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REVIEW

COPS AND ROBBERS: PUTATIVE EVOLUTION OF COPPER OXYGEN-BINDING PROTEINS

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Summary

Two closely related copper proteins, phenoloxidase and haemocyanin, are known to be involved in different physiological functions such as the primary immune response and oxygen transport. Although the proteins differ structurally, they have the same active site by which dioxygen is bound. Recent results reveal that haemocyanin also exhibits phenoloxidase activity. A scenario is proposed for the evolutionary relationships among copper oxygen-binding proteins (COPs).

Key words: copper oxygen-binding protein, evolution, phenoloxidase, haemocyanin, oxygen transport, immune response.

The earth's atmosphere changed from anaerobic to aerobic as oxygenic photosynthetic organisms evolved. Organisms had to develop different strategies to handle a final product of photosynthesis, dioxygen (O_2), as well as its highly reactive derivatives, superoxide anions, hydrogen peroxide and hydroxyl radicals. Several enzymes, such as superoxide dismutase, catalase and peroxidase, evolved that protected the organisms. A particular class of protein, the copper oxygen-binding proteins (COPs), is of special interest, since organisms used these proteins to help harness oxygen. Primitive COPs eventually developed multiple functions. COPs may first have become robbers in the form of phenoloxidases to keep the amount of free dioxygen low. The enzymatic action of phenoloxidase robs free dioxygen of its molecular status, inserting one of the oxygen atoms into a phenol and releasing the other as water. As organisms became larger and circulatory systems evolved in multicellular organisms, COPs along with iron oxygen-binding proteins became oxygen-transport proteins, storing dioxygen and moving it from places of high concentration to places of low concentration within the body. Thus, organisms used COPs both to protect themselves against the highly reactive atmospheric dioxygen and to utilize this energy source.

These considerations are supported by two recent observations that may also provide new insight into the evolutionary strategies by which organisms deal with dioxygen. During the past few years, several sequences of phenoloxidases (EC 1.14.18.1, tyrosinase) from arthropods have been published which reveal that the phenoloxidases are related to the arthropod haemocyanins (Aspan et al., 1995; Fujimoto et al., 1995). During the same period, haemocyanins from arthropods and molluscs, well-known as oxygen-transport proteins, were shown to function as phenoloxidases under some conditions (Zlateva et al., 1996; Salvato et al., 1998; Decker and Rimke, 1998).

Phenoloxidase catalyzes the incorporation of oxygen into phenolic molecules in a two-step reaction (Salvato and Beltramini, 1990; Sanchez-Ferrer et al., 1995; Solomon et al., 1996): First, a monophenol is orthohydroxylated (monophenol oxidase activity), and the resulting *o*-diphenol is then oxidized to an *o*-quinone (catecholase activity) (Fig. 1). As a result of this reaction, one oxygen of the bound dioxygen is incorporated (Mason, 1955). Thus, the enzyme is an oxygenase as well as an oxidase. The phenoloxidase reaction is found in fungi and plants as well as in animals: phenoloxidase is involved in wound healing, in skin pigmentation and in the browning of fruits and vegetables (Prota, 1992; van Gelder et al., 1997). The reaction is thought to protect plants and animals against intruders by forming melanin to encapsulate the intruders or to create an impervious scab (Anderson, 1991; Ashida and Yamazaki, 1990; Sugumaran, 1990; Barret, 1991). In insects, phenoloxidase initiates sclerotization of the new exoskeleton after moulting. In all cases, monophenolic derivatives such as L-tyrosine are the primary substrates that are converted to dopaquinone derivatives.

What do the two groups of copper oxygen-binding proteins, haemocyanins as oxygen-transport proteins and phenoloxidases as enzymes, have in common? They share several physico-chemical properties (Jolly et al., 1972; Lerch, 1981, 1987; Kuiper et al., 1980; Himmelwright et al., 1980; Salvato and Beltramini, 1990; Beltramini et al., 1990; Solomon et al., 1994, 1996; Ling et al., 1994), which led to the

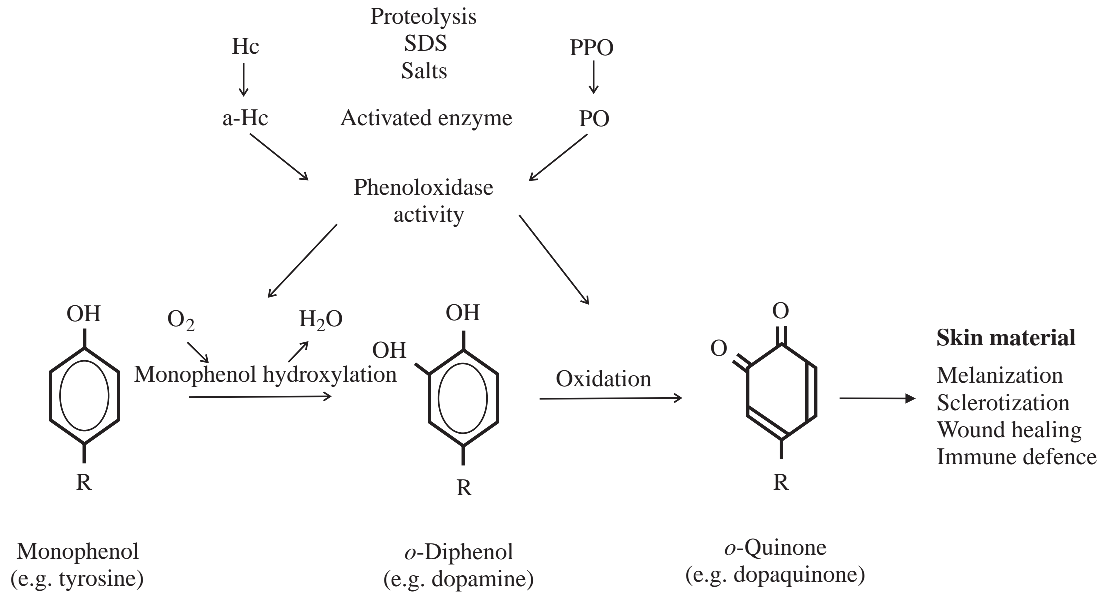

Fig. 1. Incorporation of dioxygen (O_2) into phenolic compounds. Monophenolic compounds such as tyrosine are hydroxylated by incorporation of one oxygen of the dioxygen molecule. The product, an ortho-diphenol compound such as dopamine, is then oxidized to an ortho-quinone compound. In both reactions, the monophenoloxidase and diphenoloxidase/catecholase activity, the same copper dioxygen sites of phenoloxidases/tyrosinases are involved. Hc, haemocyanin; PPO, prophenoloxidase; PO, phenoloxidase; R, alkyl group.

hypothesis that they should have a common active site, by which dioxygen bridges the two copper atoms (CuA ad CuB) in a side-on configuration (Fig. 2). This has been demonstrated by Raman spectroscopy (Ling et al., 1994), by a synthesized copper/dioxygen binding complex (Kitajima and Moro-oka, 1994) and by the X-ray structure of the oxy-form of the haemocyanin of *Limulus polyphemus* (Magnus et al., 1994). Many years ago, preliminary studies had indicated that molluscan haemocyanin had some phenoloxidase activity

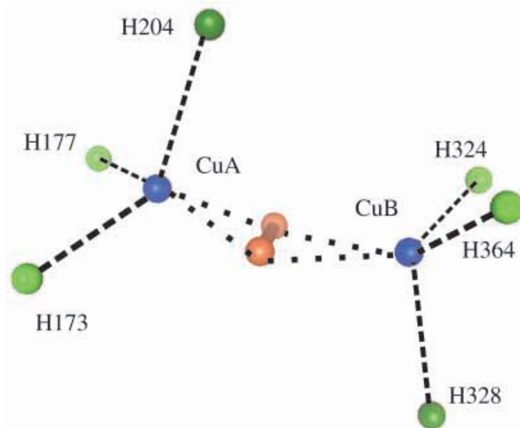

Fig. 2. The active site of copper oxygen-binding proteins. Dioxygen is bound in a side-on configuration between the two copper atoms involved according to the X-ray structure of the oxy-form from *Limulus polyphemus* haemocyanin (Magnus et al., 1994) and a synthetic compound (Kitajima and Morooka, 1994). CuA and CuB are the two copper atoms involved.

(Bhagvat and Richter, 1938; Salvato et al., 1983; Nakahara et al., 1983; Beltramini et al., 1990). The ability of both molluscan and arthropod haemocyanins to exhibit phenoloxidase activity, however, has been demonstrated in detail in the past few years (Zlateva et al., 1996; Decker and Rimke, 1998; Salvato et al., 1998). We now know that phenoloxidase activity in haemocyanins is activated by proteolysis or by partial unfolding of the protein structure in response to treatment with salts or sodium dodecyl sulphate (SDS). This allows not only molecular dioxygen but also larger phenolic compounds access to the active site by enlarging its entrance.

Despite the similarities in active site and function, a comparison of haemocyanins from arthropods and molluscs with phenoloxidases from a variety of phyla reveals that these are very different proteins with respect to size, to primary, tertiary and quaternary structure and to physiological functions. Arthropod and molluscan haemocyanins are freely dissolved in the haemolymph and reversibly bind dioxygen between two copper atoms. Each of the two copper atoms is bound in a complex manner by three histidines at either the CuA or CuB binding region within the active site (Fig. 2). The two haemocyanin types share little primary structure, however, and have completely different tertiary and quaternary structures. Arthropod haemocyanins consist of kidney-shaped subunits, each of which has three domains characterized by different folding motifs (Volbeda and Hol, 1989a; Markl and Decker, 1992). Domain I consists of 6–7 α-helices, while domain II carries the active site. Here, two copper atoms are bound by a bundle of four α-helices. Domain III consists of a seven-stranded β-barrel. According to electron micrographs,

the basic oligomer of arthropod haemocyanins consists of six subunits assembled to form a cubic structure (van Heel and Dube, 1994). Depending on the species, these hexamers (6-mers) or an integer number of them, such as 2×6-mers, 4×6-mers, 6×6-mers and 8×6-mers, are found. One active site is found per subunit with an M_r of approximately 75×10^3. In molluscan haemocyanins, 7–8 functional units are covalently linked to give a much larger subunit with an M_r of approximately 350×10^3 to 450×10^3 (van Holde and Miller, 1982, 1995). Ten of the large subunits are arranged to build a basic cylindrical structure. Depending on the species, either 10-mers or 20-mers occur in the haemolymph. A comparison of the sequences of arthropod and molluscan haemocyanins reveals that the CuB sites are more similar with respect to sequence identity between phyletic types than the CuA sites, and that the arthropod domain I is absent from each functional unit of the molluscan haemocyanin subunit (Drexel et al., 1987; Miller et al., 1998). On the basis of X-ray structures, however, the folding motifs in domains II and III seem to be very similar in the two phyla (Volbeda and Hol, 1989a,b; Hazes et al., 1993; Cuff et al., 1998). In molluscan haemocyanins, as in arthropod haemocyanins, domain II consists of α-helices carrying the dioxygen binding centre, and domain III consists of β-sheets arranged in a six-stranded β-sandwich. Its topology is a truncated version of the jelly-roll motif instead of a seven-stranded β-barrel as in arthropod haemocyanin. What is interesting is that domain I of arthropod haemocyanins and domain III of molluscan haemocyanins perform similar roles in protecting the active site, despite their structural differences, and that the two domains III of arthropod haemocyanin and mollusc haemocyanin, both β-structures, do not interact with the active site similarly.

Not as much is known about the structure of phenoloxidases. While haemocyanins are strictly conserved as hexamers or cylinders, phenoloxidases are reported to exhibit a broad variety of subunit assemblages into oligomers such as dimers, trimers and higher multiples (Jiang et al., 1997). Comparison of the primary structures of arthropod phenoloxidases with phenoloxidases from other organisms reveals a low percentage of sequence similarity, although they catalyze the same reaction (for a review, see van Gelder et al., 1997). Phenoloxidases and haemocyanins share the same active site structure with two copper atoms being bound, as revealed by their spectroscopic properties (Solomon et al., 1994, 1996) and by structural studies including a recent determination of the X-ray structure of the *met* form of an *o*-diphenol oxidase from a sweet potato (Klabunde et al., 1998). In this oxidase, the CuA and CuB sites are bound by three histidines each. Since no structure for an *o*-diphenol oxidase with bound oxygen has yet been published, a comparison of the mode of binding dioxygen is not possible. On the basis of sequence analysis, a close relationship has been reported for arthropod COPs, especially between crustacean and chelicerate haemocyanins and insect phenoloxidases (Aspan et al., 1995; Fujimoto et al., 1995; Burmester and Scheller, 1996; Durstewitz and Terwilliger, 1997; Terwilliger et al., 1999). However, phenoloxidases and haemocyanins in general show obvious differences with respect to their biological functions and chemical mechanisms. While haemocyanin transports dioxygen without changing it, phenoloxidase uses one oxygen of the dioxygen molecule for a chemical reaction.

Besides having similar copper oxygen-binding sites, phenoloxidases and haemocyanins have another feature in common: their enzymatic properties can be activated in similar ways by disturbing the protein structure. Phenoloxidases are synthesized as inactive prophenoloxidases, which are activated by serine proteases cleaving an N- or C-terminal part (van Gelder et al., 1997). In invertebrates, this is the final step of the so-called phenoloxidase-activating system of the cascade of the primary immune response (Smith and Söderhäll, 1991; Söderhäll and Cerenius, 1998). By analogy with these processes, spider haemocyanin can also be activated through proteolytic cleavage of the N-terminal part, which provides better access for large substrates to the active site (Decker and Rimke, 1998). As a phenoloxidase, haemocyanins from arthropods and molluscs also exhibit *o*-diphenoloxidase activity when exposed to salts or SDS (Inaba and Funatsu, 1964; Moore and Flurkey, 1990; Zlateva et al., 1996; van Gelder et al., 1997; Salvato et al., 1998). This type of activation is thought to be due to a distortion of the protein matrix facilitating nonspecific access of substrates to the active site of type 3 copper proteins. Therefore, SDS is used in an assay for *o*-diphenoloxidase activity (Sugumaran and Kanost, 1993). In contrast to arthropod haemocyanin, a low pH of 6 is sufficient to induce *o*-diphenoloxidase activity in mollusc haemocyanin (Salvato et al., 1998).

To explore the question of how phenoloxidases and haemocyanins evolved, one may consider that the ancient atmosphere provided a reducing environment and contained only traces of dioxygen. The occurrence of dioxygen as a consequence of photosynthesis was a threat to living organisms, which developed different strategies to neutralize this oxygen and its derivatives. One putative way to achieve this was to develop primitive metal/protein complexes that incorporated transition metals such as copper or iron. These complexes were able to bind and then release dioxygen *via* the metal ions. On the basis of currently available X-ray structures from haemocyanins and haemoglobins, a pair of antiparallel α-helices probably provided the histidines or cysteines that bound the metal ions (Volbeda and Hol, 1989a; Markl and Decker, 1992; van Holde, 1998). These proteins could be considered the first primitive oxygen-binding proteins (Fig. 3). A gene duplication and fusion (Figs 2, 3) would then have resulted in a well-known folding motif, the four α-helix bundle, which provides the correct dioxygen-binding pocket. This may have been the mode of evolution for all major oxygen-binding proteins, haemoglobins, haemerythrins and arthropod haemocyanins (Volbeda and Hol, 1989a,b). On the basis of recent sequence comparisons and the X-ray structures of a molluscan haemocyanin dioxygen-binding domain and of a plant *o*-diphenol oxidase, a gene fusion of two independently evolved mono-copper binding sites may also have occurred

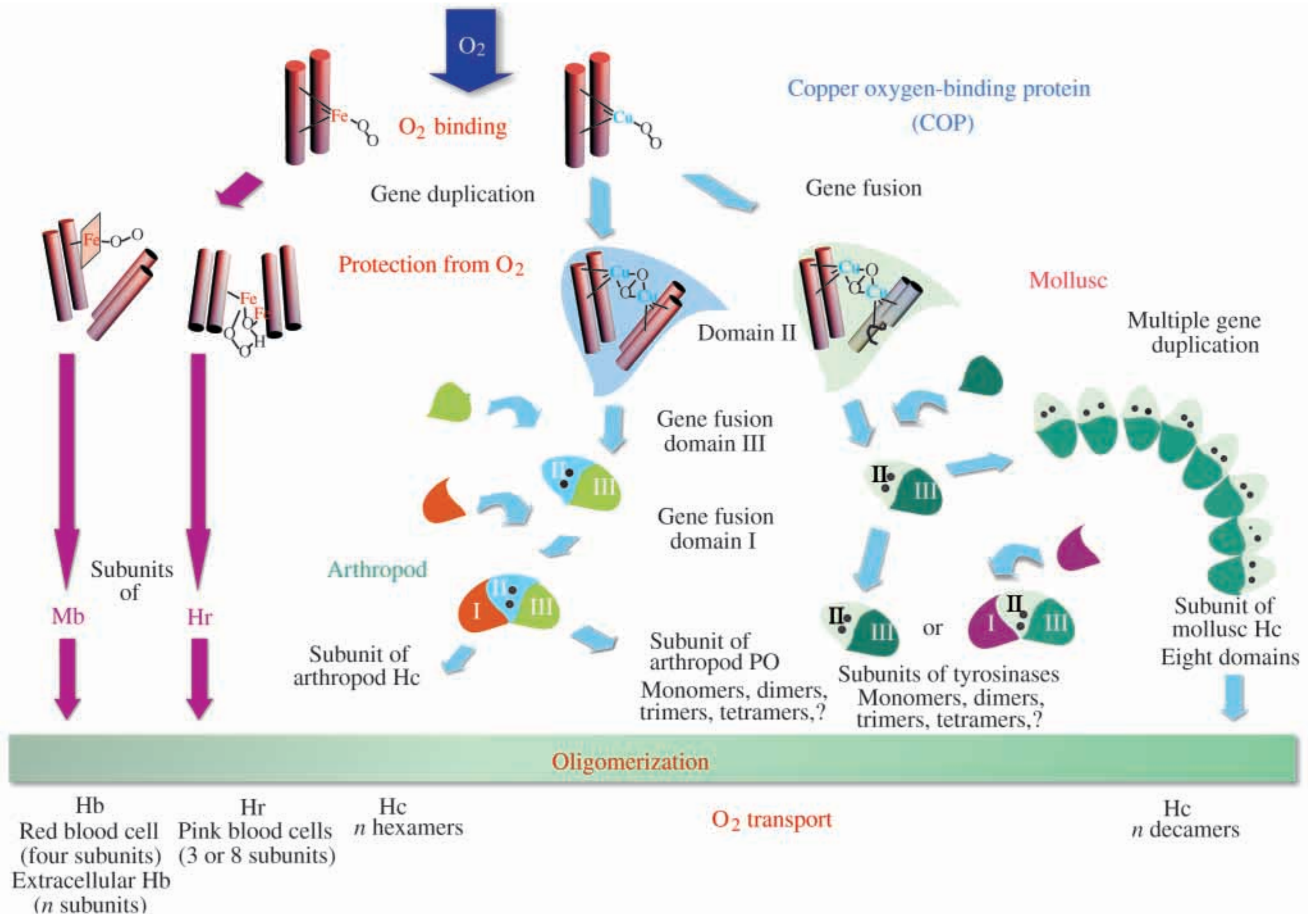

Fig. 3. Evolutionary relationships among dioxygen-binding proteins. Two different metal ions, iron (alone or as a haem group) and copper, became fixed by protein α-helices. These structures were able to bind dioxygen. Gene duplications and fusions of these structures resulted in more stable dioxygen-binding structures, providing space for the chemical reaction of phenoloxidase activity. In the case of copper oxygen-binding proteins, one or two additional domains became fused, giving rise to two basic, but different, trunks: (i) arthropod haemocyanins and phenoloxidases and (ii) molluscan haemocyanins and other phenoloxidases such as those in mushrooms or humans. Oligomerization was the essential step that provided these proteins with their known functional flexibility for binding dioxygen cooperatively. Hc, haemocyanin; PPO, prophenoloxidase; PO, phenoloxidase; Mb, myoglobin; Hb, haemoglobin; Hr, haemerythrin; *n*, an integer.

(Drexel et al., 1987; Cuff et al., 1998; Miller et al., 1998; Klabunde et al., 1998). In contrast to arthropod haemocyanin, the structure around CuA is different. One of the histidines binding CuA is not provided by an α-helix but by a loop. To stabilize the orientation of this histidine, it is covalently linked to a cysteine in an unusual thioether bond in molluscan haemocyanin and in sweet potato *o*-diphenol oxidase. The binding of dioxygen by haem or iron differs from that of COPs. In myoglobin and haemoglobin, an iron ion in the centre of the haem bridges a dioxygen and a highly conserved histidine, while in haemerythrin two iron ions are bound by the four α-helices, although the dioxygen is bound by only one iron ion. In the COPs, the dioxygen is bound to both copper ions in a 'side-on' configuration (Magnus et al., 1994), which changes the valency of Cu^{+} to Cu^{2+}. This coordination is the chemical basis for the phenoloxidase activity, in which the strategy is to split the bound dioxygen and incorporate one oxygen into phenolic compounds. Compared with synthesized copper/dioxygen complexes, the presence of a protein pocket for this reaction stabilizes binding of the substrate (Himmelwright et al., 1982).

One biological advantage of the early evolution of phenoloxidase activity would have been to 'discharge' the increasing amount of continuously produced reactive dioxygen. Whether the COPs already consisted of domains II and III or whether domain III was fused later cannot be deduced from the available data. During evolution, phenoloxidase activity became advantageous for several reasons, including initiating the primary immune response, synthesizing new biological compounds, such as melanin, and building up the exoskeleton. To activate the enzymatic reaction only at the appropriate times, a steric control mechanism developed that blocked the entrance to the active site with amino acids that could be cleaved by proteolysis, as discussed above for phenoloxidases and haemocyanins.

Depending on species and evolutionary pressures, the COPs have been modified in different ways, which could explain the low degree of sequence similarities among phenoloxidases.

Domain III in molluscan haemocyanins and domain I in arthropod haemocyanins and phenoloxidases may be responsible for protection of the active site against large substrates. This protection may have been an important step in the development of dioxygen carrier proteins since, with this protection, only small substrates such as dioxygen could be bound. The high oxygen-transport capacity of COPs was reached by a variety of strategies (Fig. 3). In the case of molluscan haemocyanin, the proteins underwent multiple gene duplications to yield a typical molluscan haemocyanin subunit, which consists of eight domains with eight oxygen-binding centres; the subunits then assemble to proteins with up to 160 oxygen-binding sites (van Holde and Miller, 1995). In the case of arthropod haemocyanins, the next step in their evolution was the fusion of domain I with domains II and III. These subunits assemble to give proteins with up to 48 oxygen-binding sites. The degree of similarity in the primary structures between arthropod haemocyanins and arthropod phenoloxidases suggests a common precurser subunit with three domains including one copper oxygen-binding site (Terwilliger, 1998). This may have been due to a common evolutionary pressure on both functions, phenoloxidase activity and oxygen transport. The development of a tracheal system in the class Insecta decreased their dependence on oxygen-transport proteins, while all classes of Arthropoda continued to require phenoloxidase participation in sclerotization of the exoskeleton after moulting. The arthropod COPs have therefore evolved into haemocyanins, whose primary function is to transport dioxygen as an energy source to the respiring tissues, and into phenoloxidases, whose function is to incorporate oxygen into new molecules essential for defence and for exoskeleton formation.

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