Inorganica Chimica Acta, 92 (1984) 209–217

The Kinetics of the Reaction of *Octopus Vulgaris* Hemocyanin with Cyanide. Its Significance for the Structure of the 11 S Subunit of Molluscan Hemocyanin

MARIANO BELTRAMINI, FERNANDA RICCHELLI and BENEDETTO SALVATO*

Centro C.N.R. per lo Studio della Biochimica e della Fisiologia delle Emocianine e di altre Metallo-proteine. Istituto di Biologia Animale, Università di Padova, Via Loredan n. 10, I-35131 Padua, Italy

Received December 6, 1983

Native Octopus vulgaris hemocyanin (Hc) reacts with cyanide stepwise. The first step involves the formation of a complex HcCN with O_2 displacement. This complex reacts further with cyanide causing the removal of one copper ion from the active site. The same reaction sequence occurs for the extraction of the second metal ion. The formation of the HcCN complex and the removal of the first and the second copper ion can be differentiated according to the KCN concentration.

The rate of metal removal is slightly affected by KCN concentration. The kinetics are dominated by site–site interactions. The kinetic curves show only slight differences when the protein is in the 11 S or 49 S aggregation states, suggesting that the site–site interactions are restricted mainly within the 11 S structure. A kinetic model describing the removal of the first copper ion is proposed assuming that the 11 S component (MW 250,000) is an annular-shaped structure made by five equivalent functional subunits (MW 50,000). An explanation for the incomplete copper removal from molluscan Hc is given. The results are compared with those previously reported for Carcinus maenas Hc.

Introduction

Hemocyanins (Hcs)† are deeply blue coloured copper-containing proteins which play the role of oxygen carriers in the hemolymph of several invertebrates belonging to the phyla of *Mollusca* and *Arthropoda* [1]. These proteins, in the oxygenated form, contain at the active site a pair of antiferromagnetically coupled Cu(II) ions. Oxygen is reversibly bound to the binuclear metal center as a peroxide bridge between the two Cu(II) ions [2]. The Cu(II)–peroxide ($2Cu:1O_2^{2-}$) complex is the chromophore responsible for the rather unusual spectral properties of oxy-Hc. In the deoxy-form, the metal is present as Cu(I) and the protein is colourless. The absorption spectrum of oxy-Hc shows a strong band at $\lambda \sim 345$ nm ($\epsilon \sim 20000\ M^{-1}\ cm^{-1}$) and a reasonably intense transition at $\lambda \sim 570$ nm ($\epsilon \sim 1000\ M^{-1}\ cm^{-1}$) [3].

Although no significant difference can be found in aminoacid composition [4] and spectral properties of the copper bands [3], molluscan and arthropod Hcs differ widely in copper [1] and sugar content [5], in the minimal molecular weight obtainable under dissociating conditions and in quaternary structure [1].

Good ligands of cupric copper as EDTA or diethyldithiocarbamate as well as ligands of cuprous copper like thiourea, thiocyanate *etc.* are unable to remove the metal from the active site in both oxy- and deoxy-Hc. Copper-free Hc is obtained only by cyanide treatment. The apoprotein maintains the tertiary and quaternary structures [6] and can be reconverted to the native protein using various Cu(I) complexes [7–9].

Some differences, however, exist between molluscan and arthropod Hc in their reactions with cyanide. Whereas in molluscan Hc the removal of copper does not exceed 85%, even after prolonged incubation with cyanide, an apoprotein containing less than 2% of the original copper can be easily obtained from arthropod Hc [1].

Previous studies [10, 11] of the reaction between cyanide and Hc indicated that the first step of the reaction is the formation of a complex between CN^- and Hc with O_2 displacement. This complex further reacts with CN^- leading to the loss of one copper ion from each active site. The same reaction sequence also occurs for the second copper ion leading to the formation of the apoprotein.

In this work the removal of the first copper ion (here called 'fast reacting copper ion') from the *Octopus v.* Hc active site has been studied at various KCN concentrations. In addition, the reaction was also carried out on the protein either in the aggregation state of 49 S (in 100 m*M* $CaCl_2$) corresponding

*Author to whom correspondence should be addressed.

†Abbreviations used: EDTA: Ethylenediamintetraacetic acid, Hc: hemocyanin.

0020-1693/84/$3.00

to the whole molecule or in the aggregation state of 11 S corresponding to a 250,000 D [12] dissociation product. The time dependence of the following parameters has been determined during the reaction with CN^-: a) absorption at 348 nm (copper–oxygen band), b) copper still bound to the protein, c) intrinsic fluorescence.

The data have been interpreted in terms of a reaction model which provides useful information on the structure of the protein and on the site–site interactions. Furthermore, the results obtained for *Octopus* Hc are compared with those reported for *Carcinus* Hc [11]. It is concluded that although molluscan and arthropod Hcs have quite similar active site organizations, they show different active site accessibilities towards external ligands which may be a consequence of the differences in their quaternary structures.

Experimental

Materials and Methods

Octopus v. Hc was prepared as described elsewhere [13]. The hemolymph was collected from living animals at the Zoological Station of Naples.

Chemicals were of analytical grade and were used without further purification.

Protein concentration was determined spectrophotometrically using the absorption coefficient $E^{0.1\,\%}_{278\,nm}$ = 1.43 in Tris/HCl buffer pH = 8.0 μ = 0.1. The absorbance was corrected for the light scattering contribution [13].

Absorption spectra were recorded with a Perkin-Elmer 576 spectrophotometer.

Fluorescence spectra were recorded with a Perkin-Elmer MPF 4 spectrofluorimeter, equipped with a thermostatted sample holder, using the 'ratio' mode to correct for the light source fluctuations. No correction was applied for the variations of the monochromator and photomultiplier response as a function of the wavelength. Sample solutions having absorbance values lower than 0.05 at the excitation wavelength were used to minimize inner filter or self-absorption effects.

Copper was determined by atomic absorption using a Perkin-Elmer Mod 300 spectrophotometer. Kinetic measurements of copper removal were done using a dialysis tube (diameter = 0.7 cm) having inside a glass rod of 0.5 cm diameter, to minimize the dialysis time of the reaction products. The efficiency of copper removal from the solution inside the dialysis tube was checked using a solution of the cyanocuprate complex. The reaction with cyanide was made using 4 ml of Hc solution (4 mg/ml) in Tris/HCl buffer pH = 8.0 μ = 0.2 containing either 100 m*M* $CaCl_2$ or 20 m*M* EDTA which was dialyzed against the same buffer containing the desired concentration of cyanide. At different reaction times, 200 μl of the Hc solution were withdrawn, diluted with 2 ml of the same buffer (without KCN) and dialyzed against 100 ml of buffer to remove the copper released and the excess cyanide. After 24 h, the absorption spectrum between 230–450 nm was taken and the copper content determined. Fluorescence spectra were obtained after dilution of 50 μl of the samples with 3 ml of buffer. The dilutions used were large enough to stop the reaction. Experiments were done also at pH 5.8 and 8.5. In some experiments the protein was directly incubated with cyanide; aliquots taken at different intervals were dialyzed and analyzed. All experiments were carried out at 20° ± 0.1 °C.

Results

In the presence of KCN concentrations lower than 1 m*M*, Hc loses the copper–peroxide absorption band (λ_{max} = 348 nm) and the rate and the extent of the process depend on the KCN concentration. The band is almost completely restored after the removal of cyanide.

At higher KCN concentrations a time dependent decrement of both the $Cu–O_2^{2-}$ absorption band and of the copper content occurs. When the experiment is carried out by direct incubation of the protein with KCN, followed by dialysis of samples at different reaction times, the kinetic curves describing the residual copper bound to the protein and the absorbance at 348 nm show a fast decrease followed by an upward curvature (Fig. 1). This kinetic profile

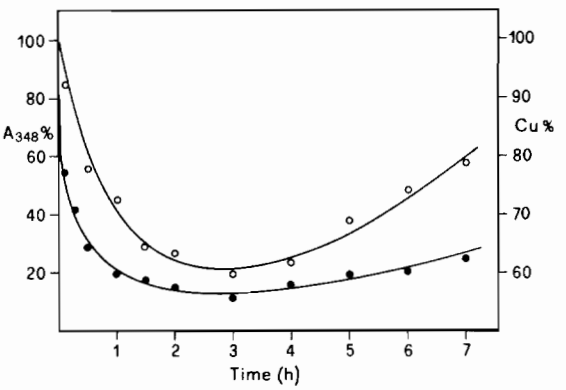


Fig. 1. Kinetic curves obtained by direct incubation of Hc with 20 m*M* KCN at T = 21.0 ± 0.2 °C. Time-course of residual copper (●) and of residual absorbance at 348 nm (○) expressed as the percentage of the initial values. Tris/HCl buffer pH = 8.0 μ = 0.2.

shows that when *Octopus* Hc is incubated with cyanide, the ligand extracts the metal forming a copper–cyanide complex. The presence of an upward curvature indicates that after a certain incubation time the apoprotein formed during the reaction is

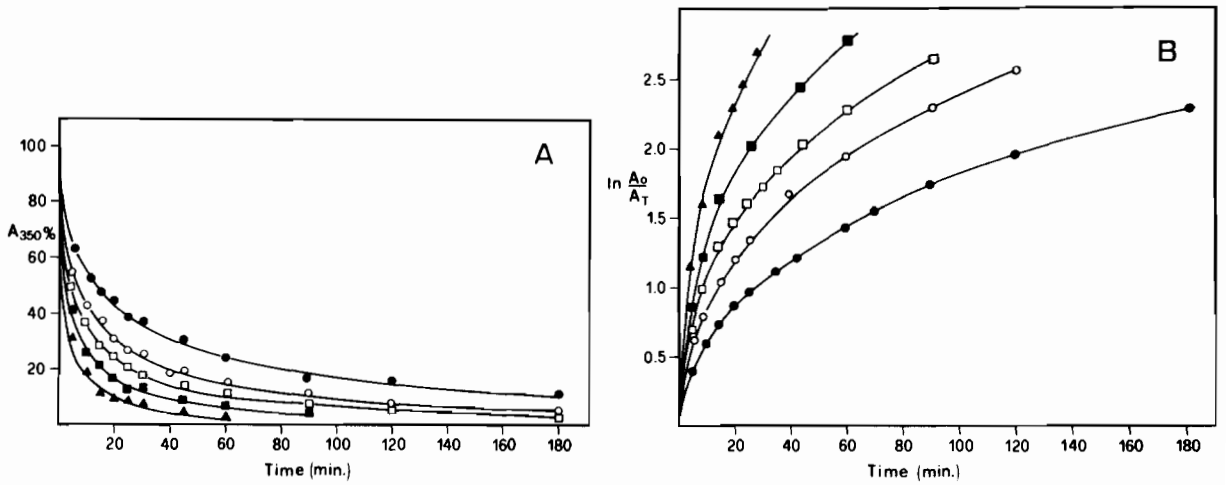


Fig. 2. A) Decrease of the 348 nm absorbance of Hc in 49 S aggregation state at different KCN concentrations: (●) 5 m*M*, (○) 10 m*M*, (□) 15 m*M*, (■) 25 m*M*, (▲) 48 m*M*. The data are expressed as in Fig. 1. Tris/HCl buffer pH = 8.0 μ = 0.2 plus 100 m*M* $CaCl_2$ T = 21.0 ± 0.2 °C. The curves are calculated according to eqn. 1 using a non-linear least-squares method. B) Semilogarithmic plots obtained from the data reported in A). A_0 and A_T indicate the absorbances at 350 nm measured at time zero and t respectively.

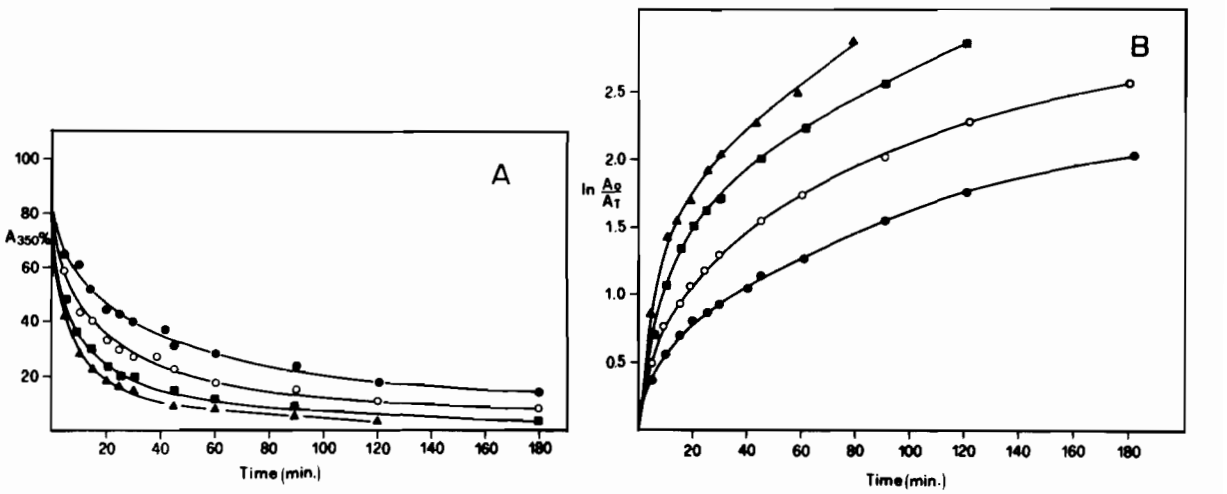


Fig. 3. A) Decrease of the 348 nm absorbance of Hc in the 11 S aggregation state at different KCN concentrations: (●) 5 m*M*, (○) 10 m*M*, (■) 20 m*M*, (▲) 30 m*M*. The data are expressed as in Fig. 1. Tris/HCl buffer pH = 8.0 μ = 0.2 plus 20 m*M* EDTA. T = 21.0 ± 0.2 °C. The curves are calculated according to eqn. 1 using a non-linear least-squares method. B) Semilogarithmic plots obtained from the data reported in A). A_0 and A_T as in Fig. 2B.

reconstituted. This effect may be due to the lower diffusion rate of the copper–cyanide complex as compared to that of the free cyanide ion and to the capability of this complex to reconstitute apo-Hc [7, 10]. When the experiment is done by continuous dialysis, the cyanocuprate complex is continuously removed and the kinetic curves do not show upward curvatures.

The removal of cyanocuprate is described by a single exponential with a $t_{1/2}$ of about 6 min, negligible compared with the time scale of the reaction between CN^- and Hc so that the procedure as above described was employed for all the experiments. The decrease of the concentration of the sites containing two metal ions can be measured by following the time dependence of the 348 nm absorption band. In Figs. 2A and 3A are reported the kinetics observed at different KCN concentrations at pH 8.0 and in the presence of either 100 m*M* $CaCl_2$ or 20 m*M* EDTA respectively. In these plots the intensity of the 348 nm band is reported as a percentage of the value measured at the time zero of reaction. The corresponding semilogarithmic plots are reported in Figs. 2B and 3B.

The reaction rate is slightly influenced by cyanide concentration and the curves cannot be described by

a single exponential. The slope of the semilogarithmic plots decreases indicating a continuous decrease of reaction rate with time. No large differences are evident either in the presence of calcium (s_{w20} = 49 S) or in the presence of EDTA (s_{w20} = 11 S).

The effect of pH (5.8 and 8.5) on the reaction rate was investigated using Hc either in the 49 S or in the 11 S aggregation states (Fig. 4A). In both cases the rate of the 348 nm absorption decrement increases with pH. This effect is, moreover, more pronounced when the protein is in the 11 S form.

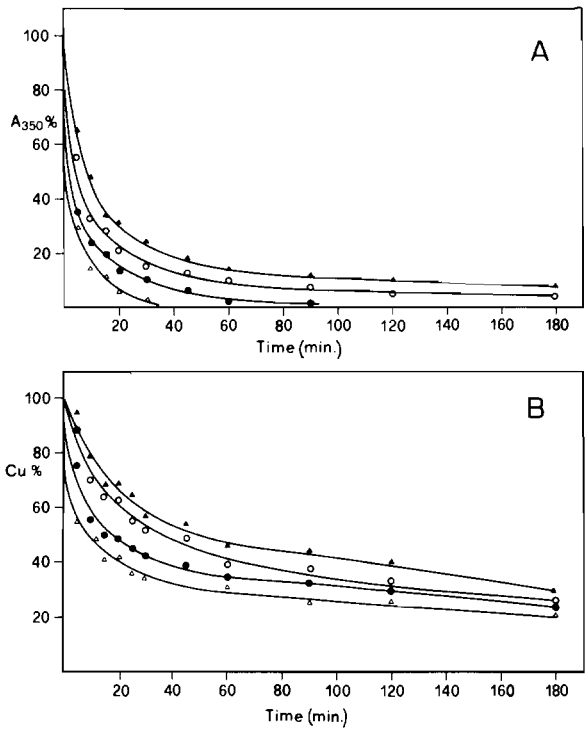


Fig. 4. A) Time-course of the 348 nm absorbance of Hc in the 49 S aggregation state at pH = 5.8 (○) and 8.5 (●) and of Hc in the 11 S aggregation state at pH 5.8 (▲) and 8.5 (△). KCN concentration = 48 m*M* in the 0.2 *M* Tris/HCl buffer or 0.2 *M* Tris/acetate. T = 21.0 ± 0.2 °C. B) Time-course of the residual copper of Hc in the 49 S or 11 S aggregation states at pH 5.8 and 8.5. Conditions and symbols as in Fig. 4A.

In Fig. 5 (A, B) are reported the kinetics of the copper removal at various KCN concentrations and using Hc in the 49 S (Fig. 5A) or 11 S (Fig. 5B). The figures show the plots of the copper still bound as a function of time. The former parameter is reported as percentage of the copper content in Hc at various times of reaction with respect to the content at time zero. The copper still bound to the protein decreases with an initial rapid phase accounting for the loss of about 50% of the copper content in native Hc, followed by a much slower phase.

The copper content determined after 24 h is essentially independent of both aggregation states

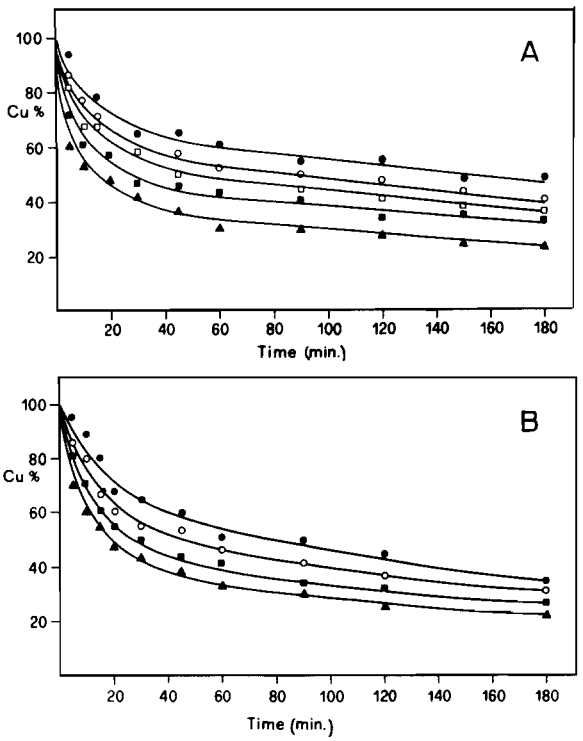
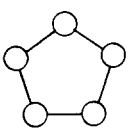


Fig. 5. A) Time-course of the residual copper of Hc in the 49 S aggregation state. Conditions and symbols as in Fig. 2. B) Time-course of residual copper of Hc in the 11 S aggregation state. Conditions and symbols as in Fig. 3.

of the protein and the cyanide concentration used and reaches a constant value of about 15–18%. The same value was observed after 96 h of dialysis.

The effect of pH on the kinetics of copper removal is shown in Fig. 4B. The results are very similar to those observed in Fig. 4A for the 348 nm band. The changes in the fluorescence emission intensity of Hc in the 11 S state during the reaction with CN^- are reported in Fig. 6 for different concentrations of the ligand. The emission intensity F is normalized to the emission of the protein in absence of CN^- (F_0). The limiting value of the ratio F/F_0 is always equal to about 5. The trend of the curves is similar, and shows that regardless of the concentration of CN^- the process involves several phases.

The experimental data have been interpreted on the basis of a kinetic model assuming that the 11 S component is an annular structure made by five



equivalent functional subunits. The extraction of one metal ion from a site leads to the abolishment of the site-equivalence by modifying in the adjacent sites the rate constant of the copper removal.

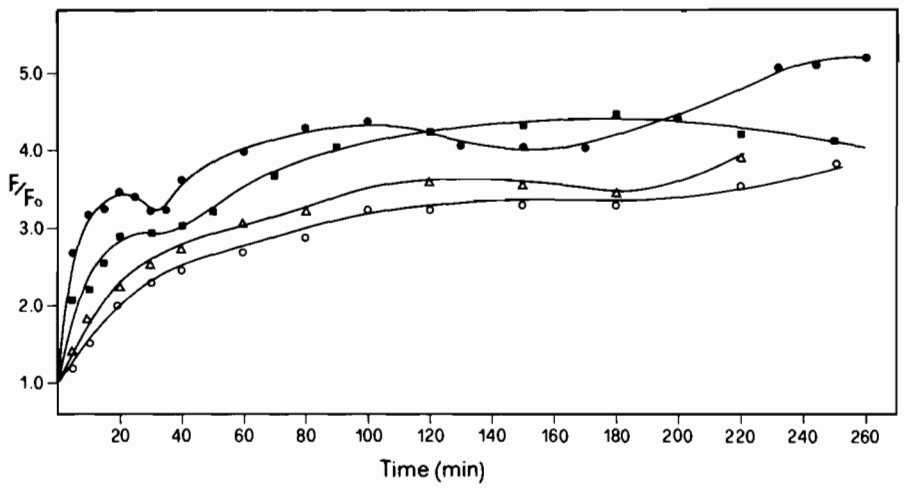
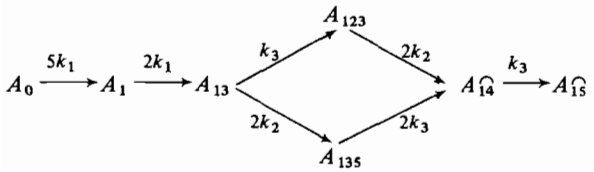


Fig. 6. Time-course of the emission fluorescence intensity of Hc in the 11 S aggregation state. F_0 is the fluorescence intensity in the absence of KCN. λ_{exc} = 295 nm; emission λ_{max} = 330 nm. KCN concentrations: (○) 5 m*M*, (△) 10 m*M*, (■) 20 m*M*, (●) 30 m*M*. The fluorescence intensities are normalized to the same protein concentration. Conditions as in Fig. 3.

Different rate constants for the reacting sites have been assumed according to the loading situation of the adjacent sites; namely: k_1 if the sites adjacent to the reacting one are still double-copper loaded, k_2 if one is double- and the other one is single-copper loaded and k_3 if both adjacent sites are single-copper loaded. Taking into account the statistical effects, the following reaction model for the loss of the 'fast reacting' copper ion is proposed:



where A_0 is the concentration of the 11 S component containing all five sites double-copper loaded; A_1, A_{13}, ..., A_{15} are the concentrations of components containing single-copper loaded sites at the position 1, 1 and 3, and 1, 2, 3, 4, 5, respectively, *etc.* As the value of k_1 can be presumed to be much higher than k_2 and k_3, some possibilities have been omitted. Furthermore, the reaction between the site and CN^- has been considered as pseudo first-order, the CN^- concentration being in large excess with respect to the site concentration. The curves (Figs. 2, 3) describing the time dependence of the $Cu{-}O_2^{2-}$ absorption band, which is proportional to the concentration of unremoved 'fast copper', have been calculated according to the following equation, obtained from the mathematical treatment of the model (see **Appendix**):

$$\alpha_1 e^{-5k_1} + \alpha_2 e^{-2k_1 t} + \alpha_3 e^{-2k_2 t} + (5 - \Sigma_i \alpha_i) e^{-k_3 t} = A \tag{1}$$

where A represents the concentration of still unreacted 'fast copper' ions at the time t (taking $A_0 = 5$ and $A_\infty = 0$) and

$$\alpha_1 = \left\{ \frac{10k_1}{(5k_1 - 2k_2 - k_3)} \left[1 - \frac{2}{3} \frac{2k_2 + k_3}{5k_1 - k_3} \right] - 1.66\bar{6} \right\} \tag{2}$$

$$\alpha_2 = \left\{ 6.66\bar{6} + \frac{10k_1}{2k_1 - 2k_2 - k_3} \left[\frac{4}{3} \frac{k_2}{2k_1 - k_3} + \right. \right.$$
$$\left. \left. + \frac{k_3(2k_1 - k_2 - k_3)}{3(k_1 - k_2)(2k_1 - k_3)} - 1 \right] \right\} \tag{3}$$

$$\alpha_3 = \frac{10}{3} \frac{k_1(k_2 - k_3)(7k_1 - 4k_2)}{(2k_2 - k_3)(5k_1 - 2k_2)(k_1 - k_2)} \tag{4}$$

It is evident that the coefficients of the various exponentials change according to the values assigned to k_1, k_2, k_3. So, this equation is more rigid than may be supposed from the number of the exponentials.

In Table I, the values of the apparent monomolecular rate constants k_1, k_2 and k_3, in different conditions, are reported together with the calculated bimolecular rate constant.

The theoretical curves of Figs. 2 and 3 have been calculated using k_1, k_2 and k_3 values arising from the application of the least squares method to all data simultaneously.

TABLE I. Pseudo-first Order and Bimolecular Rate Constants for the Reaction of *Octopus v.* Hc with Cyanide, as Calculated according to the Proposed Model.

Hc 49 S component (0.2 *M* Tris/HCl buffer, pH 8.0 plus 100 *M* $CaCl_2$), T = 21.0 ± 0.2 °C

	CN^-					
	5 m*M*	10 m*M*	15 m*M*	25 m*M*	48 m*M*	$k_{bimolecular}$
k_1 (sec^{-1})	2.5×10^{-3}	5.0×10^{-3}	7.5×10^{-3}	1.25×10^{-2}	2.4×10^{-2}	0.500 M^{-1} sec^{-1}
k_2 (sec^{-1})	2.42×10^{-4}	4.8×10^{-4}	7.25×10^{-4}	1.21×10^{-3}	2.32×10^{-3}	0.048 M^{-1} sec^{-1}
k_3 (sec^{-1})	6.76×10^{-5}	1.33×10^{-4}	2.0×10^{-4}	3.33×10^{-4}	6.4×10^{-4}	0.013 M^{-1} sec^{-1}

Hc 11 S component (0.2 *M* Tris/HCl buffer, pH = 8.0 plus 20 m*M* EDTA), T = 21.0 ± 0.2 °C

	CN^-				
	5 m*M*	10 m*M*	20 m*M*	30 m*M*	$k_{bimolecular}$
k_1 (sec^{-1})	2.5×10^{-3}	5.0×10^{-3}	1.0×10^{-2}	1.5×10^{-2}	0.500 M^{-1} sec^{-1}
k_2 (sec^{-1})	1.83×10^{-4}	3.67×10^{-4}	7.33×10^{-4}	1.1×10^{-3}	0.037 M^{-1} sec^{-1}
k_3 (sec^{-1})	4.17×10^{-5}	8.33×10^{-5}	1.67×10^{-4}	2.5×10^{-4}	0.008 M^{-1} sec^{-1}

Discussion

Native *Octopus v.* Hc reacts stepwise with KCN [10]. The first step is the formation of the colourless HcCN complex by displacement of O_2. At KCN concentrations lower than 1 m*M*, the system is represented by the following equilibria (site–site interactions are not considered):

$$Hc + O_2 \rightleftharpoons HcO_2 \quad \text{(blue)}$$

$$Hc + CN^- \rightleftharpoons HcCN \quad \text{(colourless)}$$

In agreement with the presence of two equilibria, after KCN removal the $Cu–O_2^{2-}$ absorption band centered at 348 nm is almost completely recovered.

At higher KCN concentrations, the HcCN complex further reacts with cyanide leading to the removal of one copper from each active site. Actually, no recovery of the $Cu–O_2^{2-}$ absorption band, which is a measure of the 'double-copper' loaded sites, is observed when more than 50% of the total copper is removed. The time-course of copper removal shows the kinetic inequivalence of the copper ions in the active site.

This conclusion can be inferred also by electron addition experiments [14] and by isotopic exchange using $^{64}Cu^+$ complexes [15]: only one half of the total copper of Hc is, respectively, reduced or exchanged. Clearly, one of the two copper ions in the active site is shielded from interactions with the external medium. The structure of the Hc active site has been widely investigated. In the proposed model, oxygen is bound as a peroxide bridge between two Cu(II) ions. Histidine (2 or 3 per Cu) and probably a bridging phenolate are the protein ligands [3]. According to the results presented herein, the protein moiety plays very important roles in the inequivalent shielding of the two metal ions and is responsible for the strong site–site interactions. We call 'fast reacting copper ion' the copper ion which is removed more rapidly and 'slow reacting copper ion' the other one.

The model previously proposed for interpreting the reaction between *Octopus v.* Hc and cyanide [10] assumed that the removal of the 'fast reacting' copper ion was an homogeneous process described by a single exponential. The change of the rate constant (see Figs. 2B and 3B) during the reaction course clearly shows that this process is more complex than previously reported, involving strong negative site–site interactions. Two levels of molecular organization are present in molluscan Hcs. The 49 S structure is a polymer of the 11 S component (MW ~ 250,000 D) which in turn arises from the covalent linkage of 5 functional units (MW ~ 50,000 D) [12]. The close similarities between the kinetic curves obtained for the two aggregation states suggest that the 49 S can be considered a modulating structure of the 11 S. Actually, the latter is the basic unit mainly responsible for the site–site interactions.

The same suggestion is provided by oxygen-binding experiments [16]. This allows the elaboration of a kinetic model for the $Hc–CN^-$ reaction by defining only the structural organization of the 11 S component.

The experimental data are well described by assuming that the 11 S component contains 3 fast and 2 slow reacting sites. However, this model must be discarded since this inequivalence in *Octopus v.* Hc is not supported by any experimental evidence. Also a linear structural model, made by 5 subunits linked in a necklace structure cannot be accepted. In fact, in a structure of this kind, the two end-subunits would behave quite differently in comparison to the inner ones.

Therefore, a kinetic model has been elaborated on the assumption that 11 S component is an annular structure made by five equivalent functional subunits.

Structures of this kind have been observed in electron micrographs of *Fissurella* [17] and *Octopus* Hcs [18]. The theoretical curves calculated on the basis of this structural model give a very satisfactory interpolation of the experimental data. The comparison between the k values obtained for the two aggregation states allows us to conclude that the 49 S Hc shows a small increase of the metal accessibility and a small decrease of the site–site interactions. In the 49 S form, Hc contains many Ca(II) ions linked to the protein matrix [19]. This makes the molecule more rigid and compact, leading to the observed effects on the rate of the process with CN^-. Actually it is incorrect to consider the 49 S structure as a mere aggregation of several 11 S components. In fact the polymerization occurs via Ca(II) binding which clearly introduces new constraints in the molecular organization. This may lead to a simple interpretation of pH effect. In fact the differences between the curves obtained at pH 8.5 and 5.8, in the presence of $CaCl_2$, can be attributed to the increased dissociation of the weak acid HCN. In the presence of EDTA, the additional effect of pH can be attributed to the swelling of the protein depending on the increase of the electrostatic potential and on the elimination of Ca(II) constraints. The combination of these effects would lead to greater accessibility of the sites.

The data describing the total copper removal confirm previous results on the kinetic differences between the two copper ions in the *Octopus v.* Hc active site. The difference in the extraction rate between 'fast' and 'slow' reacting copper ions is very large under both sets of conditions used. The kinetics of the 'slow reacting' copper removal have not been analyzed in detail; however it is evident from Figs. 4 and 5 that this reaction is complex too. The negative cooperation between sites would be so strong as to prevent the total removal of the metal. It must be emphasized that, when the protein still contains 15% of the original copper, 30% of sites are single-copper

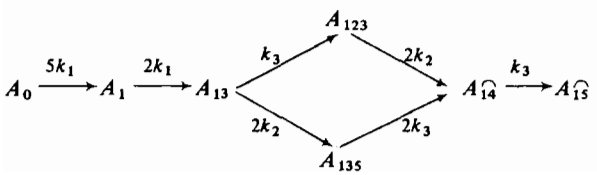
loaded. We have recently selected experimental conditions to obtain an apoprotein from molluscan Hc containing no more than 2% of the original copper and a half-apo derivative containing more than 95% of single-copper loaded sites [20]. Work is in progress to define the kinetic behaviour of the reaction between the 'slow reacting' copper ion and cyanide using half-apo Hc.

The trend of the fluorescence curves as a function of time may be interpreted on the basis of the quite different quenching effect on the intrinsic protein fluorescence exerted by the two metal ions: the fast reacting copper ion is responsible for about 95% of the quenching effect; the other one exerts no significant influence [20]. The presence in the fluorescence curves of various relative minima could imply that the removal of the 'slow reacting' copper ion may occur *via* transfer to the 'fast', free site.

Some interesting considerations can be drawn from the comparison between the results obtained for *Octopus v.* and *Carcinus m.* Hc [11]. In *Octopus v.* Hc the formation of the HcCN complex can be easily distinguished from the reaction of metal removal by the KCN concentration; furthermore, the reaction rates of the two copper ions are very different. In *Carcinus m.* Hc the above reactions are indistinguishable, as HcCN complex formation is the rate-limiting step. The kinetic difference between the two metal ions in the active site is much smaller. This observation also explains why attempts to prepare half-apo Hc have been successful only in the case of molluscan Hc [2]. Even if the kinetic model is similar for both proteins, in *Carcinus m.* Hc the apparent reaction order with respect to CN^- is 2, involving a very large effect of the KCN concentration on the reaction rate. In spite of the complexity of 16 S and 24 S components of *Carcinus m.* Hc, negligible site–site interaction has been found. The observed differences between *Octopus v.* and *Carcinus m.* Hc can be explained in terms of different accessibility of the site on the whole molecule, and of the two metal ions within the active site, even though the overall structure of the active site is similar in both Hc [3].

Appendix

The model describing the removal of the 'fast reacting' copper ion from the *Octopus v.* Hc active site is:



where A_0 is the concentration of the 11 S component containing all 5 sites double-copper loaded and A_1, A_{13}, *etc.* are the concentrations of the 11 S component containing the sites in position 1, 1 and 3 *etc.* single-copper loaded, and

$$\begin{cases} \dfrac{dA_0}{dt} = -5k_1A_0 \\ \dfrac{dA_1}{dt} = 5k_1A_0 - 2k_1A_1 \\ \dfrac{dA_{13}}{dt} = 2k_1A_1 - (2k_2 + k_3)A_{13} \\ \dfrac{dA_{123}}{dt} = k_3A_{13} - 2k_2A_{123} \\ \dfrac{dA_{135}}{dt} = 2k_2A_{13} - 2k_3A_{135} \\ \dfrac{dA_{\widehat{14}}}{dt} = 2k_2A_{123} + 2k_3A_{135} - k_3A_{\widehat{14}} \\ \dfrac{dA_{\widehat{15}}}{dt} = k_3A_{\widehat{14}} \end{cases}$$

$$A = 5A_0 + 4A_1 + 3A_{13} + 2(A_{123} + A_{135}) + A_{\widehat{14}}$$

A is the total number of the sites still double-copper loaded when the initial A_0 concentration is equal to 1. The first differential equation can be easily integrated. All the others, then, assume the form of the first-order linear differential equation:

$$y' = f(x)y + g(x)$$

whose general integral is:

$$y = \exp(\int f(x)\,dx)\{\int g(x)\exp(-\int f(x)\,dx)\,dx\} + c$$

All values A_0, A_1, ..., $A_{\widehat{14}}$ can be obtained as a function of time. The final equation for A is:

$$\left\{\frac{10k_1}{(5k_1 - 2k_2 - k_3)}\left[1 - \frac{2}{3}\frac{2k_2 + k_3}{5k_1 - k_3}\right] - 1.6\bar{6}\right\} \times$$

$$\times e^{-5k_1t} + \left\{6.6\bar{6} + \frac{10k_1}{2k_1 - 2k_2 - k_3}\left[\frac{4}{3}\frac{k_2}{2k_1 - k_3} + \right.\right.$$

$$\left.\left. + \frac{k_3(2k_1 - k_2 - k_3)}{3(k_1 - k_2)(2k_1 - k_3)} - 1\right]\right\} e^{-2k_1t} +$$

$$+ \left[\frac{10}{3}\frac{k_1(k_2 - k_3)(7k_1 - 4k_2)}{(2k_2 - k_3)(5k_1 - 2k_2)(k_1 - k_2)}\right] e^{-2k_2t} +$$

$$+ (5 - \Sigma_i\alpha_i)e^{-k_3t} = A$$

where α_i are the coefficients of the exponential terms.

References

1 K. E. Van Holde and K. I. Miller, *Quarterly Reviews of Biophysics, 15,* 1 (1982).
2 R. S. Himmelwright, N. C. Eickman and E. I. Solomon, *J. Am. Chem. Soc., 101,* 1576 (1979).
3 E. I. Solomon in 'Copper Proteins', T. G. Spiro ed., Wiley–Interscience, New York, Vol. 3, p. 41 (1981).
4 A. Ghiretti-Magaldi and G. Tamino in 'Structure and Function of Hemocyanin', J. V. Bannister ed., Springer-Verlag, Berlin, p. 271 (1977).
5 V. Albergoni, A. Cassini and B. Salvato, *Comp. Biochem. Physiol., 41B,* 445 (1972).
6 A. M. Tamburro, B. Salvato and P. Zatta, *Comp. Biochem. Physiol., 55B,* 347 (1976).
7 R. Lontie, V. Blaton, M. Albert and B. Peeters, *Arch. Int. Physiol. Bioch., 73,* 150 (1965).
8 P. Zatta and B. Salvato, *J. Inorg. Biochem., 15,* 269 (1981).
9 M. Beltramini and K. Lerch in 'Structure and Function of Invertebrate Respiratory Proteins', E. J. Wood, in : *Life Chemistry Reports,* Sup. 1, p. 323, Harwood Academic, London (1983).
10 B. Salvato and P. Zatta in 'Structure and Function of Hemocyanin', J. V. Bannister ed., Springer-Verlag, Berlin, p. 245 (1977).
11 M. Beltramini, F. Ricchelli, L. Tallandini and B. Salvato, preceding paper.
12 B. Salvato, A. Ghiretti-Magaldi and F. Ghiretti, *Biochemistry, 18,* 2731 (1979).
13 B. Salvato, A. Ghiretti-Magaldi and F. Ghiretti, *Biochemistry, 13,* 4778 (1974).
14 M. C. R. Symons and R. L. Petersen, *Biochim. Biophys. Acta, 535,* 247 (1978).
15 J. A. Cox and F. G. Elliott, *Biochim. Biophys. Acta, 371,* 392 (1974).
16 L. Tallandini and B. Salvato in 'Invertebrate Oxygen-Binding Proteins', J. Lamy and J. Lamy eds., Marcel Decker, New York, p. 727 (1981).
17 T. J. McIntosh, J. D. Robertson, H. B. Ting-Beall, A. Walter and G. Zampigh, *Biochim. Biophys. Acta, 601,* 289 (1980).
18 F. Ghiretti, A. Ghiretti-Magaldi and B. Salvato in 'Structure and Function of Invertebrate Respiratory Proteins', E. J. Wood, in: *Life Chemistry Reports,* Sup. 1, p. 397, Harwood Academic, London (1983).
19 A. Klarman, N. Shaklai and E. Daniel, *Biochim. Biophys. Acta, 257,* 150 (1972).
20 F. Ricchelli, E. Tealdo and B. Salvato in 'Structure and Function of Invertebrate Respiratory Proteins', E. J. Wood, in: *Life Chemistry Reports,* Sup. 1, p. 301, Harwood Academic, London (1983).