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1 **Structural study of *Carcinus maenas* hemocyanin by native ESI-MS:**
2 **interaction with L-lactate and divalent cations**

3

4 Short title: Study of *C. maenas* hemocyanin by ESI-MS

5

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8

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30 Abbreviations: AcNH₄, ammonium acetate; DPG, D-2,3-diphosphoglycerate; ESI-MS,

31 electrospray ionization mass spectrometry; FA, formic acid; MWC model, Monod-Wyman-

32 Changeux model; SEC, size-exclusion chromatography; TEA, triethanolamine

33

34

35 ABSTRACT

36

37 The interaction of L-lactate and divalent cations with *Carcinus maenas* hemocyanin has
38 been probed by electrospray ionization mass spectrometry under conditions preserving non-
39 covalent interactions (native ESI-MS). *Carcinus maenas* native hemocyanin in the
40 hemolymph occurs mainly as dodecamers and to a lesser extent as hexamers. A progressive
41 acidification with formic acid after alkaline dissociation resulted in the preferential
42 recruitment of the two lightest subunits into light dodecamers, a molecular complex absent
43 from native hemolymph, in addition to regular dodecamers and hexamers. Addition of
44 L-lactic acid also induced the recruitment of these subunits, even at alkaline pH. A
45 dodecamer-specific subunit is needed to enable aggregation over the hexameric state.
46 Experiments with EDTA suggested the existence of different binding sites and association
47 constants for divalent cations within hexameric structures and at the interface between two
48 hexamers. L-lactic acid specific interaction with the lightest subunits was not inhibited by
49 removal of the divalent cations.

50

51

52

53 Hemocyanin, the blue respiratory pigment responsible for oxygen transport in decapod
54 crustaceans, is a macromolecular complex comprised of 75 kDa subunits. These subunits can
55 each reversibly bind one dioxygen molecule between the two copper ions of their active site
56 and they associate into non-covalent complexes which can be hexamers (e.g. in isopods, krill,
57 shrimps, prawns, spiny lobsters), dodecamers (e.g. in brachyurans, lobsters, crayfishes, hermit
58 crabs) and in some species 24-mers (e.g. in thalassinid shrimps) (Markl, 1986; Markl &
59 Decker, 1992; Terwilliger, 1998). Hemocyanin circulates as a freely dissolved protein in the
60 hemolymph of the animals. Its functional properties can be modulated by various effectors
61 such as H⁺, divalent cations and organic ions (Bridges, 2001; Truchot, 1992). Among them,
62 the L stereoisomer of lactate is an important allosteric effector produced by the anaerobic
63 metabolism of the animal, which can be driven by either environmental or metabolic hypoxia
64 (Truchot, 1980; Truchot & Lallier, 1992). Divalent cations and pH have an effect both on the
65 structure and on the properties of the complexes since most hemocyanins can be dissociated
66 under alkaline pH and by dialysis against EDTA (Herskovits, 1988).

67 Arthropod hemocyanins are useful models for studying allosteric regulation
68 mechanisms. The existence of several types of allosteric effectors and the occurrence of huge
69 hierarchical quaternary structures in some groups (e.g. 24-mers in arachnids, 48-mers in the
70 horseshoe crab *Limulus polyphemus* (Markl & Decker, 1992)) allow for investigation of
71 allosteric mechanisms with different molecular actors and using original models such as
72 nested allostery (Decker, 1990; Menze et al, 2005). In most decapods, an increase in lactate
73 concentration increases the oxygen affinity of the pigment (Bridges, 2001; Truchot, 1980;
74 Truchot, 1992). However, some decapods exhibit low or no sensitivity to lactate (e.g.
75 thalassinid mud-shrimps (Taylor et al, 2000), the slipper lobster *Scyllarides latus* (Sanna et al,
76 2004)), notably when the lifestyle becomes more terrestrial (Bridges, 2001; Morris & Bridges,

77 1994). For one terrestrial species, *Gecarcoidea natalis*, a reverse lactate effect has been
78 observed (Adamczewska & Morris, 1998). The use of chemical analogs and the fact that
79 D-lactate does not affect O₂ affinity suggested that lactate binds stereospecifically to a
80 specific site of hemocyanin (Johnson et al, 1984) and that the binding occurs at all four
81 positions around the chiral carbon (Graham, 1985). For *Callinectes sapidus* hemocyanin,
82 lactate titrations gave values of lactate dissociation constant of 1.8 mM for the oxy-state and
83 2.2 binding sites per hexamer, and ultrafiltration techniques gave values of 3.2 mM for the
84 dissociation constant and 2.8 sites per hexamer (Johnson et al, 1984). For the spiny lobster
85 *Panulirus interruptus*, purification of each subunit type and reassembly into homohexamers
86 showed that the sensitivity to lactate was subunit-dependent: some homohexamers were
87 sensitive whereas others were not (Johnson et al, 1987). However, in native hexamers as well
88 as in sensitive reassociated homohexamers, analysis of oxygen equilibrium curves showed
89 that there was approximately only one binding site per hexamer, evidencing that the specific
90 site was not located on a single subunit but was formed within the quaternary structure of the
91 protein (Johnson et al, 1987). For *Homarus vulgaris*, ultrafiltration and equilibrium dialysis
92 using labelled ligands showed that there were approximately 2 molecules bound per
93 dodecamer, which is consistent with the finding of one site per hexamer for *Panulirus*
94 *interruptus*; the same result was observed for urate but urate and lactate binding sites were
95 different and independent (Nies et al, 1992). Moreover, lactate does not bind to hemocyanin
96 in a cooperative way, suggesting that the conformational changes induced by fixation on one
97 site does not influence the other site (Nies et al, 1992). However, a small angle X-ray
98 scattering study (SAXS) of *Homarus americanus* hemocyanin along with electron microscopy
99 showed that upon addition of 10 mM lactate the two hexamers constituting the dodecamer got
100 closer by about 0.5 nm (Hartmann et al, 2001), thus implying that even local changes at the
101 binding sites may have a global effect on the quaternary structure of the protein. Isothermal

102 titration calorimetry performed on sensible and insensible hemocyanins revealed that
103 insensible hemocyanins did not bind lactate, whereas the sensible ones did (Taylor et al,
104 2000). A recent study of O₂-dissociation curves of *Carcinus maenas* hemocyanin reported the
105 binding of 0.35 lactate ion per functional subunit, that is to say 4 sites per dodecamer or 2
106 sites per hexamer (Weber et al, 2008). This is different from the data from *Homarus vulgaris*
107 and *Panulirus interruptus* but close to the results for *Callinectes sapidus*, a brachyuran crab
108 like *Carcinus*. In this context, the precise mechanism of lactate binding, the involved amino-
109 acids and the induced conformational changes remained to be determined.

110 Many studies have also demonstrated the crucial role of calcium and pH for
111 hemocyanin structure and function. Upon dialysis against EDTA to remove calcium and pH
112 increase, complexes are dissociated and O₂-binding capacity is lost (Brenowitz et al, 1983;
113 Herskovits, 1988; Molon et al, 2000; Olianias et al, 2006). Some species show unusual
114 stability against dissociation (Beltramini et al, 2005). Usually, reassociation occurs upon
115 return to physiological pH values and addition of calcium. Divalent cations and pH are also
116 known to modify hemocyanin oxygen-binding properties (Andersson et al, 1982; Bridges,
117 2001; deFur et al, 1990; Morimoto & Kegeles, 1971; Olianias et al, 2006; Truchot, 1992).

118 In recent years, mass spectrometry (MS) has emerged as a new and potent tool for
119 structural analysis of protein complexes and protein interactions with various ligands. The
120 possibility of preserving the fragile non-covalent interactions through the electrospray
121 ionization (ESI) process has allowed probing quaternary structures of various assemblies and
122 following association and dissociation of non-covalent complexes and incorporation of small
123 organic molecules or inorganic ions (Potier et al, 1997; Rogniaux et al, 2001; Tahallah et al,
124 2002; van Duijn et al, 2006). The ESI source was also successfully used to study the
125 quaternary structure of high-molecular mass invertebrate respiratory pigments (Bruneaux et

126 al, 2008; Green et al, 2001; Green et al, 1998; Green & Vinogradov, 2004; Sanglier et al,
127 2003; Zal et al, 2002).

128 ESI-MS ability to characterize compounds by their mass with a high precision and to
129 maintain non-covalent interactions such as exist between hemocyanin and L-lactate and
130 divalent cations makes this technique a useful tool to probe the specificity of these
131 interactions and their effect on the quaternary structure of the complex. For *C. maenas*,
132 hemocyanin is the major protein found in the hemolymph and occurs mainly as dodecamers
133 and to a lesser extent as hexamers (typically 80 to 95 % dodecamers in mass). Increases of
134 pH, L-lactate concentration or divalent cations concentration are known to increase its oxygen
135 affinity (Truchot, 1975; Truchot, 1980). Its constituting subunits were characterized by ESI-
136 MS in denaturing conditions and the complexes by ESI-MS in native conditions, as described
137 in the study by Sanglier and collaborators (Sanglier et al, 2003). In this paper, we expose an
138 attempt to dissociate the subunits at alkaline pH and to probe their potential association with
139 L-lactate in non-covalent conditions. An investigation of the influence of divalent cations on
140 the reassociation of subunits was also performed using EDTA.

141

142 **MATERIAL AND METHODS**

143

144 Animal collection and hemocyanin sampling and purification

145 *Carcinus maenas* individuals were caught by baited traps in the intertidal zone in
146 Roscoff, France, and kept in running sea water at ambient temperature (salinity 35-35.4 ‰,
147 temperature 15-17.5°C). Hemolymph was withdrawn with a syringe through the articular
148 membrane of a walking leg and immediately put on ice. Samples were centrifugated for
149 10 min at 10000 g to pellet cells and coagulated proteins. For whole hemolymph study, the
150 supernatant was recovered and frozen at -20°C. For purified complexes study, dodecamers

151 and hexamers were separated by size-exclusion chromatography (SEC) using a Superose-6
152 10/300 GL column (Amersham Bioscience) at an elution rate of 0.25 ml/min with a
153 crustacean physiological saline buffer (500 mM NaCl, 10 mM KCl, 30 mM MgSO₄, 20 mM
154 CaCl₂, 50 mM Tris, pH 7.8, modified from (Chausson et al, 2004)). Collected fractions purity
155 was assessed with the same system. Purified samples were frozen at -20°C.

156 For preliminary analyses of hemocyanin in denaturing and non-covalent conditions,
157 samples were withdrawn from several individuals kept in running sea water under several
158 salinity and oxygenation conditions. For all dissociation and association experiments using
159 triethanolamine (TEA), formic acid (FA), L-lactic acid and EDTA, the whole-hemolymph
160 samples were issued from the same individual and the purified complexes were issued from
161 another single individual; those samples were withdrawn one day after catching the crabs.

162

163 Sample preparation for ESI-MS

164 All samples used for ESI-MS were desalted through concentration-dilution cycles using
165 10 kDa Microcon (Millipore) prior to analysis. Typically, 30 µl of native hemolymph diluted
166 in 470 µl 10 mM ammonium acetate (AcNH₄), pH 6.8 or 250 µl of purified sample (protein
167 concentration about 1-3 µg/µl) were first concentrated to almost dryness and then
168 resuspended in 400 µl 10 mM AcNH₄. At least 10 successive similar concentration-dilution
169 steps were performed in order to ensure that the samples were correctly desalted prior to ESI-
170 MS analysis. The samples were stored at 4°C in 10 mM AcNH₄ until analysis. The desalting
171 was realised just prior to MS analysis and desalted samples for non-covalent analysis were not
172 kept more than 2 days at 4°C because of dissociation occurring upon longer storage.

173

174 Non-covalent and denaturing ESI-MS

175 Mass spectrometry experiences were performed on a MicroTOF instrument (BRUKER
176 Daltonics, Bremen, Germany) equipped with an ESI source.

177 For non-covalent analysis, desalted samples were diluted in 10 mM AcNH₄ at a final
178 concentration of approximately 1.5 μM (for 900 kDa dodecamers). The injection rate for the
179 samples was 4 μl/min. The ESI needle voltage was set to 5 kV, nebulization gas (N₂) pressure
180 was 1 bar, drying gas flow was 4 l/min, source temperature was 200°C and the capillary exit
181 voltage was set to 400 V. The calibration was made using 1 mg/ml CsI in water/isopropanol
182 (50:50 volume). The acquisition range was 500 to 20000 m/z. Spectra were smoothed using a
183 Savitzky-Golay method and baseline subtracted. Complexes masses were estimated using a
184 built-in ruler (BRUKER DataAnalysis v3.2 software).

185 For denaturing analysis, desalted samples were diluted in a water/acetonitrile/formic
186 acid mix (H₂O/ACN/FA 50:50:1 volume) at a final concentration of approximately 4 μM (for
187 75 kDa subunits). The nebulization gas (N₂) pressure was 0.3 bar, drying gas flow was
188 3 l/min, and the capillary exit voltage was set to 160 V. Calibration was performed using
189 myoglobin (SIGMA-ALDRICH). Mass spectra were analysed using maximum entropy
190 deconvolution (BRUKER DataAnalysis v3.2 software).

191 In the following, denaturing analysis refers to ESI-MS analysis in H₂O/ACN/FA with
192 classical instrumental parameters. In these conditions, all non-covalent interactions are
193 broken. Non-covalent analysis refers to ESI-MS analysis in AcNH₄ with “gentle”
194 instrumental parameters. In this case, non-covalent interactions are preserved during the
195 analysis, even if biochemical treatments can partially dissociate the complexes before
196 analysis.

197

198 Dissociation in TEA

199 For alkaline denaturation of hemocyanin, desalted samples were diluted in 10 mM
200 AcNH₄ with various TEA concentrations (from 0.005 % to 0.05 % TEA in volume, pH 7.52
201 to 9.02 respectively) and incubated for 15 min at ambient temperature before non-covalent
202 ESI-MS analysis was performed.

203

204 Reassociation with formic acid

205 For acidic reassociation, desalted hemocyanin was first dissociated in 10 mM AcNH₄,
206 0.03 % TEA (pH 8.6) for 15 min at ambient temperature and then formic acid was added to a
207 final concentration of 0.001 % to 0.04 % (pH 8.48 to 4.22, respectively); the preparation was
208 incubated for another 15 min before performing non-covalent ESI-MS analysis to allow for
209 reassociation.

210

211 Effect of L-lactic acid

212 For investigation of L-lactic acid effect, desalted hemocyanin was first dissociated in
213 10 mM AcNH₄, 0.03 % TEA (pH 8.6) for 15 min at ambient temperature and then lactic acid
214 was added to a final concentration of 2 mM (L(+)-lactic acid, SIGMA-ALDRICH), along
215 with TEA to counterbalance the acidifying effect of lactic acid. After addition, the final TEA
216 concentration ranged from the original 0.03% to 0.07%, and pH values ranged from 5.89 to
217 8.58. The preparation was left to incubate for 15 min at ambient temperature before non-
218 covalent ESI-MS analysis.

219

220 Effect of chelation of divalent cations by EDTA

221 Divalent cations were removed using EDTA in order to investigate their structural role.
222 Two 10 mM Na⁺-EDTA, 10 mM AcNH₄ solutions were prepared with different pH, one with
223 0.11 % TEA (approximative pH 7.5) and the other with 0.15 % TEA (approximative pH 9). A

224 desalted total hemolymph sample was diluted 20-fold in each of these solutions, concentrated
225 on a 10 kDa Microcon filter, diluted in the same EDTA mix again and then washed by 6
226 concentration-dilution steps in 10 mM AcNH₄, 0.005 % TEA and 0.05 % TEA respectively in
227 order to remove the Na⁺ and EDTA salts along with the chelated cations. Non-covalent
228 analysis was performed after this step. Then, L-lactic acid was added up to 2 mM final
229 concentration with TEA to adjust pH either at 7.5 or 9 approximately and the preparation was
230 left to incubate for 15 min at ambient temperature before analysis.

231

232 **RESULTS**

233

234 Preliminary analysis of *Carcinus maenas* hemocyanin

235 28 different hemolymph samples from 14 different male individuals issued from control
236 or acclimation experiments were analysed by ESI-MS in denaturing conditions. Six main
237 subunits were most frequently observed, of which one was only observed in the dodecameric
238 fraction when analyses were performed on SEC-separated, purified complexes. No covalently
239 bound dimer was ever observed. Table I presents the masses obtained in our study for these
240 subunits and the nearest masses obtained in a previous study by Sanglier and collaborators
241 (Sanglier et al, 2003). In this previous study, a total of nine subunits were identified by the
242 authors with four of them exhibiting low intensity peaks, the other five being identical to the
243 subunits identified in our study.

244 Hemocyanin analysis by ESI-MS in non-covalent conditions produced two main
245 charge-state distributions corresponding to dodecameric and hexameric species (figure 1). The
246 observed dodecamer to hexamer ratio is similar to the one determined by SEC; this confirms
247 that the desalting steps do not markedly alter the oligomerization equilibrium provided that
248 the analysis is made just after desalting (storage shorter than 2 days at 4°C). Some high-mass

249 distributions can also be observed with a lower intensity, corresponding to 18-meric and
250 24-meric complexes (up to 13 and 7 %, respectively). SEC experiments coupled with light-
251 scattering mass determination (not presented here – personal observation) also revealed the
252 presence of a slight amount of these high molecular-mass complexes.

253

254 Alkaline dissociation of complexes by TEA and acidic reassociation by formic acid

255 A native hemolymph sample was progressively dissociated to its constituting subunits
256 by adding increasing quantities of TEA (figure 2). Before addition of TEA, one main species
257 is observed and corresponds to a 900 kDa mass (dodecamer, figure 2 panel A). A less intense
258 signal is visible corresponding to a hexamer mass around 450 kDa and a slight monomer
259 signal is also visible. It can be noted that the particular native sample shown here contains
260 almost only dodecamers and only very few hexamers. Experiments on SEC-purified
261 dodecamers and hexamers are detailed later. Upon TEA addition, the intensity of the
262 dodecamer distribution decreases while the monomers peaks get more intense (figure 2 panels
263 B,C). A second distribution with higher m/z values and lower charge states is observed;
264 precise charge-state assignment is difficult for this due to the high mass of the complex. This
265 distribution can correspond either to an 885 kDa or to an 864 kDa mass. Given that no subunit
266 of $864/12 = 72$ kDa is observed in denaturing conditions, this distribution can be identified
267 unambiguously with the 885 kDa mass. This mass fits well with a dodecamer reassociated
268 from the lightest subunits (below 74 kDa) produced by alkaline dissociation. The occurrence
269 of this species before acidic reassociation suggests that a dynamic equilibrium between it and
270 the free subunits exists as soon as dissociation of the dodecamers begins. Relative abundance
271 of residual dodecamer after alkaline dissociation could vary slightly from one experiment to
272 the other, but a massive dissociation was always observed.

273 The presence of dissociated subunits at various stages of the acidification process can be
274 monitored by examining the distribution around m/z 4400, corresponding to the monomers
275 with a 17 H^+ charge (figure 2 panels F,G,H). Six main charge-state distributions are visible,
276 corresponding to six subunits. The estimated masses are depicted in table I. They could vary
277 slightly from one experiment to another, but were almost always superior to the masses
278 observed in denaturing conditions. Interestingly, bimodal charge state distributions are
279 observed for monomers when pH increases (figure 2 panel C). These distributions suggest
280 that different conformational states exist for the dissociated monomers.

281 After alkaline dissociation of the complexes into their subunits, the preparation is
282 acidified by progressive addition of formic acid (figure 2 panel D,E). When pH decreases
283 from 9 to 7.7, a progressive and partial reassociation into complexes is observed, mainly into
284 light dodecamer (dl, 885 kDa) and dodecamer (d, 900 kDa) complexes (figure 2 panel F). In
285 the meantime, a progressive disappearance of the Cm1 and Cm2 peaks corresponding to the
286 lightest monomers suggests that these subunits are preferentially recruited for reassociation
287 into complexes (figure 2 panel H). When pH becomes strongly acidic (below the isoelectric
288 point), complexes are dissociated anew and all subunits peaks are visible again (data not
289 shown).

290 The same experiments were performed using purified dodecamers and purified
291 hexamers. Whereas dodecamers d and light dodecamers dl were obtained from dissociated
292 monomers issued from purified dodecamers, only hexamers and no dodecamers (d or dl) were
293 obtained from dissociated monomers issued from purified hexamers, indicating that the
294 dodecamer-specific subunit Cm6 is compulsory for the association into dodecamers (results
295 not shown).

296

297 Reassociation by L-lactic acid

298 L-lactic acid also induced a partial reassociation of the subunits into complexes, as
299 observed with formic acid (figure 3). The main complex observed is the light dodecamer, with
300 a relative intensity higher than in the case of formic acid reassociation (figure 3 panel B). A
301 preferential mobilization of the lightest subunits is observed again, which is consistent with
302 the appearance of light dodecamer (figure 3 panels E,F). While this preferential recruitment is
303 also observed with formic acid, there is an important difference since L-lactic acid induces an
304 immediate disappearance of the two light subunits (Cm1 and Cm2) as soon as it is added,
305 even if the pH is maintained at a dissociating value over 8.4. This specific and immediate
306 effect contrasts with the progressive effect observed with formic acid. The bimodal charge
307 state distributions of the dissociated monomers is still observed in these conditions. Cm1 and
308 Cm2 remain undetectable at pH 5.9, over the isoelectric point.

309

310 Chelation of divalent cations by EDTA

311 When divalent cations are removed by EDTA washing after alkaline dissociation of
312 whole hemolymph preparation, returning to pH 7.5 by washing with a 10 mM AcNH₄,
313 0.005 % TEA solution induces a partial reassociation mainly into hexamers and to a lesser
314 extent into dodecamers (figure 4 panel B). Interestingly, all dissociated monomers peaks
315 disappear upon reassociation except for the 4450 peak corresponding to the subunit specific
316 of the dodecameric assembly Cm6 (figure 4 panel F); the high proportion of hexamers formed
317 can be explained by the limited incorporation of Cm6 in the reassociated complexes. Addition
318 of L-lactic acid to the sample after removal of EDTA still resulted in the disappearance of the
319 lightest subunits peaks (figure 4 panels G,H) but hexamers remained the dominant complexes
320 and no light dodecamer were observed (figure 4 panels C,D). No major dissociation into
321 hexamers or monomers was observed upon addition of EDTA at neutral pH (data not shown).

322

323 **DISCUSSION**

324

325 Subunits masses in denaturing and non-covalent conditions

326 The masses observed in denaturing conditions are in good agreement with those
327 previously published by Sanglier and collaborators, also in denaturing conditions (Sanglier et
328 al, 2003). The five most abundant subunits observed in Sanglier's study are part of the six
329 subunits observed here.

330 In our study, a slight and variable difference is observed between masses estimated
331 from denaturing and from non-covalent conditions. The higher masses estimated in non-
332 covalent conditions can be accounted for by the occurrence of various adducts to the
333 dissociated subunits in these conditions, such as alkaline ions, divalent cations or unremoved
334 copper ions in the active site. These adducts are permitted by the unusual conditions used
335 here, namely an alkaline dissociation observed in ESI-MS conditions preserving the
336 remaining non-covalent interactions during the analysis. Desolvation conditions also differ
337 between ESI-MS in denaturing and in non-covalent conditions, hence the amount of the
338 remaining solvent is likely to be different.

339

340 Complexes masses and abundances in non-covalent conditions

341 The use of ESI-MS in non-covalent conditions enables to acquire signal for high-
342 molecular mass non-covalent complexes in their native state (dodecamers and hexamers). The
343 good agreement between dodecamer to hexamer ratios from ESI-MS and SEC confirms that
344 our ESI-MS data under near-native conditions are relevant for studying complexes occurring
345 in solution (figure 1). For occurrence of 18-mer and 24-mer, aggregation is known to be
346 possible during the ESI process but a slight fraction of 18-mer and 24-mer is also visible by
347 SEC coupled with a light-scattering system (personal observation for *Carcinus maenas*

348 hemocyanin; (Beltramini et al, 2005) for *Penaeus monodon* hemocyanin). However, 24-mers
349 can be detected from purified 12-mers (personal observation). This is unlikely to come from a
350 contamination during the purification step since no 18-mer is visible, but must be due to
351 aggregation of the sample during preparation or during the ESI-MS process. Whatever the
352 extent of aggregation process is for these high-mass complexes (18-mer and 24-mer), their
353 concentration is always very low compared to dodecamer and hexamer. Even if they may
354 really exist in the hemolymph of the animal and not be formed during the sampling and
355 storage, they are probably of very little physiological significance due to this low
356 concentration.

357

358 Alkaline dissociation and reassociation (native and purified)

359 Crustacean hemocyanin dissociation is known to be induced by high or low pH
360 (Herskovits, 1988), with varying stability ranges depending on the species: for example,
361 contrary to other crustacean hemocyanins, *Penaeus monodon* hemocyanin has been shown to
362 have a high stability against dissociation at high pH and in the presence of EDTA (Beltramini
363 et al, 2005). For other crustaceans, various experiments have shown that alkaline dissociation
364 was reversible and that complexes could be reassociated from whole subunit sets or from
365 selected purified subunits (Dainese et al, 1998; Johnson et al, 1987). In our study the
366 dissociation is induced by alkaline pH and yields separated subunits from the complexes. The
367 dissociation efficiency could vary from one experiment to another, and part of the complex
368 could resist the dissociation; however most of the time the dissociation was almost complete,
369 showing that ESI-MS can be used to monitor a process taking place in solution and that the
370 different aggregation states present in the aqueous phase are likely to be conserved during the
371 ionization and analysis processes. Previous studies (Chantler et al, 1973; Dainese et al, 1998)

372 also showed the dissociating effect of EDTA and alkaline pH on a close species (*Carcinus*
373 *aestuarii* also named *Carcinus mediterraneus*).

374 The occurrence of bimodal charge state distribution for dissociated monomers must
375 correspond to different conformational states of the monomers. These distributions appear at
376 high pH and bimodal distributions can correspond to partial unfolding of the subunits coupled
377 to dissociation. However, unfolding is usually associated with a shift from narrow
378 distributions of low charge-density ions towards broader distributions of higher charge-
379 density ions, reflecting that the unfolded proteins are less structured and can accommodate
380 more charges on their surface (Kaltashov 2008). In our study, lower charge-density ions
381 appear at high pH which is in contradiction with what is expected from the unfolding model.
382 Moreover, the occurring process must be reversible since the bimodal distributions almost
383 disappear when pH decreases again (figure 2, panel E). Many other processes occurring in
384 the gas-phase can also alter the charge state distribution (Kaltashov 2008) and in our case the
385 significance of the observed bimodal distributions remains unclear. A systematic study of
386 hemocyanin denaturation coupling ESI-MS analysis and circular dichroism would help to
387 resolve this issue, as already performed by another group with hemoglobin (Griffith 2003).

388 The reassociation by returning to a more neutral pH could also be monitored by ESI-
389 MS. Complete reassociation was never observed and the fact that the lightest subunits are
390 reassociated first, as evidenced by the monomers peaks and by the occurrence of the light
391 dodecamer, shows that the reassociation process after dissociation is not exactly identical to
392 the one occurring naturally. A dynamic equilibrium between subunits and light dodecamer is
393 also observed (figure 2 panel B). In several species, homohexamers could be reassociated
394 from purified subunits but reassociation into dodecamers necessitated the occurrence of
395 specific subunit types (Stöcker et al, 1988). However, it was suggested in a study of
396 *Paralithodes camtschaticae* hemocyanin that homododecamers could be formed for this

397 species but this case is original because dodecamers and hexamers seem to exist in a chemical
398 equilibrium (Molon et al, 2000). Here we cannot distinguish if two light homododecamers are
399 produced since the distributions would overlay, but the fact that the purified hexamers cannot
400 reassociate into dodecamers evidences that the dodecamer specific subunit is compulsory to
401 form dodecamers. Hence, we should have a simultaneous association of the light subunits and
402 of the dodecamer-specific subunit when reassociating dodecamers from alkaline dissociation.
403 This implies different association constants and different interactions between subunit types.

404

405 Specific effect of L-lactate

406 L-lactic acid addition has the same effect that acidification by formic acid since the
407 same light subunits are mobilized first and light dodecamers are formed first. However, the
408 fact that lactic acid has an effect even at a dissociating high pH where formic acid has no
409 effect yet shows a specific effect of the molecule. Moreover, it triggers the formation of light
410 dodecamers much more than classic dodecamers, enabling a specific stabilization/association
411 of this form. Figure 5 is a schematic summary of the steps involved in the dissociation and
412 reassociation studied here. Based on the previously published data suggesting the occurrence
413 of a site emerging from the association of several subunits and the specificity of some
414 subunits for lactate sensitivity in *Panulirus interruptus* (Johnson et al, 1987), it can be
415 hypothesized that these two lactate-sensitive subunits interact together to form a lactate-
416 binding site within the quaternary structure of the dodecamer. Another possibility is that each
417 of the lactate-sensitive subunit is self-sufficient for the formation of a lactate binding site, and
418 that two types of sites involving one or the other light subunit exist within the quaternary
419 structure of the dodecamer. The interaction of L-lactate with the binding site could stabilize
420 the subunits association and hence explain the complexes formation even at high pH (figure
421 5). For *Carcinus maenas*, 4 such lactate binding sites exist per dodecamer according to Weber

422 and collaborators (Weber et al, 2008). Since L-lactate can still promote the incorporation of
423 the light subunits Cm1 and Cm2 after washing with EDTA but mainly hexamers are observed
424 in this case, the binding sites must be located within the hexameric structure but not at the
425 interface between two hexamers. However our data are not of a type helping to resolve the
426 number of binding sites per dodecamer. As noted by Graham and collaborators (Graham,
427 1985), the existence of a binding site arising from the quaternary structure of the complex and
428 not from the tertiary structure of a single subunit would be similar to the organophosphate
429 binding site of vertebrate tetrameric hemoglobin.

430

431 Effect of chelation of divalent cations

432 Divalent cations are known to have both functional and structural roles in hemocyanins
433 (Bridges, 2001; Truchot, 1975). Here, the reassociation of subunits into complexes is still
434 possible after alkaline dissociation and chelation of the divalent cations by EDTA but the
435 reassociation tends to stop at the hexamer level, and at least part of the dodecamer-specific
436 subunit remains unassociated. The incorporation of the specific subunit must involve lower
437 association constants or need more cations than the rest of the assembly process since Ca^{2+} and
438 Mg^{2+} are removed or at least severely depleted upon chelation by EDTA. The dodecamer
439 formation process can be limited either by lower incorporation of the specific subunit in
440 hexameric assemblies or by a lower rate of hexamers association into dodecamers even with
441 the specific subunit incorporated within the hexameric structure. As underlined by Stöcker
442 and collaborators, the inter-hexamer link varies between species: it can be an ionic bond with
443 divalent cations as for *Homarus americanus* or a disulfide linked dimer as for *Astacus*
444 *leptodactylus* and *Cherax destructor* (Markl & Decker, 1992; Marlborough et al, 1981;
445 Stöcker et al, 1988). The same group also suggested that subunits could substitute during the
446 hexamer formation due to homologous interaction sites between them whereas some specific

447 interactions between precise subunits could be needed to form the dodecamer, hence
448 explaining the reassociation limited to hexamers. In *Carcinus maenas* hemocyanin, the
449 interactions between hexamers must be ionic since no covalently-bound dimer can be found.
450 The study of the hemocyanin of *Paralithodes camtschaticae* by Molon and collaborators
451 (Molon et al, 2000) also showed that after dialysis against EDTA, dodecamers were
452 dissociated into hexamers. However, the hemocyanin of this anomuran exists as a chemical
453 equilibrium between the two forms (dodecamers and hexamers) in native conditions and
454 homododecamers and homo-hexamers can be formed from one purified subunit type. In this
455 case, one type of monomer is able to form all the interactions needed to form both forms. The
456 fact that dissociated hexamers cannot reassociate into dodecamers in our study implies that
457 for *Carcinus* only some specific monomers are able to establish the interactions needed for
458 the dodecamer assembly.

459 Typically, two different types of calcium-binding sites with high and low affinities are
460 observed for crustacean hemocyanins (table II) (Andersson et al, 1982; Molon et al, 2000;
461 Sanna et al, 2004) and in the case of Andersson's study Ca^{2+} and Mg^{2+} were shown to have
462 similar affinities for the binding sites. There are usually a few sites with high affinity (1 to 3
463 sites per hexamer) and numerous with a 10 to 100-fold lower affinity (1.6 to 42 sites per
464 hexamer). Given the physiological range of calcium concentration, the sites with the highest
465 affinity are always saturated and would rather play a structural role while the sites with the
466 lowest affinity would have a modulating effect on oxygen affinity since their saturation can
467 vary depending on the calcium concentration in the hemolymph (Andersson et al, 1982;
468 Johnson et al, 1988). It is likely that the structural calcium and magnesium ions bound to the
469 high-affinity sites are retained throughout the desalting process while the others divalent
470 cations and Na^+ ions are removed (consistent with a 100-fold lower affinity of hemocyanin
471 for Na^+ (Andersson et al, 1982)). Since no EDTA effect is observed here without prior

472 alkaline dissociation, these sites must be located at the interface between different subunits
473 (Hazes et al, 1993) and be accessible to chelation by EDTA only after separation of the
474 subunits. The reassociation into hexamers rather than dodecamers after removal of the
475 structural cations can indicate that less cationic bridges are needed between subunits within a
476 single hexamer than between two hexamers implicated in a dodecamer. Another possibility is
477 the existence of some intra-hexamer sites which would retain divalent cations with a very
478 high affinity. The fact that the specific effect of L-lactate is not inhibited by EDTA shows that
479 no low-affinity bound divalent cations are necessary for the direct interaction between lactate
480 and hemocyanin.

481

482 Structure of the L-lactate binding site

483 Crustacean hemocyanin is a very complete model for the study of structural and
484 functional properties of respiratory pigments and more generally allosteric proteins. The
485 multimeric structure made of functionally different yet similar subunits and the diversity of
486 effectors and of their effect allow for numerous biochemical issues to be addressed. Here, we
487 used non-covalent ESI-MS to probe the structural effects of L-lactate and divalent cations on
488 *Carcinus maenas* hemocyanin. The specific interaction of L-lactate with 2 subunits and its
489 stabilizing effect have been evidenced, as well as the role of divalent cations for multimeric
490 assembly. The question of the precise structure of the binding site of L-lactate remains to be
491 solved. The specificity of interaction with some subunits, the symmetry of the quaternary
492 structure and the L-lactate asymmetry are to be considered (Johnson et al, 1984). The fact that
493 sensitive homohexamers only harbor one site and that the number of observed sites per
494 hexamer is about two for brachyuran crabs suggests that the sites are located on the three-fold
495 axis of the hexamer, and hence possess the same symmetry. How does the binding between
496 such a symmetric site and a chiral ligand occur? The site may present three potential positions

497 for lactate binding and the binding of one molecule on one site would prevent the binding at
498 the other sites by steric hindrance. Another possibility is that the binding site is stabilized in
499 an asymmetric conformation when L-lactate binds to it. Arnone showed for human
500 hemoglobin that the crystallographic map of the protein with the asymmetric ligand D-2,3-
501 diphosphoglycerate (DPG) showed the same non-crystallographic dyad axis as the map for
502 the protein alone, and deduced that the binding of DPG occurred in two symmetric
503 orientations related by a 180° rotation (Arnone, 1972). More recent studies with lower-salt
504 crystals or using ³¹P nuclear magnetic resonance in solution showed that the binding site of
505 DPG was actually asymmetric in the presence of the ligand (Pomponi et al, 2000; Richard et
506 al, 1993). Molecular dynamics simulations also suggested that a dynamic heterogeneity
507 existed in the hemoglobin tetramer and that DPG influences the tertiary states explored by the
508 protein (Laberge & Yonetani, 2008). A similar mechanism can be postulated for hemocyanin,
509 with the occurrence of a dynamic heterogeneity influenced by the presence of effectors such
510 as oxygen and lactate, resulting in a stabilized asymmetric binding site when L-lactate is
511 effectively bound. Performing studies similar to those made on hemoglobin and DPG with
512 hemocyanin and L-lactate would help to test this hypothesis.

513 It has been showed that the oxygenated Hc hexamer of *Panulirus interruptus* has a
514 reduced channel along the three-fold axis compared to the deoxygenated form (De Haas et al,
515 1993). Using the simple Monod-Wyman-Changeux (MWC) model for *Carcinus maenas* Hc
516 dodecamer, Weber and collaborators showed that L-lactate increases O₂ affinity in part by
517 shifting the allosteric equilibrium towards the high-affinity R state (Weber et al, 2008). From
518 these data it can be suggested that the potential binding of lactate in the reduced central
519 channel could stabilize the oxygenated R conformation. Such a hypothesis must be considered
520 with care and at the hexamer scale, since SAXS studies of the Hc dodecamer of *Homarus*
521 *americanus* have showed that the oxygenated dodecamer exists in two different forms, the

522 one without lactate and the one in the presence of lactate with the two hexamers closer by
523 0.5 nm. In this case the simple MWC model is no longer relevant and nested allostery must be
524 considered (Hartmann et al, 2001).

525 It would be of interest to perform studies similar to those conducted here using
526 hemocyanin from terrestrial crabs or mud shrimps with little or no lactate effect, and to test
527 the effect of other molecules such as the stereoisomer D-lactate or the physiological effector
528 urate.

529

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535

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739

740 **FIGURE LEGENDS**

741

742 **Figure 1:** Mass spectrum of *Carcinus maenas* native hemolymph analysed under non-
743 covalent conditions (pH 6.8). Spectra were acquired using individual whole hemolymph
744 samples. Aggregation state, estimated mass and m/z value for the main peak of each
745 distribution are indicated. Insert: size-exclusion chromatography profile of the same sample
746 with a dodecamer-to-hexamer ratio of 9:1.

747

748 **Figure 2:** Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin by
749 TEA and formic acid. Panels A to E, mass spectra of a whole hemolymph sample desalted in
750 10 mM AcNH₄ with increasing quantities of TEA (0.005, 0.03 and 0.05 % TEA for pH 7.6,
751 8.6 and 9, respectively) then formic acid (0.004 and 0.0045 % formic acid with 0.03 % TEA
752 for pH 7.9 and 7.7, respectively). m, monomers peaks, h, hexamer peaks (450 kDa), d,
753 dodecamer peaks (900 kDa), dl, light dodecamer peaks (885 kDa). Panels F to H, focus for
754 each mass spectrum on the monomer peak distributions in the 4300-4600 m/z range, in which
755 the 17⁺ charged peak of each monomer is expected. Six overlapping distributions can be
756 observed. The presence or absence of each dissociated subunit can be determined by
757 examining the peaks visible in this m/z range. Masses obtained in non-covalent and
758 denaturing conditions for each subunit are compared in table I.

759

760 **Figure 3:** Acidic reassociation of *Carcinus maenas* hemocyanin by L-lactic acid. Panel A,
761 mass spectrum of a whole hemolymph sample dissociated in 10 mM AcNH₄, 0.03 % TEA,
762 pH 8.6. Panels B and C, mass spectra of the dissociated hemocyanin treated with 2mM lactic
763 acid and with or without addition of a further 0.04 % TEA (pH 8.6 and 5.9, respectively). m,
764 monomers peaks, h, hexamer peaks (450 kDa), d, dodecamer peaks (900 kDa), dl, light

765 dodecamer peaks (885 kDa). Panels D to F, focus for each mass spectrum on the 4300-4600
766 m/z range (monomer peaks).

767

768 **Figure 4:** Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin
769 coupled with chelation of divalent cations by EDTA. Whole hemolymph sample was washed
770 by an EDTA mix (pH 9) as explained in the materials and methods section and salts were then
771 removed by washing with 10 mM AcNH₄ containing TEA at various concentrations. Panels A
772 to D, whole mass spectra, panels E to H, focus in the monomer peaks m/z range. Sample
773 preparation was as follow: panels A and E, washing with 0.05 % TEA (pH 9); panels B and F,
774 washing with 0.005 % TEA (pH 7.5); panels C and G, washing with 0.05 % TEA and
775 addition of 2 mM lactic acid (pH 8.1); panels D and H, washing with 0.005 % TEA and
776 addition of 2 mM lactic acid (pH 7.4). m, monomers peaks, h, hexamer peaks (450 kDa), d,
777 dodecamer peaks (900 kDa), dl, light dodecamer peaks (885 kDa). Panel C (pH 8.1, 2mM
778 lactate), the hexamer peaks could correspond to a classical hexamer and to a “light” hexamer.

779

780 **Figure 5:** Summary of the dissociation and association steps observed under alkalization by
781 TEA and acidification by formic acid or addition of L-lactate. In the native state before
782 dissociation, the subunit composition is unknown but two Cm6 subunits must interact to form
783 the dodecamer. Upon progressive dissociation by alkalization, dissociated monomers can
784 partially reassociate into light dodecamers by dynamic equilibrium. Monomers are fully
785 dissociated at pH 9 and can be reassociated either by adding L-lactic acid even when
786 maintaining a high pH or by progressive acidification by formic acid. L-lactic acid
787 immediately promotes the reassociation of light subunits into light dodecamer and must
788 interact with hemocyanin at two sites per hexamer, located on both sides of the three-fold axis

789 of each hexamer, at the center of each trimer. Note that partial unfolding of the dissociated
790 monomers could occur (not figured here – see the text for details).

791

792 (references cited in the tables: (Andersson et al, 1982; Brouwer et al, 1983; Johnson et al,
793 1988; Kuiper et al, 1979; Sanna et al, 2004))

794

Table I: subunit masses for *Carcinus maenas* hemocyanin obtained by ESI-MS

| Subunit name | Mass obtained by Sanglier et al. 2003 (Da) ^a | Average mass in denaturing ESI-MS (Da \pm s.d.) ^b | m/z value for the (17 H+) peak | Examples of masses estimated by non-covalent ESI-MS (Da) ^c | | Mass differences between non-covalent and denaturing ESI-MS (Da) ^d | |
|--------------|---|--|--------------------------------|---|-------------|---|-------------|
| | | | | <i>no.1</i> | <i>no.2</i> | <i>no.1</i> | <i>no.2</i> |
| Cm1 | 73931 | 73922 \pm 1.3 | 4360 | 74116 | 74090 | 194 | 168 |
| Cm2 | 74049 | 74043 \pm 1.1 | 4368 | 74218 | 74207 | 175 | 164 |
| Cm3 | 75088 | 75073 \pm 3.2 | 4418 | 75106 | 75089 | 33 | 16 |
| Cm4 | 75161 | 75187 \pm 5.8 | 4426 | 75253 | 75214 | 66 | 27 |
| Cm5 | 75234 | 75224 \pm 1.8 | 4435 | 75344 | 75355 | 120 | 131 |
| Cm6* | 75459 | 75449 \pm 0.9 | 4450 | 75684 | 75629 | 235 | 180 |

*Dodecamer specific subunit

^aNine masses were determined by Sanglier and collaborators³⁸; the masses that were the closest to those we determined are reported here

^bAverage masses and standard deviations are calculated from data for 14 different individuals; the number of values for each subunit varies from 9 to 35 depending on the occurrence of each subunit in different individuals and in purified fractions for each individual

^cThese masses were calculated from the monomers charge-state distributions from two different experiments

^dThese differences were calculated using the average masses determined in denaturing conditions in this study; molecular masses of potential adducts in non-covalent conditions are for example 127.1Da (the two Cu of the active site), 23Da (Na), 24.3Da (Mg), 40.1Da (Ca) and combinations thereof

Table II: association constants and number of sites for calcium binding with hemocyanin

| Species | High-affinity sites | | Low-affinity sites | | pH | References |
|------------------------------|---------------------|-----------------------------|--------------------|-----------------------------|------|------------|
| | $K_{Ca} (M^{-1})$ | number of sites per hexamer | $K_{Ca} (M^{-1})$ | number of sites per hexamer | | |
| <i>Panulirus interruptus</i> | 1×10^4 | | 50 | | 7.6 | (51) |
| <i>Panulirus interruptus</i> | 3×10^4 | 1 | 1.5×10^3 | 3-17 | 7.0 | (26) |
| <i>Callinectes sapidus</i> | $4-9.1 \times 10^4$ | 2.4-3 | $2-10 \times 10^2$ | 14-42 | | (52) |
| <i>Callinectes sapidus</i> | | | 7.1×10^2 | 1.6-2 ^a | 7.01 | (44) |
| | | | 3.3×10^2 | 3.5-5.4 ^a | 7.55 | |
| <i>Scyllarides latus</i> | 5.6×10^4 | | 1.3 | | 7.0 | (12) |

^aThe number of sites calculated in this study corresponds only to oxygen-linked binding sites and not to the total number of binding sites; the difference with results from (52) led the authors to the conclusion that many of the calcium binding sites did not affect oxygen binding

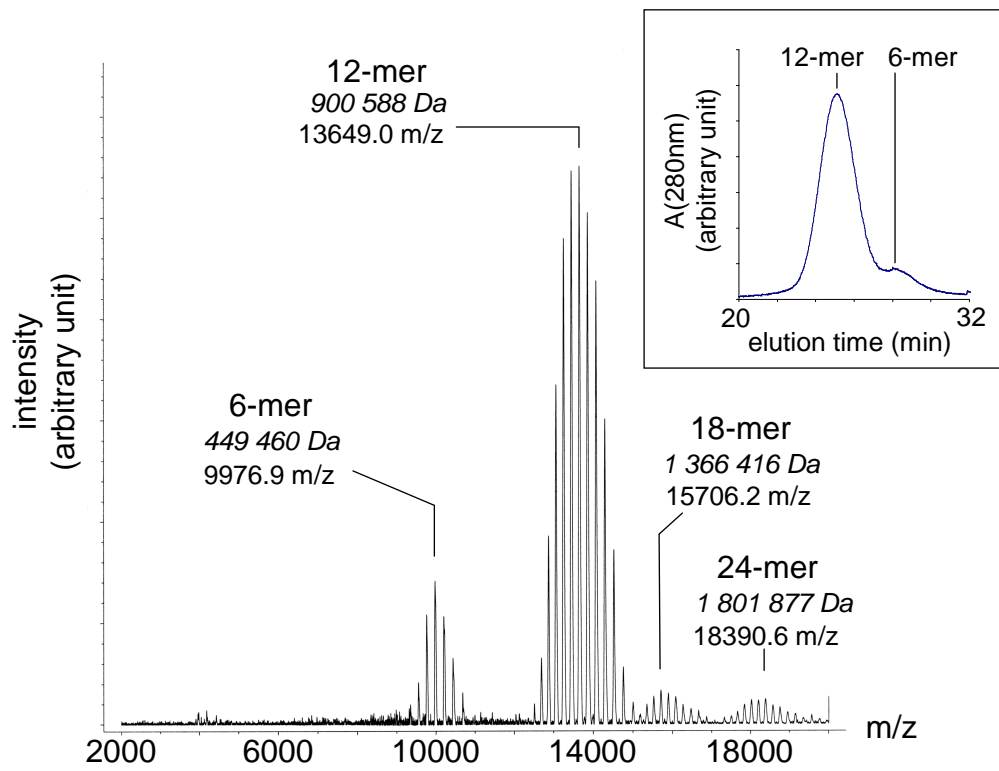
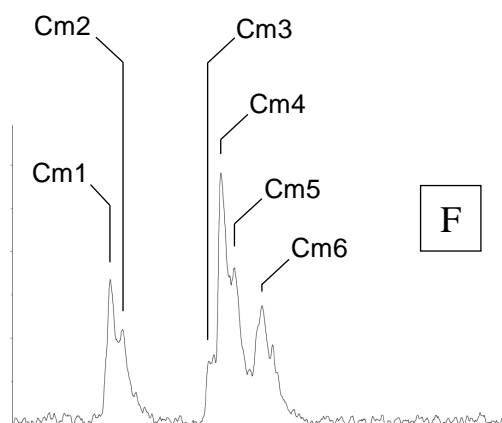
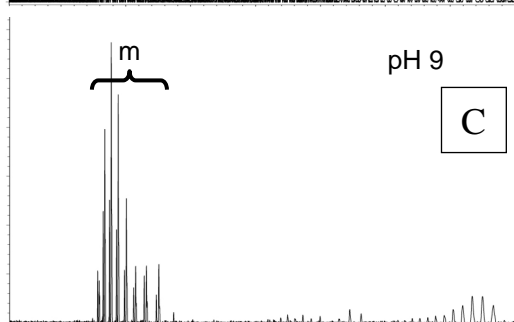
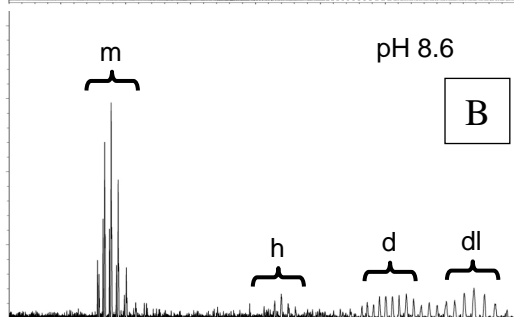
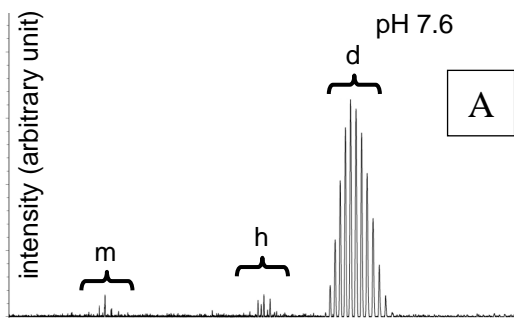


Figure 1: Mass spectrum of *Carcinus maenas* native hemolymph analysed under non-covalent conditions (pH 6.8)

ALKALINE DISSOCIATION



ACIDIC REASSOCIATION

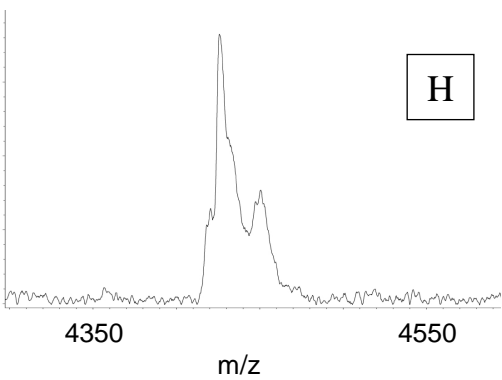
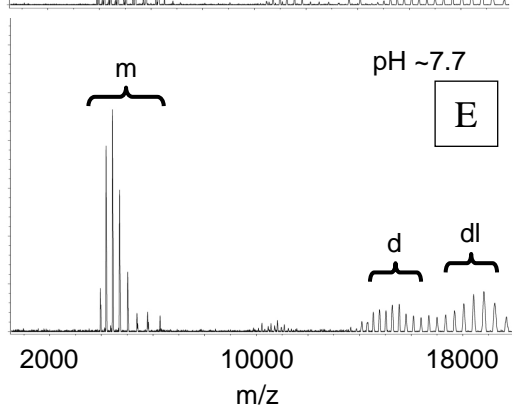
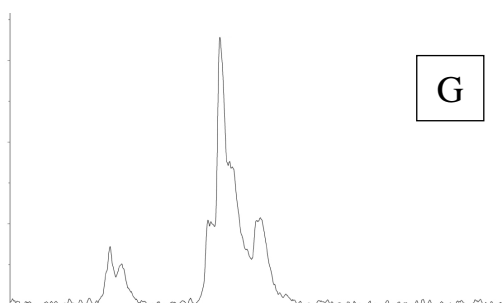
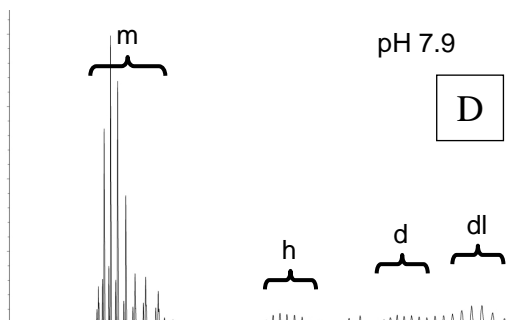


Figure 2: Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin by TEA and formic acid

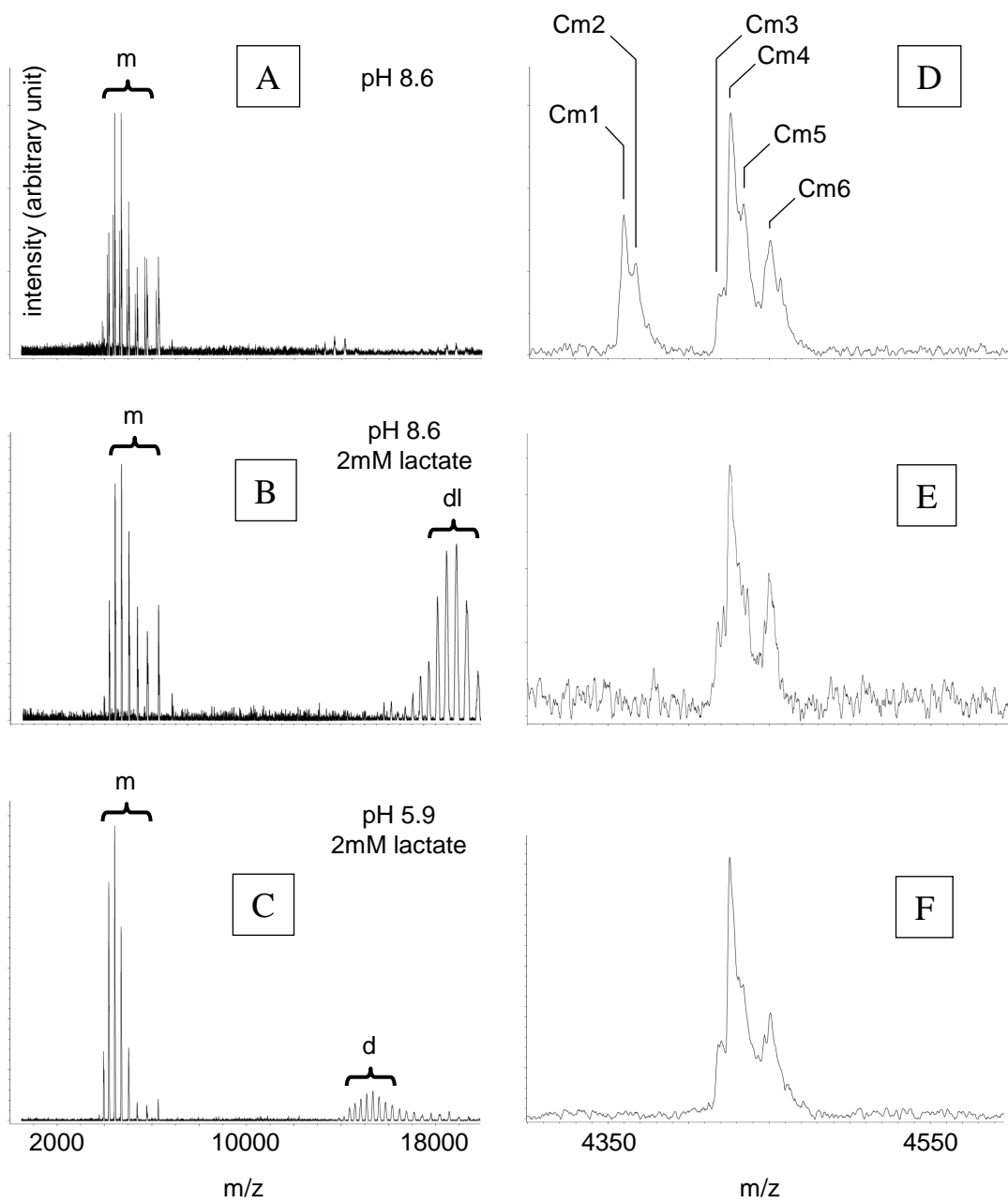


Figure 3: Acidic reassociation of *Carcinus maenas* hemocyanin by L-lactic acid

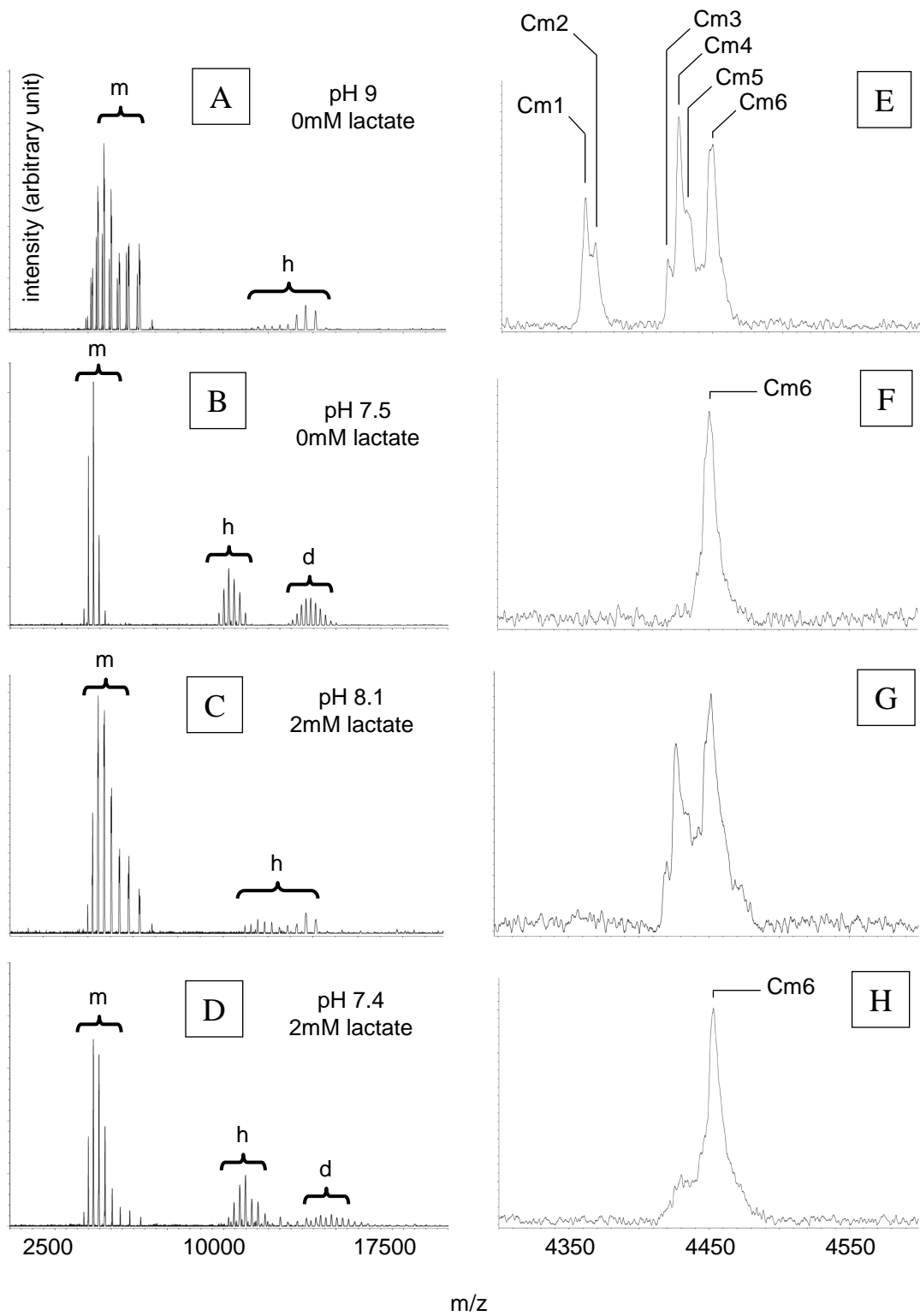


Figure 4: Alkaline dissociation and acidic reassociation of *Carcinus maenas* hemocyanin coupled with chelation of divalent cations by EDTA

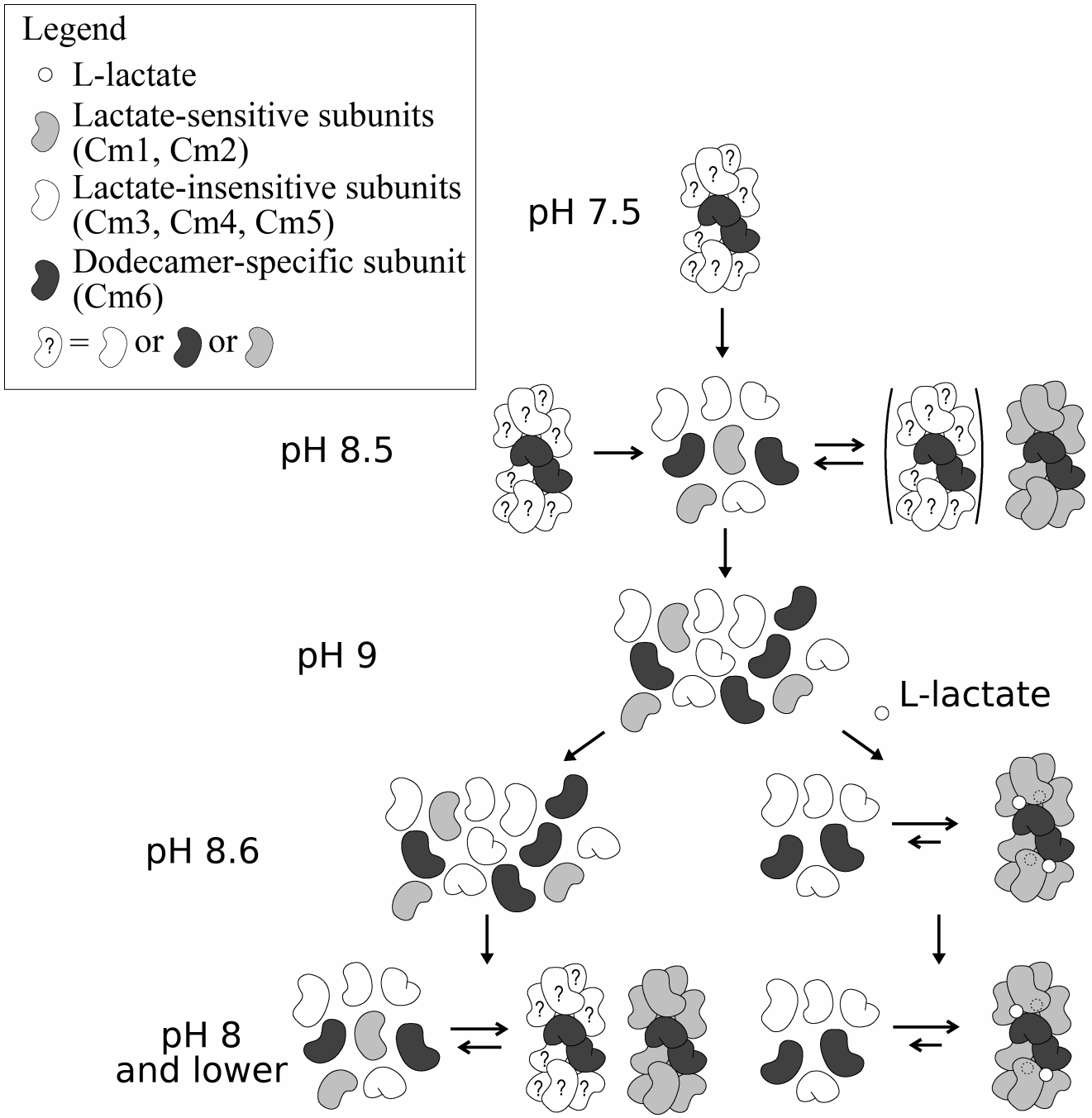


Figure 5: Summary of the dissociation and association steps observed under alkalization by TEA and acidification by formic acid or addition of L-lactate