Removal of copper from *Octopus vulgaris* haemocyanin

Preparation of the half-apo and apo derivatives

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The two copper ions bound in the active site of *Octopus vulgaris* haemocyanin can be removed by cyanide. The two metal ions react with the ligand sequentially. In this paper the preparation of *Octopus* half-apo-haemocyanin, containing at the active site a single copper ion, is described. Moreover, the conditions to obtain *Octopus* apo-haemocyanin, containing less than 3% of copper still bound, are given.

Haemocyanins (Hc) are high- M_r copper-containing proteins that play the role of O_2 carriers in some classes of molluscs and arthropods.

These proteins bind O_2 reversibly in the active site containing a pair of copper ions, which are indispensable for the formation of the complex with O_2 (Brunori *et al.*, 1980). Oxy-Hc displays typical spectroscopic features: a strong absorption band at approx. 345 nm ($\epsilon \sim 20\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$) and a reasonably intense band at approx. 570 nm ($\epsilon \sim 1000\text{ M}^{-1}\cdot\text{cm}^{-1}$). Both absorption bands are absent with deoxy-Hc or apo-Hc (Solomon, 1981). The metal can be extracted from the protein active site only by cyanide treatment (Kubowitz, 1938). However, Hc proteins from the two phyla display quite different reactivities towards the ligand: although it is possible to obtain from arthropod Hc an apo-protein containing less than 2% of residual copper, molluscan apo-Hc contains 15–20% of metal still bound (Salvato *et al.*, 1974).

In our laboratory we have undertaken a detailed study on the reaction of CN^- with the Hc proteins from *Carcinus maenas* (Arthropoda) and *Octopus vulgaris* (Mollusca). In the present paper, on the basis of a reaction model previously described (Beltramini *et al.*, 1984a,b), we define the optimal experimental conditions required to obtain the half-apo derivative of *Octopus* Hc containing a single copper ion in each active site and the apo-Hc with less than 3% of copper still bound.

Abbreviation used: Hc, haemocyanin.

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Experimental

Hc was prepared from the haemolymph of *Octopus vulgaris* collected at the Stazione Zoologica di Napoli (Naples, Italy). The protein was purified as previously described (Salvato *et al.*, 1979). Before use, the protein, stored at -20°C in the presence of 18% sucrose, was dialysed against the desired buffer.

Protein concentrations were measured spectrophotometrically by using the coefficient $A_{280}^{1\%} = 14.1$ in Tris/HCl buffer, pH 8.0 and $I/0.1$. Absorption spectra were recorded on a Perkin-Elmer 576 spectrophotometer.

Fluorescence measurements were performed on a Perkin-Elmer MPF 4 spectrofluorimeter. The solutions used for fluorescence measurements showed an absorbance <0.05 at the excitation wavelength, in order to minimize errors due to the inner-filter effect. Copper concentrations were measured either by atomic absorption spectrophotometry, with a Perkin-Elmer model 300 atomic spectrophotometer, or by chemical determination with 2,2'-diquinoline (Vogel, 1961).

Preparation of *Octopus* half-apo-Hc

The formation of half-apo-Hc during the reaction with cyanide was followed kinetically. The protein solution (approx. 4 ml, 4 mg/ml) was placed in a dialysis tube containing a glass rod to obtain a thin layer of protein. The protein was dialysed against 200 ml of Tris/HCl buffer, pH 7.0 and $I/0.1$, containing 5 mM-EDTA, 5 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and either

2.5 mM- or 6 mM-KCN (at 4°C). At different times, 200 µl of Hc solution was withdrawn, diluted in 2 ml of Tris/HCl buffer, pH 7.0 and 10.1, and dialysed against the same buffer for 24 h (at 24°C). After dialysis, the A_{345}/A_{280} absorbance ratio, the copper/protein content ratio and, after an appropriate dilution, the fluorescence emission intensity at 330 nm (excitation at 280 nm) were measured on each sample.

Preparation of *Octopus* apo-Hc

Octopus Hc (approx. 4 ml, 40 mg/ml) was dialysed at room temperature against 500 ml of Tris/HCl buffer, pH 9.0 and 10.1, containing 50 mM-KCN, 200 mM-KCl and 70 mM-(NH₄)₂SO₄. In addition, 20 mM-EDTA was included in the buffer to prevent non-specific binding of copper to the protein. After 48 h the protein was dialysed against the reaction buffer without KCN, and then against a 1:1 mixture of this buffer with Tris/HCl buffer, pH 7.0 and 10.1, for 6 h. Finally, apo-Hc was dialysed against Tris/HCl buffer, pH 7.0 and 10.1, alone for 12 h. This procedure allows a stepwise change of the protein medium to avoid the partial denaturation of the protein, which results from drastic changes in the medium composition.

Reconstitution of *Octopus* apo-Hc

Reconstitution of apo-Hc (25 mg/ml) was obtained by dialysis against Tris/HCl buffer, pH 7.0 and 10.1, containing 1 mM-CuCl₂, 800 mM-KCl, 200 mM-KSCN, 5 mM-Na₂S₂O₄ and 2.5 mM-KCN (at 4°C). Samples (50 µl) used for spectroscopic analysis were diluted in 2 ml of Tris/HCl buffer, pH 8.0 and 10.1, containing 20 mM-EDTA and dialysed against the same buffer. In all experiments, 0.02–0.04% Tween 80 was included to prevent the precipitation of the protein.

Results and discussion

When *Octopus* Hc is incubated in the presence of cyanide ([CN⁻] = 2.5 mM) a decrease of both the copper content and the absorbance at 345 nm is observed. In contrast, the fluorescence of the protein increases up to a value approx. 4.8-fold higher as compared with the native protein (Fig.

1). From the data of Fig. 1 it is evident that the copper content and the A_{345}/A_{280} ratio of the cyanide-treated Hc reach constant values that are respectively 55% and 10% of the corresponding values in the native protein.

A further treatment of this Hc derivative for 6 h with 5 mM-KCN in the same medium used for the kinetic experiment of Fig. 1 gives a half-apo derivative of *Octopus* Hc showing the chemical properties reported in Table 1.

The treatment of *Octopus* Hc with higher concentrations of cyanide (50 mM) in the presence of appropriate salts leads to the preparation of an apo-protein containing about 2% of the copper content of the native protein (Table 1). In order to exclude any irreversible modification of the protein structure occurring upon copper removal, apo-Hc, as well as the half-apo derivative, was treated with Cu(I) in the presence of CN⁻. Both derivatives are reconstituted to the native state by this procedure (Table 1), indicating that the reaction of the bound copper with CN⁻ is completely reversible.

Fig. 1. Preparation of *Octopus* half-apo-Hc

The Figure shows the kinetics of the reaction between cyanide and *Octopus* Hc as observed by monitoring the A_{345}/A_{280} absorbance ratio (●), the copper/protein stoichiometry (Cu/Hc) (○) and the fluorescence (F) of the protein (Δ). The data of A_{345}/A_{280} and Cu/Hc ratios are reported as percentages of the corresponding values in native Hc, and F_0 indicates its fluorescence. For details see the Experimental section.

Table 1. Physicochemical properties of different *Octopus* Hc derivatives

	Hc sample ...	Native Hc	Half-apo-Hc	Apo-Hc	Reconstituted Hc
A_{345}/A_{280}		0.25	0	0	0.24
Cu/protein stoichiometry (g-atoms of Cu/mol of protein)		2.0	0.9–1.1	0.04	1.9
Cu/protein (g of Cu/100g of protein)		0.25	0.123–0.128	0.005	0.24
F/F_{native}^*		1	4.8	4.9	1

* F is the fluorescence intensity measured at 330 nm ($\lambda_{\text{exc.}} = 280$ nm).

The medium used for protein reconstitution includes several agents having different effects on Hc. Thus KSCN is expected to have a swelling effect on protein conformation (Von Hippel & Wong, 1964), leading to an increased active-site accessibility, and the high ionic strength, accomplished with KCl, is required to improve the protein stability.

The affinity constant of Hc for Cu(I) is very high (10^{19} M^{-1} , Felsenfeld, 1954; 10^{26} M^{-1} , Salvato & Zatta, 1977). Thus the removal of bound copper occurs only when cyanide is in large excess with respect to the metal (Kubowitz, 1938).

The cyanide/copper ratio in the experimental conditions used to prepare apo-Hc is approx. 4000:1. In contrast, the apoprotein can bind Cu(I) ions when the cyanide/copper ratio is in the order of 2:3 (Salvato & Zatta, 1977; Beltramini *et al.*, 1984b).

The kinetic study of the removal of copper from *Octopus* Hc by cyanide showed that this reaction is rather complicated. In particular, the two metal ions in the active site are removed sequentially (Salvato *et al.*, 1974; Salvato & Zatta, 1977); this kinetic inequivalence confirms previous results obtained by using isotopic exchange with ^{64}Cu (Cox & Elliott, 1974) and electronic addition (Symon & Petersen, 1978).

The minimal structure of *Octopus* Hc consists of a subunit of 250 000 Da containing five interacting active sites (Salvato *et al.*, 1979). The differences in reactivity between the two copper ions per active site have been attributed to the presence in *Octopus* Hc of high site-site negative co-operativity. Thus the removal of one copper ion from one active site decreases the reactivity of the neighbouring sites via a conformational change (Beltramini *et al.*, 1984b). This model explains both the high reactivity of the 'fast-removed' copper ion and the high metal content of apo-Hc prepared with other methods. It is noteworthy that the presence of approx. 15% of copper still bound to apo-Hc implies that approx. 30% of the metal sites are still loaded with one metal ion.

On the basis of a well-defined reaction scheme (Beltramini *et al.*, 1984b) we are now able to prepare the half-apo and apo derivatives of *Octopus* Hc having metal contents very similar to the theoretical ones.

The concentrations of CN^- required for the removal of each copper ion are quite different. Moreover, the reactivity of the 'fast-removed' copper ion is influenced by the O_2 concentration (Beltramini *et al.*, 1984a,b). In the absence of O_2 (condition obtained by addition of $\text{Na}_2\text{S}_2\text{O}_4$) the CN^- concentration can be decreased so that the removal of the second copper ion is almost excluded.

The two metal ions bound at the Hc active site exert a considerable quenching on the protein fluorescence via both heavy-metal and paramagnetic effects (Shaklai & Daniel, 1970; Bannister & Wood, 1971; Ricchelli *et al.*, 1983). Therefore the increase of protein fluorescence observed during the CN^- reaction cannot be attributed to a conformational change but must be attributed to the metal removal itself.

It is noteworthy that, although half-apo-Hc contains only one copper atom in the active site, its quantum yield is close to that of apo-Hc. This observation strongly indicates that the quenching effect on the Hc fluorescence is almost exclusively due to the copper ion that is more quickly removed from the active site.

Octopus apo-Hc is obtained at CN^- concentrations 10-fold higher than those used to prepare the half-apo derivative. In this reaction it is not necessary to discriminate each copper ion kinetically: apo-Hc can be obtained also in the presence of O_2 , and therefore $\text{Na}_2\text{S}_2\text{O}_4$ can be omitted in the preparation. Furthermore, this preparation requires a reversible conformational modification of the protein to decrease the site-site interactions that are responsible for the high content of copper still bound in apo-Hc prepared with other methods. This can be accomplished by modulating the swelling effect of high pH (9.0) with the protecting action of SO_4^{2-} ions (Ricchelli *et al.*, 1980). These ions are known to increase the stability of the protein by modifying the capability of water to arrange the hydrophobic residues within the molecular structure (Von Hippel & Wong, 1964).

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