The respiratory proteins of insects

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Abstract

For a long time, respiratory proteins have been considered unnecessary in most insects because the tracheal system was thought to be sufficient for oxygen supply. Only a few species that survive under hypoxic conditions were known exceptions. However, recently it has become evident that (1) intracellular hemoglobins belong to the standard repertoire of insects and (2) that hemocyanin is present in many “lower” insects. Intracellular hemoglobins have been identified in \textit{Drosophila}, \textit{Anopheles}, \textit{Apis} and many other insects. In all investigated species, hemoglobin is mainly expressed in the fat body and the tracheal system. The major \textit{Drosophila} hemoglobin binds oxygen with high affinity. This hemoglobin type possibly functions as a buffer system for oxygen supply at low partial pressures and/or for the protection from an excess of oxygen. Similar hemoglobins, present in much higher concentrations, store oxygen in specialized tracheal organs of the botfly and some backswimmers. The extracellular hemoglobins in the hemolymph of chironomid midges are evolutionary derivatives of the intracellular insect hemoglobins, which emerged in response to the hypoxic environment of the larvae. In addition, several hemoglobin variants of unknown functions have been discovered in insect genomes. Hemocyanins transport oxygen in the hemolymph of stoneflies, but also in the Entognatha and most hemimetabolan taxa. Apparently, hemocyanin has been lost in Holometabola. At present, no physiological or morphological character is known that could explain the presence or loss of hemocyanins in distinct taxa. Nevertheless, the occurrence of respiratory proteins in insects adds further complexity to our view on insect respiration.

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1. Introduction

Animals that live under aerobic conditions consume large amounts of O$_2$, which is mainly used to sustain the production of ATP in the respiratory chain of the mitochondria. In Protozoa and small Metazoa, simple diffusion is usually considered sufficient for the supply of the inner layers of the body with O$_2$. Larger animals, however, require a variety of anatomical, physiological, and molecular adaptations that enhance the O$_2$ delivery to the cells and eventually to the mitochondria. These adaptations comprise respiratory organs, such as gills or lungs, circulatory systems, as well as respiratory proteins for transport or storage of O$_2$ (Willmer et al., 2000).

The largely impermeable cuticle of insects (here, the taxonomic terms “Insecta” and “Hexapoda” are used as synonyms) and other arthropods constrains the uptake of O$_2$ by diffusion across the body’s surface. Terrestrial insects and myriapods acquire O$_2$ by the aid of trachea. The tracheal system consists of highly branched air-filled tubes that connect the inner tissues with the atmosphere (Kestler, 1985; Brusca and Brusca, 2002). Aquatic insects usually have other specialized respiratory organs such as gills, tracheal gills or a plastron (Willmer et al., 2000). Within the tracheal system, O$_2$ is distributed through the body mainly by passive diffusion (Krogh, 1920; Kestler, 1985), although some active breathing may occur (Komai, 1998; Westneat et al., 2003). O$_2$ uptake by the cells takes place mainly at the tips of the smallest branches, the tracheoles, which have only a thin cuticle. In highly active organs such as the insect flight muscle, the tracheoles may even enter the cells and connect with the mitochondria.
Due to high diffusion rates and capacity coefficients, O$_2$ is delivered about 200,000–300,000 times more efficient in the tracheal air than in the aqueous environment of the hemolymph or blood (Krogh, 1920; Kestler, 1985). These features make the tracheal system an extremely efficiently transport apparatus, which has been thought to comply with the O$_2$ requirements of even the largest insect known on earth. Therefore, until recently the occurrence of respiratory proteins that would enhance O$_2$ supply has been largely unknown and considered unnecessary (Mangum, 1985; Law and Wells, 1989; Locke, 1998; Willmer et al., 2000). Only a few insect species that live in aqueous and hypoxic environments were known to harbor hemoglobin (Weber and Vinogradov, 2001; see below). However, in recent years it has become evident that O$_2$ transport and storage proteins are much more widespread among insects than previously thought (Fig. 1; Burmester and Hankeln, 1999; Hankeln et al., 2002, 2006; Hagner-Holler et al., 2004; Burmester et al., 2006). Here we summarize the present state of knowledge on the respiratory proteins of insects, and analyze their occurrence in a physiological and evolutionary context.

2. Respiratory proteins

Respiratory proteins reversibly bind molecular O$_2$ for the purpose of transport or storage. They enhance the O$_2$ transport capacitance of the body fluid, facilitate intracellular O$_2$ diffusion or enable O$_2$ storage for long or short-term periods. In the animal kingdom, three types of metal-containing respiratory proteins are known: (hemo-)globins (Hb), hemerythrins and hemocyanins (Hc). Hemerythrins are restricted to only a few animal phyla and have not been detected in insects (Vinogradov, 1985). They will not be considered here.

(Hemo-)globin (Hb) is a small globular protein, usually consisting of about 140–150 amino acids that comprise eight $\alpha$-helical segments (named A–H). It displays a characteristic 3-over-3 $\alpha$-helical sandwich structure (Dickerson and Geis, 1983; Berg et al., 2002), which includes an iron-containing heme group (Fe$^{2+}$-protoporphyrin IX). The Fe$^{2+}$ is connected to the globin chain via the proximal histidine in F8 (i.e., 8th amino acid of helix F). The gaseous ligand, typically O$_2$, but also CO or NO, can bind between the Fe$^{2+}$ ion and the distal amino acid at position E7. Hbs are phylogenetically ancient molecules, whose complex

![Fig. 1. Distribution of respiratory proteins in insect orders. The phylogenetic tree of the insects is based on Hennig (1969), Kukalová-Peck (1991) and Wheeler et al. (2001).]
evolution is demonstrated by their widespread occurrence in bacteria, fungi, plants, invertebrate and vertebrate animals (Weber and Vinogradov, 2001; Vinogradov et al., 2005). Although the various Hbs may display only little sequence resemblance, tertiary structures are usually conserved. Invertebrate Hbs may assemble to multimers of up to 144 Hb subunits or domains. Hbs are involved in many different aspects of O2 supply. In almost all vertebrates and many invertebrates, Hb is present in the circulatory system. Hbs may either be contained in specialized cells (erythrocytes) or are freely dissolved in the blood. Hbs may also occur in distinct tissues. The functions of these “tissue-globins” are not always obvious. The best-studied example is myoglobin, a tissue-globin that meets the high O2 demands of muscle tissues in both vertebrates and invertebrates. Although there is no doubt that it has respiratory functions, it is still not clear whether myoglobin facilitates O2 diffusion from the cell surface to mitochondria, or whether it acts as a short-term O2 store (Wittenberg and Wittenberg, 2003). The physiological role of other specialized tissue-globins, like neuroglobin in the nervous system of vertebrates (Burmaster et al., 2000) and cytoglobin in various cell types (Burmaster, 2002), is still a matter of debate (Burmaster and Hankeln, 2004; Hankeln et al., 2005).

Hcs are copper-containing respiratory proteins that only occur in the hemolymph of several arthropod and mollusk species, but not outside these phyla (Markl and Decker, 1992; van Holde et al., 2001). Although arthropod and mollusk Hcs have similar binuclear Cu-binding centers, these proteins are most likely phylogenetically not related and have emerged from different types of copper-proteins (Burmaster, 2001, 2002). Arthropod Hcs form either hexamers or multi-hexamers of six similar or identical subunits, with structures up to 8 x 6-mers (Markl and Decker 1992; van Holde et al., 2001). A typical arthropod Hc subunit comprises about 650 amino acids (~75–80 kDa polypeptide). Each subunit consists of three structural domains and harbors two Cu+ ions, which can bind one O2. Each Cu+ ion is coordinated by three histidines, which reside in domain 2. In contrast to some Hbs, Hcs are never intracellular proteins and always occur freely dissolved in the hemolymph. In an evolutionary perspective, arthropod Hcs derived from phenoloxidases, Cu+ containing enzymes of the melanin-pathway which are involved in immune response, wound-healing and cuticle formation (Burmaster and Scheller, 1996; Burmaster, 2001, 2002). Hcs are also related to some non-respiratory proteins: crustacean pseudo-hemocyanins, insect hexamerins and dipteran hexamerin receptors (Beintema et al., 1994; Burmaster and Scheller, 1996; Burmaster, 2001, 2002). These proteins have lost the ability to bind Cu+, and thus O2, and are mainly used for amino acid and energy storage, but also have other functions (Burmaster, 1999a, b, 2002; Terwilliger et al., 1999).

The multi-subunit structure of Hc and many Hbs enhances the O2 transport capacity (Willmer et al., 2000; Berg et al., 2002). The combination of O2 with one subunit may increase the O2-affinity of the other subunits (and vice versa), thus enhancing O2 uploading at high PO2 and O2 unloading at low PO2 (positive cooperativity). In many cases, O2-binding can also be modulated by changes of the pH (Bohr effect). E.g., a low pH may increase O2 release in the metabolic active tissues, which tend to be acidic. Both cooperativity and Bohr effect enhance efficiency of respiratory proteins in delivering O2.

3. The extracellular hemoglobins of the Chironomidae

As early as in the 19th century, scientists noted that the bright red color of the aquatic larvae of chironomid midges (Diptera: Nematocera) is due to Hb (Rollett, 1861; Lankester, 1872). According to our present knowledge, the Chironomidae are the only insects that have Hbs in their hemolymph. The larvae of many chironomid species live in the sediment of eutrophic and polluted waters, sometimes reaching considerable depths. In this often chronically hypoxic environment the ambient O2 partial pressures range from 10 to 50 Torr (1.3–7 kPa) (Osmulski and Leyko, 1986), and the larvae absorb O2 via their cuticular surface. There is little doubt that chironomid Hb has a respiratory function analogous to vertebrate Hb. It enhances the O2 capacitance of the hemolymph, thus enabling O2 transport inside the larvae. In addition, Hb may serve as a short time oxygen store during rhythmic periods of hypoxia, which result when the larvae stop irrigating inside their dwelling tubes (Walshe, 1951). Accordingly, poisoning of the Hb with CO reduces filter-feeding in Chironomus plumosus larvae (Walshe, 1951) and larvae increase Hb synthesis in poorly aerated water (Fox, 1955).

Genomic analyses in diverse chironomid species have revealed that the number of recognized Hb protein variants is even outnumbered by the number of non-allelic Hb gene copies (Kao et al., 1995; Trewitt et al., 1995; Hankeln et al., 1997, 1998). We have counted more than 40 Hb genes in the genome of Chironomus tentans (T. Hankeln, H. Friedl and E. R. Schmidt, unpublished), making this the largest Hb gene family reported so far in any organism. The Hb gene duplicates are clustered at two separate chromosomal loci (Hankeln et al., 1988). In few cases, chironomid Hb genes have turned into non-functional pseudogenes (Gruhl et al., 2000) or appear to have experienced accelerated sequence evolution indicative of novel functional properties (Hankeln et al., 1998). While systematic data on expression patterns/levels and ligand binding features of all Hb variants are missing, the intriguing Hb multiplicity in chironomids remains unexplained at the functional level. Preliminary indications for a differential regulation of Hb gene variants at the mRNA and protein levels exist (Saffarini et al., 1991; Green et al., 1998). It remains to be shown whether Hb gene copies are evolutionarily preserved in chironomid genomes due to a “distribution of tasks” (sub-functionalization; Force et al., 1999) or
simply due to a more general, selected increase in Hb protein dosage (Gruhl et al., 1997).

Hb synthesis in Chironomidae appears to be largely restricted to the aquatic larval and pupal stages (Osmulski and Leyko, 1986), although Hbs (for unknown reasons) have also been found in adult ovaries and eggs (Trewitt et al., 1986). Hbs are produced mainly by the fat body cells (Bergtrom et al., 1976) and are exported into the hemolymph by the means of N-terminal signal peptides. Hb decomposition takes place in the Malphigian tubules (Jarial, 1988). The Chironomus Hb polypeptides comprise about 140 amino acids plus N-terminal signal peptides of about 20 amino acids. In all known Chironomus thummi Hbs the distal position E7 is occupied by a standard histidine residue; only in the Hb variant IIIa this residue is a glutamine. By contrast, the Hb variants of Tokunagayusurika akamusi, a Japanese chironomid, either have histidine, leucine or isoleucine at the E7 distal site (Fukuda, 1993). The functional consequences of these differences are unknown. Hb chains are monomers, form homodimers or occur in a monomer–dimer equilibrium (Goodman et al., 1983; Weber and Vinogradov, 2001). Other Chironomus species have distinct Hb aggregation levels, ranging from exclusively monomers up to tetramers (Weber and Vinogradov, 2001). This demonstrates the evolutionary flexibility of Hb organization in the Chironomidae. The chironomid Hbs bind O2 with high affinity (half-saturation pressure \( P_{50} = 0.5–1.5 \text{Torr} \)) and in a non-cooperative manner (Table 1) (Amiconi et al., 1972; Weber et al., 1985). Measurements of pH-dependence of O2-binding revealed that both monomeric and dimeric Ch. thummi Hbs exhibit a mild Bohr effect (Weber et al., 1985; Ruf et al., 1994). The crystal structure of Hb chain III from C. thummi shows a protein with a typical globin fold, but a reversed orientation of the heme (compared to vertebrate Hb) and an unusual open conformation of the ligand binding site (Huber et al., 1971).

### 4. Intracellular insect hemoglobins with specialized O2 storage function

In some insects, specialized tissues store O2 by the virtue of intracellular Hb (Weber and Vinogradov, 2001). The presence of these Hbs can be easily recognized by the red color of particular tissues, e. g. in the larvae of the horse botfly Gasterophilus intestinalis (Diptera) (Keilin and Wang, 1946) and in backswimmers (Hemiptera) (Miller, 1964; Bergtrom, 1977; Wells et al., 1981). There is little doubt that the intracellular Hb of G. intestinalis has a myoglobin-like role. The larvae of G. intestinalis live for 7–8 months as parasites in the alimentary tract of horses. The early larvae are bright red due to the presence of Hb in the fat body, in the parietal musculature and in the hypodermis (Keilin and Wang, 1946). In older larvae, Hb becomes concentrated at the posterior body part in specialized tracheal cells, which are thought to be derivatives of the fat body. The Hb assists in the extraction of O2 from air bubbles, which have been engulfed by the horses and which are only temporarily available in the semi-fluid environment. The Hb stores O2 that is subsequently released during anoxic periods. Biochemical evidence and the crystal structure showed that the Hb of G. intestinalis is a dimer, which is encoded by at least two distinct genes (Dewilde et al., 1998; Pesce et al., 2005). The protein measures 152 amino acids and harbors histidines in proximal position F8 and in distal position E7. G. intestinalis Hb binds O2 in a non-cooperative manner (Table 1). Early measurements of O2 binding kinetics suggested a moderately high affinity of \( P_{50} = 4.9 \text{Torr} \) at 39 °C (Keilin and Wang, 1946), while more recent data obtained by flash photolysis showed a higher affinity of \( P_{50} = 0.15 \text{Torr} \) at 25 °C (Dewilde et al., 1998). The reason for this discrepancy is unknown.

Backswimmers are diving predators that breathe under water by the aid of an abdominal air bubble that had been captured at the surface. Anisops and Buenoa (Notonectidae) and Macrocorixa Geoffroyi (Coryxidae) have Hbs at high concentrations (~20 mM) in tracheal organs, which are penetrated by tracheoles (Hungerford, 1922; Miller, 1964, 1966; Bergtrom, 1977; Wells et al., 1981; Matthews and Seymour, 2006). Poisoning of the Hb with CO reduces dive durations dramatically (Miller, 1964, 1966; Wells et al., 1981). A recent study employing the Australian backswimmer Anisops deanei has demonstrated that during the initial phase of the dive O2 is taken mainly from the bubble, which thus looses volume (Matthews and Seymour, 2006). When the PO2 in the bubble falls below about 30 Torr (4.3 kPa) at a later phase of the dive, Hb becomes

### Table 1

Biochemical characteristics of selected insect respiratory proteins

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Localization</th>
<th>Structure</th>
<th>( P_{50} ) (Torr)</th>
<th>( n_{50} )</th>
<th>pH</th>
<th>T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisops assimilis</td>
<td>Hb</td>
<td>Tracheal organ</td>
<td>Monomer, hexamer</td>
<td>30–40</td>
<td>5.2</td>
<td>6.9</td>
<td>25</td>
</tr>
<tr>
<td>Gasterophilus intestinalis</td>
<td>Hb</td>
<td>Musculature, hypodermis, fat body, tracheal cells</td>
<td>Dimer</td>
<td>4.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>7.3</td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Hb</td>
<td>Tracheal cells, fat body</td>
<td>Monomer</td>
<td>0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>7.0</td>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chironomus thummi</td>
<td>Hb</td>
<td>Hemolymph</td>
<td>Monomers, dimers</td>
<td>0.32–1.3</td>
<td>1</td>
<td>7.0</td>
<td>25</td>
</tr>
<tr>
<td>Perla marginata</td>
<td>Hc</td>
<td>Hemolymph</td>
<td>Hexamer</td>
<td>8</td>
<td>2</td>
<td>7.8</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Keilin and Wang (1946).

<sup>b</sup>Dewilde et al. (1998).
the main source of $O_2$. This strategy helps to stabilize the volume of the air bubble and to maintain neutral buoyancy. Sequences of backswimmer Hbs are yet unknown, but biochemical studies in *Anisops* or *Buenoa* showed 17 kDa Hb monomers. In their oxy-form, the Hbs are mainly monomeric, while in the deoxy-form, the Hb occurs as a mixture of monomers and hexamers (Bergtrom, 1977; Wells et al., 1981). *Anisops* Hb has a low $O_2$ affinity in the range $P_{50} = 30–40$ Torr and a high cooperativity (Wells et al., 1981; Matthews and Seymour, 2006). This $P_{50}$ correlates with the critical $P_{O_2}$ in the bubble, when Hb becomes the main source of $O_2$ (Matthews and Seymour, 2006).

5. Intracellular insect hemoglobins at lower concentrations

While the Hbs of *Gasterophilus* and the backswimmers can easily be linked to their specialist lifestyle, the recent identification of intracellular Hbs in other insect species was unexpected. Hbs have been discovered in the Drosophilidae (Diptera, Brachycera) (Burmester and Hankeln, 1999; Hankeln et al., 2002; Burmester et al., 2006), the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Diptera, Nematocera) (Burmester et al., in press) and the honeybee *Apis mellifera* (Hymenoptera) (Hankeln et al., 2006). In addition, Hb sequences are present in the genomes or ESTs (expressed sequence tags) of the hemipterans *Acrystosiphon pisum* and *Aphis gossypii*, the coleopterans *Dascillus cervinus* and *Tribolium castaneum*, the silkworm *Bombyx mori* (Lepidoptera) and the fly *Glossina morsitans* (Diptera) (Hankeln et al., 2006). The conservation of amino acids in key positions of the globin chain suggests that these Hbs are actually functional in binding $O_2$ (Table 2).

The *Drosophilidae* have three distinct Hb genes (*glob1–3*), of which *glob1* features the highest level of expression (Burmester et al., 2006). *A. gambiae* and *A. aegypti* each harbor two Hb genes that duplicated early in the evolution of mosquitoes (Culicidae) (Table 1). Hb expression in *D. melanogaster*, *A. gambiae* and *A. mellifera* is mainly associated with the tracheal system. Hb mRNA has been detected in the walls of tracheal tubes and in terminal cells (Hankeln et al., 2002, 2006; Burmester et al., 2006). In all three species other sites of Hb synthesis have been identified in organs such as the fat body and pharynx muscle in *D. melanogaster*, the visceral muscles in *A. gambiae* and the Malphigian tubules in *A. mellifera*. The intracellular concentrations of these Hbs in these tissues are unknown; a rough estimate by comparing Western blot signal intensities and EST numbers suggests that the *glob1* protein of *D. melanogaster* makes up about 0.1% of all adult proteins (Burmester et al., 2006). Taking into account the restricted cellular expression of this protein, this is certainly a significant amount. *Glob1* expression regulation was studied under experimental hypoxia employing transfected S2 cell lines (Gorr et al., 2004) or living embryonic, larval and adult *Drosophila* (unpublished data). The data agree in that low $O_2$ conditions appear to trigger a down-regulation of *glob1* mRNA, although there are differences among developmental stages (unpublished data).

Biochemical and crystallographic studies demonstrated that *D. melanogaster* *glob1* is a monomer, which covers 153 amino acids (Hankeln et al., 2002; de Sanctis et al., 2005). It has an $O_2$ affinity of $P_{50} = 0.15$ Torr (Hankeln et al., 2002), which is similar to that of the *Chironomus* and *Gasterophilus* Hbs (Table 1). However, *D. melanogaster* *glob1* displays a so-called hexacoordinate binding scheme in its deoxy-state, in which the Fe$^{2+}$ is bound to the distal histidine residue at helix position E7. Interestingly, the closely related *G. intestinalis* Hb shows the “classical” pentacoordination (Fe$^{2+}$ is only bound to four N-atoms of the heme ring plus the proximal histidine at F8) (Pesce et al., 2005). The functional consequences of hexacoordination

Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>A12</th>
<th>B10</th>
<th>CD1</th>
<th>E7</th>
<th>E10</th>
<th>E11</th>
<th>F8</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Human Mb</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Thr</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Chironomus Hb</em></td>
<td>Trp/Phe/Tyr</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Arg</td>
<td>Ile/Val</td>
<td>His</td>
</tr>
<tr>
<td><em>G. intestinalis</em> Hb</td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Arg</td>
<td>Ile</td>
<td>His</td>
</tr>
<tr>
<td><em>Drosophila glob1</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Arg</td>
<td>Ile</td>
<td>His</td>
</tr>
<tr>
<td><em>Drosophila glob2</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Ala</td>
<td>Met</td>
<td>His</td>
</tr>
<tr>
<td><em>Drosophila glob3</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Arg</td>
<td>Phe</td>
<td>His</td>
</tr>
<tr>
<td><em>Apis mellifera glob1</em></td>
<td>Trp</td>
<td>Met</td>
<td>Phe</td>
<td>His</td>
<td>Gly</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Anopheles gambiae glob1</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Asn</td>
<td>Leu</td>
<td>His</td>
</tr>
<tr>
<td><em>Anopheles gambiae glob2</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>Gln</td>
<td>His</td>
<td>Ile</td>
<td>His</td>
</tr>
<tr>
<td><em>Aedes aegypti glob1</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Asn</td>
<td>Leu</td>
<td>His</td>
</tr>
<tr>
<td><em>Aedes aegypti glob2</em></td>
<td>Trp</td>
<td>Met</td>
<td>Phe</td>
<td>His</td>
<td>Asn</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Acrystosiphon pisum glob1</em></td>
<td>Trp</td>
<td>Val</td>
<td>Phe</td>
<td>His</td>
<td>Lys</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Aphis gossypii glob1</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Lys</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Dascillus cervinus glob1</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>Gln</td>
<td>Ser</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Tribolium castaneum glob1</em></td>
<td>Trp</td>
<td>Phe</td>
<td>Phe</td>
<td>His</td>
<td>Asn</td>
<td>Val</td>
<td>His</td>
</tr>
<tr>
<td><em>Bombyx mori glob1</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Asn</td>
<td>Ile</td>
<td>His</td>
</tr>
<tr>
<td><em>Glossina morsitans glob1</em></td>
<td>Trp</td>
<td>Leu</td>
<td>Phe</td>
<td>His</td>
<td>Arg</td>
<td>Ile</td>
<td>His</td>
</tr>
</tbody>
</table>
are unknown, although this structure is also known from other globins such as vertebrate neuroglobin and cytoglobin (Pesce et al., 2003; de Sanctis et al., 2004).

6. Evolutionary conservation and diversity of insect globins

Although being identified only in Eumetabola (Fig. 1), it can be assumed that Hb gene(s) belongs to the standard repertoire of insects. Insect Hbs are most similar to crustacean Hbs. Phylogenetic studies suggest a complex pattern of evolution (Fig. 2). Drosophila glob1-type Hbs are orthologous to the G. intestinalis Hb. The Drosophila glob2 and glob3 genes are of ancient evolutionary origin, as confirmed by their ancestral exon–intron pattern (Burmester et al., 2006). Interestingly, mosquito and the chironomid Hb genes are not orthologous, but have divergent evolutionary origins before the radiation of Diptera (Burmester et al., in press). Chironomid Hbs are associated with G. intestinalis Hb, G. morstians Hb and Drosophila glob1 proteins, whereas the A. gambiae and A. aegypti Hbs have a more basal position within the insect Hb tree. Thus the extracellular Hbs of the Chironomidae evolved from an intracellular Hb after the separation of the Chironomidae (Nematocera) and the brachyceran flies (Burmester and Hankeln, 1999; Burmester et al., 2006). An orthology of Chironomus and vertebrate Hbs, and thus an ancient origin of the Chironomus Hbs, as originally proposed, e.g. by Goodman et al. (1983), is not evident. The phylogenetic history of insect Hb genes can also be traced by the aid of intron positions (Hankeln et al., 1997; Burmester et al., in press). Introns in helix-positions B12.2 (i.e. intron inserted between the second and third bp of codon 12 of globin helix B) and G7.0 are most likely ancestral in the globin gene lineage (Hardison, 1996). The Hb genes of T. castaneum, B. mori, the mosquito glob1 genes and Drosophila glob2 and glob3 genes indeed have introns in B12.2 and G7.0 (Fig. 2). This may be interpreted as an indication of a basal position of these genes within the insect globin tree. Mosquito glob2 genes have lost the intron in G7.0, while A. aegypti glob1 has acquired an additional intron in E18.0. This complex pattern shows the dynamics of intron gain and loss in the insect globin gene lineage. G. intestinalis Hb and the Drosophila glob1 have introns in D7.0 and G7.0, indicating a loss of the B12.2 intron and an acquisition of an intron in D7.0 during the evolution of the Brachycera (Burmester and Hankeln, 1999; Burmester et al., 2006). Furthermore, this pattern confirms the evolutionary orthology of G. intestinalis Hb and Drosophila glob1 genes. Most known present-day chironomid Hb genes do not harbor any introns, but during evolution some have acquired introns in central positions E9.1, E15.0 and E12.2 (Hankeln et al., 1997; and our unpublished data). In some cases, introns may have laterally spread to neighbor gene duplicates by gene conversion-like processes.

7. Intracellular hemoglobins as ‘standard’ respiratory proteins in insects

The apparently universal presence of intracellular Hbs among insects is striking and requires a physiological interpretation. At the first glance, it may seem improbable that Hbs expressed at low concentrations have a myoglobin-style O2 supply function, as it has been demonstrated for botfly and backswimmer Hbs. This is mainly due to the apparent lack of need of a respiratory protein in insect taxa that live under largely normoxic conditions. Nevertheless, at least in Drosophila, a respiratory function of glob1 is conceivable (Hankeln et al., 2002; Burmester et al., 2006): (1) although the cellular concentration of glob1 is unknown, Western blot signals and EST numbers suggest that local amounts are likely sufficient to contribute significantly to O2 supply. (2) Embryos and larvae of Drosophila compete efficiently with microorganisms for a limited supply of O2 within fermenting fruits. Therefore, an Hb in the tracheal system that enhances the extraction of O2 from the environment would almost certainly be beneficial. The same might be true for adult flies which possibly meet short-term hypoxia when closing their spiracles during flight in order to prevent excessive desiccation (Lehmann, 2001). (3) Drosophila glob1 and G. intestinalis Hb display similar tissue expression patterns and O2-binding kinetics. In an evolutionary perspective, G. intestinalis Hb and Drosophila glob1 are orthologues (Fig. 2). Thus, it is most parsimonious in an evolutionary sense to propose that glob1 fulfills a respiratory role just like the botfly Hb.

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**Fig. 2. Phylogeny of insect hemoglobins.** An alignment of insect Hb amino acid sequences was analyzed by MrBayes 3.1.1. A simplified tree is displayed; branches with support values <0.9 have been collapsed.
However, alternative physiological functions of intracellular insect Hbs may also be considered. Some globins carry out enzymatic functions, such as the detoxification of nitric oxide (NO; Flögel et al., 2001) and possibly other noxious reactive nitrogen and oxygen species, or they may serve as O₂ sensing molecules (Freitas et al., 2005). A role of glob1 as an O₂ sensor is unlikely, because the observed ligand affinity is too high for a sensor that works under cellular O₂ concentrations. By contrast, a hypothetical function of glob1 in detoxification of reactive oxygen species (ROS) would be in line with the observed tracheal expression pattern and gene regulation data (unpublished results). It must be considered that the cells adjacent to the tracheal tubes may experience unusually high O₂ partial pressures, which are close to the atmospheric level (Krogh, 1920), whereas inner tissues normally work at PO₂ in the range of few kPa. High PO₂, however, leads to the generation of ROS and cell damage (Halliwell and Gutteridge, 1999). In fact, it has been proposed that under certain conditions the tracheal system has to adapt to avoid the influx of an excess of O₂ (Burmester, 2005; Hetz and Bradley, 2005). The Hbs in the insect tracheal cells may therefore scavenge ROS directly by transforming them into harmless compounds.

One can also combine the oxygen-supply and protection hypotheses. In this scenario, Hb in the tracheal walls may act as an O₂ buffer system that on the one hand supports a constant flow of O₂ from the trachea to the inner cells, but also protects the cells from an excess of O₂ that would lead to the generation of ROS. In this context it is noteworthy that intracellular Hbs at high levels are located in the tracheal system of insects that experience intermittent hypoxia, which results in dramatically changing PO₂ in the tracheal system. A similar cyclic pattern of oxygen availability also exists in insects that periodically close their spiracles. When the spiracles are closed, the PO₂ in the terminal cells slowly decreases, and O₂ is released from the Hb. With open spiracles, Hb binds to O₂ and impedes its flow from the atmosphere to the inner organs, thus protecting the cells from an excess of O₂.

8. Insects with blue blood

Since many years, the presence of Hc in the hemolymph of most spiders (Chelicerata) and the malacostracan Crustacea is common knowledge (for review, see: Markl and Decker, 1992; van Holde et al., 2001). It has been demonstrated that Hcs also occur in Onychophora and some Myriapoda (Jaenicke et al., 1999; Kusche and Burmester, 2001; Kusche et al., 2002, 2003). Hcs had been unknown in insects, although hexamerins, storage proteins that had derived from Hc, are widespread in this taxon (Telfer and Kunkel, 1991; Burmester et al., 1998; Burmester, 1999a). Hexamerins and Hcs can easily be distinguished by the absence of the conserved Cu-binding histidines in hexamerins. In 1998, however, Sánchez and colleagues identified an Hc-like protein in the embryonic hemolymph of the grasshopper Schistocerca americana. This “embryonic hemolymph protein” (EHP) measures 674 amino acids (including an N-terminal signal peptide of 20 residues) and shows conservation of the three structural domains and the six Cu-binding histidines. Although this finding was the first indication that true Hcs may occur in insects, a role of EHP in O₂ transport was uncertain because neither O₂-binding nor its expression in later developmental stages could be demonstrated (Sánchez et al., 1998).

Recently, however, the presence of a true Hc was demonstrated in the stonefly Perla marginata (Plecoptera) (Hagner-Holler et al., 2004). This Hc occurs in the hemolymph at moderately high concentrations in nymphs and adults, and there is no doubt that it acts as respiratory protein. P. marginata Hc binds O₂ reversibly with a P₅₀ of 8 Torr and shows moderate cooperativity. Thus the O₂ affinity of P. marginata Hc is lower than that of Chironomus Hb, which is likely related to the higher PO₂ in the stonefly’s environment. Stonefly Hc is a hexamer with a mass of about 460 kDa, which consists of two distinct subunits that assemble in unknown stoichiometry. Both subunits contain N-terminal signal sequences for transmembrane transport. The native Hc subunits comprise 659 and 655 amino acids with molecular masses of 77.2 and 76.3 kDa, respectively. In both subunits the six Cu-binding site histidines in the second domain, as well as phenylalalanines in the first and second domain that stabilize the binding of O₂, are conserved. Isolated subunits have high O₂ affinities of P₅₀ = 0.063 and 0.023 Torr. Interestingly, the two Hc subunits appear to have divergent origins. While Hc1 resembles the EHP of S. americana (thus providing further indirect evidence for a respiratory function of this protein) and forms a common clade with the insect hexamerins, Hc2 has a more ancient evolutionary origin and diverged before Crustacea and insects split (Fig. 3).

The leading phylogenetic hypothesis suggests a close relationship between insects and crustaceans, or even the inclusion of the insects within a Pancrustacean taxon (Hwang et al., 2001; Giribet et al., 2001). This view is also supported by hemocyanin phylogeny (Fig. 3). Fundamental changes in the mechanisms of gas exchange must have accompanied the diversification of insects from crustaceans and their invasion of terrestrial and aerial environments (Kukalová-Peck, 1991). Nevertheless, Hcs are still present in many lower insects. This finding shows that neither terrestrialization nor the evolution of a tracheal system for efficient O₂ supply was accompanied by the loss of a hemolymph-based respiratory protein. A similar observation has been made in Onychophora and Myriapoda, which possess both trachea and Hc (Jaenicke et al., 1999; Kusche et al., 2002, 2003).

We have also investigated other insect species for the presence of Hc (C. Pick and T.B., unpublished data). Although the picture is far from being complete, the data show that Hcs are common in many different “lower”
Phylogeny of insect hemocyanin subunits

Fig. 3. Phylogeny of insect hemocyanin subunits. A Bayesian phylogenetic tree was deduced from the analyses of an amino acid alignment of insect hexamerins and hemocyanins. All nodes are supported with >0.95. Insect hemocyanin subunits are shaded in gray.

insect taxa (Fig. 1). Hc sequences have been identified in Collembola, Diplura, Archaeognatha, Zygentoma, Plecoptera, Orthoptera, Phasmida, Dermaptera, Isoptera and Blattodea, but appear to be missing in Eumetabola. The latter statement is confirmed by the absence of Hc in the genomes of *D. melanogaster* and other Drosophilids, *A. mellifera*, *T. castaneum*, *A. aegypti* and *B. mori*, and in the ESTs from *T. castaneum*, *A. aegypti* and *A. gambiae*. Some other “lower” taxa apparently have lost Hc as well. E.g., Hcs could not be identified so far in larvae and adults of dragonflies (Odonata) and mayflies (Ephemeroptera). Whether this finding is due to the actual lack of Hc genes in these species or to Hc expression only in particular developmental stages remains to be investigated. At the moment, no environmental, morphological or physiological character is known that could explain the sketchy pattern of presence or absence of Hc in insects. However, once lost, Hc could not be regained. When insects entered hypoxic environments that required a respiratory protein to enhance O2 supply (such as in case of the backswimmers, *Gasterophilus* and the chironomids), they had to recruit Hb from the intracellular globin gene that appears to be available in the genome of all insects.

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