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T. Burmester

Origin and evolution of arthropod hemocyanins and related proteins

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Abstract Arthropod hemocyanins are large, multimeric, (n×6) copper-containing proteins that deliver oxygen in the haemolymph of many chelicerate, crustacean, myriapod, and also possibly some insect species. The arthropod hemocyanins belong to a large protein superfamily that also includes the arthropod phenoloxidases, certain crustacean and insect storage proteins (pseudo-hemocyanins and hexamerins), and the insect hexamerin receptors. Here I summarise the present knowledge of the origin, functional adaptations, and evolution of these proteins. Arthropod and mollusc hemocyanins are, if at all, only distantly related. As early as in the arthropod stem line, the hemocyanins emerged from a phenoloxidase-like enzyme. The evolution of distinct hemocyanin subunits, as well as the formation of multi-hexamers occurred independently within the arthropod subphyla. Hemocyanin subunit evolution is strikingly different in the Chelicerata, Myriapoda and Crustacea. Hemocyanins individually gave rise to two distinct copper-less storage proteins, the insect hexamerins and the crustacean pseudo-hemocyanins (cryptocyanins). The receptor responsible for the uptake of hexamerin by the larval fat body of the insects emerged from a hexamerin-precursor. Molecular phylogenetic analyses show a close relationship of the crustacean and insect proteins, providing strong support for a pancrustacean taxon, while structural data suggest a myriapod-chelicerate clade.

Keywords Arthropoda · Hemocyanin · Hexamerin · Hexamerin receptor · Phenoloxidase

Abbreviations MYA million years ago · PPOs prophenoloxidases

Introduction

The methods of molecular phylogeny have revolutionised our knowledge of animal systematics, as well as our understanding of the evolution of proteins and protein functions (e.g. Swofford et al. 1996; Pagel et al. 1999). It is well established that proteins that serve very different functions may share significant identities at the molecular level. Such sequence similarities reflect a relationship in evolution (Doolittle 1981, 1989). Groups of proteins that share a common ancestry can be classified in families and superfamilies (Dayhoff et al. 1975). The phylogeny of the members of such protein (super-) families can be inferred analogous to those of living species (e.g. Swofford et al. 1996). Protein functions and adaptations at the molecular level cannot be understood without considering species phylogeny. In fact, the proteins of an organism share its phylogenetic history, and physiological adaptations that have occurred during evolution have driven and have been driven by the changes in the protein sequences.

The hemocyanins are large copper-containing proteins that transport oxygen in the haemolymph of many arthropod and mollusc species (Markl and Decker 1992; van Holde and Miller 1995). The arthropod hemocyanin superfamily includes four other classes of proteins that share significant sequence similarities but serve distinct functions (Table 1; Beintema et al. 1994; Burmester and Scheller 1996; Burmester 2001). The phenoloxidases are enzymes involved in the melanisation pathway. The non-respiratory crustacean pseudo-hemocyanins (cryptocyanins) and the insect hexamerins serve mainly as storage proteins. The insect hexamerin receptors are responsible for the uptake of hexamerins by the larval fat body. Previous reviews on arthropod hemocyanins (van Holde and Miller 1982; Ellerton et al. 1983; Linzen et al. 1985; Salvato and Beltramini 1990; Markl and Decker 1992;
van Holde and Miller 1995), phenoloxidases (Söderhäll and Cerenius 1998), hexamerins (Telfer and Kunkel 1991; Haunerland 1996; Burmester 1999a) or hexamerin receptors (Burmester and Scheller 1999) have mainly focussed on protein function. Markl and co-workers (Markl 1986; Markl et al. 1986) have carried out an immunological approach to elucidate hemocyanin relationship and evolution within the Crustacea and Chelicerata. With the amount of sequence data that have accumulated in recent years, as well as the advances in the development of molecular phylogenetic methods, comparative sequence studies have gained much attention (e.g. Beintema et al. 1994; Burmester and Scheller 1996; Burmester et al. 1998; Durstewitz and Terwilliger 1997a; Burmester 2001). Here I will discuss these findings and will present a comprehensive review of the current knowledge on the evolution of the arthropod hemocyanin superfAMILY.

The origin of the arthropod hemocyanin superfAMILY

The delivery of oxygen within the animal’s body is an essential process that is accomplished by three different types of metal-containing proteins. While haemoglobins are widespread throughout the animal kingdom, haemerythrins and hemocyanins are restricted to only a few phyla (Vinogradov 1985; Terwilliger 1998; Kurtz 1999). Hemocyanins are large proteins that may assemble to complexes of up to several million Daltons. These copper-proteins only occur in the body fluid of some arthropod and mollusc species (Markl and Decker 1992; van Holde and Miller 1995). Hemocyanins bind oxygen by means of two Cu²⁺ ions, each of which is co-ordinated by three histidines in two distinct binding sites (CuA and CuB) (Fig. 1). There are striking structural and functional similarities among such “type III” copper-binding centres, and also certain sequence resemblance in the CuB site. It is not surprising that some confusion arose due to these apparent similarities. The common nomenclature of mollusc and arthropod hemocyanins even led to the misinterpretation of a close relationship of the two phyla within the Prostostomia (Kim et al. 1996). A common origin of these two different classes of hemocyanins from an oxygen-binding ancestor has been repeatedly proposed in the literature (e.g. Müller et al. 1988; Kawabata et al. 1995; Durstewitz and Terwilliger 1997a; Terwilliger et al. 1999), while others have emphasised the fundamental differences in the construction of the native hemocyanins of the two phyla (e.g. Markl and Decker 1992; van Holde and Miller 1995; van Holde et al. 2001). In fact, statistical analyses have demonstrated that even the sequence similarities between the mollusc and arthropod hemocyanin superfamilies are not significant (Burmester 2001). Thus the mollusc and arthropod hemocyanins

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**Table 1** Structure and properties of arthropod hemocyanins, phenoloxidases, pseudo-hemocyanins, hexamerins and hexamerin receptors

<table>
<thead>
<tr>
<th>Main function</th>
<th>Occurrence</th>
<th>Structure</th>
<th>Cu²⁺</th>
<th>Length (aa)</th>
<th>Signal peptide</th>
<th>Evolution rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenoloxidase</td>
<td>Tyrosinase</td>
<td>Crustacea</td>
<td>1±6?</td>
<td>yes</td>
<td>~660–690</td>
<td>no²</td>
</tr>
<tr>
<td></td>
<td>Myriapoda</td>
<td>Insecta</td>
<td>n.d.</td>
<td>yes</td>
<td>~650</td>
<td>no²</td>
</tr>
<tr>
<td>Hemocyanins</td>
<td>Oxygen transport</td>
<td>Chelicerata</td>
<td>1–4±6</td>
<td>yes</td>
<td>~650–660</td>
<td>yes</td>
</tr>
<tr>
<td>Pseudo-hemocyanins</td>
<td>Storage protein (cryptocyanins)</td>
<td>Crustacea (Decapoda)</td>
<td>1±6</td>
<td>no</td>
<td>~660–750</td>
<td>yes</td>
</tr>
<tr>
<td>Hexamerins</td>
<td>Storage protein</td>
<td>Insecta</td>
<td>1±6 or 2±6</td>
<td>no</td>
<td>1010–1240</td>
<td>yes</td>
</tr>
<tr>
<td>Hexamerin receptors</td>
<td>Hexamerin uptake</td>
<td>Insecta (Diptera?)</td>
<td>n.d.</td>
<td>no</td>
<td>~74 kDa</td>
<td>yes</td>
</tr>
</tbody>
</table>

¹Subunits; excluding signal peptides
²Synthesised as pro-phenoloxidases
³Substitutions per site and year

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**Fig. 1A–D** Structure of arthropod hemocyanin. **A** O₂-binding site. **B** subunit. **C** simplified hexamer structure. **D** simplified multi-hexamer (arachnid 4×6 hemocyanin)