Hemoglobin From a Deep-Sea Hydrothermal-Vent Copepod

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Abstract. Deep-sea hydrothermal-vent fauna live in a highly variable environment where oxygen levels can be very low, and carbon dioxide and sulfide can reach high concentrations (1). These conditions are harsh for most aerobic metazoans, yet copepods can be abundant at hydrothermal vents. Here we report the structure and functional properties of hemoglobin extracted from the copepod Ben-thoxynus spiculifer, which was found in large numbers in a paralvinellid/gastropod community collection made during a cruise to the Juan de Fuca Ridge in 1998. Although hemoglobin has been reported in some littoral copepods (2), this is the first study of the structure and functional properties of copepod hemoglobin. Hemoglobin represents about 60% of the total soluble proteins extracted from B. spiculifer, and although it imparts a red color to the copepod, it does not provide a significant storage pool of oxygen. It is a 208-kDa protein, composed of 14 globin chains—7 of 14.3 kDa and 7 of 15.2 kDa. The hemoglobin has a very high and temperature-sensitive oxygen affinity, with no cooperativity or Bohr effect. These properties are adaptive for an animal living in a low-oxygen environment in which the primary function of the hemoglobin is most likely oxygen acquisition to support aerobic respiration.

Copepods occur in both freshwater and marine environments that range from pelagic to benthic and littoral to deep-sea (3). Red copepods have been observed at hydrothermal vents of the Mid-Atlantic Ridge, Juan de Fuca Ridge, and East Pacific Rise (SH, pers. obs.). However, the number of animals collected has previously been too small for a study of their oxygen-binding protein. On dive 3259 of the DSRV Alvin, a paralvinellid (worm) / gastropod community (similar to Community III described by Sarradin et al. [4]) was collected from the base of the S & M chimney on the main field of the Endeavour segment of the Juan de Fuca Ridge. In that community, the animals are probably exposed to temperatures ranging from 10° to 25°C (4). The collection was made using a new device, nicknamed the Chimney Master, which is a hydraulically actuated net lined with 62-μm mesh and suspended in an aluminum frame. The 30-cm-diameter open end of the device is placed over a community to be collected, and then the net is drawn closed by a stainless steel cable while the frame is held firmly against the substrate by the submersible. In an appropriate environment, the Chimney Master removes and collects all attached and associated fauna from the substrate along with a surface layer of loose rocks and sulfides.

The collection contained many specimens of the copepod Benthoxynus spiculifer. Examination of the animals revealed that their deep-red color was not due to the gut content (which consisted of white filamentous material resembling bacteria) but rather to a soluble pigment distributed throughout the rest of the body. About 6000 specimens were separated from the collection, using a pipette; these were rinsed, concentrated by centrifugation, and frozen at -70°C in several cryovials.

The hemoglobin was purified from an extract of about 4000 animals that were thawed; homogenized in an extraction buffer containing 1 μM PMSF (phenylmethanesulfonyl fluoride) and 1mM EDTA in 50 mM Tris, pH 8; and then centrifuged to remove animal debris. The extract was puri-
fied by size exclusion chromatography (see legend of Fig. 1). The pigment eluted as a single pink band that represented about 55% to 60% of the total soluble proteins in the extract. Using proteins of known molecular weight for calibration, we estimated the apparent native molecular weight of the pigment to be 208 kDa. This pure fraction was used for further studies.

The light absorbance spectrum of the 208-kDa fraction showed the typical peaks for oxy-hemoglobin: α, β, and δ (Soret's band) peaks at 578 and 544 and 414 nm, respectively (Fig. 1). The γ and the protein peaks were present at 348 nm and 270 nm, respectively. The absence of a methemoglobin peak at 630 nm confirmed that little or no hemoglobin had been oxidized. The ratio α/β was 0.83, smaller than unity, as reported for some other extracellular hemoglobins (see [5]). The presence of hemoglobin has previously been reported in other copepods from reduced environments by Fox (2), who detected this protein in vivo using a microspectrophotometer. To determine the subunit structure of the 208-kDa fraction, it was further fractionated by SDS polyacrylamide gel electrophoresis in the presence and absence of β-mercaptoethanol (Fig. 2). Two bands, of 14.3 and 15.2 kDa, were resolved under both conditions. This suggests that the native molecule is composed of monodomain globin chains that are not linked by disulfide bonds. The bands were of similar intensity, suggesting that the intact hemoglobin molecule is composed of 14 chains (7 of each type), with a calculated mass of 206.5 kDa. This agrees well with the native mass estimated by gel filtration of 208 kDa. This structure is unusual for an arthropod hemoglobin (Table 1). Insect hemoglobins are generally much smaller (15-30 kDa), whereas those of Crustacea have a high molecular weight (220-4000 kDa). The mass of B. spiculifer hemoglobin is at the lower limit of those observed among crustaceans. The subunits are monodomain globins and not multidomain globins as is more normal for crustaceans (Table 1). With regard to subunit mass, B. spiculifer hemoglobin is similar to that found in Rhizocephala, although the native mass is 5 to 20 times smaller.

The functional properties of the hemoglobin were studied using the step-by-step procedure (6) in a modified diffusion chamber (7). Under the conditions we used, the oxygen affinity of the hemoglobin is extremely high, with $P_{50}$ values at pH 7.3 of 0.05, 0.13, and 0.35 mm Hg at 10°, 20°, and 30°C, respectively (Fig. 3A). These affinities are among the highest reported for arthropod hemoglobins (8). Among the arthropods, only the conchostracan Cyzicus hierosolymitanus (9) has hemoglobin with higher affinity ($P_{50} = 0.035$ mmHg at 28°C and pH 7.2, Table 1). Both species, B.
**COPEPOD HEMOGLOBIN**

**Table 1**

<table>
<thead>
<tr>
<th>Occurrence and structural and functional characteristics of hemoglobin in arthropods</th>
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<td>SbP</td>
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<td><strong>Uniramia</strong></td>
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<td><em>Hexapoda</em></td>
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<td><strong>Crustacea</strong></td>
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<td><em>Branchiopoda</em></td>
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<td>Siphonostomatoida</td>
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<td><em>Malacostraca</em></td>
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<td>Eumalacostraca</td>
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<td>Peracarida</td>
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SbP: subphylum; C: class; SbC: subclass; SpO: superorder; O: order. Modified after Terwilliger (16). Conchastracan Hb. \( P_{50} \) measured at 28°C. all other \( P_{50} \) measured at 20°C.

*B. spiculifer* and *C. hierosolymitanus*, have \( P_{50} \) values 20 to 600 times smaller than the \( P_{50} \) values of other arthropod hemoglobins. The affinity of *B. spiculifer* hemoglobin for oxygen is also higher than that reported for the hemocyanins of other hydrothermal vent crustaceans, although their hemocyanin \( P_{50} \) values are quite low (reviewed in [10]).

*Benthoxynus spiculifer* hemoglobin lacks cooperativity (\( n_{50} = 1.0 \)) over the range of temperature (10°C to 30°C) and pH (6.7 to 8.1) examined. The hemoglobin components of *Chironomus thummi thummi* (Insecta) also lack cooperativity; however, in contrast to *B. spiculifer* hemoglobin, they are monomeric or dimeric (Table 1). The other arthropod hemoglobins are multimeric and exhibit some cooperativity (\( n = 1.6 \) to 2.3). Like the hemoglobin of most crustaceans, that of *B. spiculifer* does not exhibit a significant Bohr effect (\( \Phi = +0.04 \)) (Table 1). However, the hemocyanins of hydrothermal vent crustaceans often show substantial Bohr effects (10), as do the hemoglobin components of the arthropod *C. thummi thummi* (\( \Phi = -0.9 \)). *B. spiculifer* hemoglobin thus does not show any homotropic or heterotropic interactions and behaves like a myoglobin. Temperature has a strong effect on *B. spiculifer* hemoglobin, as shown by the apparent \( \Delta H \) value of -69 kJ · mole⁻¹ (Fig. 3B). This value is higher than that of other arthropod hemoglobins (Table 1) and is consistent with the absence of a Bohr effect (oxygenation-linked proton dissociation that is endothermic and decreases the overall exothermic heat of oxygenation) in *Benthoxynus* hemoglobin (11).

To determine the in vivo hemoglobin concentration, a small group of copepods (about 400 animals) was weighed and homogenized in a ground-glass tissue homogenizer, and the hemoglobin content of the homogenate was determined using the cyan-met-hemoglobin method and a millimolar absorption coefficient of 11 cm⁻¹ at 540 nm (12). The estimated in vivo concentration of 0.95 mM heme explains the conspicuous red color of the animals. Large hemoglobin pools can play an important role in oxygen storage in some situations. However, assuming a respiratory rate similar to that of littoral harpacticoid copepods (13) and an abrupt switch from aerobiosis to anaerobiosis, we estimate that the quantity of hemoglobin present would support aerobic respiration for less than 2 min at 15°C and about 30 s at 25°C. Thus the hemoglobin pool is insufficient to allow the copepod to make more than short forays into anaerobic microhabitats without relying on anaerobic respiration. Another role of high affinity hemoglobins has been theorized to be detoxification of free radicals from oxygen or nitrogen monoxide (14). Although free radicals do form in sulfidic systems, and some vent animals have detoxification mechanisms (15), we consider it more likely that the hemoglobin of *B. spiculifer* functions primarily in oxygen acquisition from the environment. The other hemoglobin-containing copepods identified by Fox (2) were collected from muddy and reduced environments with low levels of oxygen and high levels of sulfide. The adaptive significance of hemoglobin for acquisition of oxygen in these environments is
Figure 3. (A) Oxygen equilibrium curves of Benthoxynus spiculifer hemoglobin at 10°, 20°, and 30°C, measured as previously described (5), and (inset) arrhenius plot showing calculated values of the apparent oxygenation enthalpy values (ΔH). (B) Variation of P_{SO2} and n_{SO2} values with pH and temperature.

apparent, and the very high affinity of the hemoglobin in B. spiculifer probably also reflects the very low oxygen tensions this species experiences in its hydrothermal vent microhabitat. In this context it is relevant that hemoglobins or hemocyanins with high oxygen affinity characterize many hydrothermal vent animals (10).

Acknowledgments

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