Comparative ESI-MS Study of ~2.2 MDa Native Hemocyanins from Deep-Sea and Shore Crabs: From Protein Oligomeric State to Biotope

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In the past years, the potential of electrospray ionization mass spectrometry (ESI-MS) for the observation of intact weak interactions, such as non-covalent protein-ligand, protein-protein, protein-DNA complexes, has spread out. The coupling of ESI with time-of-flight (TOF) and quadrupole-time-of-flight (Q-TOF) analyzers has even enabled the detection of larger complexes with molecular weights greatly higher than 200 kDa. In this paper, we report a comparative ESI-MS study on the protein quaternary structure of native hemocyanins (Hc) from crabs living in different biotopes: a shore crab (*Carcinus maenas*) and two deep-sea crabs (*Segonzacia mesatlantica* and *Bythograea thermydron*). Hc is an extracellular blood protein, composed of several protein chains which can associate in large multimers. The goal of this study is to point out that the oligomerization state of native Hcs is biotope-dependent. Depending on the crab, ESI-MS analyses under non-denaturing conditions reveal different oligomeric forms present in equilibrium in solution. Molecular weights up to 2,235 kDa were measured for the associations of 30 subunits of the *Bythograea thermydron* Hc. Thanks to ESI-MS analyses, it could be concluded for the first time that the oligomerization state of native Hcs is dependent on the crab environment. The investigation of these different non-covalent self-assemblies is very important for the life history of crabs, since they are directly related with different oxygen binding abilities and thus, with their ability to colonize habitats with different oxygen contents. (J Am Soc Mass Spectrom 2003, 14, 419–429) © 2003 American Society for Mass Spectrometry

Reversible non-covalent interactions play a prominent role in a wide range of biological relevant processes. The structural determination of proteins and protein complexes plays an important role in the fundamental understanding of biochemical pathways. Especially assemblies of multiple proteins are often required for target recognition, binding, transport and function. The non-covalent association of different protein subunits to form multimers serves for the stabilization of proteins [1–3] or allows the possibility of allosteric interactions between subunits leading to cooperative binding of substrate molecules (metallic ions, cofactors, ligands, etc.) [4]. Common techniques used to determine the oligomerization state of a protein include size exclusion chromatography (also called gel filtration) [5], centrifugation techniques [5], light scattering measurements [6] or when available crystallographic studies [7, 8]. However the first methods cannot directly and precisely provide a precise quantification and an accurate determination of the molecular weight of oligomeric forms present in solution. On the contrary, X-ray crystallography is the unique method to obtain high resolution structural information on individual protein or well-defined multi-protein complexes through a precise picture of the active site or of the surfaces involved in the interaction. But obtaining high-quality crystals of such large macromolecules is often a limiting step, both time and material consuming.

Over the past several years, electrospray ionization mass spectrometry (ESI-MS) has emerged as a powerful tool for the investigation of supramolecular complexes, i.e., edifices maintained by non-covalent interactions (for review, see [9–12]). Indeed supramolecular mass

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spectrometry is far from a routine technique, since a careful optimization of the operating conditions is needed. In the biological field several studies convincingly demonstrate that under carefully controlled operating conditions the weak intermolecular interactions involved in the constitutive formation of specific non-covalent complexes in solution, such as protein/protein, protein/ligand or protein/DNA complexes, can survive the ESI ionization/desorption process [13–17]. ESI-MS has the ability to directly give information on the stoichiometry of the interactions formed in solution with a far better precision than common approaches such as gel shift electrophoresis or centrifugation methods (for review, see [10,11]). Some studies also report that ESI-MS can be used to determine the relative binding affinities in solution [17–21]. More recently, ESI-MS has been proposed as a novel approach to easily probe cooperativity in the binding of a ligand to a multimeric enzyme [22].

Another emerging application of ESI-MS is the detection of very large non-covalent multimeric protein complexes in their native state (at near physiological pHs) which is still a challenging area for mass spectrometrists. Continuous improvement in instrumentation, especially the development and commercialization of orthogonal time-of-flight (TOF) analyzers, have resulted in a dramatic extend of the experimentally available *m/z* range from 4,000 to 90,000 [23–25], allowing the observation of large multi-protein assemblies in the gas phase. Currently ESI-TOF and ESI-Q-TOF mass spectrometers, allying sensitivity, resolution and an extended mass range, are predominantly used for studying non-covalent protein complexes. ESI-MS was used by Green et al. [26] to study large non-covalent multiprotein complexes of globin subassemblies comprising 12 globin chains with molecular weights varying from 204 to 214 kDa. A few years before, with the advent of nanospray, the team of Robinson was able to determine the molecular weights of very large protein complexes such as the chaperone GroEL (a 14-mer assembly of 800 kDa) [27] or ribosomes (850 kDa for the 30S subunit) [28]. Tito and co-workers [29] obtained a mass spectrum for the intact assembly of the bacteriophage MS2 virus with a measured molecular weight of 2,484,700 ± 25,200 Da, which is to date the largest complex observed by ESI-MS. Another example is the detection of well-resolved species of an octamer-trimer (24-mer) of vanillyl-alcohol oxidase at 1,525,600 ± 1,000 Da [30]. More recently Zal and co-workers reported an ESI-MS analysis of a purified hemocyanin from *Bythograea thermydron* (deep-sea crab) which was shown to exist as several oligomeric forms, the highest one corresponding to the association of eighteen subunits (18-mer) with a molecular weight of 1,354,940 ± 480 Da [31]. Nevertheless because such ESI-MS experiments need a careful control of all the experimental conditions (desalting procedure, pH, buffer, the accelerating voltage and the pressure in the interface region of the mass spectrometer) some publications are more pessimistic and avoid the sole use of mass spectrometry for the precise determination of the oligomeric form of a protein [32]. In all cases, the most important question remains: can the measurements in the gas phase reflect solution phase behavior? As illustrated by several authors [33–38], at least some structural elements are preserved upon coming through the gas phase. Finally, the applicability of mass spectrometry to investigate non-covalent biological complexes seems to be strongly dependent on the nature of the interactions between the partners: electrostatic interactions are believed to be strongly emphasized while coming from the solution to the gas phase whereas most of the hydrophobic interactions are disrupted. So if hydrophobicity plays a prominent role in the binding, the chance to preserve the edifice during the ESI-MS analysis is highly compromised [39–41]. Thus the existence of hydrophobic contributions in the stability of a specific non-covalent complex is a prominent factor for the investigation of the given non-covalent edifice by ESI-MS.

In this paper, we investigate the oligomeric state of native hemocyanins (Hc) from deep-sea and shore crabs living in different biotopes. Because classical techniques used for the determination of the oligomerization state of proteins failed in case of those Hcs, ESI-MS was used as an alternative method. Mass spectrometry yields a direct control of both formation and integrity of all oligomeric forms that exist in solution. The relevance of interface conditions in the mass spectrometer, the difficulties related to the measurements of high masses and the question of the specificity of the detected interactions are also addressed. More precisely, the aim of this study is to better understand the molecular mechanisms that allow organisms to modulate the structures of their respiratory pigments in function of variations of environmental physicochemical parameters (i.e., in function of their biotope). To date we expect that oligomerization state variations of Hcs, related to environmental adaptation, play a key role in the regulation of their activities and more precisely for the oxygen affinity of the molecules. To achieve our target, three biological models were chosen: two deep-sea crabs (*Bythograea thermydron*-ByTh- and *Segonzacia mesatlantica*-SeMe-) which live in different biotopes and a reference species, a shore *crab (Carcinus maenas*-CaMa-). ByTh is an endemic species of vent crabs that lives on the walls of hydrothermal sources located on the East Pacific ridge at ~2,600 m deep. SeMe crabs are another species of vent crabs, which were collected on the Mid-Atlantic ridge off the Azores at ~800 m deep. Finally, CaMa (also called European green crab) is the most common species of shore crabs living in rocky or sandy areas of Atlantic coasts. Hcs are blue extracellular proteins which are present in high concentrations in the blood of various mollusks and arthropods [42]. Among the latter family, hemocyanins are present in spiders, scorpions, limulus, crustaceans and at least in two species of myriapods. Comparable to hemoglobin, Hc is an oxygen-carrier protein: the difference lies in the nature of

the metallic ions that coordinate oxygen, which is iron in case of haemoglobin whereas it is copper for hemocyanins. Chelating oxygen happens through two copper ions, each metallic ion interacting with Hc via three histidine residues. Hc is reported in literature to be composed of several quite similar polypeptidic chains that can form large non-covalent subassemblies [42]. For example, Hcs from arthropods usually form hexamers or multi-hexamers, each constitutive subunit weighting ~75 kDa with one single oxygen-binding site via two copper ions per monomer.

EXPERIMENTAL

Animal collection and hemolymph sampling

Segonzacia mesatlantica were collected during ATOS'01 cruise realized during the summer 2001 on the Mid-Atlantic Ridge. Samples of *Bythograea thermydron* were collected during PHARE'02 expedition which took place on 13°N East-Pacific Ridge, during May 2002. Animals were collected with baited traps or biological vacuum manipulated by the French ROV Victor 6000. Collection sites were "Genesis" (12°48', 66N, 103°406, 43W, ~2600 meter of depth) and Menez Gwen (37°51, 60'N, 31°31, 30'W; 850 meters of depth) for Pacific and Atlantic oceanographic cruises, respectively. *Carcinus maenas* were collected at low tide near Roscoff (Penpoull Beach), Nord Finistère, France. Hemolymph was quickly sampled with a syringe through the arthropodial membrane at the base of the fourth pair of legs. Hemolymph samples were frozen immediately in liquid nitrogen. Before use, samples were thawed at 4 °C, centrifuged 15 minutes at 12,000 rpm, and the supernatant collected.

Electrospray Ionization Mass Spectrometry

Prior to mass analysis, the samples were desalted on Centricon PM10 (cut off = 10,000 Da) micro-concentrators (Amicon, Millipore, Bedford MA, USA) in 10 mM ammonium acetate (pH 6.8). Eight dilution/concentration steps were performed at 4 °C and 6,000 trs/min. Ammonium acetate enables native structures of proteins to be preserved and is compatible with ESI-MS analyses.

ESI-MS measurements were performed either on a ESI-TOF (LCT, Micromass, Altrincham, UK) or on a ESI-Q-TOF (Q-TOF II, Micromass, Altrincham, UK) mass spectrometer. Both instruments were fitted with standard Z-spray source and have extended mass range of 42,000 *m/z* for the LCT and 25,000 *m/z* for the Q-TOF II. In case of the hybrid quadrupole time-of-flight instrument, mass spectra were recorded at the exit of the TOF analyzer, using the first quadrupole in the "RF-only" mode.

Purity and homogeneity of the Hcs samples were estimated by mass analysis in denaturing conditions: Hcs were diluted to 10 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% formic acid. In these conditions the non-covalent interactions are disrupted, which allows the measurement of the molecular weight of the constitutive monomeric polypeptidic chains with a good precision (better than 0.01%). Mass spectra were recorded on the LCT in the positive ion mode on the mass range 500–4,000 *m/z*, after calibration with horse heart myoglobin diluted to 2 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% formic acid. Accelerating voltage was set to 40 Volts and the pressure in the interface region of the mass spectrometer was 2.5 mbar.

In non-denaturing conditions, the mass measurement of native Hcs were performed in ammonium acetate (10 mM–pH = 6.8) to preserve its native conformation in solution. Concentrations of the samples after desalting were estimated to be 5×10^{-4} mol/l for monomers. Samples were diluted two to three times in the previous buffer to a final concentration of about 20 pmol/μl for a 1,000 kDa complex and continuously infused into the ESI ion source at a flow rate of 5 μL/min. Great care was exercised so that the non-covalent interactions survive the ionization/desorption process. Particularly in order to preserve the integrity of the non-covalent assemblies and to enhance the sensitivity of the detection, the pressure in the interface between the atmospheric source and the high vacuum region was increased to 6.5 mbar by throttling the pumping line. The accelerating voltage applied on the sample cone ranged from 120 to 200 V (optimal value was 200 V) and both source and desolvation temperatures were 100 °C. Experiments were performed on both instruments, ESI-TOF an ESI-Q-TOF. Because the quality of the data was far better on the ESI-Q-TOF than on the ESI-TOF, only native mass spectra recorded on the ESI-Q-TOF are presented here. Clusters of cesium iodide $Cs_{(n+1)}I_n$ (separate injection of a solution of 1 mg/mL CsI in 50% aqueous isopropanol) were used for the calibration of the extended mass range in the high *m/z* region. Calibration and sample acquisitions were performed in the positive ion mode on the mass range *m/z* 2,000–25,000, with a manual pusher value of 250 μs. Data were accumulated over 5 minutes, smoothed with the Savitzky Golay method, the background subtracted and the masses finally calculated. Molecular species were assumed to be represented by series of peaks corresponding to multiply protonated ions. The mass of each species is expressed as a mean of the masses calculated from the series of ions ± standard deviation (SD). Charge state assignments were those that gave minimum standard deviation. The maximum entropy-based software (MaxEnt) was used only to find the approximate mass of each subassembly and hence the charge on each multiply charged peak [43]. Because the MaxEnt software fits symmetrical Gaussian peak shapes to the experimental data, it could not be used to establish the accurate mass, since the peaks were asymmetrical due to adduct formation.

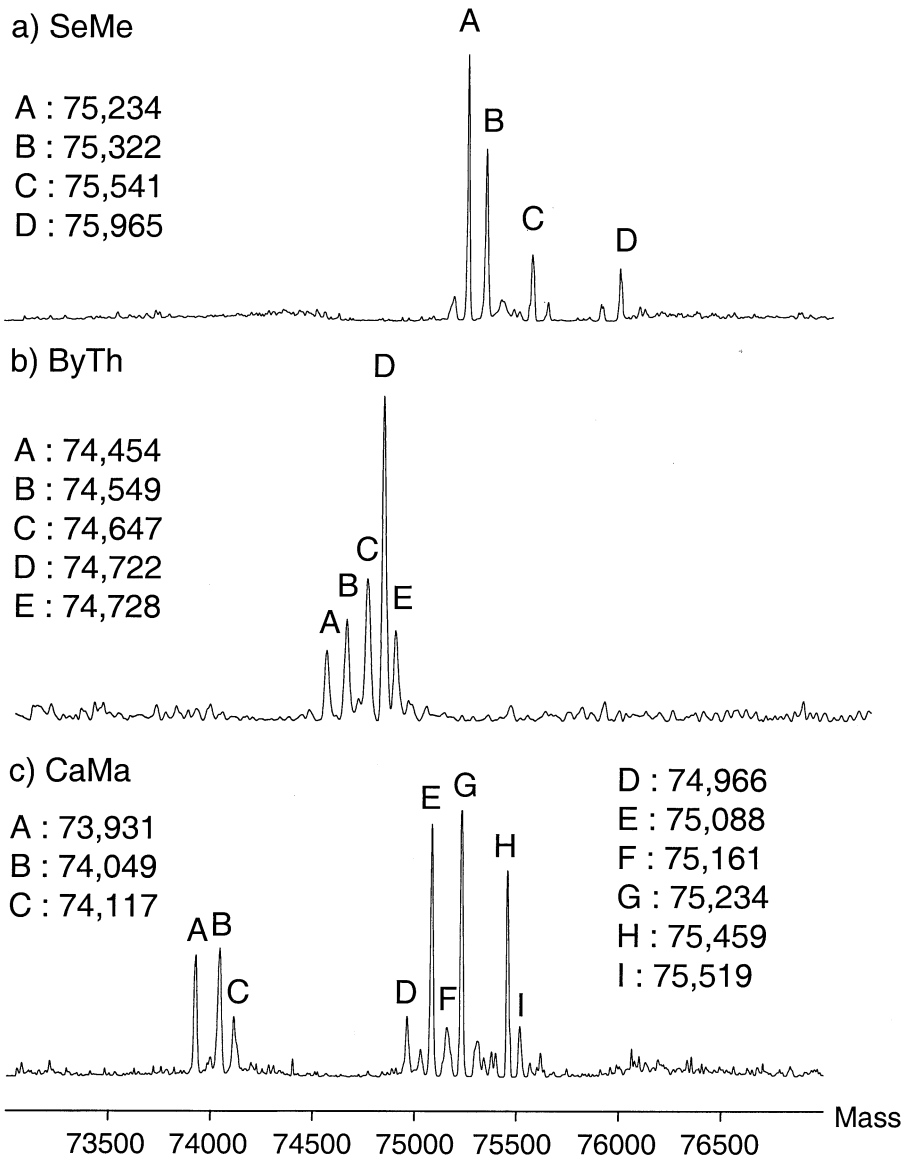

Figure 1. MaxEnt deconvoluted ESI mass spectra obtained under denaturing conditions : **a)** SeMe Hc; **b)** ByTh Hc; **c)** CaMa Hc. Hcs were diluted to 10 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% formic acid (pH 2.3).

Results and Discussion

ESI-MS Analysis of Hcs Under Denaturing Conditions

Before any analysis in native conditions, the individual Hcs were first analyzed by ESI-MS in denaturing conditions, a technique which allows the determination of masses with a far better precision than other techniques (precision of 0.01%). After the previously described desalting and concentration procedures (see Experimental part), ESI-MS analysis in denaturing conditions revealed highly pure but heterogeneous Hcs samples. Figure 1 presents the ESI mass spectra obtained after MaxEnt deconvolution [43] of the raw data. Indeed, deconvolution was necessary for interpretation because of the heterogeneity of the samples: numerous monomeric species (at least 4 species) are present on each mass spectrum. The masses of all polypeptidic chains (subunits) are summarized in Table 1. Relevant information regarding the heterogeneity of Hcs chains were obtained. The number of polypeptidic chains is increasing from SeMe to ByTh and CaMa, with the last one showing the greatest heterogeneity with nine different chains. These observations might be related to the crab ability to adapt to different environments as already suggested for the blue crab *Callinectes sapidus* which colonizes estuarine and seaside environments [44]. Both deep-sea crabs are strictly endemic of the hydrothermal vent environment, which is characterized by non-predictable physicochemical variations in very short time

Table 1. Masses of the polypeptidic chains of the different Hcs determined by ESI-MS under denaturing conditions

Species	Biotope	Number of monomeric subunits observed	Measured masses (Da)[a]
Segonzasia mesatlantica (SeMe)	Deep-sea crab	4	75,234
			75,322
			75,541
			75,967
Bythograea thermydron (ByTh)	Deep-sea crab	5	74,454
			74,549
			74,647
			74,722
			74,728
Carcinus maenas (CaMa)	Shore crab	9	73,931
			74,049
			74,117
			74,966
			75,088
			75,161
			75,234
			75,459
			75,519

[a]Masses are obtained after MaxEnt deconvolution of the raw ESI mass spectra. Only species presenting relative ion intensities after deconvolution above 15% are considered as representative of the sample.

scale. In contrast, the green crab CaMa colonizes the intertidal area characterized by seasonal variations. Consequently, we can suppose that CaMa, in contrast to the deep-sea crabs, is able to colonize a large range of habitats by modifying its hemocyanin phenotype as it has already been reported for the blue crab [45]. In *Callinectes sapidus*, a variation in hemocyanin subunit composition is accompanied by a shift of oxygen affinity. This phenomenon is reversible between estuarine and seaside specimens and witnesses a very short term adaptive process developed during the time course of evolution. In contrast, the deep-sea crabs seem only to be able to colonize more restrictive habitats.

ESI-MS Analysis of Hcs Under Non-Denaturing Conditions

Figure 2 shows the ESI mass spectra obtained for the Hcs of the different crabs after dilution in a 10 mM ammonium acetate buffer (pH 6.8) and under carefully controlled experimental conditions (the accelerating voltage/voltage applied on the sample cone, Vc, was set to 200 V and the pressure in the interface region of the mass spectrometer, Pi, was set to 6.5 mbar). It was noted that ESI-MS experiments were reproducible from scan-to-scan (each ESI mass spectrum is the sum of 30 10-sec scans), and from sample-to-sample (relative abundances varied by less than 5%). For SeMe (Figure 2a and Table 2), four ion series corresponding to the monomer, 6-mer, 12-mer and 18-mer, with ions ranging from *m/z* 4,000 to *m/z* 18,000, were detected: 1) a first minor ion series corresponding to monomeric ions was detected in the mass range 4,000–5,000 *m/z*; 2) the major set of peaks with a charge state distribution ranging from 40+ to 49+ (the 44+ charge state being the most abundant) was observed in the mass range *m/z* 9,000-11,000 and led to a molecular weight of 453,889 ± 45 Da corresponding to the non-covalent association of six subunits; 3) the third distribution, with charge states ranging from 61+ to 73+, corresponded to the formation of a 12-mer detected in the mass range 12,000–15,000 *m/z* with an estimated molecular weight of 909,628 ± 157 Da; 4) the fourth distribution in the mass range *m/z* 15,000-18,000 could be related to the association of 18 subunits leading to a mass of 1,364,968 ± 264 Da. No ions corresponding to any intermediate stoichiometry (heptamer, pentamer, etc.) resulting from any statistical aggregation were observed on the mass spectrum, suggesting that the species detected by ESI-MS faithfully reflect the species present in solution. One interesting point concerns the wide distribution of the charge states (especially the left part of the distribution) observed for the 12-mer and 18-mer. One possible explanation for this peak broadening could be the overlapping of two ion series, the resolution of our instrument being insufficient to distinguish the two series. The hypothesis of gas-phase dissociation in the interface of the mass spectrometer is quite improbable, since experiments were performed at different cone voltages, for which peak broadening is still observed. The fact that under denaturing conditions, a strong heterogeneity of the constitutive subunits was observed (4 different subunits) strongly support the first explanation. As a consequence, it could be imagined that different 12-mer, for instance, resulting from different subunit associations would result in heterogeneity inside the same oligomeric state.

The mass spectrum obtained under native conditions

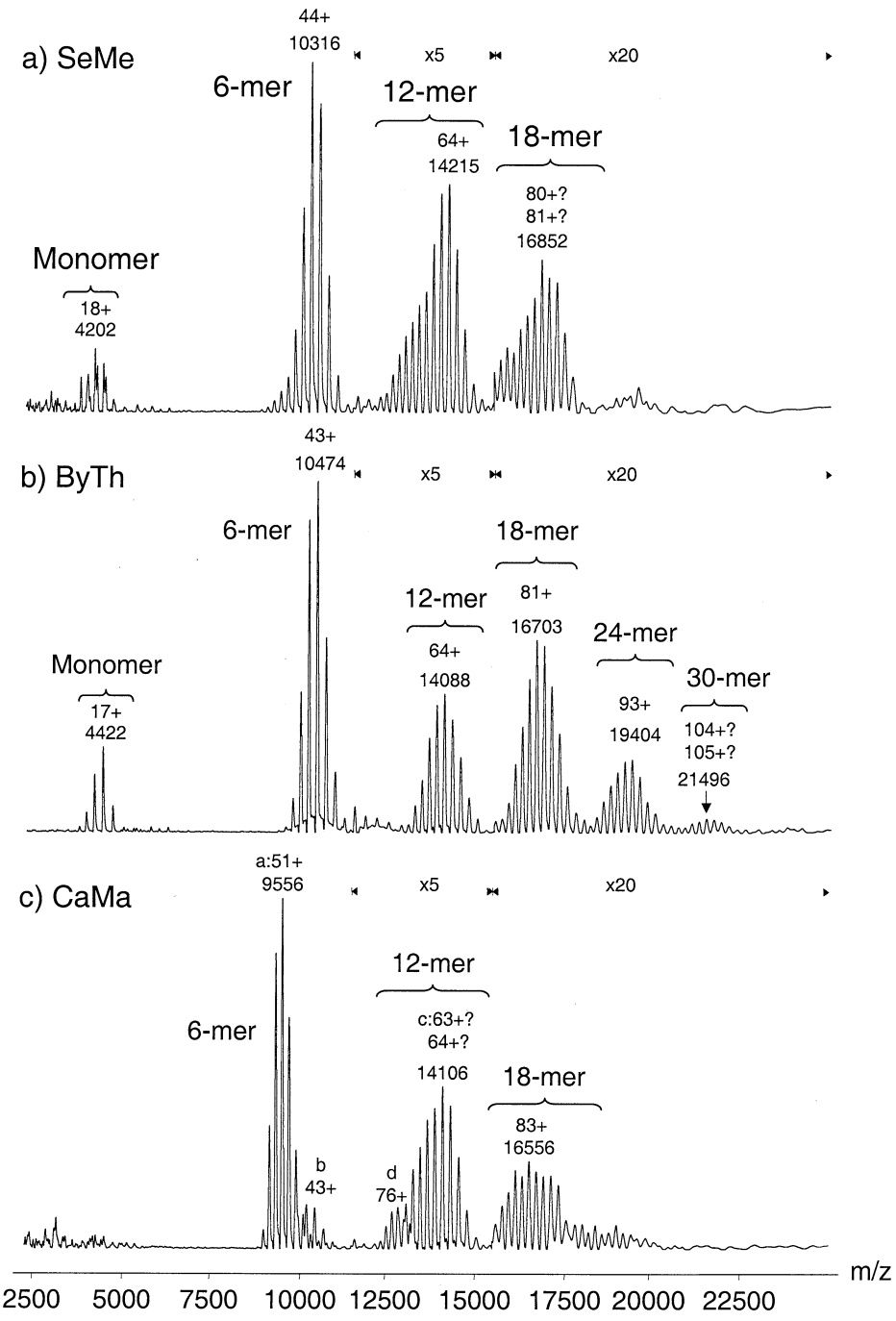

Figure 2. ESI mass spectra of native Hcs obtained under non-denaturing conditions : **a**) SeMe Hc; **b**) ByTh Hc; **c**) CaMa Hc. Hcs were diluted to 5×10^{-4} mol/l (concentration in monomeric species) in 10 mM ammonium acetate buffer (pH 6.8). The charge state of the most intense peak of each ion series is indicated.

for ByTh Hc (Figure 2b) revealed six ion series in the mass range *m/z* 4,000–25,000, multimeric species being present on the upper mass range (*m/z* 10,000–25,000). Those ion series correspond respectively to monomer, hexamer, 12-mer, 18-mer, 24-mer and 30-mer present in equilibrium in solution. Table 2 summarizes the masses calculated from the charge state distribution: molecular weights up to 2,235,051 ± 303 Da were measured for the 30-mer.

Figure 2c presents the native ESI mass spectrum of CaMa Hc. Three main ion series are detected in the mass range *m/z* 9,000–20,000: the hexamer, the 12-mer and the 18-mer with a measured mass of 1,374,136 ± 280 Da for the latter one (Table 2). As observed for SeMe Hc, two different types of hexamers and 12-mers are clearly present on the mass spectrum. However this heterogeneity inside one oligomeric form could not be

Table 2. Masses of the non-covalent assemblies of the different Hcs determined by ESI-MS under non-denaturing conditions[a]

Species	Oligomeric Form	Measured Mass (Da)[a]	Standard Deviation (Da)
Segonzacia mesatlantica (SeMe)	6-mer	453,889	45
	12-mer	909,628	157
	18-mer	1,364,968[b]	264
		1,347,998[b]	300
Bythograea thermydron (ByTh)	Monomer	75,094	56
	6-mer	450,333	45
	12-mer	901,605	190
	18-mer	1,352,823	221
	24-mer	1,804,368	284
	30-mer	2,235,051[b]	303
		2,256,550[b]	396
Carcinus maenas (CaMa)	6-mer	487,337(a)	35
		450,541(b)	44
	12-mer	975,322(d)	151
		888,652(c)[b]	299
		902,675(c)[b]	392
	18-mer	1,374,136	280

[a]Mean masses derived from multiply charged series observed under non-covalent conditions.
[b]The mean mass of the non-covalent assembly cannot be given without any ambiguity, due to an incapacity in a precise charge state assignment. Thus, the different possible mean values are reported in the table. However this lack in mass accuracy has no consequence on the determination of the oligomeric form. Letters in brackets refer different types of subunit association for the same oligomeric state (see Figure 2).

detected anymore for the 18-mer, due to the insufficient resolution of the instrument for this purpose.

Problems Related to Calculations of High Molecular Weights

ESI-MS is a widely recognized technique for mass measurements of individual proteins with high precision (0.01%). In case of large non-covalent macromolecules (mega-Dalton proteins), the determination of the masses is not trivial at all. In our experience, the main difficulty in the mass calculation process is the ability to make the correct charge state assignment for each peak present on the ESI mass spectrum. For instance, in case of SeMe Hc, the resolution of the 2nd order equation resulting in the determination of the charge state of the main peak (*m/z* 16,851.8) of the 18-mer of SeMe Hc was not possible: an ambiguity between an assembly with 80, 81 or 82 charges remains. Consequently, in a first step, the mean value for the molecular weight is obtained for the charge state distribution with the minimum standard deviation (SD). In our case, SD does not represent the mass error compared to the theoretical mass calculated from the amino acid sequence or to the mass measured in denaturing conditions, but rather the accuracy of the mass calculation from *m/z* values reported from the ESI mass spectrum. However in some cases this minimum SD criterion is even not sufficient to assign clearly the charge state of a peak. This was still the case for the 18-mer of SeMe Hc, the 30-mer of ByTh Hc and the 12-mer of CaMa Hc. For example, in the case of the 18-mer of SeMe Hc, two masses present very close SDs (M_1 = 1,347,998 ± 300 Da and M_2 = 1,364,968 ± 264 Da) and the minimum SD criterion is obsolete. However it should be noticed that despite the impossibility of having an estimation of the average molecular weight for the 18-mer of SeMe Hc, there is no ambiguity for the determination of the oligomeric form of the protein, the mass difference between the two values (~18 kDa) being smaller than the mass of one monomeric subunit (~75 kDa). When the SD criterion is not appropriate, the next step consists in using a deconvolution software, such as MaxEnt [43], to be able to estimate the molecular weight of the species. The comparison of the masses obtained with both methods (SD criterion and MaxEnt approach) can help to determine the most probable charge state for a given peak. However the MaxEnt software is not suited for such applications, i.e., deconvolution on large *m/z* ranges of very high molecular weights edifices. Finally if a MaxEnt deconvolution is unsuccessful or cannot be processed because the limits of the software are reached, the minimal SD criterion is retained, knowing that the error on the mass calculation can be within ~20 kDa in the case of Hcs.

Another point that needs to be focused on in case of high molecular masses, is the fact that masses are generally higher than those expected from the amino acid sequence or from the ESI-MS analysis in denaturing conditions. This can be related to the heterogeneous non-covalent binding of small molecules, metallic ions or non volatile salts to the non-covalent edifice. As reported by Green et al. [26, 46] and others [29, 30], at low Vc values, the desolvation process is incomplete, the energy communicated to the ions being insufficient to disrupt those weak interactions. By increasing the Vc voltage, desolvation is much more efficient but not necessarily complete as the non-covalent complex can be dissociated upon gas phase collisions before the disruption of the weak interactions involving those

small molecules or ions. In the case of Hcs, it is quite obvious that at least copper ions, that help to coordinate oxygen, contribute to an unknown extend to this peak width.

Relevance of Experimental Parameters of the Mass Spectrometer for the Mass Detection of Large "Structurally-Specific" Non-Covalent Complexes

The potential of ESI-MS to gain information on the oligomeric state of proteins is an interesting field for mass spectrometrists. Several publications report the ability of ESI-MS to record spectra of intact macromolecules [22, 26–32, 34–36, 47]. However all authors agree with the fact that the detection of non-covalent high molecular weight complexes is not easy at all, since those macromolecules should be driven from the solution to the gas phase without destruction of the weak framework. So the essential prerequisite to the use of ESI-MS for the determination of the oligomeric structure of an enzyme is that observed peaks on ESI mass spectra in vacuo are reliable to species effectively present in solution. Great care in the data acquisition as well as in the interpretation and mass calculation must be taken, since it is quite established that the solution-phase image might be distorted due to several factors during the ESI-MS analysis. In particular during the evaporation of the ions in the gas phase, or during the transfer from the ion source to the analyzer through the interface region of the mass spectrometer. As reported by several studies [29, 32, 47–49], the careful adjustment of the interface conditions is a prerequisite for the transfer of intact non-covalent complexes from the solution to the gas phase. Especially the role of the accelerating voltage Vc and the pressure in the interface region Pi of the mass spectrometer are critical parameters that need to be optimized for each new sample. We recently detailed the influence of Vc and Pi on the detection of the 310 kDa non-covalent hexamer of HPr kinase/phosphatase [47]. We [22, 31, 34–36, 47] and others [10, 27, 29, 30, 32, 48, 49] demonstrated that increasing the pressure in the interface greatly helps in the detection of very large non-covalent protein complexes. An increase in Pi (at least 5 mbar on our ESI-TOF or ESI-Q-TOF instruments) induces a better transmission of high *m/z* ions due to the collisional cooling effect [48] but also because higher Pi values seem to reduce fragmentation processes. In the same way, the accelerating voltage has several effects: 1) a transmission effect: high *m/z* ions require higher initial kinetic energies to be able to reach the detector; 2) a desolvation effect: higher Vc values lead to a most efficient desolvation of the ions; 3) a dissociation effect: an increase in Vc induce more energetic collisions with residual N_2 molecules in the interface of the mass spectrometer, that may lead to possible in-source dissociation of weak interactions. Consequently a compromise between sufficient desolvation, optimal transmission of intact high *m/z* ions and non-destructive gas-phase collisions needs to be set to be able to detect specific non-covalent edifices of high molecular weights. A careful optimization of the interface pressure and the accelerating voltage, different for each non-covalent assembly, is necessary to get best results. Thus performing systematically control experiments in which both Vc and the Pi vary are a prerequisite to unambiguously conclude for an ESI-MS detection of specific non-covalent interactions.

Another crucial point that needs to be addressed when analyzing non-covalent complexes by ESI-MS is the important issue of binding-specificity. One needs to unambiguously distinguish between "structurally-specific" non-covalent complexes and between non-specific non-covalent complexes resulting from any gas-phase or in-solution artefactual association. As precisely detailed by Smith et al. [50], several control experiments need to be performed in order to assume the detection of specific interactions by ESI-MS. The fact that different species of crabs display different multimeric forms is a first good evidence for a specific interaction. Moreover adjustments of the interface conditions in the mass spectrometer did not induce any change on the mass spectra, no higher oligomeric states than those already detected by ESI-MS were observed, which also strongly supports "structurally-specific" interactions. A third point which accounts for the detection of "structurally-specific" interactions is the fact that, under strictly identical experimental conditions (sample preparation, buffer, interface conditions—especially accelerating voltage and pressure in the interface region, etc.), no statistical stoichiometry of association was detected (no pentameric or heptameric ions were detected, for instance). Thus it could be concluded that the associations observed by ESI-MS can be related to "structurally-specific" interactions that preexist in solution and do not result from any artefactual aggregation.

Hcs Exhibit Different Oligomeric States Depending on the Crab's Biotopes

In this paper, we unambiguously demonstrate that the non-covalent associations of Hcs subunits are strongly dependent on the biotopes colonized by the crabs. The study of these different non-covalent self-assemblies is very important for the life history of crabs since they are directly related to different oxygen binding abilities and consequently with the ability to colonize different habitats with different oxygen contents. The simplest structure was found for *Segonzacia* Hc with only four different polypeptidic chains, which can form three non-covalent assemblies. However, it seems highly probable that the 12-mer and the 18-mer exist under two different forms increasing consequently the phenotype plasticity of SeMe Hcs. Furthermore, recent physiological study realised under pressure with different oxygen conditions revealed very slight changes of aggregation states between experiments conducted during six hours (i.e.,

anoxia and hyperoxia conditions [51]. However, the experiment time was probably too short to induce phenotype modifications of Hc. Indeed, 8 days of adaptation between experimental conditions were necessary to observe changes for *Callinectes sapidus* Hc collected on same individuals [44]. Recently Zal et al. reported gel filtration and ESI-MS analyses of Hc from *Bythograea thermydron* [31]. In this study, only ions corresponding to the monomer (18%), the hexamer (67%), 12-mer (13%) and 18-mer (1%) were detected by both techniques, whereas no ions corresponding to a higher degree of oligomerization (24-mer and 30-mer) were observed. The discrepancies between this previous study and our current investigation could have the following explanation. This sample came from another specimen of *Bythograea* collected at 13°N East Pacific Rise (EPR, Genesis site) whereas the previously analyzed Hc sample was collected at 9°50'N EPR (M-Vent site), two sites separated of hundreds nautical miles away from each other on the Pacific Ridge. *Genesis* is an old site in comparison with *M-Vent* and most probably the hydrothermal vent emissions are different by their chemistry. Consequently, the microhabitat of each crab may differ, even if its biotopes are similar. Therefore, the Hc structure of these crabs would be modified in order to act with local exigency

Finally, CaMa presents the highest Hc structure with nine different polypeptidic chains. Nevertheless, the non-covalent association is similar as the one found for SeMe with also a double distribution for the 6- and 12-mer. These data strongly suggest that CaMa Hc assemblies could be distinct depending of the environmental conditions, allowing this crab to colonize a broad range of habitats characterized by different physicochemical parameters.

CONCLUSIONS

In this study, we used the benefits of non-denaturing ESI-MS to unambiguously determine the quaternary structures of Hcs of different crabs. Molecular weights up to 2,235 kDa were measured for the associations of 30 subunits of the *Bythograea thermydron* Hc. In that way, supramolecular mass spectrometry seems to be particularly well-suited for the measurement of high masses in the mega-Dalton range. Thanks to ESI-MS analyses, it could be concluded for the first time that the oligomerization state of native Hcs is dependent on the crab environment. The investigation of these different non-covalent self-assemblies is very important for the life history of crabs, since they are directly related with different oxygen binding abilities and thus, with their ability to colonize habitats with different oxygen contents.

However, the investigation of high molecular weight non-covalent complexes is not straightforward as a careful control of all experimental conditions (pressure and accelerating voltage in the interface region of the mass spectrometer, concentration conditions, desalting, pH, buffer, etc.) is necessary to detect large "structurally-specific" non-covalent assemblies by mass spectrometry. Moreover great caution needs to be exercised for the estimation of the high molecular masses of such edifices. In the case of Hcs, non-denaturing ESI-MS was the only method that enables a fast, direct and unambiguous determination of their oligomerization states. Thus supramolecular mass spectrometry seems to be particularly a powerful alternative or at least complementary technique to gain information on the association state of proteins. However, as previously explained, great care needs to be exercised to ensure that the detected species faithfully reflect the species present in solution. Thus control experiments will always be needed to conclude about the specificity of the ESI-MS detected interactions.

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