The *o*-diphenol oxidase activity of arthropod hemocyanin

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Abstract Arthropod hemocyanin (isolated from the crab *Carcinus maenas* and the lobster *Homarus americanus*) is usually referred to as an oxygen transport-storage protein. The protein, however, also catalyses with low efficiency the oxidation of *o*-diphenol to quinone, similarly to tyrosinase (monophenol,*o*-diphenol:oxygen oxidoreductase). The enzymatic parameters of hemocyanin are affected by the aggregation state of the protein; namely V_{max} exhibited by a dissociated subunit is one order of magnitude greater than that of aggregated species. The reaction velocity is increased by the presence of perchlorate, an anion of the Hofmeister series. The results are also discussed on the basis of active site accessibility in comparison with tyrosinase.

Key words: Hemocyanin; Copper active site; Dioxygen; Catechol; (*Carcinus maenas*); (*Homarus americanus*)

1. Introduction

The hemolymph of several arthropods and molluscs is characterised by the presence of a high molecular weight copper protein, hemocyanin (Hc).

The biological role of Hc, referred to as an oxygen carrier/storage protein, is based on the capability of reversibly binding dioxygen to a dinuclear copper site [1]. However, several physico-chemical properties of Hc are remarkably similar to those of another copper protein, tyrosinase (Ty; monophenol,*o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1), widely distributed in animals and plants where it catalyses the *o*-hydroxylation of monophenols and the O_2-dependent oxidation of *o*-diphenols to quinones (monophenolase and diphenolase activities, respectively) [1,2]. In both proteins each metal ion is bound by three imidazole ligands to yield a dinuclear site having approximately the same metal-metal distance of about 3.6 Å [3,4]. Comparative studies on the interaction of Hc and Ty with exogenous molecules showed that the active site of both proteins reacts in the same way with respect to exogenous species: like deoxy-Hc, deoxy-Ty binds reversibly molecular dioxygen at the di-cuprous site to yield the reactive form of the enzyme, oxy-Ty, containing a di-cupric peroxide adduct [1,2]. Beside molecular oxygen, deoxy-Ty and deoxy-Hc reversibly bind carbon monoxide with concomitant development of a typical luminescence characterized by an unusually large Stoke's shift [5,6]. The site can be chemically modified to yield half-met derivatives whose dinuclear cuprous-cupric sites display essentially the same EPR spectrum [7]. Cyanide reacts with the copper active site of both proteins competitively with respect to dioxygen but is also capable of displacing the two metal ions from the active site. In all different experimental approaches, however, the reactions on the dinuclear site occur at a much higher rate with Ty than with Hc. These results convincingly showed that the Ty active site is much more accessible to exogenous molecules as compared to Hc [8,9].

On the basis of kinetic, equilibrium and spectroscopic studies it has been proposed that the enzymatic activity of Ty is founded on its capability to coordinate phenolic substrates at the copper site. The transient adduct is suited either to chemical attack at the *o*-position (monophenolase activity) and/or to electron transfer involving the metal (catecholase activity) [1,2,10].

Arthropod Hcs are characterised by a rather complicated quaternary structure exhibiting cooperative behaviour in oxygen binding. The basic structure is a 16S species ($M_r \sim 450$ kDa) which is a hexamer of subunits ($M_r \sim 75$ kDa) containing one active site each and arranged so as to form a trigonal antiprism. The 16S species is the component responsible for the generation of the higher aggregation forms namely: the 24S (2×6-mer, $M_r \sim 900$ kDa), 37S (4×6-mer, $M_r \sim 1800$ kDa) and 62S (8×6-mer, $M_r \sim 3600$ kDa) [11]. On the basis of the previous comparative results between Hcs and Ty on the interaction with exogenous copper ligands [8] and on consideration of the possibility to change the aggregation state of Hc, we have investigated the catechol oxidation reaction, or pseudo-tyrosinase activity, exhibited by two arthropod Hcs (from the crab *Carcinus maenas* and from the lobster *Homarus americanus*) using isolated subunits or aggregated forms of the proteins and in the presence of agents capable of perturbing protein conformation.

2. Materials and methods

Hc was isolated from the hemolymph of *C. maenas* and *H. americanus*. Hcs from both species were prepared as described by Bubacco et al. [12]. Preparations of native Hc exhibited ratios of absorbance at 337 and 278 nm (A_{337}/A_{278}) of 0.21 in the case of both *Carcinus* and *Homarus* Hcs, indicating the complete purification of the protein [11].

Before activity determinations, the protein was dialysed at 4°C against 20 mM phosphate buffer containing 10 mM EDTA at pH 7.5 for *Homarus* and at pH 7.0 for *Carcinus* and then agaist EDTA-free buffer. The concentration of the protein was determined spectrophotometrically using an extinction coefficient E=1.24 ml mg^{-1} cm^{-1} and a molecular mass value of 75 kDa relative to the functional subunit containing one active site.

Separation of dissociated components of arthropod Hcs was achieved by a combination of gel filtration and ion exchange chromatography using a Pharmacia FPLC system. The elution was monitored at 280 nm.

Native *Homarus* Hc was stripped by dialysis against 0.05 M glycine-NaOH buffer at pH 9.6 containing 10 mM EDTA. The sample was size-fractionated with a Superdex 200 column (26×60 cm) previously equilibrated with the same buffer and eluted at a flow rate of 120 ml/

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Abbreviations: Hc, hemocyanin; Ty, tyrosinase.

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h. Ion exchange chromatography was performed on a Sepharose Hi-Load column (26×10 cm) previously equilibrated with the buffer used for gel filtration and the protein was eluted at a flow rate of 420 ml/h by applying a linear gradient of 0–1 M NaCl. In the case of *Carcinus* Hc the same methods and equipment were used as described for *Homarus*. The dissociation of the native *Carcinus* Hc was carried out in 0.05 M Tris-HCl buffer pH 9.2, containing 10 mM EDTA. The buffer used for reassociation experiments was Tris-HCl, pH 7.5 containing 20 mM $CaCl_2$.

The purity of all aggregation states of Hc was assessed by gel filtration using an analytical Superdex 200 HR 10/30 column and the FPLC system (Pharmacia). The column was calibrated with HMW and LMW gel filtration calibration kits (Pharmacia).

The *o*-diphenol oxidase (catecholase) activity of Hc was routinely assayed at 20°C in 3 ml of 20 mM phosphate buffer at pH 6.0. This pH was chosen to minimise autooxidation of the substrate. In all cases, however, the kinetics of quinone formation were corrected for this effect. Experiments were also carried out in the presence of 50 or 200 mM sodium perchlorate in the case of *Homarus* and *Carcinus* Hc, respectively. Because reassociated *Homarus* Hc was unstable at high ionic strength, a 2 mM phosphate buffer pH 6.0 was used with this Hc.

The reaction was started by the addition of 0.3–3.0 mg of Hc to the substrate solution, whose concentration ranged between 6.0 and 33 mM, and it was followed by means of quinone formation by recording the increase of absorbance at 400 nm (ε = 1417 M^{-1} cm^{-1} for *o*-quinone). The reaction velocity was calculated from the initial, quasi-linear portion of the plot. Kinetic parameters K_m (mM) and V_{max} (mM min^{-1} mg^{-1}) for Hc species were determined under the standard assay conditions using Lineweaver-Burk plots.

Absorption measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer equipped with a thermostatted sample holder.

All reagents were of the best grade commercially available. The *o*-diphenol was used after purification by double vacuum sublimation and the white crystalline powder was stored at −20°C until use.

3. Results

The native Hc from both *Carcinus* and *Homarus* is present in Tris-HCl buffer pH 7.5, containing 10 mM EDTA, as a 16S hexameric species (native 16S). The protein can be dissociated, 'stripped', into the subunit polypeptide chains carrying one active site by dialysis at high pH, against a buffer containing EDTA. However, the dissociation process leads to a stable mixture of 16S and 5S whose relative amounts differ from species to species. Following gel filtration chromatography on the Superdex 200 column under stripping conditions two fractions consisting of 16S (16S*) and 5S components were collected with both Hcs. From the 5S gel filtration pool of *Carcinus* Hc two monomers named *Cm*ss2 and *Cm*ss3 are isolated, in a 1:1.5 relative ratio, by means of ion exchange chromatography on a Superose Q column. The 5S fraction of *Homarus* Hc yields one markedly predominant component. The reassociation experiments performed in 20 mM phosphate buffer at pH 7.5, containing Ca^{2+} ions, show that the *Cm*ss3 component was completely converted to the hexamer, in contrast to *Cm*ss2 that does not give reassociation. For *Homarus* monomer a quantitative reassociation to 16S occurs after removal of EDTA and decreasing the pH in the presence of Ca^{2+}. Thus, by combination of gel filtration and ion exchange chromatography it is possible to obtain hexameric and monomeric forms of both *Carcinus* and *Homarus* Hcs. The hexameric species is derived either from native proteins or by reassociation of purified monomers. Each form was used for catechol oxidation experiments.

The oxidation of catechol in the presence of either *Carcinus* or *Homarus* Hcs is manifested by the time-dependent increase of absorbance at 400 nm reported in Fig. 1.

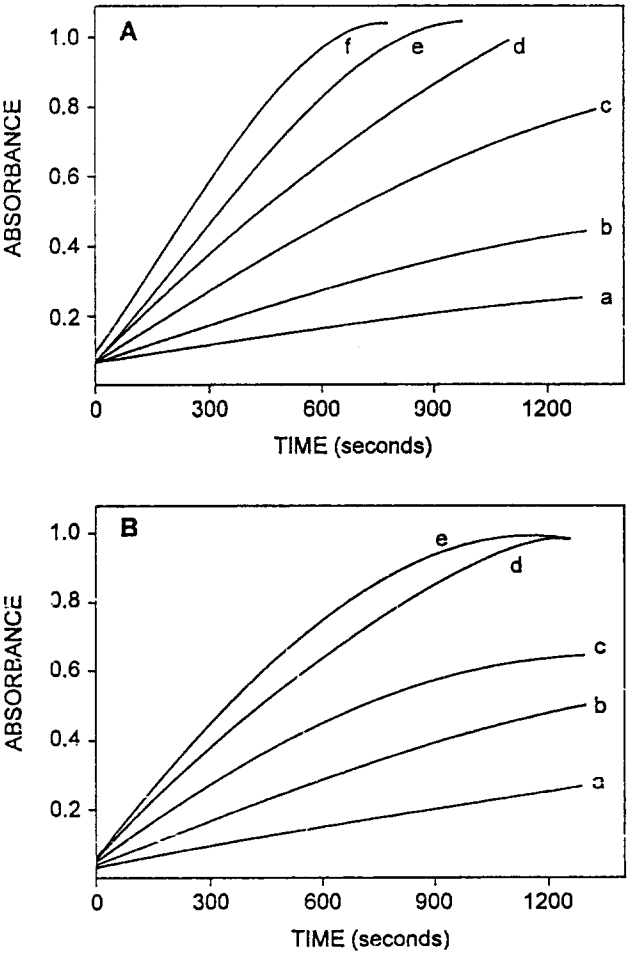

Fig. 1. Hc catalyzed oxidation of catechol. Time courses of quinone formation in the presence of: (A) *Carcinus* Hc (1.9 mg/ml, 25.6×10^{-6} M) and concentrations of catechol (a) 3.33×10^{-3} M, (b) 6.67×10^{-3} M, (c) 13.33×10^{-3} M, (d) 20.0×10^{-3} M, (e) 26.7×10^{-3} M, (f) 33.33×10^{-3} M; (B) *Homarus* Hc (1.5 mg/ml, 20.0×10^{-6} M) and concentrations of catechol as indicated in A. Buffer 20 mM phosphate pH 6.0 containing 200 mM (in A) and 50 mM (in B) perchlorate; t = 20°C.

The catecholase activity of both arthropod Hcs is enhanced by addition of perchlorate in the assay medium. In Fig. 2 the dependence of reaction velocity as a function of perchlorate concentration is shown. Control experiments show that the presence of perchlorate does not lead to changes of the Hc aggregation state. With both Hcs sigmoidal curves are obtained showing that the reaction velocity undergoes about a 5-and 1.5-fold increase with *Carcinus* and *Homarus* 16S Hcs, respectively, on increasing the perchlorate concentration. The concentration of anion to be included in the assay medium to increase the reaction velocity (50 or 200 mM sodium perchlorate in the case of *Homarus* and *Carcinus* Hcs, respectively) was taken from the plateau region of the curves. In Fig. 3, the dependence of the initial reaction velocity is reported versus protein concentration in the case of the hexameric forms of *Homarus* and *Carcinus* Hcs as well as *Carcinus* monomeric *Cm*ss2. All plots are linear with remarkably different slopes for the hexameric versus the monomer forms.

The kinetic parameters for all species, as determined from Lineweaver-Burk plots, are summarised in Table 1. The Michaelis constant K_m reveals only minor alterations in the binding affinity of Hcs both as 16S hexamers and 5S monomers. In

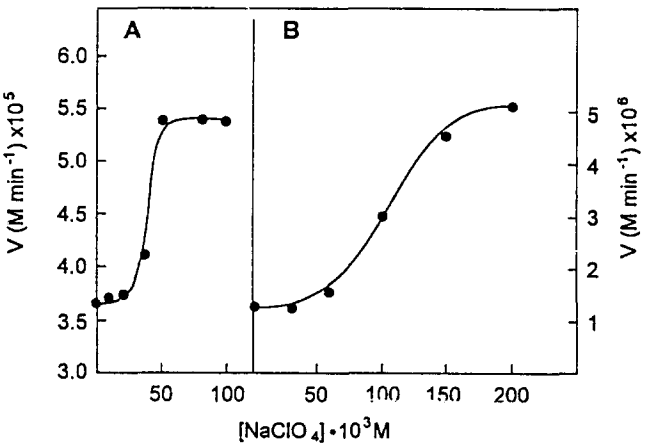

Fig. 2. Hc catalyzed oxidation of catechol. Effect of perchlorate on the reaction velocity. (A) *Homarus* 16S native Hc ([catechol] = 20.0 mM, [Hc] = 1.7 mg/ml); (B) *Carcinus* 16S native Hc ([catechol] = 1.4 mM, [Hc] = 2.0 mg/ml). Buffer 20 mM phosphate pH 6.0; t = 20°C.

contrast, marked changes in the V_{max} values are observed. The V_{max} found for the *Carcinus* monomer (*Cm*ss2) is about 11-fold higher than that of the native 16S hexamer and about 6-fold with respect to reassociated *Cm*ss3. We were not able to determine kinetic data for *Homarus* subunit because, upon removal of EDTA and decrease of the pH, the association process occurs. The kinetic parameters of reassociated *Homarus* Hc are remarkably similar to those of the native 16S hexamer.

4. Discussion

The efficiency of catechol oxidation by arthropod Hcs is very low, however, an increase of catalytic activity is observed in the presence of perchlorate. Previous studies [13] have shown that the presence of such an anion has the effect of increasing the rate of copper removal reaction by cyanide. In particular, perchlorate was found to be one of the most effective anions of the Hofmeister series in increasing the Hc active site accessibility. Accordingly, the peculiar sigmoidal shape observed for the plots of Fig. 2 can be interpreted by assuming that perchlorate induces a change in Hc conformation with concomitant increase of catechol oxidase activity. The effect is disclosed by an increase of V_{max} in the case of *Carcinus* and *Homarus* 16S Hcs when perchlorate concentration is raised to 200 mM (with *Carcinus* Hc) or 50 mM (with *Homarus* Hc). The possibility that the increase of activity is related to Hc dissociation induced by perchlorate is excluded by gel filtration control experiments. Thus, perchlorate was constantly included in the assay medium in order to improve the catalytic efficiency of Hc.

The plots of Fig. 3 together with the data of Table 1 give a clear indication that the isolated subunit has a greater efficiency than 16S species generated either from the native protein or by reassociation of dissociated subunits. The V_{max} of *Cm*ss2 is approximately one order of magnitude higher than that of *Carcinus* native 16S and approx. 6-fold as compared with reassociated *Cm*ss3 or native *Homarus* 16S Hc. Since the subunit can be isolated after alkaline treatment of the native protein, the question arises as to whether the observed differences in catalytic efficiency are related to irreversible conformational changes occurring on raising the pH. This point can be specifically addressed by comparing the results obtained with 16S*, remaining in the hexameric form after alkaline treatment, and native 16S. The data of Table 1 show that the two forms of the protein have quite similar properties (either in the absence or presence of perchlorate). Furthermore, the treatment of native Hc with stripping conditions followed by equilibration with the reassociation buffer does not affect the catechol oxidation parameters (data not shown). These pieces of evidence show that the exposure to alkaline pH does not affect the enzymatic efficiency, at least as far as the quaternary structure is retained. A somewhat higher enzymatic efficiency is exhibited by the *Carcinus* 16S species obtained by reassociation of *Cm*ss3, in comparison with native 16S (Table 1). The difference is probably related to the heterogeneity in subunit composition in the latter 16S in contrast with the single subunit type present in the former case. In agreement, with *Homarus* Hc, where one subunit is predominant, the catecholase efficiency is the same for native 16S or reassociated form.

Previous experiments addressed to the study of the hydrogen peroxide disproportionation by scorpion Hc [14] showed no significant differences between native proteins, subunits or proteolytic fragments in catalytic properties. In this case, however, since the steric hindrance of the substrate is comparable

Table 1
Kinetic parameters of catechol oxidation by arthropod hemocyanins in different aggregation states

Hemocyanin		K_m (mM)	V_{max} (mM min^{-1} mg^{-1})
Carcinus maenas			
16S native	$NaClO_4$	182	0.091
	–	142	0.023
16S*[a]	$NaClO_4$	167	0.033
	–	200	0.025
16S (*Cm*ss3)	$NaClO_4$	250	0.150
5S (*Cm*ss2)	$NaClO_4$	219	1.010
Homarus americanus			
16S native	$NaClO_4$	200	0.147
	–	250	0.074
16S*[a]	$NaClO_4$	192	0.042
5S reassociated	–	257	0.103

Reaction buffer: 20 mM phosphate pH 6.0 in the presence or absence of 50 mM perchlorate with *Homarus* or 200 mM perchlorate with *Carcinus* (for details see section 2).
[a]16S form isolated after 'stripping' native protein at alkaline pH in the presence of EDTA.

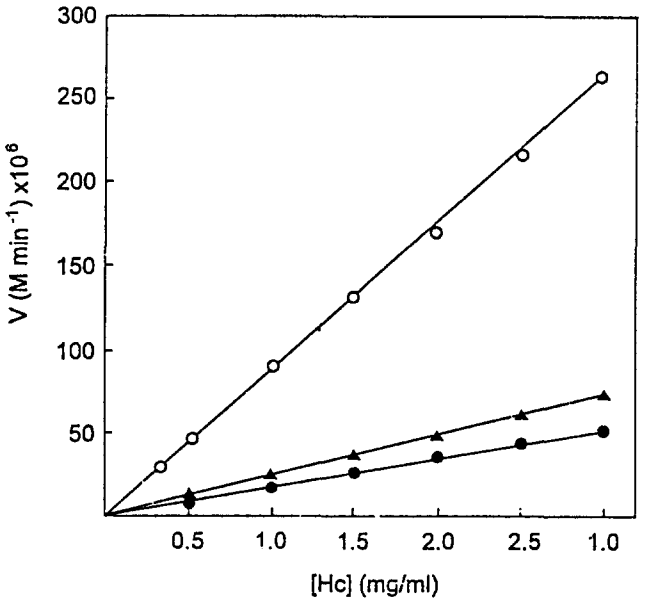

Fig. 3. Dependence of the rate of quinone formation versus protein concentration; (●) *Carcinus* 16S native Hc ([catechol] = 13.3 mM), (▲) *Homarus* 16S native Hc ([catechol] = 13.3 mM) and (○) *Carcinus CmSS2* 5S species ([catechol] = 10.0 mM). Buffer 20 mM phosphate pH 6.0; t = 20°C.

with dioxygen no differences are expected as long as the copper coordination sphere of native protein is retained upon treatment.

The elucidation of the tyrosinase gene sequence of *Neurospora crassa* has revealed that the protein is synthesised as a polypeptide with a C-terminal elongation of 200 amino acids as compared to the mature enzyme. This region shows distinct similarities with the C-terminal part of arthropod Hc (domain 3) folded so as to form a long protein loop shielding the active site [15].

The coordination of dioxygen as peroxide in a dinuclear copper active site as it is found in Ty and Hc has the ultimate effect of activating the ligand with respect to redox reactions involving *o*-diphenols. Steric effects are expected to play an important role in modulating the catalytic efficiency by affecting the accessibility of the active site towards exogenous molecules. Thus, the catecholase activity exhibited by molluscan [16] and arthropod Hcs should be considered a property of the protein resulting from the peculiar coordination of dioxygen strongly affected by the low accessibility of the active site to substrate molecules. This activity of Hc, therefore, can be considered as a residual property after the functional divergence of Hc from the more ancient tyrosinase.

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