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Does cobalt really bind tightly to hemocyanin active sites?

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Abstract. The analysis of Co(II)-apoHc complexes of two arthropodan species (freshwater crayfish): *Orconectes limosus* and *Astacus astacus* enabled to reach some conclusions about possible cobalt binding sites in the hemocyanin molecules. The occurrence of binding sites for Co(II) at sites other than the active center has been demonstrated. We excluded the possibility of strong binding of EDTA-non-removable cobalt ions in the binding sites occupied by copper. There were no differences between apoHc and the Co(II)apoHc complex in terms of the amount of bound Cu(I) ions and the kinetics of Cu(I) ion reconstitution.

Key words: Hemocyanin – Cobalt binding – Active site

Introduction

The investigation of the spectroscopic properties of hemocyanin active sites substituted with an inorganic anion for oxygen is a widely used method for the study of the structure of oxygen binding sites in hemocyanin (Hc) (Himmelwright et al. 1980; Gondko et al. 1985). Attempts to introduce Co(II) ions into the copper binding sites have been reported recently (Suzuki et al. 1982a, b; Witters and Lontie 1983; Salvato et al. 1986; Lorosch and Haase 1986). The values reported for the strength of the binding and the number of binding sites for Co(II) vary considerably. This metal seems to be a good substitute for copper at the active site, as shown by the characteristic spectroscopic properties (strongly influenced by the geometry of complex) and by the existence of a number of synthetic complexes that transport oxygen (Jones et al. 1979).

It was also found that the protein moiety of Hc can bind other metal ions at sites other than the active site. The hemocyanins or marine crustaceans are thought to accumulate trace metals and, as has been shown in the case of Hg(II) and Cd(II), to exhibit considerable affinity in their binding (Brouwer and Engel 1982; Belleli et al. 1985).

The aim of this study was to investigate the binding of cobalt ions to hemocyanins of fresh water crayfish *Orconectes limosus* and *Astacus astacus*, and, first of all, to check their ability to bind Co(II) ions in the active sites. The effect of cobalt bound to apoHc on the incorporation of copper into the active site of the hemocyanin was also studied.

Experimental

Hemocyanins were obtained from hemolymph of crayfish *Orconectes limosus* and *Astacus astacus*. After removing the clot, the hemolymph was centrifuged (150 000 × *g*, 4 h) and the sedimented Hc was purified on Ultrogel AcA 22 (LKB, France) (Gondko et al. 1985). Next, the Hc solution was dialyzed against 20 m*M* EDTA (pH 7.7, 0.1 *M* *Tris*-HCl buffer) to remove weakly bound metals. Apohemocyanin (apoHc) was obtained by overnight dialysis of oxyHc solution (pH 10, 0.1 *M* *Tris*-HCl) against 10 m*M* NaCN. The copper content in such an apoHc did not exceed 3.5% in relation to the initial content in oxyHc.

As some cobalt binding sites with different binding constant are likely to be present in the protein, Hc solutions were dialyzed for two days against complexed or free Co(II) ions. The Co(*His*)$_2$ complex was used in 50 m*M* *Tris*-HCl buffer, pH 7.5. In order to prevent free Co(II) ions from being oxidized to Co(III) in *Tris*-HCl buffer, the dialysis was carried out in an atmosphere of argon. Cobalt concentrations used were 2- to 5-fold greater than the concentration of copper required for complete saturation of copper binding sites in the active site of the oxyHc form. Because of turbidity of apoHc

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Abbreviations: Hc, hemocyanin; apoHc, apohemocyanin; oxyHc, oxyhemocyanin; Co-Hc, hemocyanin complex with cobalt ions

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samples dialysed against a buffer containing cobalt ions, in particular against free Co(II) ions, the samples were subsequently dialysed 2–3 times against buffer, until they became completely transparent. As an indicator of specific binding of cobalt to protein we took the resistance of cobalt ions to removal by dialysis against EDTA.

In order to determine the ability of Co(II) to influence the reaction of Cu(I) with apoHc, cobalt treated apoHc (Co-apoHc) was dialyzed against a buffer containing Cu(I) $(CH_3CN)_4ClO_4$ (Konings et al. 1969), as well as 1 *M* NaCl and 0.05% Tween 80, added to prevent protein precipitation (Salvato et al. 1986). To stop the reaction of Cu(I) with apoHc or Co-apoHc, we raised the pH to 9 and and added 20 m*M* EDTA (1 : 1 v/v) to the solution. The solution of hemocyanin was dialyzed repeatedly against 0.1 *M* *Tris*-HCl, pH 7.7 and then clarified by centrifugation (20 000 × *g*, 20 min).

For each sample of apoHc treated with Co(II) the value of the ratio $[Co]/A_{280}$ was determined and compared with the value of the ratio $([Cu]/A_{280})^{oxy}$ for the oxyHc form. On this basis the percentage of bound cobalt in relation to the total content of copper bound in oxyHc was calculated. The amount of the oxyHc form was measured by comparing the values of the ratio (A_{340}/A_{280}) in a given sample and the ratio $(A_{340}/A_{280})^{oxy}$ relevant to the oxyHc form, taking into account the residual absorption at 340 nm originating from broad absorption band at 280 nm (the presence of 340 nm band is characteristic only of the oxyHc form).

The initial oxyHc concentration was from 1 to 2 m*M* in respect to copper.

Absorption measurements were carried out on an SP 1700 spectrophotometer (Pye Unicam, England), cobalt and copper contents having were determined using an atomic absorption spectometer AAS 1 (Carl Zeiss Jena, GDR).

Results

The dialysis of apoHc solution, conducted in an atmosphere of argon, against a buffer containing Co(II) ions, followed by the removal of excess cobalt ions, caused a change of the apoHc color to pale pink, this color originates from bound cobalt ions. The difference spectrum of apoHc (of both species) after dialysis against a Co(II) containing buffer versus free apoHc revealed a broad absorption band of very low intensity in the range 550–650 nm and similar alterations were found in oxyHc. The amount of bound cobalt ions reached about 250% relative to the number of copper binding sites in the case of Hc of *Orconectes limosus*, and up to 430% in Hc of *Astacus astacus* (Table 1). Dialysis of the Co-apoHc form against EDTA caused the amount of cobalt bound with apoHc and not complexed by EDTA to be reduced to 49% and 68%, respectively (Table 1).

To find out whether the bound cobalt ions inhibit the rebinding of copper, which would confirm that they can be incorporated in copper binding sites, the same solution was dialyzed against the monovalent copper complex: $Cu(CH_3CN)_4ClO_4$. It was found that Co-apoHc binds more than 90% of the initial copper content, in spite of the fact that cobalt was previously bound. The cobalt content decreased drastically and the final content of the oxyHc form was above 30% (Table 1). Analogous measurements were carried out for apoHc dialyzed against Co(II) in complexes with histidine (Table 2). In this case the amount of bound cobalt ions, non-removable by dialysis against EDTA, was 46% in *Orconectes limosus* Hc and 54% in *Astacus astacus* Hc. Hemocyanin reconstitution by means of Cu(I) was about 96% efficient and the oxyHc content after copper binding was above 30%.

The measurements of the amount of cobalt bound to oxyHc were carried out in parallel using the same proce-

Table 1. The amount of copper and cobalt bound to apoHc and oxyHc after dialysis against Co(II) ions and following subsequent treatment with EDTA and $Cu(CH_3CN)_4ClO_4$ in relation to the initial copper content in oxyHc

Hc form	Metal	Content after treatment		
		Co(II) in Ar	EDTA	Cu(I) complex
Orconectes limosus				
apoHc	Co	250%	49%	8%
	Cu	2.8%	2.7%	96% (35%)[a]
oxyHc	Co	160%	43%	–
	Cu	100%	99%	–
Astacus astacus				
apoHc	Co	430%	68%	6%
	Cu	3.2%	3.0%	93% (33%)[a]
oxyHc	Co	320%	63%	–
	Cu	98%	98%	–

[a] The content of oxyHc form is given in parentheses

Table 2. The amount of copper and cobalt bound to apoHc and oxyHc after dialysis against Co(*His*)$_2$ complex and following subsequent treatment with EDTA and $Cu(CH_3CN)_4ClO_4$ in relation to the initial copper content in oxyHc

Hc form	Metal	Content after treatment		
		Co-*His* complex	EDTA	Cu(I) complex
Orconectes limosus				
apoHc	Co	110%	46%	10%
	Cu	2.9%	2.5%	95% (32%)[a]
oxyHc	Co	105%	44%	–
	Cu	99%	98%	–
Astacus astacus				
apoHc	Co	130%	54%	5%
	Cu	3.4%	3.1%	97% (34%)
oxyHc	Co	140%	57%	–
	Cu	98%	96%	–

[a] The content of oxyHc form is given in parentheses

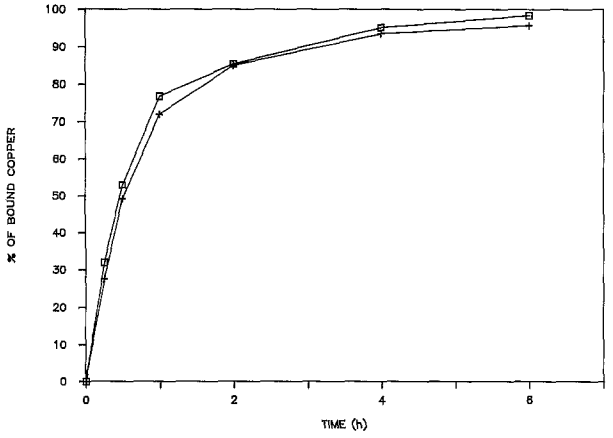

Fig. 1. Kinetics of Cu(I) binding to apoHc (□) and Co-apoHc (+) of *Astacus astacus*

dure. The results are shown in Tables 1 and 2. In the case of both free Co(II) ions and cobalt complexed in Co(*His*)$_2$, the amount of cobalt, non-removable by dialysis against EDTA, was similar to that in apoHc.

Because of lack of inhibition of copper incorporation into Co-apoHc active sites, we carried out comparative studies of the kinetics of Cu(I) binding to apoHc and Co-apoHc. If the binding of EDTA-resistant cobalt ions by the active center of hemocyanin does, in fact, occur one might expect considerable differences in the rate of binding of Cu(I) ions to apoHc and Co-apoHc. This was not found to be the case, the reaction half time was estimated to be equal at about half an hour (Fig. 1).

The investigation of hemocyanins of these two species of arthropods has revealed the presence of three components with very different extents of aggregation (Gondko and Michalak 1981). Using analytical ultracentrifugation we failed to detect any differences in component proportions in oxyHc, apoHc and EDTA-treated Co-apoHc. However, considerable aggregation, appearing as an increase in turbidity, was found when dialyzing Hc against a buffer containing free Co(II) ions.

None of the cobalt-Hc forms obtained showed ESR signals at 123 K, typical for low sin cobalt complexes binding oxygen (Jones et al. 1979).

Discussion

The complex of cobalt(II) ions with hemocyanin of squid *Sepioteuthis lessoniana* was reported by Suzuki et al. (1982a). They found that the amount of Co(II) bound to apoHc was 1.3-fold higher than the number of copper binding sites. After treatment by Chelex resin the amount of bound cobalt dropped to about 50%. The authors suggested binding of one Co(II) ion per Hc active center. Similar investigations were carried out with four species of horseshoe crab. The amount of cobalt(II), resistant to removal by Chelex, was 85–100% of total amount of copper binding sites in oxyHc (Suzuki et al. 1982b). In both cases, inhibition of copper rebinding was found and after the reconstitution the content of oxyHc reached about 20%.

These results differ from those reported earlier by Witters and Lontie (1983), who found the binding of 49% of EDTA-resistant cobalt with hemocyanins of mollusks and arthropods and suggested the incorporation of one cobalt(II) ion for every two active centers. The Hc cobalt form obtained showed only a high spin ESR Co(II) spectrum at 4.8 K.

Salvato and co-workers have reported the binding of EDTA-resistant cobalt ions to the native Hc of *Carcinus maenas* at the ratio of 1 Co : 2 Cu, suggesting the incorporation of the cobalt ions into the copper binding sites. As in this study the resulting complex lacked the low spin Co(II) ESR signal (Salvato et al. 1986).

The results differ from the data of cobalt binding to hemocyanin of *Limulus polyphemus* reported recently by Lorosch and Haase (1986). These authors suggest that free Co(II) ions bind to the protein moiety at non-copper binding sites at the [metal]/[protein] ratio of 4.5, and also find binding of one cobalt ion to every copper binding site of apoHc, easily removable by ion exchangers.

Our results on the binding of cobalt ions to various forms of hemocyanin clearly point to the ability of cobalt ions to bind to the protein moiety of both oxyHc and apoHc. Since the amount of apoHc-bound with the respect to oxyHc-bound cobalt ions varies by about 90–110% in terms of Cu sites (Table 1), this indicates that a portion of cobalt ions bound to apoHc is most likely located in the sites occupied by copper in oxyHc. This peculiarity is common as well to hemocyanins of *Limulus polyphemus* and *Sephioteutis lessoniana*. The majority of the ions bound are removable by either Chelex 100 resin (Suzuki et al. 1982a, Lorosch and Haase, 1986) or EDTA (Tables 1 and 2). The EDTA-resistant Hc-bound cobalt in the two species examined amounts about 43–68%, not only in apoHc but also in oxyHc, the active centers of which are completely saturated with Cu(II) ions. This value is approximately equal to a cobalt/copper ratio of 0.5.

The ability of oxyHc to bind other metals has been reported. Brouwer and Engel (1982), studying the association of sea water trace metals with hemocyanins of numerous marine arthropods, have found that up to 17 or 7 Hg(II) ions can be bound per subunit of Hc of *Callinectes sapidus* and *Limulus polyphemus*, respectively. The sites of ion binding exhibited distinctly marked variability as to binding constants and a single high-affinity site of binding, predominantly Cd and Hg, was found compared with two Cu sites.

Belleli et al. (1985), investigating the effect of cadmium, mercury and chromium on the structural and functional properties of *Palinurus interruptus* oxyHc, found that the amount of EDTA-non-removable metal was, counted for two copper binding sites, 4, 1 and 9 for mercury, cadmium and chromium, respectively.

For both Hc forms (apoHc and oxyHc) the amount of bound Co non-removable by EDTA was almost the same (Table 1 and 2). Neither decreasing the amount of copper rebinding, owing to the presence of apoHc bound Co(II) ions, nor the kinetic inhibition of Cu(I) incorporation into active sites of Co-apoHc were observed. The amount of oxyHc thus obtained reached 32–35%, i.e. slightly more

than estimated for other hemocyanins (Suzuki et al. 1982a, b). This incomplete recovery of oxyHc from Co-apoHc dialysed against Cu(I)-acetonitrile complex should not be attributed to the inhibition of copper binding in active centers, owing to their occupation by cobalt. The above results from the fact that the oxyHc so formed contains nearly all the initial amount of copper (Table 1, 2). The remaining copper ions are most likely associated with the protein methemocyanin (metHc), incapable of oxygen binding, in which copper ions are also bivalent. The suggestion is also supported by the observation of the faint green colour of Hc solutions (Himmelwright et al. 1980).

The comparison of the data presented in tables for oxyHc and apoHc indicates the loss of previously bound cobalt ions during the dialysis against the Cu(I) complex. The plausible explanation for the removal of protein-bound cobalt ions during the reaction of Co-apoHc with acetonitrile-Cu(I) complex might be the formation of acetonitrile complexes with cobalt, in which cobalt ions are bound more strongly (greater binding constant) than they bind to apoHc. Considering the above, we conclude that the remaining metal ions (not removed by EDTA) are not bound at the active site. This is further confirmed by the absence of any significant difference in the kinetics of Cu(I) ion binding to apoHc or Co-apoHc (Fig. 1).

The differences between the amounts of Hc-bound cobalt, obtained by dialysis against free Co(II) ions and cobalt complexed with histidine (Table 1 and 2) together with the fact of their partial removal by EDTA, point to the occurrence of various binding sites with different binding constants. A similar phenomenon was reported for the interaction of hemocyanins with other metal ions (Belli et al. 1985; Brouwer and Engel 1982).

On the basis of the investigation of so called "spectral derivatives" some significant differences in the properties of molluscan and arthropodan Hc active site have been reported (Himmelwright et al. 1980; Witters and Lontie 1975). Amongst arthropodan hemocyanins, the hemocyanins of *Merostomata* had different properties (Himmelwright et al. 1980). The results obtained do not point to EDTA-resistant cobalt substitution at the active sites of Hc of *Orconectes limosus* and *Astacus astacus*. The incorporation of cobalt into copper binding sites in the hemocyanin molecule seems to be possible only when dialyzing apoHc against free Co(II) ions. The subsequent treatment with EDTA or Chelex removes cobalt ions from active centers. This accordance was also found for the hemocyanin of *Limulus polyphemus*, as reported by Lorosch and Haase (1986).

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