

Functional Changes in the Family of Type 3 Copper Proteins During Evolution

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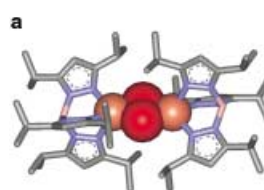
Introduction

When life developed on earth, it happened in a reducing atmosphere where oxygen was only a trace element. Thus, the first cells and organisms produced metabolites necessary for life under anaerobic conditions. Some 3500 million years ago some bacteria began to meet their energy needs by photosynthesis.^[1] Dioxygen as well as its highly reactive derivatives, hydrogen peroxide, superoxide anions, and hydroxyl radicals, were released as by-products and became a severe poison for the hitherto anaerobic cells. To cope with these highly reactive oxygen species and minimize the damage caused by them, different kinds of enzymes emerged. These enzymes, such as superoxide dismutase, catalase, peroxidase, monooxygenases, and dioxygenases, react with oxygen to yield harmless end products.

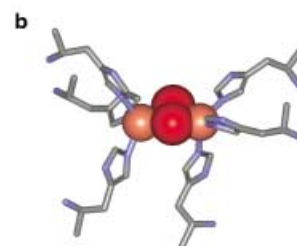
Many of these enzymes, such as tyrosinase (EC 1.14.18.1), incorporate oxygen into organic compounds by hydroxylation. This enzyme catalyzes two reactions, the hydroxylation of phenolic compounds in the *ortho* position (cresolase activity) and subsequently oxidation of diphenolic products (catecholase activity).^[2–4] Tyrosinase as well as catecholoxidase (EC 1.10.3.1), which catalyzes only the oxidation, belong to the group termed phenoloxidases. Their presence in all phyla of living organisms demonstrates their early origin in the history of life. Tyrosinases and catecholoxidases bind oxygen at copper-containing active sites, called type 3 copper sites. This distinction was originally based on their specific EPR spectra.^[2, 5] Their active site is a binuclear copper center consisting of two copper atoms, CuA and CuB, each coordinated by three histidines. The histidines are provided by two pairs of α -helices forming a four α -helix bundle motif (Figure 1).^[6, 7] Upon oxygen binding, a change of oxidation state is observed from Cu⁺ to Cu²⁺.^[2, 8]

The type 3 copper-protein family includes not only tyrosinases and catecholoxidases but also hemocyanins, which are found extracellularly in the hemolymph of various mollusks and arthropods. Hemocyanins are responsible for the precise delivery of oxygen, in analogy to the hemoglobins.^[7, 9–11] Tyrosinases and hemocyanins have been investigated independently over the years, although comparative data have been reported.^[3, 10–15] The increasing volume of sequences and biophysical/biochemical data have suggested a study of both proteins, in a comparative way. Such comparative studies of type 3 copper protein sequences have revealed the existence of two different protein classes, which differ in their CuA and CuB environments.^[7, 11, 16, 17] CuA denotes the copper binding site closer to the N terminus, whereas CuB is the copper binding site closer to the C terminus.

i) Dinuclear Cu Center

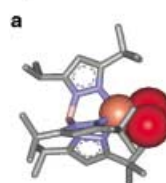


$[\text{Cu}(\text{HB}(3,5\text{-IPr}_2\text{pz})_3)]_2(\text{O}_2)$

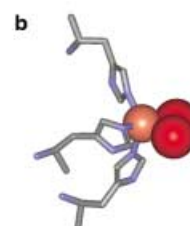


Limulus Hemocyanin

ii) Mononuclear Cu Centers



$\text{Cu}(\text{HB}(3\text{-tBu-5-IPr}_2\text{pz})_3)(\text{O}_2)$



primitive Cu Center

Figure 1. Structure of mono- and binuclear copper centers. i) Binuclear copper centers (orange) bind dioxygen (red) in a “side-on” coordination between two copper atoms as shown by a bioinorganic complex (ia) and the active site of the hemocyanin of the horseshoe crab *Limulus polyphemus* (ib).^[26, 30] ii) Crystallographic structure of a bioinorganic complex (iia), which is equivalent to a half active site of *Limulus polyphemus* hemocyanin (iib). It reveals that even a mononuclear copper center can bind oxygen in a “side-on” coordination.^[29] To date no protein has been described containing such a mononuclear active site.

Spectroscopic and EPR methods applied to hemocyanin and phenoloxidases strongly favor a very similar oxy structure of the active sites.^[2, 5, 18–24] According to X-ray data of crystal structures of arthropod and mollusk hemocyanins, a dioxygen molecule binds in the same way as a peroxide, that is, in a $\mu\text{-}\eta^2\text{-}\eta^2$ side-on coordination between the copper ions (Figure 1).^[25–28]

Thus, at least two different protein families exist within the phenoloxidase/hemocyanin protein family, depending on the

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origin of the binuclear copper site. In this review, we primarily focus on phenoloxidases and hemocyanins from arthropods. In this phylum the events of structural evolution can be best illustrated due to accumulation of functional and structural data in recent years. Both proteins fold in three domains with different folding motifs, where domain II always carries the active site.^[7]

In evolution only structures with essential biological function have been strongly conserved. Sequence comparisons and the unlikelihood of the binuclear type 3 copper site being formed instantaneously, suggest that the ancestral protein had a fold that bound only one copper. However, it has not yet been possible to answer this question by experimental evidence, since no enzyme has been found with a mononuclear center. In addition, the following theoretical question needs to be addressed: what function could such a putative mononuclear copper center, from which binuclear type 3 centers could evolve, have had? It should be pointed out that synthetic bioinorganic complexes with mononuclear copper centers that can coordinate oxygen in a way similar to binuclear centers, have been reported (Figure 1).^[29, 30] The advantage of a binuclear center over a mononuclear center is twofold. Firstly, the precise orientation of bound oxygen within the hydrophobic apolar pocket allows the existence of the Cu^+ state, which seems to be essential for the complex reaction of tyrosinases. Secondly, the spatial position of phenolic substrates can be better controlled by the protein matrix surrounding the binding pocket. Both

enhance the efficiency of an oxygen-metabolizing enzyme, since the correct orientation of the substrates with respect to one another is crucial for effective enzymatic catalysis.

Constitution of domain II: Mini-tyrosinases

Only a few options seem reasonable for the evolution of a binuclear center. One is gene duplication of a mononuclear center to create a minimal phenoloxidase.^[31] This mini-phenoloxidase essentially represents the second domain of today's arthropod hemocyanins (Figure 2). However, the active site of this small structure would exhibit uncontrolled access for phenolic substrates. Thus, this mini-phenoloxidase was always active, detoxifying dioxygen in the cell and keeping oxygen concentrations low. However, in the course of evolution it became advantageous to regulate this enzymatic activity. When aerobic metabolism evolved, it was no longer necessary or even advantageous to detoxify all the oxygen, since it was now used to produce large amounts of ATP. By that time, however, phenoloxidase would have acquired additional functions in other essential physiological processes such as in primary immune response, for example, by encapsulation of microbial invaders, sclerotization of the cuticle after molting, wound healing, and protective coloration. Thus, the possibility for regulation and discrimination between various functions became necessary.

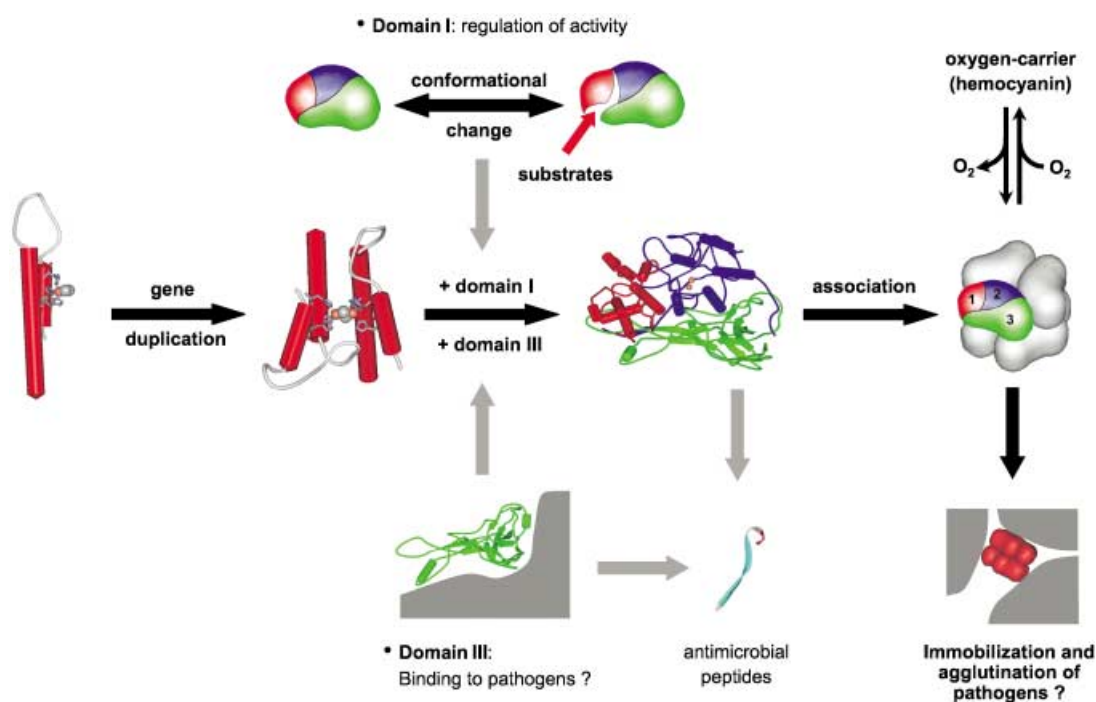


Figure 2. Structural evolution of the arthropod hemocyanin superfamily. The first type 3 copper proteins developed by gene duplication from mononuclear copper proteins which were already able to bind dioxygen. These minimal phenoloxidases detoxified oxygen but lacked regulation. They would have been comparable to domain II of present-day hemocyanin. Later domain I was added at the N terminus by gene fusion. Domain I regulates activity by controlling access of bulky phenolic substrates to the active site. Another gene fusion at the C terminus added domain III with an immunoglobulin folding motif which is a putative binding site to various surfaces and also the source of antimicrobial peptides in hemocyanins. Phenoloxidases aggregated to hexamers by self-assembly. This increased the concentration of active sites on pathogen surfaces and putatively enabled agglutination of pathogens. From this structure, cooperative oxygen carriers evolved.

Activity of domain I: Regulation

A mechanism of regulation was acquired by fusion with another α -helical protein at the N terminus (domain I) (Figure 2).^[32] This domain regulated enzymatic activity by controlling access to the active site for bulky phenolic substrates. The structural basis for the activation mechanism of inactive prophenoloxidase was deduced by comparison of phenoloxidase and hemocyanin sequences, based on resolved hemocyanin crystal structures.^[32, 33] A conformational change seems to be crucial for activation and this can be triggered in two different ways: either by proteolysis by specific proteases or binding of lipophilic substances.^[34–39] This conformational switch opens a binding pocket by exposing the chemically inert Phe49, which is conserved among all arthropod phenoloxidases and hemocyanins and serves as a “placeholder” for the tyrosine substrate, with its hydroxyl group pointing toward the bound dioxygen (Figure 3).^[33, 40]

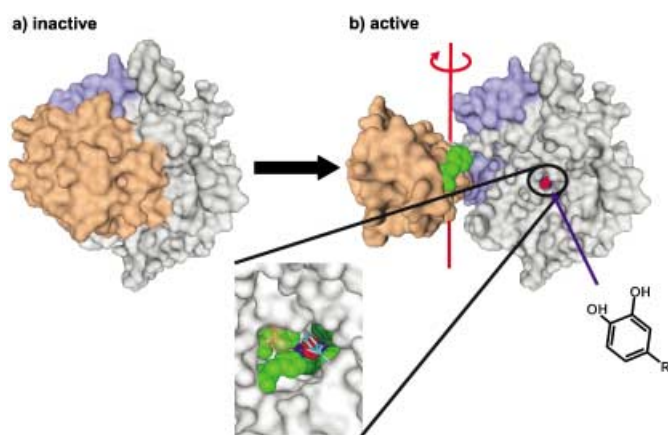


Figure 3. Activation of hemocyanin and phenoloxidase by removing the first domain shielding access to the active site. Arthropod phenoloxidase and hemocyanin are activated by a conformational change of the N-terminal domain as suggested for hemocyanin based on the crystal structure of the horseshoe crab *Limulus polyphemus*.^[40] a) The inactive form is activated by a rotation or removal of the N-terminal domain relative to domains II and III. Phe49, which normally blocks the active site as a pseudosubstrate, is removed by this rotation. b) View into the active site. Bulky phenolic substrates can clearly access the active site. Insert: enlarged view into the active site. The tyrosine substrate (turquoise), takes the place of Phe49. Histidines (bright green = CuA, dark green = CuB) coordinate the copper atoms (blue). Oxygen (red) is bound in a side-on configuration between the coppers.

The necessity of a conformational change even for proteolytic activation is also supported by experimental data on phenoloxidase from *Drosophila melanogaster* and *Bombyx mori*.^[39, 41] Although an N-terminal 50-amino-acid fragment is cleaved, this is not sufficient to sterically unblock the entrance to the active site. To achieve this, the complete N-terminal domain I (180 amino acids) has to be removed. It seems more likely that proteolysis releases tension in the backbone of the first domain, inducing a conformational change that causes activation.

Activation can also be achieved by lipophilic substances, such as lysolecithin, and detergents, such as sodium dodecylsulfate

(SDS) or cetylpyridinium chloride (CPC).^[34, 36] The molecular mechanism here is unclear. It is thought that these substances distort the protein matrix to favor a more relaxed conformation, thereby opening an entrance to the active site for substrates (Figure 3). While detergents are commonly used for in vitro activation, activation by physiological substances, such as lysolecithin, could be involved in wound repair as it may be released by cell rupture at the point of injury.^[34]

Addition of domain III: Support of the immune response

To produce the phenoloxidase existing today this two-domain phenoloxidase must have genetically fused a third domain containing a folding motif resembling immunoglobulins (Figure 2).^[42] The function of this domain is less obvious than for domain I. However, we want to present a hypothesis for the function of the third domain based on the fact that immunoglobulins belong to a protein family that is well known for its involvement in binding reactions and the observation that phenoloxidases are “sticky” enzymes that bind to a variety of surfaces.^[43–45] We propose that the function of domain III is to complement the enzymatic activity of phenoloxidase in immune response and wound healing by mediating a binding reaction to surfaces. This guarantees that an active phenoloxidase stays bound to a surface and will not float freely in the hemolymph. This hypothesis is further supported by recently published data that hemocyanin binds strongly to chitin when mediated by antimicrobial peptides such as tachyplesin.^[43] Furthermore, fungicidal peptides can be obtained from the third domain of hemocyanin by limited proteolysis.^[46, 47] These peptides must somehow interact with microorganisms in order to harm them. A common mode of action for antimicrobial peptides is to destroy the integrity of the cellular membrane by interacting with it in a detergent-like manner.^[48] The mode of action of antimicrobial peptides derived from hemocyanin is not known yet; it is likely that the structures of antimicrobial peptides may change in solution after they are cleaved off hemocyanin. However, Lee et al. (2003) observed that the antimicrobial peptide Astacidin1 from the crayfish *Pacifastacus leniusculus*, has a β structure in solution.^[47] Before cleavage, this peptide should also have a β -strand structure as inferred from comparison with the closely related hemocyanins from the spiny lobster (*Panulirus interruptus*) and the horseshoe crab (*Limulus polyphemus*).^[25, 42, 49] Therefore, we postulate that these antimicrobial peptides arose from a binding site for pathogen surfaces in domain III, which later in evolution acquired a new function by being cleavable, and therefore became a “free binding site” as an antimicrobial peptide. In this context it should be noted that hemocyanin itself exhibits antimicrobial activity which could be interpreted as a binding site for microbes on the hemocyanin, although the antimicrobial activity is about 50 times lower than that of the free peptides.^[50] Antimicrobial peptides cleaved from hemocyanin could also explain the subunit polymorphism observed with arthropod hemocyanins, which could then provide antimicrobial peptides with different specificities.

Hexamer assembly: Origin of cooperativity

The three-domain phenoloxidases of arthropods then gained the ability to form hexamers by self-assembly as demonstrated for two crustacean phenoloxidases by electron microscopy (Figure 2).^[51] However, was there any advantage in forming hexamers?

Firstly, forming a multimer increased stability against proteases, since the total surface of the almost-spherical hexamers is much smaller than that of the free kidney-shaped subunits. In addition, in the case of hexameric hemocyanin, most cleavage sites are hidden in the interior. Proteolysis of hemocyanins for at least three hours does not destroy the quaternary structure.^[52] Therefore, hexamers should be less prone to degradation by any proteases present in the hemolymph or secreted by invading microorganisms. However, during evolution the site for proteolytic activation was not hidden. Secondly, forming hexamers can produce a simple form of "cooperativity". In this context the term "cooperativity" is not used for homo- and heterotropic interactions with respect to oxygen or substrate binding. We use the term in the sense that binding of a subunit to a pathogen not only attaches the active site of one subunit on the pathogen, but brings the five other active sites of a hexamer into the close vicinity of the pathogen, thus increasing enzymatic activity. In addition, if two or more subunits bind to the same pathogen, an increase in binding strength, analogous to the IgM-pentamer, would also take place. Furthermore, when two subunits of a hexamer bind to different pathogens then agglutination of pathogens will occur.

Based on this reasoning, evolutionary pressure favored hexamer assembly to gain a more efficient immune response, and not to favor establishment of cooperative oxygen binding as is commonly discussed for hemocyanins. As discussed below, the structural basis for cooperative oxygen binding was already established in phenoloxidase.

Conversion of phenoloxidase to hemocyanin during evolution

Phenoloxidases have acquired additional properties but lost some original characteristics during their evolution into hemocyanin. They seem to have had the ideal predisposition for being converted to an oxygen-transporting protein. Only three major changes were necessary:

- 1) Phenoloxidase had to be permanently inactivated, so that the active site was no longer accessible to any ligands larger than gas molecules such as oxygen. To achieve this, only small changes in domain I were necessary: either the proteolytic cleavage site for activation of prophenoloxidase had to be removed or interaction between the first and second domains had to be strengthened to make activation impossible.
- 2) To improve reversible oxygen binding and reduce enzymatic activity, a change in the electronic structure of the active site had to take place. Phenoloxidases are already able to bind oxygen reversibly.^[2, 5] An essential difference from hemocyanins is that the active site of phenoloxidase exists to a large extent in the oxidized 'met' state, which cannot bind

dioxygen.^[53, 54] However, the differences in electronic structure between the active sites of phenoloxidases and hemocyanins are not at all clear.

- 3) Cooperative binding of oxygen to hemocyanins had to be established to ensure efficient and flexible oxygen transport. The most essential prerequisite for cooperativity, the ability to assume at least two conformational states, was already established in the phenoloxidase hexamer by adopting an active or inactive state. A comparison between the oxygenated and deoxygenated states of arthropod hemocyanins has revealed that the main difference between the two states, apart from a movement of the coppers at the active site by more than 1 Å, is a slight rotation of the first domain by 8° relative to the second (Figure 4).^[26] The crystal structure also

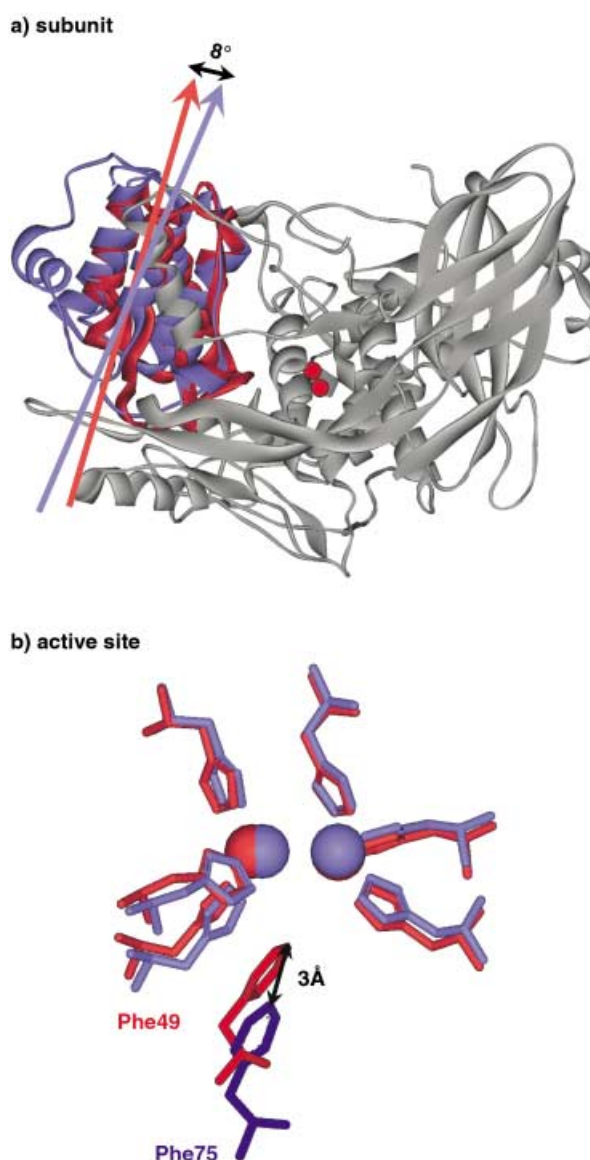


Figure 4. Movement of domain I in arthropod hemocyanin upon oxygenation. a) Domain I of the subunit is rotated by 8° relative to domain II and domain III based on the comparison of crystal structures. b) This movement withdraws Phe49 (or its equivalent Phe75) by almost 3 Å from the active site. Red = oxy structure (*Limulus polyphemus*), blue = deoxy structure (*Panulirus interruptus*).^[25, 26]

reveals a large movement of the essential and conserved Phe49. However, a pronounced movement of the first domain must occur to pull out the placeholder Phe49 and open the entrance for substrates (Figure 3). The tight hexamer assembly would then transfer the conformational change of one subunit to the other subunits, as is known for hemocyanins.

Phenoloxidase activity of hemocyanin

The obvious structural similarity between hemocyanins and phenoloxidases is demonstrated by the fact that, for almost all hemocyanins, a phenoloxidase activity can be induced by detergents or proteases.^[49] In most cases, especially in crustaceans, the activity is very weak. This is believed to be due to the fact that the binding site for phenolic substrates has degenerated during evolution.

However, in some chelicerates, a strong phenoloxidase activity has persisted in hemocyanins until the present. Tarantula hemocyanin from *Eurypelma californicum* is comparable to phenoloxidases based on activation mechanism, substrate specificity and inhibition.^[55, 56] The same holds for hemocyanins from the horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus*.^[43, 46, 55] All three are ancient chelicerate species and unlike more modern crustaceans, myriapods and insects, no true phenoloxidases have been found in their hemolymphs to date. However, since these arthropod animals also need a phenoloxidase, it has been proposed that hemocyanin fulfills the functions of phenoloxidase in these chelicerates.^[33, 43, 46, 55] The lack of a signal peptide and synthesis in hemocytes of phenoloxidases (as is also the case for chelicerate hemocyanins), emphasizes the close tie between phenoloxidases and chelicerate hemocyanins.^[16] In contrast, modern crustacean hemocyanins have signal peptides and are synthesized in the hepatopancreas.^[16]

However, in *Eurypelma californicum* and *Limulus polyphemus* phenoloxidase activity is confined to only a few subunit types. In both species these subunits link hexamers, which build up the multihexamers (24-mer and 48-mer, respectively).^[55] For the hemocyanin of *Eurypelma californicum*, sequences of all subunit types are known, and phylogenetic analysis has revealed that the two subunits possessing phenoloxidase activity separated from the others quite early in evolution.^[57] This implies that some original properties of phenoloxidases were conserved in these two subunits, although they gained the functional property of the other subunits to bind oxygen reversibly, as an oxygen carrier with similar oxygen affinities.^[58] Therefore, these two subunit types may be considered as "transitional" structures between phenoloxidases and hemocyanins.

In vivo role of hemocyanins in the immune response

One may still ask whether the phenoloxidase activity of hemocyanins induced by substances such as detergents, proteases, alcohols, or salts is an in vitro experimental artifact or if it can also be found in vivo. Horseshoe crabs are model organisms for the innate immune response, and their blood-

coagulation cascade has been investigated in great detail since it is widely used as an assay for bacterial endotoxins.^[59, 60] In horseshoe crabs, hemocyanins can be activated either by activated factor B of the clotting cascade or the clotting enzyme itself.^[46] Although both proteins are active proteases, they do not activate hemocyanin by proteolysis, but by forming a 1:1 complex with hemocyanin. A common structural feature of clotting enzyme and factor B is a clip domain, which, it has been suggested, mediates the interaction with hemocyanin and subsequently causes a conformational change in the hemocyanin.^[46] The formation of a 1:1 complex instead of proteolytic activation could be a regulation mechanism to confine activation of hemocyanin at the site of injury in vivo. Hemocyanin also seems to be activated by several antimicrobial peptides such as tachyplesin, tachystatins and big defensin, which are also released from hemocytes like proclotting enzyme and factor B when a defense reaction is initiated.^[43, 61] When antimicrobial peptides such as tachyplesin are bound to pathogens they might mediate the binding of hemocyanins to these pathogens and induce the activation of hemocyanin. Thus, in addition to limited proteolysis and lipophilic substances, another new activation mechanism for hemocyanin has been described: the binding of special activation proteins and/or peptides.^[43, 46] If this kind of activation is also possible for phenoloxidases remains to be investigated.

The role of hemocyanins in immune response seems to be twofold and not only present in chelicerates but also in crustaceans. Whereas hemocyanin can act as a phenoloxidase in chelicerates, in crustaceans antimicrobial peptides can be cleaved from the C-terminal domain of hemocyanin. The specificity of the antimicrobial peptides depends on the species. While peptides originating from hemocyanin of *Penaeus vannamei* and *Penaeus stylirostris* are antifungal, those from *Pacifastacus leniusculus* are antibacterial.^[47, 50] Antimicrobial peptides are liberated upon immunological challenge of crustaceans. Nevertheless, the mechanism to produce these antimicrobial peptides is not known yet. Interestingly hemocyanin itself could be an antimicrobial substance. The antifungal action of hemocyanin is 50 times less than that of the antimicrobial peptides derived from hemocyanins. But hemocyanin concentration is much higher, and concentrations of 20 mg mL⁻¹ are commonly found in the hemolymph.^[50] Thus, hemocyanin could very well be an antimicrobial substance in the hemolymph, when mass action is accounted for.

Role of hemocyanin in wound repair and molting

Besides the immune response, hemocyanins in chelicerates are likely to be involved in wound repair and molting. Although hemocyanin from *Tachypleus tridentatus* itself has no affinity to bind to chitin, it readily binds to chitin coated with the antimicrobial peptide tachyplesin and is subsequently activated to become a phenoloxidase.^[43] Two major precursors for cuticle tanning in insects, *N*- β -alanyldopamine and *N*-acetyl-dopamine are good substrates for *Tachypleus* hemocyanin activated by tachyplesin. *N*-acetyldopamine is also a preferred substrate for

hemocyanin of the tarantula *Eurypelma californicum* when activated with mild proteolytic treatment or the detergent SDS.^[40, 43, 56] Hemocyanin fragments of the same tarantula species were identified as a component of the protein matrix of the cuticle.^[62] Hence, hemocyanin might be involved in two different ways in wound healing. When cuticle chitin is exposed by injury, hemocyanin seems to be activated by the clotting cascade, and be bound to exposed chitin primed by tachyplesin. During the sclerotization reaction, hemocyanin itself is thought to be incorporated into the cuticle as part of the protein matrix.^[62] In the absence of an injury, however, hemocyanins are not in contact with the cuticle, which is shielded from the hemolymph by the epidermis. Nevertheless, phenoloxidases from the silkworm *Bombyx mori*, which are synthesized in hemocytes are transported via the epidermis to the cuticle.^[63] A similar but still unclear mechanism could also be possible for hemocyanins.

Thus, hemocyanin might not only be involved in sclerotization during wound repair but also after molting.^[64] Note that, in this context, it has been suggested that the hemocyanin of the tarantula *Eurypelma californicum* binds ecdysteroids by the first domain.^[65] However, it is still not known whether binding of an ecdysteroid molecule in the first domain is involved in inducing conversion of hemocyanins to a phenoloxidase.

Evolution of mollusk-related phenoloxidases

Our hypothesis on the functional conversion of phenoloxidase to hemocyanin in arthropods does not yet explain the corresponding evolution of catecholoxidase/tyrosinase to mollusk-related hemocyanin, although, a crystal structure of catecholoxidase from potatoes has been resolved.^[6] In contrast to arthropod hemocyanin subunits (~72 kDa) the subunits from molluskan hemocyanin are very large (~400 kDa) and fold in 7–8 structurally comparable functional units (FU).^[7, 11, 66] These functional units (~50 kDa) are functionally comparable to subunits from arthropod hemocyanin and both possess one active site, which binds one molecule of dioxygen reversibly (Figure 5). The crystal structures of functional units are almost superimposable with the structure of catecholoxidase.^[67] These proteins are characterized by two domains, lacking domain I of arthropod hemocyanins.^[27, 28] In addition, the CuA environment differs from the corresponding arthropod site as it involves an odd covalent cysteine–histidine bond. This bond stabilizes the pair of α -helices at the copper site, which in these proteins are very short and therefore not stable.^[6, 27] Thus, a gene fusion of the two different copper sites (CuA and CuB), rather than a gene duplication, is assumed to have yielded the type 3 copper centers in these proteins. In order to control the entrance to the active site, a C-terminal domain was genetically added that has the structure of a squeezed β -barrel. However, this domain III takes the topological position occupied by domain I in arthropod hemocyanins (Figure 5). As in arthropod hemocyanins, a conformational switch of this regulatory domain is necessary to control the access of phenolic substrates to the copper center.^[33] This hypothesis is supported by a comparison of the crystal

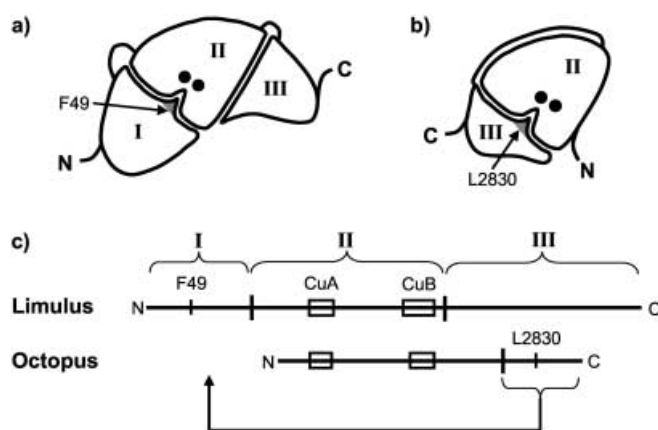


Figure 5. Scheme of the topological positions of the three domains in arthropod hemocyanins and the two domains in a functional unit of molluskan hemocyanin. Domain II carries the active site with the two copper atoms. Domain III in the functional unit (b) takes the topological position of domain I in arthropod hemocyanin (a). Depending on the phylum, Ph49 or L2830 sterically closes the entrance to the active site.^[33] N and C, denote the N and C termini.

structures of molluskan hemocyanins in the oxy- and deoxy-states, where upon deoxygenation a large channel to the active site becomes visible.^[27, 28] Thus, the activation mechanisms of molluskan- and arthropod-related tyrosinases and hemocyanins seem to be based on a very similar conformational transition, albeit involving very different protein structures.

Open question

Various crystal structures of type 3 copper proteins have been resolved. Nevertheless a difference between the simple oxidation of diphenols and the chemically very demanding hydroxylation of phenols in the *ortho* position cannot be deduced on a molecular basis yet.^[2, 5, 23] Elucidation of the latter enzymatic activity will only be possible by future analysis of the tyrosinase crystal structure. However, based on analysis of catecholoxidase and hemocyanins some possible chemical reaction mechanisms can already be excluded.^[68]

Keywords: antimicrobial peptides • hemocyanin • innate immunity • metalloproteins • molecular evolution • phenol-oxidase

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