Comparison of Hemocyanin from Taiwan Snails with a Synthetic Dicopper(I) Oxygen Carrier

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Abstract

Some properties and reactions of *Achatina fulica* hemocyanin (Hc) from Taiwan snails have been compared with those of a synthetic dicopper oxygen carrier, $Cu_2(EDTB)(ClO_4)_2$, (where EDTB is *N,N,N',N'*-tetrakis(2-benzimidazolylmethyl)-1,2-ethanediamine), serving as an active-site model. The colorless solution of $Cu_2(EDTB)(ClO_4)_2$ in dimethylsulfoxide shows an uptake of oxygen to give a green solution with an electronic spectrum characteristic of Cu(II). Addition of ascorbic acid, AA, results in decolorization of the solution, and the cycle can be repeated 3–4 times. This is similar to the effect of AA on Hc, except that oxyhemocyanin is blue instead of green. The solids of green and colorless forms of the synthetic compound give ESCA Cu($2P_{3/2}$, $2P_{1/2}$) main peaks in the same positions. However, only the green solid spectrum shows 'shake-up' satellites, indicating Cu(II). By treatment with KCN, Cu can be removed from Hc and from $Cu_2(EDTB)(ClO_2)$, and Cu can be reconstituted into both. Properties and reactions of the reconstituted Hc and native Hc are compared.

Introduction

Dicopper coordination compounds with formulas $Cu_2(EDTB)(ClO_4)_2$ and $Cu_2(EDTB)Cl_4$ have been prepared (EDTB=*N,N,N',N'*-tetrakis-(2-benzimidazolylmethyl)-1,2-ethanediamine). Reedijk and coworkers [1] have discussed the importance of $Cu_2(EDTB)^{2+}$ as a potential model for hemocyanin (Hc). The purpose of the research was to investigate the reactions of $Cu_2(EDTB)(ClO_4)_2$ with several ligands and compare these with the reactions of hemocyanin from Taiwan snails (*Achatina fulica* [2]) with the same ligands. The Cu(II) compound was studied as a comparison. By treatment with KCN, Cu can be removed from Hc and $Cu_2(EDTB)(ClO_4)_2$, and Cu can be reconstituted into both. Properties and reactions of the reconstituted Hc and native Hc are compared.

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Experimental

Preparations

EDTB was prepared as described by Hendriks *et al.*, [3] with a final yield of 50%. $Cu_2(EDTB)(ClO_4)_2$ was prepared by mixing under N_2, 20 ml of hot ethanol solution containing 2 mmol EDTB and 2 ml hot acetonitrile solution containing 4 mmol $Cu(CH_3CN)_4ClO_4$ (prepared as described by Lontie *et al.* [4]). The dicopper(I) EDTB compound was filtered off under N_2, washed with cold absolute alcohol and diethyl ether. After drying *in vacuo* at 50 °C, white powders were obtained, which were not air-sensitive. The copper content was 13.9%, which agrees with the calculated value, 14.0%, in the formula $Cu_2C_{34}H_{32}N_{10}(ClO_4)_2$. Elemental analyses of C, H, N for the compound have been published [1b].

Dicopper(II) coordination compound of EDTB was prepared by reacting with $CuCl_2$ using Cu:EDTB ratio of 2:1. A solution of EDTB in hot ethanol was added to an ethanol solution of $CuCl_2$. After reflux for 30 min, crystals of the coordination compound were formed. These were filtered, washed with ethanol and diethyether, and then dried *in vacuo* at 60 °C. The yellow compound obtained was $Cu_2(EDTB)Cl_4$. The copper content was 15.3% (calc., 15.0%).

Copper can be removed from $Cu_2(EDTB)(ClO_4)_2$ by adding KCN/H_2O dropwise to a $(CH_3)_2SO$ solution of the compound until the color changed to slight brownish-yellow. Addition of water resulted in a white turbid suspension. The aqueous cuprous cyanide complex formed was filtered off and the precipitate, EDTB, was dried and dissolved in hot ethanol. By treating the alcohol solution with a 2.5-fold excess of $Cu(CH_3CN)_4ClO_4$ in hot acetonitrile in a N_2 atmosphere, we obtained $Cu_2(EDTB)(ClO_4)_2$ again. The white powder was filtered off, washed with ethanol and vacuum dried at 60 °C.

Hemocyanin from Taiwan snails was prepared and purified in the manner previously described [2]. Cu can be removed from Hc by KCN treatment [4]. For the reconstitution of Hc, the Cu-free solution was treated at pH 5.7 for 24 h at 4 °C in N_2 atmosphere with an amount of solid $Cu(CH_3CN)_4ClO_4$

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corresponding to twice the copper concentration of Hc. The reconstituted hemocyanin (R-Hc) was dialyzed against 0.025 M EDTA in 0.1 M acetate buffer, pH 5.7 in N_2 atmosphere and then against the acetate buffer alone.

The native Hc has a Cu content of 0.24%. After reconstitution, a Cu content of 0.21% was found, corresponding to a reconstitution factor of 87.5%.

Physical Measurements

NMR spectra were obtained on a Varian EM-390 spectrometer. UV–Vis spectra were obtained on a Perkin-Elmer Lambda 5 spectrophotometer. The metal contents were determined by neutron activation analysis, using the University reactor. The electron spectrum for chemical analysis (ESCA) was obtained using a Perkin-Elmer 560 spectrometer using Mg Kα radiation. For ESCA experiments, solid samples were fixed on scotch tape, maintained at 22 °C during analysis, and the vacuum in the sample chamber was 1.9×10^{-10} torr. The energy of the exciting X-rays was 1253.6 eV. X-ray source of 10 kV and 20 mA was used. For $Cu_2(EDTB)(ClO_4)_2$, data of 160 scans, while for $Cu_2(EDTB)Cl_4$, 90 scans were collected. ESR experiments were carried out using a Bruker ER 200D spectrometer operating at 9.1 GHz at room temperature.

Results and Discussion

Hendriks *et al.* [1a] previously reported that the colorless solution of $Cu_2(EDTB)(ClO_4)_2$ in Me_2SO showed an uptake of dioxygen of 0.96 mol/Cu_2, resulting in a green solution with maximum absorption at 690 nm, characteristic of Cu(II). These data suggest the formation of a Cu–O_2–Cu species, as is also found in oxyhemocyanin. The addition of small amount of ascorbic acid (AA) to 0.005 M $Cu_2(EDTB)(ClO_4)_2$ in Me_2SO after exposure to air (green solution), results in decreased absorbance at 690 nm, and subsequent bubbling with O_2 raises the absorbance. The cycle can be repeated 3–4 times. After 3–4 cycles, further addition of small amount of AA results in smaller decrease of absorbance at 690 nm. Addition of sufficient ascorbic acid to the green solution results in a complete decolorization of the solution. An absorption maximum of *Achatina fulica* oxyhemocyanin in aqueous medium occurs at 345 nm and the effect of adding AA in decreasing absorbance is the same. Lowering of A_{690} also occurs on adding KCN to 0.0065 M $Cu_2(EDTB)(ClO_4)_2$ in Me_2SO, which had been exposed to air (green solution). However, no increase in absorbance occurs after adding oxygen (see Fig. 1). Chen *et al.* [2] have shown that addition of KCN (up to 1 mM) to *Achatina fulica* oxyhemocyanin causes a drop in absorbance at 345 nm, due to reduction of Cu(II) to Cu(I), with the copper still bound to the protein. Above 1 mM, copper begins to be removed from the protein, forming aqueous cuprous cyanide complexes. The effects of adding ascorbic acid and KCN to the green dicopper EDTB compound and to oxyhemocyanin in decreasing absorbances, are quite similar, except for differences in the absorption maxima. Experimental evidence for the reaction of CN^- with Hc has previously been given by Salvoato *et al.* [5, 6] which indicates the occurrence of two processes: the loss of color due to formation of a Hc–CN complex and its decomposition which leads to Cu displacement. The same processes apparently occur in the reaction of CN^- with the dicopper EDTB compound. Evidence for strong binding of CN^- to Hc has also been given by Borke *et al.* [7] from oxygen-binding and ion-exchange experiments.

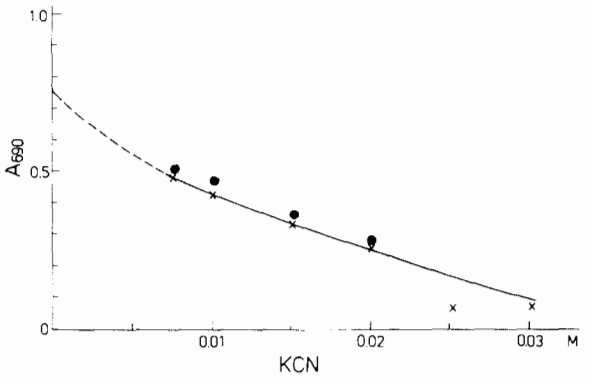


Fig. 1. Decrease in absorbance at 690 nm of 0.0065 M $Cu_2(EDTB)(ClO_4)_2$ (green solution in $(CH_3)_2SO$) after adding KCN (×), bubbling O_2 after adding KCN (•). The dashed line extrapolates to absorbance at 690 nm before adding KCN.

Figure 2 shows that the solution of $Cu_2(EDTB)(ClO_4)_2$ in Me_2SO, after exposure to air (green solution) gives distinct ESR signals. The spectrum of the green oxygenated complex has the appearance of a doublet signal in the g-perpendicular region suggestive of some triplet character, *i.e.* some Cu(II)···Cu(II) interaction. One day after adding KCN (0.01 M), only one broad ESR signal remains. One day after adding KCN (0.04 M), the solution becomes completely ESR-silent, which suggests that paramagnetic Cu(II) has been converted to diamagnetic Cu(I) for both coppers. Oxyhemocyanin is ESR-silent because there is strong antiferromagnetic coupling between the two copper(II) ions with a separation close to 3.6 Å [8, 9]. We have found that when KCN is added to *Achatina fulica* oxyhemocyanin (CN^- concentration from 10^{-5} M to 0.02 M), the solution is always ESR-silent. Both coppers in oxyhemocyanin are accessible to CN^-.

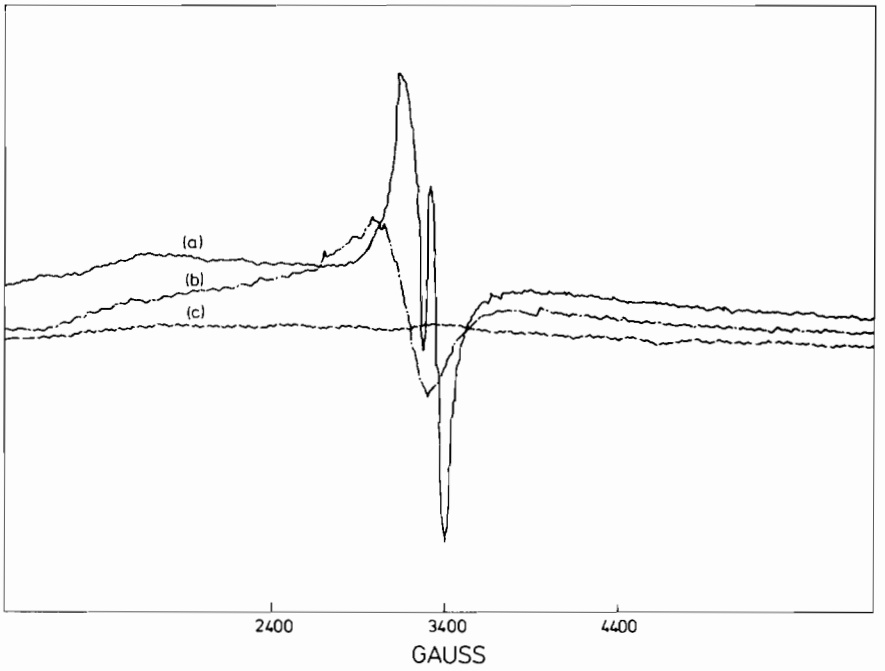


Fig. 2. ESR spectra, obtained at 9.1 GHz and room temperature. (a) $Cu_2(EDTB)(ClO_4)_2$ (0.007 M) in $(CH_3)_2SO$, green solution; (b) as in (a) and 0.01 M KCN, after 1 day; (c) as in (a) and 0.04 M KCN, after 1 day.

Our data are in agreement with Salvato's model [5] for Hc–CN^- interaction: $Hc + CN^- = HcCN$; $HcCN + CN^- = Hc^0 + Cu(CN)_2^-$; $Hc^0 + CN^- = Hc^0CN$; $Hc^0CN + CN^- = Hc^{00} + Cu(CN)_2^-$ where Hc^0 and Hc^{00} represent Hc with one copper and both coppers removed, respectively.

Figure 3a shows the Cu($2P_{3/2}$, $2P_{1/2}$) part of the ESCA spectrum of colorless solid $Cu_2(EDTB)(ClO_4)_2$ with binding energies at 934.2 and 954.2 eV. The bands for Cl 2p, C 1s, N 1s and O 1s occurred at 206.8, 284.0, 399.8, and 532.8 eV, respectively. When this colorless dicopper(I) coordination compound was suspended in a small amount of absolute ethanol and oxygen was bubbled through, the color changed to green. After filtration, the green solid was dried at 70 °C for 6h. Figure 3b gives the Cu-($2P_{3/2}$, $2P_{1/2}$) part of the ESCA spectrum of the green compound, with binding energies at 934.6 and 954.4 eV as the main $2P_{3/2}$ and $2P_{1/2}$ peaks. In addition, there are shake-up satellites at around 944.8 and 964. 2 eV (higher binding energies than the corresponding main peaks). Since the shake-up satellites usually occur with paramagnetic states, Fig. 3b suggests that part of the Cu in the green compound has been oxidized to the cupric state. This is confirmed by comparison with Fig. 3c, yellow solid $Cu_2(EDTB)Cl_4$. In this compound, both coppers are completely cupric and all the peaks (main peaks at 935.2 and 955.2 eV for $2P_{3/2}$, $2P_{1/2}$, as well as the shake-up satellites) have larger binding energies than the green solid shown in Fig. 3b. In the complete ESCA spectrum (0–1000 eV) of Cu_2-$(EDTB)Cl_4$ the O 1s peak is absent, which indicates the cupric compound does not absorb oxygen.

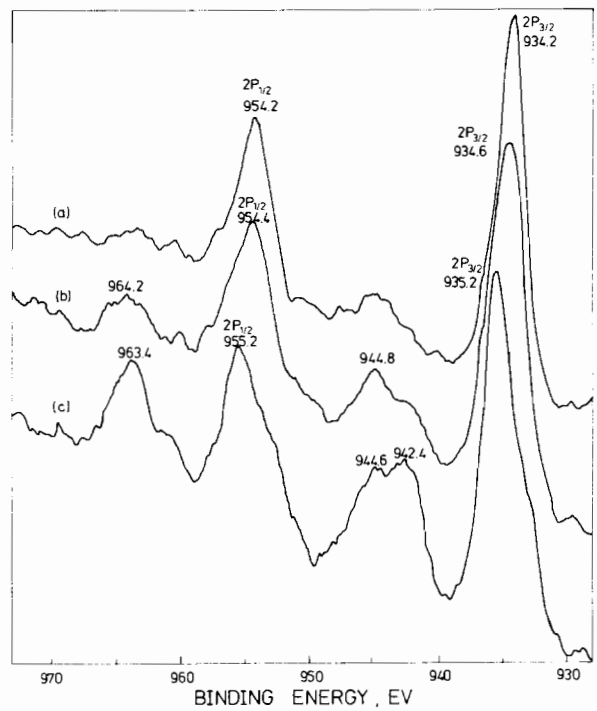
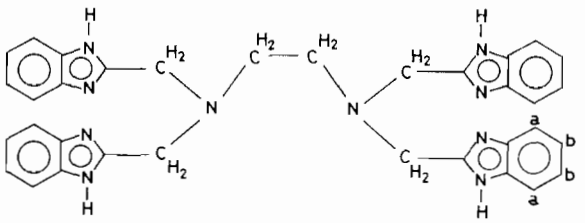


Fig. 3. ESCA spectra of solids: (a) colorless $Cu_2(EDTB)$-$(ClO_4)_2$, (b) green form of $Cu_2(EDTB)(ClO_4)_2$, (c) Cu_2-$(EDTB)Cl_4$.

Van der Deen *et al.* [10] reported that the binding energies of the $2P_{3/2}$, $2P_{1/2}$, and $3P_{3/2}$ levels of Cu in *Helix pomatia* Hc are 931, 951, and 73 eV, respectively. Their ESCA data suggested that Cu in oxyhemocyanin is cupric, because the Cu peak values were nearly the same as for Cu in superoxide dismutase [10]. These authors hoped that a ESCA study of binuclear Cu(I) and Cu(II) complexes would give additional proof for this suggestion. Our present ESCA results show that the Cu peaks of the dicopper compounds of EDTB are nearly the same as for Cu in Hc, and show that in changing a compound from Cu(I) to partly Cu(II), no drastic effect at the peak positions of Cu occurs. The 'shake-up' satellites are of help in determining the state of copper. Since the sensitivity of the spectrometer is not high enough to detect the satellites in the protein, this constitutes one example of how a study of model compound can be of help in unravelling the state of copper in the active-site in a protein.



The ^1H NMR spectrum of EDTB in $(CD_3)_2SO$ is identical with that previously reported [3], multiplicity, δ ppm (relative intensity: m, 7.5(8); m, 7.1(8); s, 4.06(8); s, 2.94(4). A colorless solution of $Cu_2(EDTB)(ClO_4)_2$ in $(CD_3)_2SO$ develops a green color in 30 min, and the multiplet signal at 7.5 ppm (ascribed to protons 'a' in EDTB) becomes greatly broadened. The coppers bind to EDTB through the nitrogens of benzimidazole (from crystal structure studies [1]), so that protons 'a' are closer to copper than protons 'b' and the signal at 7.5 ppm becomes preferentially broadened when cuprous changes to paramagnetic cupric. Starting with a green solution of $Cu_2(EDTB)(ClO_4)_2$, (all EDTB signal are broadened because the coppers are partly cupric), addition of ascorbic acid reduces cupric to diamagnetic cuprous and the signal of protons 'b' at 7.1 ppm begins to appear first. This illustrates another advantage of studying small model compounds for the active site, because in the change between deoxyhemocyanin and oxyhemocyanin, no difference in NMR spectra can be detected. The high molecular weights of the aggregates in hemocyanins preclude NMR spectroscopy on the protein itself.

By treatment with KCN, Cu can be removed from Hc and $Cu_2(EDTB)(ClO_4)_2$, and Cu can be reincorporated by treatment with $Cu(CH_3CN)_4ClO_4$. Both Hc and the dicopper compound have the same general properties before and after treatments with KCN and $Cu(CH_3CN)_4ClO_4$. Figure 4 shows the effects of adding ascorbic acid and subsequent bubbling with oxygen on absorbance of a green solution of reconstituted $Cu_2(EDTB)(ClO_4)_2$. The same general effects of ascorbic acid and of KCN on the original dicopper(I) compound, Hc, and reconstituted Hc have been noted, except for the difference in λ_{max} (690 nm for $Cu_2(EDTB)(ClO_4)_2$ and 345 nm for Hc). The 345 nm band of oxyhemocyanin corresponds to a charge transfer (CT) from oxygen to copper. This CT band is not evident in the model complex because of intense absorption throughout the entire 250–400 nm region.

With oxyhemocyanin it is generally accepted that the coppers are Cu(II), with the bound dioxygen as peroxide. The latter form of dioxygen has been demonstrated from resonance Raman spectroscopy. For *Busycon* and *Cancer magister* oxyhemocyanins, there are Raman peaks at 749 and 744 cm^{-1}, respectively, ascribed to O_2^{2-} vibration [11]. In our laboratory, Chen *et al.* [2] found the O_2^{2-} vibration for *A. fulica* oxyhemocyanin to be 752 cm^{-1}, which is quite close to the value reported for *Busycon* oxyhemocyanin. We plan to carry out resonance Raman studies of the dicopper compound in the presence and absence of dioxygen, in the hope that a dioxygen complex can be demonstrated as has already been done with oxyhemocyanin.

Native oxy Hc is ESR-silent, so is the oxy form of R-Hc. There are however, significant differences in the rate of KCN reaction with the two species of

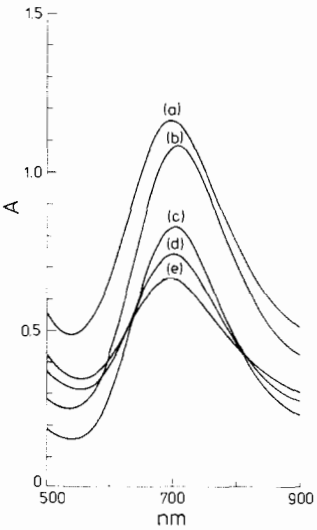


Fig. 4. Visible absorption spectra of the dicopper (EDTB) compound, prepared from treating $Cu_2(EDTB)(ClO_4)_2$ with KCN and subsequently $Cu(CH_3CN)_4ClO_4$ (see text). (a) 0.01 M oxy form (green), (b) after bubbling O_2 through (c), (c) 2 ml (a) + 0.01 g ascorbic acid, (d) after bubbling O_2 through (e), (e) 2 ml (b) + 0.01 g ascorbic acid.

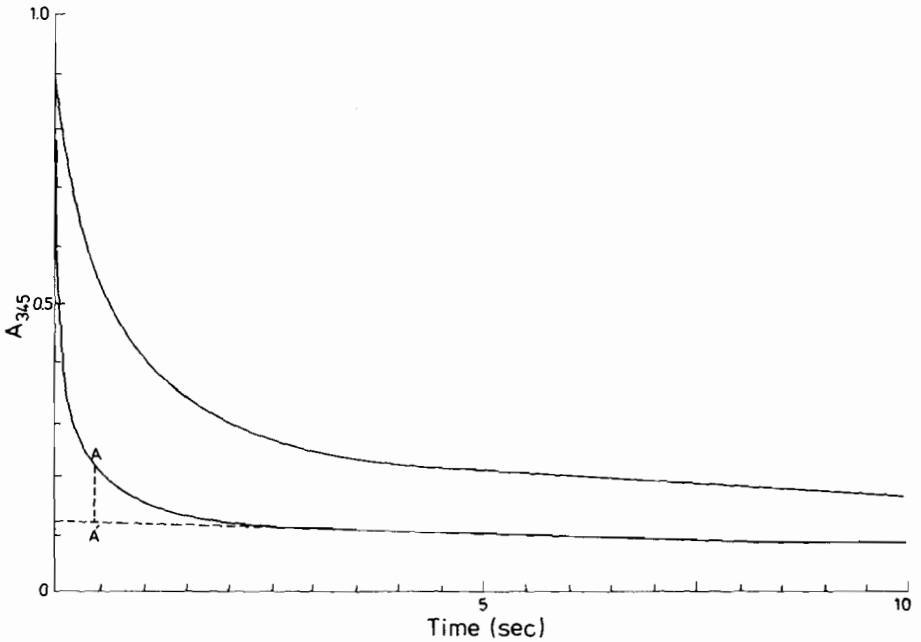


Fig. 5. Decrease in absorbance at 345 nm *vs.* time for KCN added to buffered solutions of oxyhemocyanin at pH 5.7 at 25 °C: top curve, R-Hc; bottom curve, native Hc. For both, Hc conc. 1×10^{-5} M, KCN conc. 0.08 M, data from ref. 12. A and A' are defined as drawn.

A. fulica Hc. Addition of KCN to oxyhemocyanin causes a decrease in absorbance around 345 nm. The loss of absorbance occurs in two stages: a rapid primary reaction followed by a much slower secondary reaction shown graphically in Fig. 5. Linear plots of $-\ln(A-A')$ *vs.* time (for definition of A and A', see Fig. 5) were obtained for the primary reactions of KCN with native and reconstituted hemocyanins. For a solution containing 0.14 M KCN, pH 5.7 (predominant cyanide form, HCN), the rate constants for the primary reactions with native and reconstituted hemocyanins (both 1.0×10^{-5} M) at 25 °C are 3.6 s^{-1} and 0.4 s^{-1}, respectively (stopped-flow measurements [2]). This represents a 10-fold difference in rates, and may be ascribed in part to a difference in copper affinities to the protein ligands in the active-sites of native and reconstituted hemocyanins. Moreover, prior to preparing the latter species, KCN had been used in preparing the copper-free hemocyanin. Douglas *et al.* [13], working with *Busycon* hemocyanin, had reported that cyanide causes extensive fragmentation, which is a subunit dissociation phenomenon and this fragmentation of Hc may lead to a different degree of Hc–CN^- interaction. We have previously reported [2] for *A. fulica* Hc, that in the presence of 0.001 M KCN it was not possible to obtain a Hc-pellet even after ultracentrifuging at 100 000 g for more than 3 h, whereas for native Hc, a pellet was obtained after ultracentrifuging under the same condition for 2 h. This means that the KCN treatment must have drastically reduced the molecular weight of the protein.

There are other differences in the extent of reaction between ligands and the two species of hemocyanin. For instance, fluorescence enhancements on addition of Tb^{3+} to the proteins (excitation 295 nm, emission 543 nm) are in the order: reconstituted hemocyanin(R–HcO_2) > native HcO_2. Also, there are differences in oxygen affinities as measured by P_{50} (partial pressure of O_2 required to half saturate the protein) for reconstituted and native hemocyanins. Differences between native Hc and R-Hc deserve further study and are under active investigation in this laboratory.

Acknowledgements

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