Identification, Structure, and Properties of Hemocyanins from Diplopod Myriapoda*

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Hemocyanins are copper-containing, respiratory proteins that occur in the hemolymph of many arthropod species. Here we report for the first time the presence of hemocyanins in the diplopod Myriapoda, demonstrating that these proteins are more widespread among the Arthropoda than previously thought. The hemocyanin of Spirostreptus sp. (Diplopoda: Spirostreptidae) is composed of two immunologically distinct subunits in the 75-kDa range that are most likely arranged in a 36-mer (6 × 6) native molecule. It has a high oxygen affinity ($P_{so}$ = 4.7 torr) but low cooperativity ($h$ = 1.3 ± 0.2). Spirostreptus hemocyanin is structurally similar to the single known hemocyanin from the myriapod taxon, Scutigera coleoptrata, indicating a rather conservative architecture of the myriapod hemocyanins. Western blotting demonstrates shared epitopes of Spirostreptus hemocyanin with both chelicerate and crustacean hemocyanins, confirming its identity as an arthropod hemocyanin.

In the hemolymph of many arthropod species oxygen is transported by large respiratory copper proteins that are termed hemocyanins (1, 2). The principal structure of a hexamer of six similar or identical subunits in the 75-kDa range is conserved within all arthropod hemocyanins, although in many cases these hexamers associate to quaternary structures containing up to 8 × 6 subunits (3). Each subunit carries one oxygen molecule by the virtue of two copper ions that are coordinated by six histidine residues (4). Hemocyanins are members of a functionally and structurally diverse protein superfamily that includes arthropod tyrosinases (prophenoloxidases), crustacean nonrespiratory pseudo-hemocyanins (cryptocyanins), insect hexamers, and dipteran hexamerin receptors (5–10).

Hemocyanins had long been unknown among the Myriapoda (classes: Chilopoda, Diplopoda, Symphyla, and Pauropoda), whereas these proteins have been studied in detail in many different chelicerate and crustacean species. Respiratory proteins were considered unnecessary in the Myriapoda because these animals possess, similar to the insects, a well developed tracheal system (11). However, several years ago Mangum et al. (12) demonstrated the existence of a true, 36-mer hemocyanin in the hemolymph of the common house centipede Scutigera coleoptrata. The exceptional presence of a hemocyanin in the scutigeromorph Chilopoda has been attributed to the high activity and peculiar tracheal system of these species rather than representing a general feature of the Myriapoda (13). However, here we show that high amounts of hemocyanins are also present in the Diplopoda, indicating that these respiratory proteins are more widespread among the Myriapoda than previously thought.

EXPERIMENTAL PROCEDURES

Animals—Living male and female specimens of three diplopod species from the family of Spirostreptidae (two different species of the genus Spirostreptus, named “A” and “B” here, Telodeinopus aoutii) were obtained from the Aquazoo in Düsseldorf (Germany). The hemolymph was withdrawn from the dorsal intersegmental regions with a syringe, immediately diluted in one volume of 100 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 5 mM CaCl$_2$, and centrifuged for 10 min at 10,000 × g to remove hemocytes and tissue contamination. Purified hemolymph was stored at 4 °C or frozen at −20 °C. Protein concentrations were determined according to the method of Bradford (14).

Hemocyanin Purification and Analysis—About 30 ml (~1.5–2 mg of total protein) of freshly collected hemolymph of Spirostreptus B were applied to a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) and eluted with 100 mM Tris-HCl, pH 7.5, 20 mM MgCl$_2$, 20 mM CaCl$_2$, at a flow rate of 0.2 ml/min. Proteins were detected in a flow cell at 280 nm. The molecular masses of the fractions were estimated after calibration with native hemocyanin molecules with known sizes: Limulus polyphemus (48-mer, 3600 kDa), Euryperla Californica (24-mer, 1800 kDa), Astacus leptodactylus (12-mer, 900 kDa), and Panulirus interruptus (6-mer, 450 kDa). Electron microscopic analysis of the hemolymph samples was performed by negative staining with uranylacetate (15). UV-visible spectra were recorded on a Hitachi U-3000 spectrometer. Oxygen-binding curves at pH 7.5 in 100 mM Tris-HCl, 20 mM MgCl$_2$, 20 mM CaCl$_2$ were obtained using the polarographic-fluorometric method described by Loeve (16).

Cell Electrophoresis and Western Blotting—About 400 μg of purified Spirostreptus B hemocyanin were used to raise polyclonal antibodies in a guinea pig. Crossed immunoelectrophoresis was carried out according to Weeke (17). SDS-PAGE (Fig. 1A) was performed on a 7.5% gel (18). For Western blotting, the proteins were transferred to nitrocellulose after negative staining with uranylacetate in a guinea pig. Crossed immunoelectrophoresis was carried out according to Weeke (17). SDS-PAGE was performed on a 7.5% gel (18). For Western blotting, the proteins were transferred to nitrocellulose at 0.8 mA/cm$^2$. Nonspecific binding sites were blocked by 5% nonfat dry milk/TBST. The filters were washed three times for 20 min in TBST, incubated for 1 h with the appropriate secondary antibody conjugated with alkaline phosphatase, and diluted in 5% nonfat dry milk/TBST. The membranes were washed as above and the detection was carried out using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Identification of Putative Hemocyanins in Diplopoda—The hemocyanin of male and female adult specimens of three juliform Diplopoda from the family of Spirostreptidae (Spirostreptus A and B, T. aoutii) was investigated for the presence of hemocyanin-like proteins. In all species, prominent polypeptides with apparent molecular masses between 70 and 85 kDa were observed in SDS-PAGE (Fig. 1A), which is in the expected range.
range of arthropod hemocyanin subunits. In all three species, electron microscopic investigations of the hemolymph revealed the presence of protein particles that resemble the unique appearance of $6 \times 6$ S. coleoptrata hemocyanin (12, 19). This was the first indication that hemocyanin-like proteins may be present in the Diplopoda. In Fig. 1B, an electron micrograph of the hemolymph of Spirostreptus species B is displayed. This species was chosen for further investigations because of the large size of the adult animals (about a 10–15-cm body length, 1-cm diameter) and the high content of putative hemocyanin molecules in the hemolymph (about 40–50 mg/ml).

**Purification and Properties of Spirostreptus Hemocyanin**—Size-exclusion gel chromatography on a Superose 6 HR 10/30 column yields three peaks of about 2400, 1250, and 390 kDa (Fig. 2A). The first two peaks ($P_1$ and $P_2$) both contain two polypeptides of about 75 kDa, whereas the third peak consists of four other proteins between 85 and 200 kDa (Fig. 2B). Electron-microscopic investigations show no differences between the proteins of peak one and two with particles resembling the putative hemocyanin observed in the raw hemolymph samples, but also particles that probably correspond to monohexamers (not shown). The third peak does not contain any hemocyanin-like structures. Assuming a mean molecular mass of about 75 kDa/subunit (as revealed by SDS-PAGE), the first peak indicates a protein consisting of more than 30 subunits. Because arthropod hemocyanins only elute as multimers of six subunits and considering the similarities to Scutigera hemocyanin, a 36-mer ($6 \times 6$) molecule is the most probable structure of Spirostreptus hemocyanin. This assumption is also consistent with the appearance of this molecule in the electron microscopic images. The estimated molecular mass of the second peak ($P_2$) is about half of the first, suggesting a stable 18-mer dissociation product ($3 \times 6$).

Column-purified hemocyanin was used to raise specific antibodies in guinea pigs (Charles River, Germany). Then the hemocyanin was dissociated into subunits by dialysis against 0.13 M glycine/NaOH buffer for 2 days at pH 9.6 and applied to crossed immunoelectrophoresis (17). Although the dissociation of the hemocyanin was incomplete even after prolonged incubation at a high pH, we observed several precipitation lines, indicating the presence of at least two immunologically distinct polypeptides in the hemocyanin sample (Fig. 2C).

**Oxygen-binding Properties of Spirostreptus Hemocyanin**—In spectroscopic analysis, Spirostreptus hemocyanin shows a peak at 336 nm, which is typical for oxygenated hemocyanins and reflects the formation of the copper-oxygen complex (Fig. 3A). However, as already observed in the centipede S. coleoptrata, the $A_{340}/A_{280}$ ratio is much lower than in the other arthropod hemocyanins. This may be explained in part by its high extinction coefficient at 280 nm (1 mg/ml corresponds to 1.8–2 OD), which indicates a highly aromatic protein. The oxygen affinity of Spirostreptus hemocyanin was measured in Tris buffer at pH 7.4 according to Loewe (16) and yields a $P_{50}$ of 4.7 torr (Fig. 3B). A low cooperativity (Hill coefficient, $h = 1.3 \pm 0.2$) was detected. The hemolymph of Spirostreptus has an extraordinarily high protein content up to 100 mg/ml. We estimated that hemocyanin represents about 40–50% of the total hemolymph proteins (Figs. 1 and 2), indicating a hemocyanin content up to 50 mg/ml. Because every subunit of about 75 kDa can carry one oxygen molecule, an oxygen transport capacity of about 20 ml/liter hemolymph was calculated. This value is comparable with those of other Arthropoda.

**Spirostreptus Hemocyanin Cross-reacts with Antibodies Against Other Arthropod Hemocyanins**—Several specific antibodies directed against hemocyanins of Crustacea and Chelicera have been described in the literature; in this study, five different rabbit antibodies have been tested for cross-reaction with the Spirostreptus hemocyanin by Western blotting (Fig. 4). Two of the antisera were raised against chelicerate hemocyanins (E. californicum and Androctonus australis), and three were raised against hemocyanins of malacostracan Crustacea (A. leptodactylus, Carcinus maenas, and Homarus americanus). About 2.5 μg of total hemolymph proteins from Spirostreptus species B were applied/lane; the different antibodies were used in 1:5000 to 1:10,000 dilutions. Signals in the 75-kDa range were observed in all cases, although most of the antibodies cross-react differentially with
the two hemocyanin polypeptides. This is consistent with the assumption that they correspond to immunologically distinct hemocyanin subunits (see also Fig. 2C). None of the other proteins in the Spirostreptus hemolymph were detected by the anti-hemocyanin antibodies, demonstrating the specificity of the immunological relationship.

**DISCUSSION**

The hemocyanins of Arthropoda have been the subject of detailed functional, structural, and evolutionary studies mainly in the Chelicerata and Crustacea (1–5). This paper is the first report of the presence of hemocyanins in the diplopod Myriapoda. All three investigated species belong to the Spirostreptidae, a large family of mainly Afrotropical and Neotropical juliform Diplopoda. The hemocyanin of Spirostreptus sp. displays the typical arthropod hemocyanin features in terms of subunit size, structural appearance, and spectroscopic properties. Moreover, it shares similar antigenic determinants with crustacean and chelicerate hemocyanins. Therefore, the identity of the O₂-binding protein of Spirostreptus as a genuine hemocyanin should be considered as conclusive.

The presence of a typical hemocyanin in the Diplopoda is rather surprising. This protein appears to be structurally very similar to the hemocyanin from the centipede, S. coleoptrata (12, 13, 20). In this species, the presence of an oxygen-carrying hemocyanin was considered as an exception related to the peculiar blind-ending tracheal system in the Scutigeramorpha (13). In contrast, although the tracheal system of the Spirostreptidae is normal and similar to those of the other Diplopoda, a high hemocyanin concentration was observed in most specimens. Therefore, the evolution of trachea is not necessarily accompanied by a loss of specific respiratory proteins. This suggests that hemocyanins may be found in other Myriapoda as well and that these proteins are more widespread among the Arthropoda than previously thought.

Chilopod and diplopod Myriapoda probably diverged in the Devonian or an earlier period (21). However, whereas the subunit arrangement within the chelicerate and crustacean hemocyanins is variable, ranging from 1 × 6 to 8 × 6 subunits in the native molecule, the unique 6 × 6 organization of subunits appears to be a conservative feature of the Myriapoda that has been conserved for at least 400 million years. Nevertheless, the oxygen-binding properties of the hemocyanins from Scutigera and from Spirostreptus are strikingly different. Whereas Spirostreptus hemocyanin has a high oxygen affinity (P₅₀ = 4.7 torr at pH 7.5) and a low cooperativity (h = 1.3 ± 0.2), the situation is reversed in Scutigera hemocyanin, which has a low oxygen affinity (P₅₀ = 55 torr at pH 7.5) and high cooperativity (h = 8.9 at pH 7.5) (12). The high oxygen affinity of Spirostreptus hemocyanin may be related to the low oxygen conditions of the subterrestrial environment, where these species remain most of the time during daylight hours.

Recent molecular phylogenetic studies (22, 23) shed doubt on the monophyly of the taxon “Tracheata” or “Antennata,” which combines the Myriapoda with the Hexapoda (11). Although there is increasing evidence for a sister group relationship of the Hexapoda and Crustacea (23, 24), the actual systematic position of the Myriapoda became uncertain. Although we observed cross-reactivity of the Spirostreptus hemocyanin with antibodies that have been raised against either chelicerate or crustacean hemocyanins (Fig. 4), such data are not sufficient to determine the exact evolutionary relationships among the arthropods. However, at least some antigenic structures have been conserved among myriapod, crustacean, and chelicerate hemocyanins because of their divergence in the Cambrian period more than 520 million years ago. As already mentioned by Mangum et al. (12), the presence of hemocyanins (and related proteins, e.g. hexamericins) is an autapomorphic character of the Arthropoda, providing strong evidence in favor for a monophyletic origin of this phylum. These proteins have also been successfully used to investigate arthropod relationships (5–7). Hence, the determination of the cDNA sequence of Spirostreptus hemocyanin will not only provide insights in a complete new class of hemocyanins but will probably also allow the elucidation of the phylogenetic affinities of the Myriapoda.
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REFERENCES
1. Markl, J. (1986) *Biol. Bull. (Woods Hole)* 171, 90–115
2. van Holde, K. E., and Miller, K. I. (1995) *Adv. Protein Chem.* 47, 1–81
3. Markl, J., and Decker, H. (1992) *Adv. Comp. Environ. Physiol.* 13, 325–376
4. Linzen, B., Soeter, N. M., Riggs, A. F., Schneider, H. J., Schartau, W., Moore, M. D., Behrens, P. Q., Nakashima, H., Takagi, T., Nemoto, T., Vereijken, J. M., Bak, H. J., Beintema, J. J., Valbeda, A., Gaykema, W. P. J., and Hol, W. G. J. (1985) *Science* 229, 519–524
5. Beintema, J. J., Stam, W. T., Hazes, B., and Smidt, M. P. (1994) *Mol. Biol. Evol.* 11, 493–503
6. Burmester, T., and Scheller, K. (1996) *J. Mol. Evol.* 42, 713–728
7. Burmester, T., Massey, H. C., Zakharin, S. O., and Benes, H. (1998) *J. Mol. Evol.* 47, 93–108
8. Burmester, T. (1999) *J. Biol. Chem.* 274, 13217–13222
9. Burmester, T. (1999) *Eur. J. Entomol.* 96, in press
10. Terwilliger, N. B., Dangott, L. J., and Ryan, M. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2013–2018
11. Brusca, R. C., and Brusca, G. J. (1990) *Invertebrates* Sinauer Associates, Sunderland, MA
12. Mangum, C. P., Scott, J. L., Black, R. E. L., Miller, K. I., and van Holde, K. E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 3721–3725
13. Mangum, C. P., and Godette, G. (1986) in *Invertebrate Oxygen Carriers* (Linzen, B., ed.) pp. 276–280, Springer-Verlag, Berlin
14. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
15. Harris, J. R., and Agutter, P. S. (1970) *J. Ultrastruc. Res.* 33, 219–232
16. Loewe, R. (1978) *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 129, 161–168
17. Weekes, B. (1973) *Scand. J. Immunol.* 2, Suppl. 1, 47–56
18. Laemmli, U. K. (1970) *Nature* 227, 680–685
19. Boisset, N., Taveau, J.-C., and Lamy, J.-N. (1990) *Biol. Cell* 86, 73–84
20. Gebauer, W., and Markl, J. (1999) *Naturwissenschaften* 86, in press
21. Shear, W. A. (1997) in *Arthropod Relationships* (Fortey, R. A., and Thomas, R. H., eds) pp. 211–219, Systematic Association Special Volume Series 55, Chapman and Hall, London
22. Turbeville, J. M., Pfeifer, D. M., Field, K. G., and Raff, R. A. (1991) *Mol. Biol. Evol.* 8, 669–686
23. Friedrich, M., and Tautz, D. (1995) *Nature* 376, 165–167
24. Boore, J. L., Lavrov, D. V., and Brown, W. M. (1998) *Nature* 392, 667–668

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