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The molecular heterogeneity of hemocyanin: Structural and functional properties of the 4×6-meric protein of *Upogebia pusilla* (Crustacea)

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Abstract

The structural properties of the hemocyanin isolated from the Mediterranean mud shrimp, *Upogebia pusilla* (Decapoda: Thalassinidea), were investigated. Our intent was to make use of the *U. pusilla* case to perform a structural comparison between crustacean and chelicerate 4×6-meric hemocyanins. The thalassinidean hemocyanin appears similar in size but different in structural organization compared to the chelicerate 4×6-mer. Ultracentrifuge analyses on the purified protein revealed a sedimentation coefficient of 39S, typical of 4×6 hemocyanins. Electron micrographs are in agreement with a model in which four 2×6-meric building blocks are arranged in a tetrahedron-like quaternary structure and not in the quasi-square-planar orientation characteristic of the chelicerate protein. Size-exclusion chromatography-fast protein chromatography analysis showed elevated instability of the protein in absence of divalent ions or at pH values higher than 8.0. This analysis also shows that the dissociation of the *U. pusilla* 4×6-meric hemocyanin into hexamers occurs without any intermediate 2×6-meric state, in contrast with the dissociation profile of the chelicerate protein exhibiting several dissociation intermediates. The oxygen-binding properties of *U. pusilla* hemocyanin were studied to disclose possible effects by the typical allosteric effectors that modulate the functional properties of crustacean hemocyanin. A marked Bohr and lactate effect, but no significant influence of urate, on the oxygen affinity of *U. pusilla* hemocyanin were found.

Keywords: Hemocyanin; Quaternary structure; Dissociation; Oxygen binding; Catalytic properties

1. Introduction

Hemocyanins (Hcs) are giant oligomeric oxygen carriers present in the hemolymph of arthropods and molluscs. In arthropods, polypeptide chains with M_R of about 75 kDa, each containing one dinuclear copper oxygen-binding site, represent the functional and structural subunits. Arthropod Hcs, however, are generally found as oligomers where 6 subunits aggregate to form in general hetero2×6-meric complexes. More hexamers may eventually be combined into 2×6, 4×6, 6×6 or 8×6 oligohexamers. The particular aggregation form, in which the oxygen carrier is found, is highly species dependent, and more than one form may coexist in the same species (Markl and Decker, 1992). Significant differences in primary structures exist within the different subunits building the heterohexamers found in one species as well as within the Hcs subunits of different species. Within Crustacea, 2×6 and hexameric (1×6) Hcs are generally found: notably the former is the characteristic aggregation form of portunid crabs, the latter of caridean, peneid, pagurid and palinuran decapod species (Markl, 1986).

In the context of Crustacea, the thalassinidean shrimps represent a unique case since they carry a 4×6-meric Hc. Comparative studies on the association state of these Hcs revealed a main component with 39S sedimentation coefficient, which corresponds to the 4×6-mer of chelicerates (Roxby et al., 1974; Miller and van Holde, 1974; Miller et al., 1977; Ellerton et al., 1983; Miller and van Holde, 1982; Taylor et al., 2000). In addition, a minor pool of Hc in the 1×6-meric form (17S) was described. A 2×6-meric intermediate form (25S) was found notably for members of the family of Upogebiidae, *U. pugettensis* (Miller et al., 1977) and *U. deltaura* (Taylor et al., 2000).

Abbreviations: EDTA, ethylene diamine tetra-acetic acid; EM, electron microscopy; FPLC, fast protein liquid chromatography; Hc, hemocyanin; SEC, size-exclusion chromatography; TEM, transmission electron microscopy.

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The 4×6-meric Hcs are present also in chelicerates. The physico-chemical properties of the protein isolated from the tarantula *Eurypelma californicum*, have been thoroughly investigated. While the native protein is characterized by a sedimentation coefficient of 37S, several oligomers can be singled out in dissociation experiments (Markl et al., 1982; Savel-Neimann et al., 1988; Voit et al., 2000).

Two models are known for the ikosatetrameric Hc: the first one refers to chelicerate 4×6-mers, fully described for the scorpion *Androctonus australis* (Lamy et al., 1981) and the tarantula *E. californicum* (Erker et al., 2006; Voit et al., 2000); the second model has been proposed to describe the thalassinidean shrimps Hc, notably in *Callianassa californiensis* (Cavellec et al., 1990). It was shown by multivariate statistical analysis applied to computer aligned electron microscopic images that the 4×6 Hcs of *A. australis* and *E. californicum* are very similar (Bijholt et al., 1982) and fit the model earlier derived for the 4×6 half molecules of *Limulus polyphemus.* Such 4×6-mers are quasi-square-planar and comprise two 2×6-meric halves in an antiparallel arrangement that come in contact edge-to-edge, leaving a "deep cleft" between them (Martin et al., 2007). No high-resolution structure is available for the crustacean 4×6-mer but electron microscopy (EM) analysis revealed a completely different quaternary structure. EM analysis displayed a characteristic triangular shape and a three-dimensional model was constructed to combine a 4×6 aggregation state with the triangular profiles observed in the electron micrographs. The model consists of four 1×6-meric building units associated in a tetrahedron-like structure (Cavellec et al., 1990). Thus, while both in thalassinidean shrimps and chelicerates 4×6-meric Hcs exist, their quaternary structures seem to be very different.

The oxygen-binding properties of Hcs, that define their physiological role as oxygen carriers, are modulated by protons and organic ions such as lactate and urate. The effects of pH and organic ions were intensively studied on crustacean and chelicerate Hcs and different responses in the two *phyla* were described. Protons affect the oxygen-binding properties of both crustacean and chelicerate Hcs. In contrast, lactate and urate exert their effect on crustacean Hcs but not on chelicerate proteins. Within Crustacea, however, not all species exhibit this effect: in particular, no lactate effect was reported for thalassinid shrimps *Colocaris macandreae* (Taylor et al., 2000) and *Neotrypaea* (*Callianassa*) *californiensis* (Mangum, 1983).

Our focus in this work was the structural characterization of the Hc isolated from the thalassinid shrimp *Upogebia pusilla* in order to generalise the model of a thalassinid-specific hexamers arrangement, the association–dissociation behaviour and the oxygen-binding properties of this 4×6-meric Hc in comparison to the 24-mers found in chelicerates. Furthermore, we have investigated the oxygen-binding properties of *U. pusilla* Hc, with the aim to add further information on the lactate and urate effect on thalassinid shrimps Hcs.

2. Materials and methods

Living specimens of *U. pusilla* were purchased from local fishermen at Porto Levante on the Adriatic Sea in the proximity of the delta of the river Po. The hemolymph was collected by means of a needle inserted into the pericardium of living animals. The hemolymph, sampled from N=250/300 individuals, was stored at 0 °C after addition of protease inhibiting cocktail (Sigma-Aldrich Art. P2714). The samples were successively dialyzed against stabilization buffer (Tris 100 mM, $CaCl_2$ 20 mM, pH 7.6) and subsequently centrifuged at 45,000 *g* for 25 min (Beckman J2-21) for the removal of cells and other suspended material. The Hc was finally isolated by sedimentation at 296,000 *g* for 5 h in a Beckman ultracentrifuge XL-70. After purification, the pellet was resuspended in the same buffer and stored at −20 °C in the presence of 20% (w/v) sucrose. Before use, Hc was further purified by size-exclusion chromatography (SEC) carried out with a Sephacryl S-300 XK 26/60 column (Pharmacia). Elution was performed at low pressure and regulated by a BIORAD BioLogic DuoFlow FPLC device with stabilization buffer at 1.3 mL/min. Before chromatography, the protein was dialyzed against stabilization buffer. Fractions exhibiting an absorbance ratio A_{338}/A_{280}=0.21–0.25 were pooled and used for further analysis.

Both the Bradford and the Lowry assays were employed to determine the extinction coefficient of *U. pusilla* Hc. Two different Hcs standards were used to calibrate the colorimetric assay: *E. californicum* Hc, which has about the same size of *U. pusilla* Hc but belongs to the group of chelicerates; and *Carcinus aestuarii* Hc, a 2×6-mer but phylogenetically more closely related to the studied Hc. The concentrations of standard Hcs in the samples used to calibrate the assays were determined using the coefficients: 1.10 mL mg^{-1} cm^{-1} for *E. californicum* Hc (Loewe, 1978) and 1.24 mL mg^{-1} cm^{-1} (Dainese et al., 1998) for *C. aestuarii* Hc. The Bradford and the Lowry assays were used to determine the concentration of *U. pusilla* Hc solutions used to record the absorption spectrum. By this approach it was possible to calculate an extinction coefficient at 278 nm of 1.11±0.05 mL mg^{-1} cm^{-1} for *U. pusilla* Hc.

Aggregation state was determined by comparing *U. pusilla* Hc SEC-FPLC elution profile with the elution profiles of *Palinurus elephas* 1×6 Hc, *Astacus leptodactylus* 2×6 Hc and *E. californicum* 4×6 Hc. Chromatography was performed in the buffer above described with a Sephacryl S-300 XK 26/60 column (Pharmacia). Dissociation experiments were carried out with the column as above using 50 mM Tris/HCl buffer at various pH values and in the presence of 10 mM EDTA. Ion exchange chromatography was performed on a Resource Q column equilibrated with 50 mM Tris/HCl, 10 mM EDTA pH 9.2.

The aggregation state was also determined by sedimentation velocity analysis performed with a Beckmann XL-I analytical ultracentrifuge. For each analysis, 400 μL of 0.7 mg/mL purified sample were employed. The experiments were carried out at 25,000 rpm (rotor Beckman type 60 TI) for 100 min and 50 measurements (at 2 min intervals each) were performed at wavelengths of 278 (and 338 nm). The data were processed with the freeware UltraScan II v.7.2 (Borries Demeler and University of Texas System). The sedimentation constants given correspond to the values extrapolated for 20 °C and water ($S_{20°,w}$).

Transmission electron microscopy (TEM) analysis was carried out on a Hitachi H600 electron microscope. Diluted aliquots of Hc