

Coordination Studies of the Metal Center of Hemocyanin by $^{199\text{m}}\text{Hg}$ Nuclear Quadrupole Interaction

W. Tröger, B. Ctortekca, P. Faller^a, H. Decker^b, and the ISOLDE Collaboration^c

Nukleare Festkörperphysik, Universität Leipzig, FRG

^a Biochemisches Institut, Universität Zürich, CH

^b Institut für Molekulare Biophysik, Universität Mainz, FRG

^c ISOLDE Collaboration, CERN, Geneva, CH

Reprint requests to Dr. W. T.; E-mail: troeger@physik.uni-leipzig.de

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The nuclear quadrupole interaction of $^{199\text{m}}\text{Hg}$ monitored by time differential perturbed angular correlations of γ -rays was employed to investigate the binding of Hg(II) to the binuclear metal site in the copper protein hemocyanin *Eurypelma californicum*. The data indicate that Hg(II) occupies the two metal sites and alters the metal site geometry from a trigonal to a digonal coordination.

Key words: Hemocyanin; Hg(II) Coordination; Perturbed Angular Correlation of γ -rays.

1. Introduction

Hemocyanins [1, 2] are blue copper proteins serving as oxygen carrier in the blood of arthropods. The reversible binding of oxygen is performed by a binuclear copper center generally referred as a “type 3” copper center (see Fig. 1). The classic type 3 copper center is found in Ascorbate Oxidase in which each Cu is coordinated by three histidines (His) in a trigonal prismatic geometry; in hemocyanin the same ligands are arranged in a trigonal antiprismatic coordination sphere [3]. The histidines are bound via a nitrogen atom to the metal ion. The binuclear metal center in hemocyanins has been investigated by several spectroscopic methods like Raman [4], X-Ray absorption [5] or ultraviolet / visible light absorption [6].

Triggered by our previous investigations of the metal coordination in the type 3 copper center in the blue copper proteins Ascorbate Oxidase and Laccase by the nuclear quadrupole interaction of the probe $^{199\text{m}}\text{Hg}$, monitored by Time Differential Perturbed Angular Correlation (TDPAC) [8], we extended these investigations to hemocyanins (Hc). Arthropod hemocyanins are hexamers. Each subunit contains a binuclear copper site and has a molecular weight of 75 kDa. For our studies, Hc from the

tarantula *Eurypelma californicum* is used. It consists of 4 hexamers, i. e. a “4 × 6” hemocyanin (“24-mer”), which contains 48 copper atoms in 24 binuclear centers [9] (see Fig. 2). Heavy metals influence the function of hemocyanins, but the underlying process is not understood [10 - 13].

2. Experimental

Hemocyanin was isolated and purified from the blood of the tarantula *Eurypelma californicum* by standard procedures. The copperfree hemocyanin, the so-called apo-hemocyanin, was obtained by dialysis. Holo-hemocyanin (the copper containing protein), as well as apo-hemocyanin are quite stable and were stored in TRIS buffer (0.1 M, TRIS/HCl, pH = 8.2) at 5 °C. The dissociation of the 24-mer form in monomeric units was achieved by exchanging the TRIS buffer against a 0.05 M glycine / NaOH solution (pH = 9.6) and a subsequent temperature rise to 52 °C. This temperature was kept for 1 hour. After that the protein was again transferred to the TRIS buffer. The concentration of the 24-mer was 14.6 mg/l.

The $^{199\text{m}}\text{Hg}$ activity was supplied by the on line-isotope separator ISOLDE / CERN at Geneva: $^{199\text{m}}\text{Hg}$ from the radioactive beam was implanted into ice from deionized ultra pure water at ≈ 150 K.

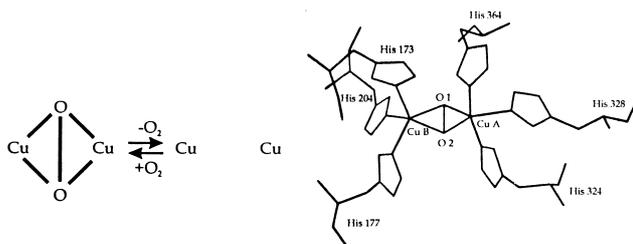


Fig. 1. The biological function and the active binuclear metal site of hemocyanin (adopted from [1]). The binding of dioxygen as a peroxide in a side on coordination leads to a change in valence from Cu(I) to Cu(II) and therefore to a different coordination at the active site [7].

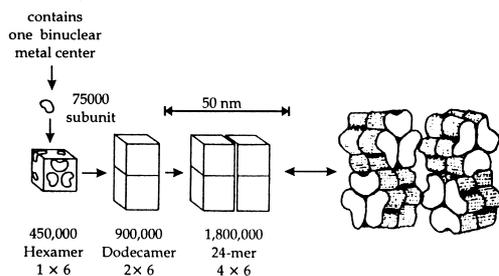


Fig. 2. The principal quaternary structure of the arthropod hemocyanin. The numbers are molecular weight of each unit.

Melting the ice gives a pure aqueous $^{199}\text{mHg(II)}$ solution of 50 to 150 μl , the only metal contaminant being $^{199}\text{Hg(II)}$ in the ground state; this solution is referred to as “no carrier added” or “n.c.a.”.

The proteins were incubated at room temperature with either the n.c.a. $^{199}\text{mHg(II)}$ solution or with stoichiometric amounts of a 0.1 M $^{199}\text{mHg/HgCl}_2$ solution. Typically, 200 μl protein solution and 100 - 150 μl Hg(II) solution were used for the incubation. After the incubation the proteins were immobilized by shock-freezing in liquid nitrogen or by adding sufficient amounts of sucrose to increase the viscosity of the protein solution.

The TDPAC measurements were performed using the high efficiency TDPAC-Camera [14] equipped with BaF_2 scintillation detectors to achieve a sufficient time resolution of 600 ps for (i) the rather short half-life of 2.3 ns of the intermediate state of 375 - 158 keV γ - γ -cascade used for the TDPAC measurements and (ii) the usually very high NQI precession frequencies of the TDPAC probe ^{199}mHg (> 1 Grad/s). The time dependence of the anisotropy was monitored for ≈ 20 ns, i. e. 9 half-lives of the intermediate state, and per TDPAC spectrum 10 to 16 million coinci-

Table 1. The set of NQI 1 and NQI 2 and the additional NQI 3 which allowed to analyze all hemocyanin spectra. ω = NQI precession frequency, η = asymmetry parameter, δ = Lorentzian line broadening, ν_Q = nuclear quadrupole coupling constant, $|V_{zz}|$ = absolute value of the largest component of the EFG tensor.

Signal	ω [Grad/s]	η	δ [%]	ν_Q [GHz]	$ V_{zz} $ [10^{21} V/m 2]
NQI 1	1.35(4)	0.12(5)	4(1)	1.41(5)	86.5(5)
NQI 2	1.66(7)	0.07(9)	4(1)	1.75(8)	107.5(7)
NQI 3	1.27(2)	0.23(4)	1(2)	1.28(3)	78.3(6)

dences were recorded. For a detailed description of the TDPAC technique and data analysis see, e. g. [15].

Ten TDPAC experiments with the following Hg(II) to hemocyanin (Hc) stoichiometries were carried out: seven experiments with apo-hemocyanin $\text{Hg} : \text{Hc} = “0”$ (n.c.a.), 1 (1/48 equivalent Hg for all metal sites), 24 (1/2 equivalent Hg for all metal sites), 48 (1 equivalent Hg for all metal sites), 100 (≈ 2 equivalents Hg for all metal sites); two experiments with holo-hemocyanin: $\text{Hg} : \text{Hc} = “0”$, 48; one experiment with dissociated apo-hemocyanin: $\text{Hg} : \text{Hc} = 48$. The measuring temperatures were 4 $^\circ\text{C}$, -30 $^\circ\text{C}$, -196 $^\circ\text{C}$.

3. Results

With two exceptions, all TDPAC spectra could be analyzed with an identical set of two NQI signals (NQI 1 and NQI 2) which are given in Table 1, these NQIs are equally populated within the error margins.

The first exception in this series of hemocyanin experiments is that with apo-hemocyanin, $\text{Hg} : \text{Hc} = 48$, -30 $^\circ\text{C}$. Here, the NQI 2 is replaced by NQI 3 (see Table 1). Furthermore, this spectrum is dominated by NQI 2 whose amplitude is by a factor of 3 higher than that of NQI 3. The second exception is the experiment with the excess of Hg(II) (apo-hemocyanin, $\text{Hg} : \text{Hc} = 100$, -30 $^\circ\text{C}$). Here, NQI 2 dominates the spectrum and has high line broadening of 13(4)%.

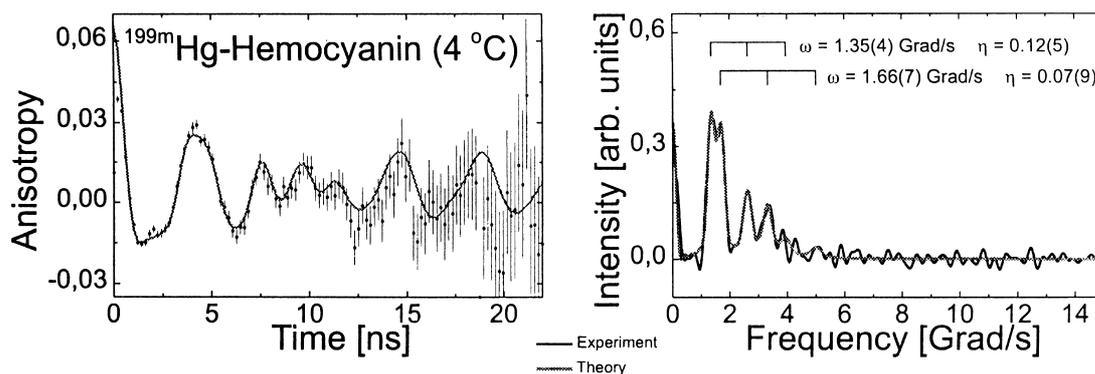


Fig. 3. A $^{199\text{m}}\text{Hg}$ time sum spectrum (left) and its Fourier transform (right) in a highly viscous sucrose solution at 4 °C. The sum spectrum contains the spectra of three $^{199\text{m}}\text{Hg}$ -TDPAC experiments with apo-hemocyanin at Hg : Hc = 1, 24, 48. The NQI parameters precession frequency ω and the asymmetry parameter η of each site are given in the Fourier spectra. Each site has a Lorentzian line broadening of $\approx 4\%$.

In all $^{199\text{m}}\text{Hg}$ -TDPAC spectra of hemocyanin no unbound or unspecifically bound Hg(II) were observed, there were also no significant changes of the signals in the temperature range from 4 °C to -196 °C.

In Fig. 3 a sum spectrum and its Fourier transform of three single TDPAC spectra (apo-hemocyanin, Hg : Hc = 1, 24, 48) are displayed. The Fourier transformed spectrum shows clearly that the main peak between 1 Grad/s and 2 Grad/s consists of two different NQIs. The first harmonics of the two NQIs are also clearly visible, whereas the second harmonics are quite reduced in intensity due to the line broadening and the limited time resolution of the spectrometer.

4. Discussion and Conclusions

The detected NQIs (see Table 1) are completely different from those we found in ascorbate oxidase for the “classic” type 3 metal centers ($\omega = 1.12(1)$ Grad/s, $\eta = 0.83(2)$) or laccase ($\omega = 0.95(1)$ Grad/s, $\eta = 0.66(3)$) [8]. The higher frequencies in Hc together with the significantly lower asymmetry parameter indicate a twofold coordination geometry in contrast to the expected trigonal coordination.

However, ascorbate oxidase and laccase contain also two other metal sites, one of them, the so-called “type 2” site has an almost linear (His)N-Hg-N(His) coordination. The NQI parameters of these sites are: $\omega = 1.35(1)$ Grad/s and $\eta = 0.16(2)$ for laccase; $\omega = 1.44(1)$ Grad/s and $\eta = 0.17(2)$ for ascorbate oxidase [8]. These NQIs agree well with NQI 1 found in hemocyanin. Therefore, NQI 1 can be attributed to a digonal (His)N-Hg-N(His) coordination.

The unusual high frequency of NQI 2 together with the low asymmetry parameter indicates a linear Hg(II) coordination with unusual short bond lengths.

In LAC and AO, the metal ions are coordinated in a trigonal prismatic coordination sphere, whereas hemocyanin exhibits a trigonal antiprismatic metal site coordination with one more distant histidine ligand at each metal site. Since Hg(II) prefers twofold coordination, it is most probable that Hg(II) resides in the two metal sites of the type 3 metal center, but each Hg(II) in a digonal coordination neglecting the third more distant histidine.

The two exceptions mentioned above can be explained as follows: NQI 3 is quite similar to the NQI detected for HgCl₂ [16]. Since HgCl₂ was used as carrier, it might be possible that during the sample preparation a precipitation of the solved HgCl₂ occurred. The excess of Hg(II) together with its strong affinity for digonal coordinations with SH-, NH-, and OH- groups leads to a variety of different linear coordinations resulting in broadened NQIs with high frequencies and low asymmetry parameters. These broadened NQIs can not be separated from NQI 2 due to the limited frequency resolution of the TDPAC probe $^{199\text{m}}\text{Hg}$.

Since the TDPAC experiments with apo- and holo-hemocyanin show the same results, Hg(II) is able to kick out the bound Cu(II) ions in hemocyanin.

Due to the fact that NQI1 and NQI2 are almost equally populated there might also be a “cooperative binding” of Hg(II) to the binuclear metal center in hemocyanin, i. e. in one protein always the two sites have to be empty or occupied by metal ions.

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