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Quadrupole Time-of-Flight Mass Spectrometry of the Native Hemocyanin of the Deep-Sea Crab *Bythograea thermydron*

Franck Zal,*,† Fabienne Chausson,† Emmanuelle Leize,‡ Alain Van Dorsselaer,‡ François H. Lallier,† and Brian N. Green§

Equipe Ecophysiologie, UMR 7127 CNRS/UPMC, Station Biologique, BP 74, 29682 Roscoff Cedex, France, Laboratoire de Spectrométrie de Masse Bio-Organique, Université Louis Pasteur−CNRS UMR 7509, ECPM, 25 rue Becquerel, 67087 Strasbourg Cedex, France, and Micromass UK Ltd., Tudor Road, Altrincham, Cheshire WA14 5RZ, U.K.

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Since electrospray ionization mass spectrometry (ESI-MS) has demonstrated capabilities for observing intact, weak interactions, there has been increasing interest in studying by this method noncovalently bound complexes. In this communication, we report for the first time the structure obtained by a commercial ESI quadrupole time-of-flight spectrometer on a native hemocyanin of deep-sea crab *Bythograea thermydron* with a molecular mass of 1.3 MDa. ESI-MS analysis of the native hemocyanin revealed the formation of a 18-mer noncovalent assembly with a measured molecular mass of 1 354 940 ± 480 Da. ESI-MS data also revealed that this huge structure is an equilibrium with several assemblages, dodecamer (measured molecular weight = 902 570 ± 110 Da), hexamer (measured molecular weight = 450 310 ± 260 Da), and monomeric structures (measured molecular weight = 74 999 ± 85 Da).

In recent years, mass spectrometry has become one technique of choice for structural biology studies.[1,2] More recently, the combination of electrospray ionization (ESI) with the orthogonal acceleration time-of-flight (Tof) mass analyzer was introduced as a new type of mass spectrometer suitable for protein and peptide analysis.[3,4] This type of instrument is particularly well-suited to the study of noncovalent interactions between proteins in large complexes because of its high sensitivity and wide mass range. Some examples are available in the literature on noncovalently assembled large biomolecules witnessing the novelty of this research area.[5−7] The largest molecule so far observed by ESI mass spectrometry (MS) is the intact MS2 virus capsid of mass 2484.7 kDa.[8] In this study, the multiply charged ions representing the intact molecule were barely resolved, and only part of the final spectrum was shown. The measured mass was 0.55% higher than that predicted from its component protein. Another example showed the GroEL molecule, partly resolved at 803.7 kDa and 0.37% higher than that predicted from its component parts.[9] In contrast, well-resolved species were observed up to an octamer−trimer (24-mer) of vanillyl alcohol oxidase at 1530 kDa.[7] In this case, the measured mass was remarkably close to that predicted from its component parts. In this communication, we report for the first time on the structure of a purified hemocyanin (Hc) analyzed on a commercial ESI-QTof instrument (Q-Tof 2, Micromass, U.K.) using the ESI Z-spray source. To preserve the integrity of the noncovalently assembled species and enhance their sensitivity, the pressure in the interface between the atmospheric source and high vacuum was increased to 5 mbar (normally ∼2 mbar) by throttling the pumping line.[10,11] The cone voltage (declustering potential) was 200 V.

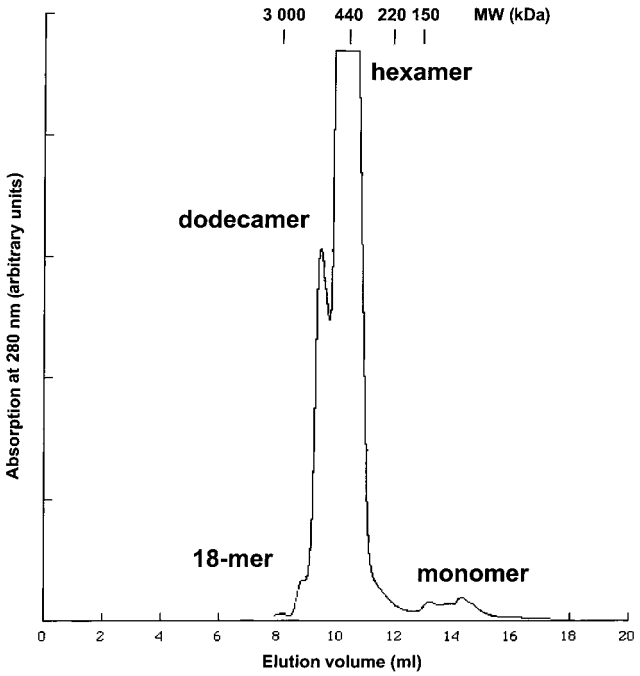

Figure 1. Elution profile of native hemolymph obtained from *Bythograea thermydron* and monitored at 280 nm showing the different hemocyanin aggregations. Due to its high concentration the hexamer peak is outside the range of detection. The proportions of the different species were deduced from the FPLC data, assuming that absorption of 18-mer, 12-mer, and hexamer were 18 times, 12 times, and 6 times, respectively, more intense than the absorption of the monomer.

* To whom correspondence may be addressed. Phone: (33) 2 98 29 23 09. Fax: (33) 2 98 29 23 24. E-mail: zal@sb-roscoff.fr.

† Equipe Ecophysiologie, UMR 7127 CNRS/UPMC.

‡ Laboratoire de Spectrométrie de Masse Bio-Organique, Université Louis Pasteur−CNRS UMR 7509, ECPM.

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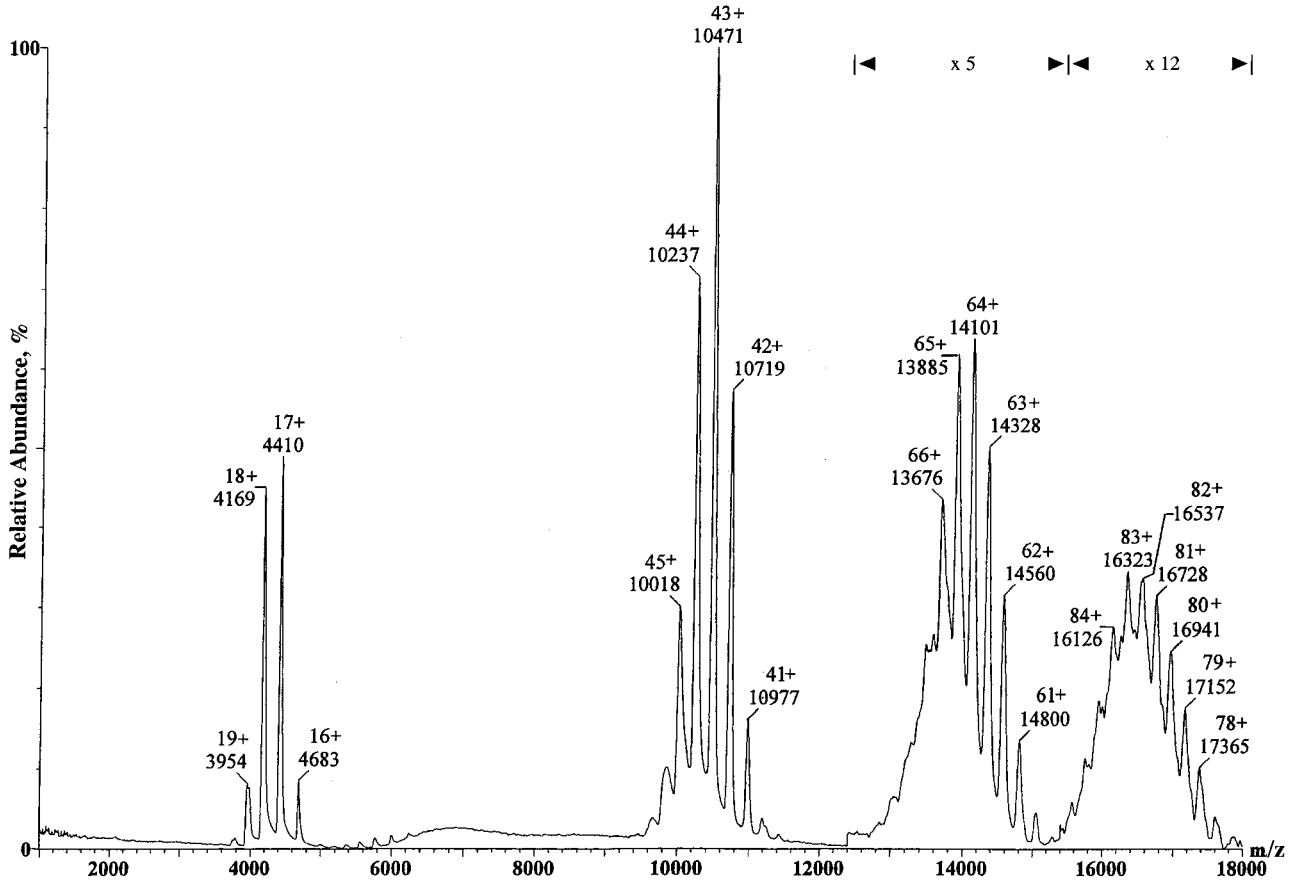

Figure 2. ESI mass spectrum of native *Bythograea thermydron* hemocyanin analyzed at 2 μM concentration in 10 mM ammonium acetate (pH 6.5). The figures above the *m*/*z* values at the tops of the peaks indicate the number of positive charges on the ions.

Hc is an extracellular blue protein that occurs at high concentration in the blood of many molluscs and arthropods.[12] Among this latter group, it is present in spiders, scorpions, horseshoe crabs, crustaceans, and at least in two centipedes. This protein serves as an oxygen carrier, but instead of iron as in hemoglobin, the oxygen-binding site consists of a pair of copper atoms held in place by three histidine residues. Hc's comprise a number of subunits that noncovalently assemble into extremely large macromolecular entities. Arthropod Hc's generally occur as hexamers and oligohexamers assembled from 75 kDa polypeptide chains, each containing a single oxygen binding site.[12] In the hemolymph of the deep-sea crab, *Bythograea thermydron*,[13] an equilibrium mixture of monomer (18%), hexamer (67%), dodecamer (13%), and octadecamer (1%) was observed by gel filtration at room temperature, Figure 1.[14] The proportions of these multi-subunit associations appear to vary with concentration and temperature. The ESI mass spectrum obtained under noncovalent conditions from *Bythograea thermydron* Hc displayed four groups of peaks (Figure 2).[15] The peaks in each group represent a series of positive ions of the same molecular species in which the charge on each ion in the series differs by one charge from its immediate neighbors. This relationship between adjacent ions allows the number of charges on each ion to be established, and hence several values of the molecular weight (mass) can be derived from each series. The mean masses thus derived from each series are summarized in Table 1, assuming that the charges result from multiple protonation. The Hc was also analyzed in denaturing solvent, from which the average mass of the monomer was determined to be 74 706.9 Da. This mass is assumed to correspond to the monomer lacking the pair of copper atoms. A comparison between multiples of the denatured monomer mass (plus the mass of two copper atoms, 74 834 Da) and the masses of the four molecular species determined under noncovalent conditions shows that the closest agreement occurs for 1, 6, 12, and 18 monomers (Table 1). It can be seen from the difference column in the Table 1 that the masses measured under noncovalent conditions are 0.2−0.6% higher than those predicted from the denatured Hc. These mass differences are similar to other measurements made on noncovalent assemblies[8,9] and are presumably due to the inclusion of low molecular weight ligands, e.g., water and cations, that are not present in the denatured monomer. Despite these differences, there is no doubt that the four species observed under noncovalent conditions correspond to the monomer, hexamer, dodecamer

Table 1. Masses of *Bythograea thermydron* Hc Species Determined by ESI-MS

species	measd mass ± SD (Da)[a]	predicted mass (Da)[b]	difference (%)
monomer	74 999 ± 85	74 834	0.22
hexamer	450 310 ± 260	449 004	0.29
dodecamer	902 570 ± 110	898 008	0.51
octadecamer	1 354 940 ± 480	1 347 012	0.59

[a] Mean derived from multiply charged series observed under noncovalent conditions. [b] Predicted from mass of denatured Hc plus two copper atoms (74834 Da).

(probably hexamer-dimer), and octadecamer (probably hexamer-trimer). These results also confirm that the principal noncovalent assembly is indeed a hexamer of 75 kDa subunits with lesser amounts of the other species. This is the first time that the noncovalent assemblies present in an arthropod Hc have been observed and their molecular weights determined by ESI-MS.

The ESI-MS result implies that the *Bythograea thermydron* Hc exists as a mixture in equilibrium of four species, namely, monomer (25%), hexameric (60%), dodecameric (10%), and octadecamer (5%), which show a good agreement with the proportions determined by fast protein liquid chromatography (FPLC), particularly with respect to the monomer. The difference in the proportion determined by the two techniques could be due in part to the different concentrations and buffers used. The use of saline buffer in FPLC and ammonium acetate buffer in ESI-MS could induce different levels of dissociation. It is also possible that some fragmentation of noncovalent assemblies occurred within the mass spectrometer. Nevertheless, despite the different proportions observed by the two techniques, there is no doubt about the composition of the four components observed by ESI-MS. Overall, these initial results demonstrate that ESI-MS has potential for the structural study of Hc's, provided that their basic subunits have similar homogeneity to the 75 kDa subunits in *Bythograea thermydron* Hc. A prerequisite for the accurate determination of the molecular weight of proteins by ESI-MS is that ions in the multiply charged series are adequately resolved from one another. Ultimately, this requirement depends on the homogeneity of the proteins comprising the noncovalent assembly.

Conclusions

The results of the present study revealed that viable data from these large molecules can be generated by the standard Z-spray ESI source which we have found to be more routine to operate, although it has a higher sample consumption rate than the nanospray source. Future studies on native *Bythograea thermydron* Hc would allow the proportions of the different aggregation states to be further investigated and quaternary models for this multimeric blue protein proposed. These results may have implications on the oxygen binding properties of this respiratory pigment and consequently on the adaptation strategies developed by this species to suit various habitats.

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(13) Individuals of *Bythograea thermydron* were collected during the "HOT 96" and "HOPE 99" oceanographic cruises with a baited trap from the "M-Vent" site (09°50′N, 104°16′W; 2510 m in depth) located on the East Pacific Rise. Hemolymph was quickly sampled with a syringe through the arthropodial membrane at the base of the fourth pair of legs. Samples were frozen immediately in liquid nitrogen. Before use, samples were thawed at 4 °C and centrifuged for 15 min at 12 000 rpm, and the supernatants were collected.
(14) Analytical gel filtration of hemolymph was performed on a Superose 6-C column using a low-pressure FPLC system (Pharmacia). The column was equilibrated with a physiological saline buffer (400 mM NaCl, 10 mM KCl, 30 mM $MgSO_4$, 20 mM $CaCl_2 \cdot 2H_2O$, and 50 mM Tris, pH 7). The eluate was monitored with a UV detector at 280 and 337 nm. To estimate the molecular weight of each fraction, the column was calibrated with the vascular hemoglobin of *Riftia pachyptila* obtained from stored samples and determined previously SV1 (3503 kDa), SV2 (433 kDa), SV3 (220 kDa), and SV4 (150 kDa). Zal, F.; Lallier, F. H.; Wall, J. S.; Vinogradov, S. N.; Toulmond, A. *J. Biol. Chem.* **1996**, *271*, 8869.
(15) ESI mass spectrometry was performed on a Q-Tof II instrument fitted with a standard Z-Spray source (Micromass, Altrincham, U.K.). To preserve the integrity of the noncovalently assembled species and enhance their sensitivity, the pressure in the interface between the atmospheric source and high vacuum was increased to 5 mbar (normally ∼2 mbar) by throttling the pumping line. The cone voltage (declustering potential) was 200 V, and the source and desolvation gas temperatures were 100 °C. Mass scale calibration used the $Cs_{(n+1)}I_n$ ions from a solution of CsI (2 mg/mL in 2-propanol/water (1:1)). Sample (2 μM concentration based on a mass of 450 kDa, pH 6.5) in 10 mM aqueous ammonium acetate was introduced at a 5 μL/min flow rate using a syringe pump (type 22, Harvard Apparatus Inc., South Natick, MA). Desalting was necessary in order to remove alkali metal adducts and hence reduce the peak width. This was undertaken by shaking the Hc solution prior to adding the ammonium acetate for 1 min with a few milligrams (5−10 mg/200 μL of solution) of washed (twice with water) mixed bed ion-exchange resin beads (AG 501-X8, catalog no. 143-7424, Bio-Rad). Data were acquired over m/z 1000−18000 for 8 min and smoothed. Molecular species were assumed to be represented by a series of multiply protonated peaks. The mass of each peak was determined from its peak top m/z value using mass = $n(m/z + H)$, where n is the number of charges and H the mass of the proton. The mass of each species is expressed as a mean of the masses calculated from the series of ions plus standard deviation. Charge state assignments were those that gave minimum standard deviation.[8] Ambiguities were resolved by using the maximum entropy based deconvolution software supplied with the mass spectrometer to establish the approximate mass from which the charge states were derived.

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