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Copper isotope composition of hemocyanin

Marine Paquet^{1*}, Toshiyuki Fujii², Frédéric Moynier¹

¹ Université de Paris, Institut de Physique du Globe de Paris, CNRS, UMR 7154, 75005 Paris, France

*Corresponding author: [Marine Paquet \(paquet@ipgp.fr\)](mailto:paquet@ipgp.fr)

² Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Background

Copper is a metal that plays a central role in biology, for example, as co-factor in various redox enzymes. Its stable isotopic composition is being used as tracer of its transport in living organisms and as a biomarker for diseases affecting its homeostasis. While the application of copper stable isotopes to biological studies is a growing field, there are presently no biological standards that are systematically analyzed in the different laboratory, as it is the case for geological samples (e.g., by using widely available basalt samples). It is therefore paramount for the community to establish such standard. Copper also binds oxygen in the respiratory protein, hemocyanin, in the hemolymph of mollusks and arthropods and is thus critical to respiration for these species.

Methods

Here, the Cu isotope composition of hemocyanin of different modern species of mollusks and arthropods (*Megathura crenulate* Keyhole limpet, *Limulus polyphemus* Horseshoe crab and *Concholepas concholepas* Chilean abalone), as well as theoretical constraints on the origin of these isotopic fractionations through ab initio calculations are reported.

Results

The isotopic fractionation factors for Cu(I) and Cu(II), both in hemocyanin and in seawater, predict an enrichment in the lighter isotope of Cu in the hemocyanin by over 1 permil compared to seawater. The hemocyanin of Chilean abalone and Horseshoe crab have Cu isotope compositions ($\delta^{65}\text{Cu} = +0.63 \pm 0.04\text{‰}$ and $+0.61 \pm 0.04\text{‰}$, respectively, with $\delta^{65}\text{Cu}$ the permil deviation of the $^{65}\text{Cu}/^{63}\text{Cu}$ ratio from the NIST SRM 976 standard), similar to that of the octopus reported in literature ($+0.62\text{‰}$), that are undistinguishable from seawater, suggesting quantitative Cu absorption for these organisms. Conversely, the Keyhole limpet is enriched in the lighter isotope of Cu, which is in line with the ab initio calculation and therefore Cu isotopic fractionation during incorporation of Cu into the hemocyanin.

Conclusions

Because these hemocyanin standard samples are widely available, they could serve in the future as inter-laboratory standards to verify the accuracy of the Cu isotopic measurements on biological matrices.

88 Introduction

89 Copper has two naturally occurring stable isotopes, ^{63}Cu and ^{65}Cu , with relative abundances of 69.17%
90 and 30.83% respectively. Copper is also a key micronutrient in seawater for all organisms in the food
91 chain [e.g., 1, 2, 3, 4], but can be toxic at high concentrations of its free Cu^{2+} form [e.g., 1, 5].
92 Measurement of Cu isotope composition in seawater is challenging due to low Cu concentrations,
93 especially for surface seawater [e.g., 6, 7]. However, recent analytical improvements have allowed the
94 measurement of seawater samples, with for example an estimate for the global surface seawater isotope
95 composition of $\delta^{65}\text{Cu} = +0.49 \pm 0.04 \text{‰}$ ([8], with $\delta^{65}\text{Cu}$ the permil deviation of the $^{65}\text{Cu}/^{63}\text{Cu}$ ratio from
96 the NIST SRM 976 standard), within variability reported in different ocean basins (+0.51 to +1.25 ‰;
97 [9]). Alternatively, deep sea corals have been used to estimate the Holocene and modern seawater
98 composition, and provide similar isotopic composition as deep seawater samples [10]. It would, however,
99 be important to develop a complementary proxy for the Cu isotopic composition of the seawater through
100 time.

101
102 Hemocyanins are the respiratory proteins that turn blue upon oxygen binding in the hemolymph of some
103 mollusks (Protobranchi) and arthropods [11], equivalent to hemoglobin for vertebrates. In hemocyanins,
104 the active site corresponds to two copper ions complexed by six histidine residues, that can bound a
105 dioxygen molecule (Figure 1) to form a Cu_2O_2 cluster. Hemocyanins of mollusks and arthropods appeared
106 ~740 Ma [12, 13] and ~600 Ma [14] ago, respectively. Both type of hemocyanins have similar active sites
107 and are large multimeric extracellular proteins composed of many subunits: molluscan hemocyanins are
108 hollow cylindrical decamers or multidecamers (~4 MDa or > 8 MDa), whereas arthropod hemocyanin are
109 hexamers or multihexamers (~450 kDa). For arthropods the functional unit corresponds to a subunit
110 whereas a molluscan subunit can contain up to 8 functional units. Finally, the primary, ternary and
111 quaternary structure of molluscan and arthropod hemocyanin is different and they are part of two distinct
112 protein superfamilies [11].

113
114 Biological processes are known to have the ability to fractionate isotopes and create isotopic fractionation:
115 Zn, Fe, Cu and Mg isotopic fractionation between plants and soils [e.g., 15- 24], between animals and
116 their environment, or between organs in mice, dogs or humans and due to diseases affecting their
117 homeostatis (e.g., 10, 24-43]. While the Cu stable isotopic measurements are routine for geological
118 materials, with widely available geological samples analyzed systematically in all laboratories (e.g.,
119 United States Geological Survey basaltic rocks such as BHVO-2 or BCR-2), there are presently no
120 biological standard that plays this role in this new field of isotope metallomics, and metal isotope
121 compositions of certified reference materials of biological origin remain very scarce [e.g., 38, 44-46].
122 Given that more and more laboratories are not working on such topics, it is therefore paramount that the
123 community use biological standards. Hemocyanin standards are widely available and given their high Cu
124 content could play this role.

125
126 However, apart from one measurement done on octopus (mollusk) hemocyanin ($\delta^{65}\text{Cu} = +0.62\text{‰}$; [47]),
127 these respiratory proteins have not been studied using Cu isotopic methods yet. This value is close to the
128 global seawater Cu isotopic composition, further suggesting the possibility to use hemocyanin as a proxy
129 for seawater $\delta^{65}\text{Cu}$. Given the difference of structure between the hemocyanin of arthropods and mollusks,
130 it would be important to evaluate the composition of some arthropods hemocyanins. Moreover, non-
131 traditional metal stable isotope systems, such as Cu and Zn, are good tools to monitor anthropogenic metal
132 bioaccumulation into marine environments, notably in bivalve mollusk soft tissues due to homeostasis and
133 species-specific physiological processes [e.g., 48-50].

134
135 Here we report new Cu isotope composition in three certified hemocyanin standards (Sigma
136 Aldrich/Merck, Darmstadt, Germany) of different species (*Megathura crenulate* keyhole limpet [H7017],
137 *Limulus polyphemus* Horseshoe crab [H1757] and *Concholepas concholepas* Chilean abalone [B8556]),
138 and compare it with octopus hemocyanin Cu isotopic data from literature [47]. In addition of establishing

139 the Cu isotopic composition of both arthropods and mollusks, these values from certified standards could
140 be used in the future as reference value for interlaboratory comparison for Cu isotopic work on biological
141 standards. Moreover, in order to predict the theoretical isotopic fractionation between seawater and
142 hemocyanin, the first ab initio calculations on hemocyanin are computed. The combination of these
143 calculations with future natural Cu isotopic data in hemocyanin may allow to better constrain the Cu
144 isotope composition of seawater in the past.

145

146 **Analytical methods**

147 Copper purification and isotopic measurements were performed at the Institut de Physique du Globe de
148 Paris (IPGP). Acids used in this study were distilled (4 times sub-boiled) from BASF Selectipur® AR
149 grade acids (69% HNO₃; 37% HCl) (BASF, Ludwigshafen, Germany). Dilutions used ultra-pure (18.2
150 MΩ.cm resistivity) Milli-Q water. Between 2.5 and 19.7 mg (~0.6 to 7.3 μg of Cu) of the hemocyanin
151 samples were dissolved using a mixture of 16 mol.L⁻¹ HNO₃ and a Fischer Chemicals Seastar® optima
152 grade hydrogen peroxide solution (30-32% H₂O₂) (Thermo Fisher Scientific™, Bremen, Germany).
153 Copper was then purified by ion exchange chromatography following the same procedure as described in
154 Moynier et al. [34-35]. Hemocyanin samples were loaded in 1 mL of 7 mol.L⁻¹ HCl on columns filled up
155 with 1.6 mL of AG-MP1 resin (BioRad, Hercules, California, USA). After washing the resin with 7 mL of
156 7 mol.L⁻¹ HCl, the Cu was collected in 22 mL of 7 mol.L⁻¹ HCl. The procedure was repeated twice to
157 ensure a clean Cu fraction. Copper isotope compositions were determined using a Neptune Plus Multi-
158 Collector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS) (Thermo Fisher Scientific™,
159 Bremen, Germany) located at IPGP, using the same method as in Moynier et al. (2019, 2020). Samples
160 were introduced into the instrument using an ESI Teflon micro-nebulizer (100 μL.min⁻¹) and a glass spray
161 chamber. The MC-ICP-MS was operated at low resolution, with the ⁶⁵Cu and ⁶³Cu isotope beams
162 collected in the L1 and L3 faraday cups, respectively. Matrix elements such as ⁶²Ni and ⁶⁴Zn were
163 monitored in L4 and L2 cups in the same setup. The sample dilutions were adjusted to match the
164 concentration of the standard (within ~5%). Under typical running conditions, a 50 ppb Cu solution
165 generated a 5-6 V total signal using 10⁻¹¹Ω resistors. Copper concentrations have been estimated by
166 voltage equivalence on the MC-ICP-MS, by comparing sample Cu voltage to that of the IPGP-Cu
167 standard of known concentration (Table 1). The total procedural blank contained ~0.3 ng of Cu, which is
168 negligible in comparison to the amount of Cu present in each sample (Chilean abalone: 7.3 μg; Horseshoe
169 crab: 3.4 μg; Keyhole limpet: 0.6 μg).

170

171 Throughout the paper, all copper isotope data are reported relative to the SRM 976 standard (National
172 Institute of Standards and Technology, Gaithersburg, Maryland, USA). Each sample was analyzed
173 between 6 and 12 times, and the reported error is the 2 Standard Deviation (2SD) of these repeated
174 measurements. We used a standard bracketing method that consists in measuring a standard before and
175 after each sample and use the average of the two standards for the normalizing ratio [51]. During analysis
176 session, we used the Cu-IPGP standard (Thermo Fisher Scientific™, Bremen, Germany), which has a
177 δ⁶⁵Cu of +0.271 ± 0.006‰ relative to the NIST SRM976 standard [34]. Thus, all the data presented here
178 were converted to δ⁶⁵Cu relative to NIST SRM 976 by adding 0.271. To ensure the quality of the
179 measurements, we also processed the biological standard ERM CE464 (Tuna fish; Sigma Aldrich/Merck,
180 Darmstadt, Germany), which has been previously analyzed for its Cu isotopic composition by multiple
181 laboratories [38].

182

183 We also define Δ⁶⁵Cu_{X-Y}, the difference of δ⁶⁵Cu between two species X and Y as:

$$184 \Delta^{65}\text{Cu}_{X-Y} = \delta^{65}\text{Cu}(X) - \delta^{65}\text{Cu}(Y)$$

185

186

187

188 **Computational methods**

189 Orbital geometries and vibrational frequencies of Cu species were computed using the density functional
190 theory (DFT) as implemented by the Gaussian09 code [52, 53]. The DFT method employed here is a
191 hybrid density functional consisting of Becke's three-parameter non-local hybrid exchange potential (B3)
192 [54] with Lee-Yang-and Parr (LYP) [55] non-local functionals. The DFT computational protocol was the
193 same with our previous studies [56, 57]. The 6-311+G(d,p) basis set, which is an all-electron basis set,
194 was chosen for H, C,N, O, and Cu. Molecules were modeled without any forced symmetry. An "ultrafine"
195 numerical integration grid was used and the SCF (self-consistent field) convergence criterion was set to
196 10^{-9} .

197
198 Logarithm of isotope fractionation factor $\ln \beta$ which is identical with the \ln RPF (reduced partition
199 function ratio) defined by Bigeleisen and Mayer [58] and Urey [59] was evaluated by the same manner
200 with Fujii et al. (2013). In previous studies, various Cu complexes have been computed [51, 56, 57, 60-
201 62]. Here, we present the result computed for Cu histidylhistidine, which is considered to be an analogue
202 of hemocyanin [63] (Table 1). Complexation of Cu(I) with ligands forms a linear structure [56]. The linear
203 structure tends to remain via the oxidation of Cu(I) to Cu(II) [60] and predict that the strength of the bond
204 increases with ionization energy or electronegativity from S to N and O. Thus, Cu binding with histidine,
205 such as in superoxide dismutase, is expected to be isotopically heavy (high $\delta^{65}\text{Cu}$), whereas Cu binding
206 with cysteine, such as in ceruloplasmin, will be isotopically light (low $\delta^{65}\text{Cu}$). The model molecule of Cu
207 histidylhistidine is shown in Figure 3. The Cu atom is surrounded by three N-donors of histidines. A linear
208 structure of N-Cu(I)-N with the angle of $\angle\text{NCuN}=178^\circ$ has been reported by Adam et al. [63], for which
209 our DFT calculation resulted in $\angle\text{NCuN}=162^\circ$. Oxidation of Cu(I) to Cu(II) changes the angle to
210 $\angle\text{NCuN}=173^\circ$, which agreed with the literature value of $\angle\text{NCuN}=171^\circ$ [63].

211 212 **Results**

213 First, the biological standard ERM CE464 has a $\delta^{65}\text{Cu}$ value (0.19 ± 0.03 2SD) which is consistent within
214 error with previously reported values (e.g., 0.11 ± 0.14 2SD, [38]). The Cu isotopic data for Keyhole
215 limpet [H7017], Horseshoe crab [H1757] and Chilean abalone [B8556], are reported in Table 2 and in
216 Figure 2. Hemocyanins for Chilean abalone and Horseshoe crab are similar within error, with $\delta^{65}\text{Cu}$ values
217 of $+0.63 \pm 0.04\text{‰}$ (2SD) and $+0.61 \pm 0.04\text{‰}$ (2SD), respectively. These values are also similar within
218 error with the Cu isotopic composition measured in a Mediterranean octopus hemocyanin by Zhu et al.
219 [47] ($+0.62 \pm 0.05\text{‰}$, 2SD, Figure 2). It is worth noting that this later value is similar to the Mediterranean
220 surface seawater value ($+0.48 \pm 0.11\text{‰}$; [64]) within error. Conversely, the Keyhole limpet hemocyanin
221 gives an isotopically lighter value of $-0.29 \pm 0.06\text{‰}$.

222
223 To estimate the isotopic effect of Cu incorporation into the hemocyanin we have calculated the theoretical
224 isotopic fractionation between hemocyanins (containing Cu(I) or Cu(II)) and seawater (at 277K, see Table
225 3 and Figure 3). Here we have approximated the structure of the hemocyanin by histidyl histidine (Figure
226 1). Our calculations are therefore first order prediction of the isotopic effect, but it has been shown in
227 previous studies that doing such approximations was correct [e.g., 33]. These calculations predict an
228 isotopic fractionation of $\Delta^{65}\text{Cu} \sim +1.5\text{‰}$ between Cu(II) hydrated (the dominant form of Cu in seawater,
229 whereas Cu(I) is non mobile) and the reduced and oxidized forms of hemocyanins at $\sim 4^\circ\text{C}$. The
230 temperature-dependence of the calculations are reported in Figure 4.

231 232 **Discussion**

233 The Cu isotopic composition for surface seawater (<100 m depth) is very variable, ranging between +0.47
234 and $+1.2\text{‰}$ [e.g., 8-10, 24, 64-67] with an average of $\delta^{65}\text{Cu} = +0.49 \pm 0.04\text{‰}$ for the global surface
235 seawater [8] (Figure 2). Thus, it appears that unlike what is predicted by ab initio calculations, there are
236 very limited Cu isotopic fractionation between seawater and hemocyanin ($+0.61$ to $+0.63\text{‰}$) for all
237 animals except the Keyhole limpet (-0.29‰). This result is in agreement with average Holocene and
238 modern coral Cu isotope composition being similar to the mean published deep seawater $\delta^{65}\text{Cu}$ values
239 [10], even though mollusks and arthropods considered in this study live in much shallower waters (up to

240 40 m deep versus 170-2260 m for the corals). This absence of large isotopic fractionation (at the exception
241 of the Keyhole limpet) could also suggest that the ab initio calculations are too approximated, but it would
242 be rather unlikely that these calculations were wrong by $> 1.5 \text{ ‰}$, or more likely that the incorporation of
243 Cu from seawater to hemocyanin is quantitative. In other words that the organisms absorbed most of the
244 Cu available in their environment.

245
246 The lighter isotopic composition of the Keyhole limpet ($\delta^{65}\text{Cu} = -0.29 \pm 0.06\text{‰}$) compared to seawater
247 ($\delta^{65}\text{Cu}$ between $+0.47$ and 1.2‰) (see Figure 2) is consistent with the predicted isotopic fractionation
248 between Cu(II) hydrated and hemocyanins. The different Cu isotopic composition of the Keyhole limpet
249 may either reflect (1) some interspecies variability or (2) that the Cu absorption was not quantitative
250 unlike what is observed in the other animals or (3) that there was an isotopic fractionation processes
251 during sample preparation as the hemocyanins considered in this study were purchased certified standards.
252 These hypotheses could be tested in the future by laboratory control growth of the animals in Cu-rich
253 medium together with systematic measurements of the Cu isotopic composition of the seawater in which
254 the organisms grew up in, in order to better constrain the origin of such interspecies variability.

255 256 **Conclusion**

257 The Cu isotopic composition of the hemocyanin from a Keyhole limpet [H7017], a Horseshoe crab
258 [H1757] and a Chilean abalone [B8556] as well as ab initio calculation for the Cu isotopic fractionation
259 between hemocyanin and hydrated Cu are reported. The Cu isotopic composition of the Horseshoe crab
260 and Chilean abalone were indistinguishable from seawater composition, while ab initio calculation would
261 predict an enrichment in the lighter isotopes in the hemocyanin. This likely reflect quantitative absorption
262 of Cu in the organisms. Conversely, hemocyanin from the Keyhole limpet is enriched in the lighter
263 isotope of Cu compared to seawater, in line with the theoretical prediction. Finally, as these hemocyanin
264 standards are widely available, this dataset could be used for future interlaboratory comparisons, as well as
265 additional studies related to organotropism in marine invertebrates or aquarium-controlled
266 experimentation to elucidate Cu isotope fractionation between environment and hemocyanin. If the
267 conservative transposition of Cu isotope composition in seawater to hemocyanin is confirmed in further
268 studies, it could lead to biomonitoring studies designed to detect anthropogenic Cu emissions.

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510

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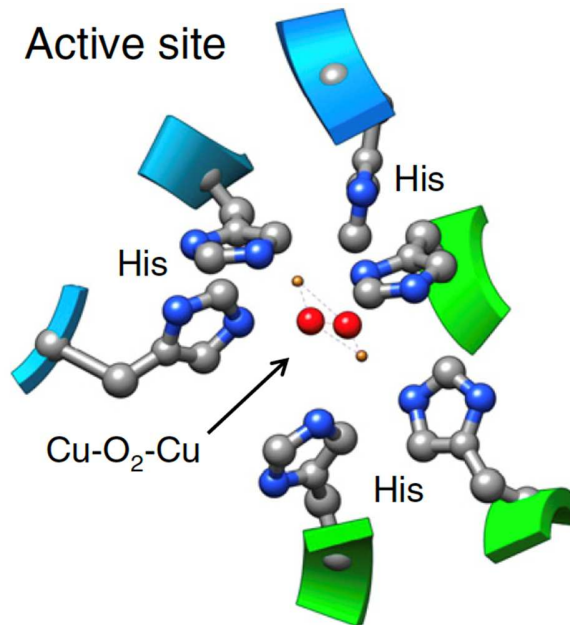
521 **Author contributions**

522 F.M. designed the project. T.F. performed the calculations. M.P. performed the isotopic measurements.
523 M.P wrote the manuscript with inputs from T.F. and F.M.
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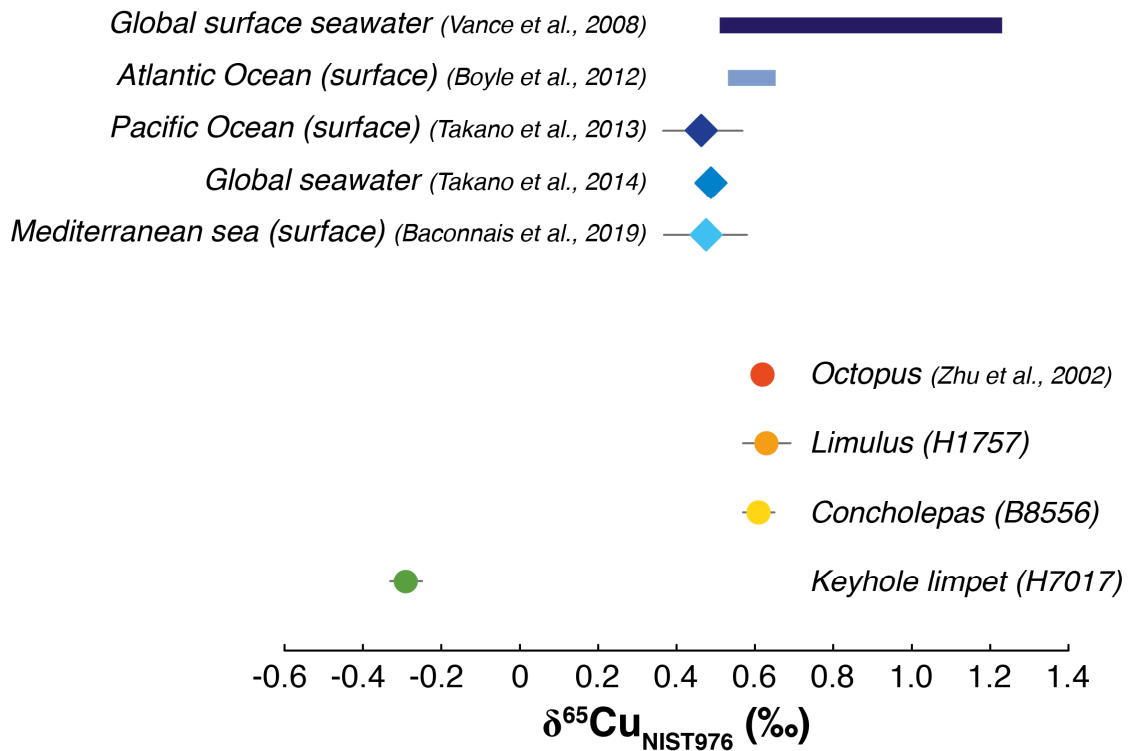
525 526 **Declaration of interests**

527 The authors declare that they have no known competing financial interests or personal relationships that
528 could have appeared to influence the work reported in this paper.
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540 **Figures and figure captions**
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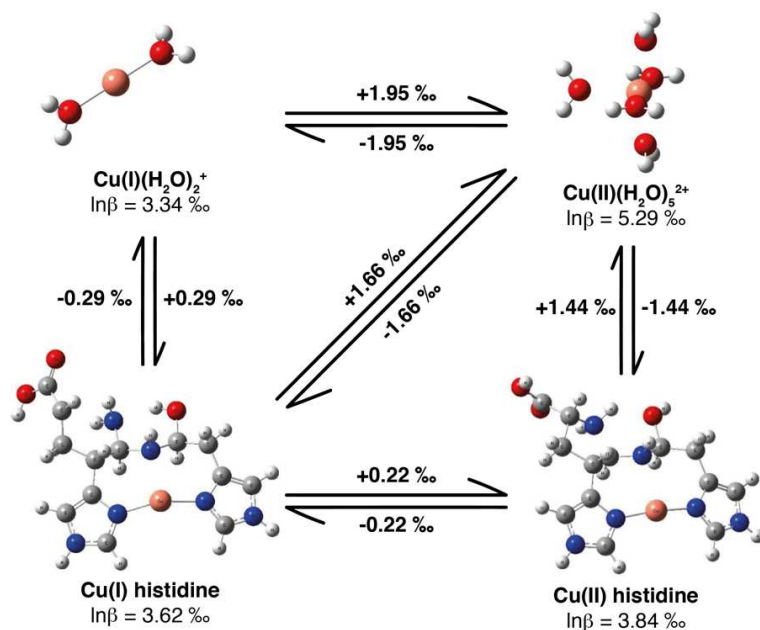


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 544 **Figure 1.** Active site with two copper ions (orange), six histidine residues and a bound dioxygen molecule
 545 (red) [11].
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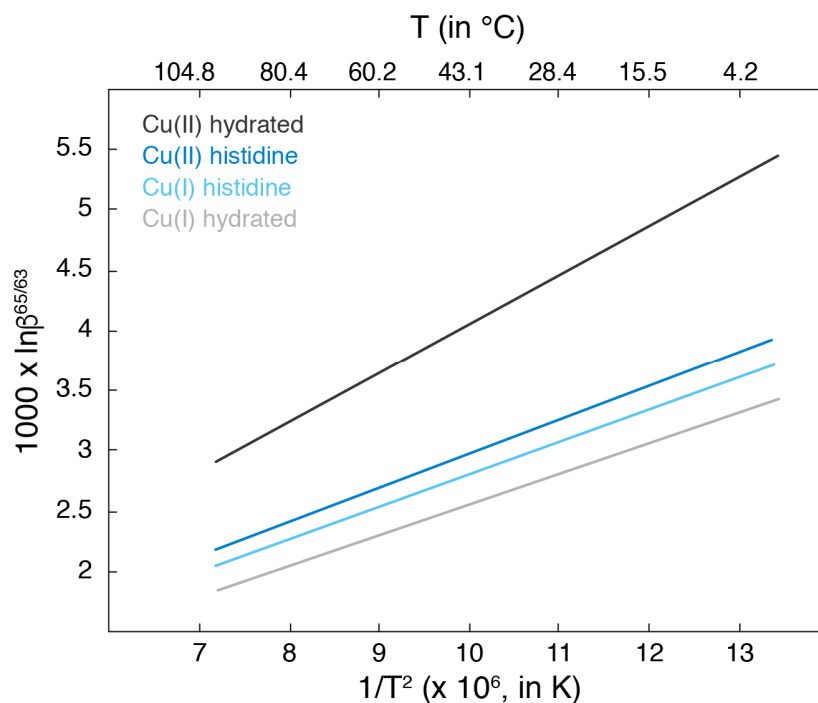
548
 549 **Figure 2.** $\delta^{65}\text{Cu}$ values for hemocyanins and seawater.
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Figure 3. Theoretical isotopic fractionation between different forms of hemocyanins and seawater at 277K.



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Figure 4. Temperature dependence of $\ln\beta$. The $\ln\beta^{65/63}$ values of copper in water and hemocyanin shown as linear functions of T^{-2} .

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Table and table captions

568 **Table 1.** Logarithm of the reduced partition function, $\ln \beta$ (‰), for copper in seawater and hemocyanin.

569 **Table 2.** Copper isotope compositions in hemocyanins.

570 **Table 3.** Theoretical isotopic fractionation between different forms of copper and hemocyanin at 277K.

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574 **Table 1.** Logarithm of the reduced partition function, $\ln \beta$ (‰), for copper in seawater and hemocyanin.

Species	$\ln\beta^{65/63}$ (277K)	$\ln\beta^{65/63}$ (310K)
Cu(I) hydrated	3.34	2.66
Cu(II) hydrated	5.29	4.22
Cu(I) histidine	3.62	2.92
Cu(II) histidine	3.84	3.10

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577 **Table 2.** Copper isotope compositions in hemocyanins.

Sample	ID	Mass (mg)	n	$\delta^{65}\text{Cu}_{\text{CuIPGP}}$			$\delta^{65}\text{Cu}_{\text{SRM976}}$	Reference	
				Mean	2σ	SE			
<i>Concholepas concholepas</i> Chilean abalone	Mollusk	B8556	19.7	12	0.36	0.14	0.04	0.63	This study
<i>Limulus polyphemus</i> Horseshoe crab	Arthropod	H1757	3.3	6	0.33	0.10	0.04	0.61	This study
<i>Megathura crenulate</i> Keyhole limpet	Mollusk	H7017	2.5	10	-0.56	0.19	0.06	-0.29	This study
Octopus	Mollusk							0.62	[47]

σ is the standard deviation; SE is the standard error, which corresponds to $2\sigma/\sqrt{n}$

Relative to NIST SRM 976, the Cu_IPGP standard has $\delta^{65}\text{Cu} = +0.271 \pm 0.006\text{‰}$ (2σ ; $n = 55$) [34].

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580 **Table 3.** Theoretical isotopic fractionation between different forms of copper and hemocyanin at 277K
581 calculated in this study.

Species	$\Delta^{65}\text{Cu}$
Cu(I) hydrated vs Cu(II) hydrated	1.95
Cu(I) histidine vs Cu(I) hydrated	0.29
Cu(I) histidine vs Cu(II) hydrated	1.66
Cu(II) histidine vs Cu(II) hydrated	1.44
Cu(I) histidine vs Cu(II) histidine	0.22

582