset, N.; Taveau, J.; Lamy, J.; Wagenknecht, T.; Radermacher, M.; Frank, J. *J. Mol. Biol.* **1990**, *216*, 743–760.
(38) Makino, N. *FEBS Lett.* **1988**, *173*, 431–435.
(39) Monod, J.; Wyman, J.; Changeux, J. P. *J. Mol. Biol.* **1965**, *12*, 88–118.
(40) Johnson, B. A.; Bonaventura, C.; Bonaventura, J. *Biochemistry* **1988**, *27*, 1995–2001.
(41) Robert, C. H.; Decker, H.; Richey, B.; Gill, S. J.; Wyman, J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1891–1895.
(42) Savel-Neimann, A.; Markl, J.; Linzen, B. *J. Mol. Biol.* **1988**, *204*, 385–395.
(43) Wyman, J. *Curr. Top. Cell. Regul.* **1972**, *6*, 207–223.
(44) Wyman, J. *Q. Rev. Biophys.* **1984**, *17*, 453–488.
(45) Leidescher, T.; Decker, H. *Eur. J. Biochem.* **1990**, *187*, 617–625.
(46) Decker, H.; Sterner, R. *Naturwissenschaften* **1990**, *77*, 561–568.
(47) Decker, H.; Connelly, P. R.; Robert, C. H.; Gill, S. J. *Biochem.* **1988**, *27*, 6901–6908.
(48) Decker, H. *Biophys. Chem.* **1990**, *37*, 257–263.
(49) Connelly, P. R.; Gill, S. J.; Miller, K. I.; Zhou, G.; Van Holde, K. E. *Biochemistry* **1989**, *28*, 1835–1843.
(50) Zhou, G.; Ho, P. S.; Van Holde, K. E. *Biophys. J.* **1989**, *55*, 275–280.
(51) Brouwer, M.; Serigstad, B. *Biochemistry* **1989**, *28*, 8819–8827.
(52) Lamy, J. N.; Lamy, J. In *Invertebrate Dioxygen Carriers*; Preaux, G., Lontie, R., Eds.; Leuven University Press: Lovain, Belgium, 1990; pp 183–184.
(53) Volbeda, A.; Feiters, M. C.; Vincent, M. G.; Bouwman, E.; Dobson, B.; Kalk, K. H.; Reedijk, J.; Hol, W. G. J. *Eur. J. Biochem.* **1989**, *181*, 669–673.
(54) Brenowitz, M.; Bonventura, C.; Bonventura, J.; Gianzza, E. *Arch. Biochem. Biophys.* **1981**, *210*, 748–761.
(55) Sullivan, B.; Bonaventura, J.; Bonaventura, C.; Godette, G. *J. Biol. Chem.* **1976**, *251*, 7644–7648.
(56) Nakashima, H.; Behrens, P. Q.; Moore, M. D.; Riggs, A. F. *J. Biol. Chem.* **1986**, *261*, 10526–10533.
(57) Bonaventura, C. Duke University Marine Laboratory, personnel communication, 1993.
(58) Magnus, K.; Hazes, B.; Ton-That, H.; Bonaventura, C.; Bonaventura, J.; Hol, W. G. J. *Proteins*, in press.
(59) Pate, J. E.; Ross, P. K.; Thamann, T. J.; Reed, C. A.; Karlin, K. D.; Sorrell, T. N.; Solomon, E. I. *J. Am. Chem. Soc.* **1989**, *111*, 5198–5209.
(60) Tainer, J. A.; Getzoff, E. D.; Beem, K. M.; Richardson, J. S.; Richardson, D. C. *J. Mol. Biol.* **1982**, *160*, 181–217.
(61) Hazes, B.; Hol, W. G. J. *Proteins* **1992**, *12*, 278–298.
(62) Lello, Z.; Giambelli, L.; Maurizio, B. *Biochim. Biophys. Acta* **1990**, *1037*, 165–169.
(63) Volbeda, A.; Hol, W. G. J. *J. Mol. Biol.* **1989**, *206*, 531–546.
(64) Magnus, K. A.; Lattman, E. E.; Volbeda, A.; Hol, W. G. J. *Proteins* **1991**, *9*, 240–247.
(65) Brenowitz, M.; Bonaventura, C.; Bonaventura, J. *Biochemistry* **1984**, *23*, 879–888.
(66) Brown, J. M.; Powers, L.; Kincaid, B.; Larrabee, J. A.; Spiro, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 4210–4216.
(67) Tahon, J.-P.; Van Hoof, D.; Vinckier, C.; Witters, R.; De Ley, M.; Lontie, R. *Biochem. J.* **1988**, *249*, 891–896.
(68) Tahon, J.-P.; Gielens, C.; Vinckier, C.; Witters, R.; De Ley, M.; Preaux, G.; Lontie, R. *Biochem. J.* **1989**, *262*, 253–260.
(69) Tahon, J.-P.; Maes, G.; Vinckier, C.; Witters, R.; Zeegers-Huyskens, T.; De Ley, M.; Lontie, R. *Biochem. J.* **1990**, *271*, 779–783.
(70) Andrew, C. R.; McKillop, K. P.; Sykes, A. G. *Biochim. Biophys. Acta* **1993**, *1163*, 17–25.
(71) Salvato, B.; Giacometti, G. M.; Beltramini, M.; Zilio, F.; Giacometti, G.; Magliozzo, R. S.; Peisach, J. *Biochemistry* **1989**, *28*, 680–684.
(72) Felsenfeld, G.; Printz, M. P. *J. Am. Chem. Soc.* **1959**, *81*, 6259–6264.
(73) Topham, R. W.; Tesh, S.; Bonaventura, C.; Bonaventura, J. *Arch. Biochem. Biophys.* **1988**, *261*, 299–311.
(74) Huyart, N.; Calvayrac, R.; Briand, J.; Goyffon, M.; Vuillaume, M. *Comp. Biochem. Physiol.* **1983**, *76B*, 153–159.
(75) Vuillaume, M.; Ducancel, F.; Calvayrac, R.; Rabilloud, T.; Hubert, M.; Goyffon, M. *Comp. Biochem. Physiol.* **1989**, *92B*, 17–23.