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Folding of the polypeptide chain(s), conformational flexibility and reactivity of the metal active site of hemocyanin and tyrosinase

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Summary. The comparative accessibility of the active sites of hemocyanin and tyrosinase, two proteins containing a binuclear type-3 copper site, has been investigated. The approaches were: (a) the kinetic study of the reaction of hemocyanin with cyanide in the presence of conformation perturbants; (b) the comparison between the kinetic parameters of the cyanide reaction on hemocyanin and tyrosinase; (c) the study of the efficiency and reaction mechanism of hemocyanin interaction with a typical tyrosinase substrate like catechol. The results indicate that the active site of tyrosinase is much more exposed than that of hemocyanin.

Key words: Hemocyanin - Tyrosinase - Protein conformation - Active site

Introduction

Tyrosinase (Ty) and hemocyanin (Hc) are two members of a large family of metalloproteins which contain a binuclear copper (type-3) active site but perfom different biological functions. Particularly strong similarities exist between Ty and Hc in that they contain exclusively 'type-3' copper sites, in contrast to the proteins generally referred to as 'blue' copper oxidases in which additional metal sites (type-1 and type-2 sites) are also involved in the catalytic cycle. The type-3 site of Ty and Hc bind molecular oxygen reversibly. In the deoxy form, the metal is present in the cuprous state and both proteins are diamagnetic and colourless. Binding of O_2 occurs via electron transfer from each Cu(I) ion to dioxygen; the resulting complex can be depicted as $[Cu(II) \cdot O_2^{2-} \cdot Cu(II)]$ where the peroxide dianion binds both cupric ions as a μ-peroxo-bridge (Solomon 1981; Lerch 1981). Both oxy-Ty and oxy-Hc are characterized by a unique absorption spectrum in the near ultraviolet-visible region displaying a strong band at approximately 340 nm ($\varepsilon \approx 18000\ M^{-1}\ cm^{-1}$ for each binuclear site) and a broader feature at approximately 570 nm ($\varepsilon \approx 1000\ M^{-1}\ cm^{-1}$ for each binuclear site).

Recent sequence comparisons have shown that Ty and Hc exhibit a remarkable degree of similarity in the regions containing histidinyl residues which are involved in metal coordination ('Cu_A' and 'Cu_B' amino acid clusters; Huber and Lerch 1986; Lerch and Germann 1988). In spite of the large similarity of their copper sites, Hc and Ty serve different biological functions: the former is an oxygen carrier whereas the latter utilizes dioxygen to *o*-hydroxylate monophenols (monophenolase activity) and/or to oxidize *o*-diphenols to quinones (catecholase activity). Kinetic, equilibrium and spectroscopic studies have shown 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 *ortho* position of the aromatic ring by peroxide (monophenolase activity) and/or to electron transfer involving copper (catecholase activity). Furthermore, it appears that the protein pocket surrounding the active site contributes to stabilizing the substrate binding (Lerch 1981; Wilcox et al. 1985). The greater buried nature and rigidity of the Hc active site, rather than intrinsic structural differences, are proposed to be responsible for the very low levels of tyrosinase-like activity in Hc (Nakahara et al. 1983; Wilcox et al. 1985; Huber and Lerch 1986). In this work, we have studied the reaction of the Ty active site with cyanide, a strong field ligand of Cu(I) capable of giving both a ligand-ligand competition reaction with oxygen and of extracting the metal from the active site. The interaction of both molluscan and arthropodan Hcs with the typical Ty substrates was also studied.

Materials and methods

Hc (from *Octopus vulgaris* or *Carcinus maenas*) and Ty (from *Neurospora crassa*) were prepared and characterized according to Beltramini et al. (1984a, b) and Lerch (1987) respectively. Protein concentrations were calculated spectrophotometrically at 278 nm

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using the absorption coefficients 1.41 ml mg^{-1} cm^{-1}, 1.24 ml mg^{-1} cm^{-1} and 2.28 ml mg^{-1} cm^{-1} for *Octopus* Hc, *Carcinus* Hc and *Neurospora* Ty, respectively (Beltramini et al. 1984a,b; Lerch 1987). Ty activity was measured according to Lerch (1987). Catechol oxidation to *o*-quinone by Hc was followed spectrophotometrically at 400 nm ($\varepsilon = 1417\ M^{-1}\ cm^{-1}$) in 20 mM phosphate pH 6.0 ($t = 21°$ C). Experiments with CN^- were performed in 0.1 M Tris/HCl pH 8.0 ($t = 21°$ or 4° C) in the case of Hc and 0.1 M phosphate pH 8.0 ($t = 4°$ C) with Ty. The reaction with CN^- was followed either by measuring the decrease of absorbance at 345 nm or the loss of Ty specific activity (the value for native Ty is 1,200 U/mg; Lerch 1987).

Results and discussion

The reaction of native Hc with CN^- involves two processes: a competition reaction between two ligands (O_2 and CN^-) for the binuclear cuprous site of deoxy-Hc and the stepwise removal of the metal from the active site according to Eqs. (1-5) (Beltramini et al. 1984):

$$\mathrm{Hc} + \mathrm{O_2} \overset{K_{O2}}{\rightleftharpoons} \mathrm{HcO_2} \quad (1)$$

$$\mathrm{Hc} + \mathrm{CN^-} \overset{K_{CN}}{\rightleftharpoons} \mathrm{HcCN^-} \quad (2)$$

$$\mathrm{HcCN^-} + \mathrm{CN^-} \overset{k_1}{\rightleftharpoons} \mathrm{Hc^\circ} + [\mathrm{Cu(CN)_2}]^- \quad (3)$$

$$\mathrm{Hc^\circ} + \mathrm{CN^-} \overset{K^\circ_{CN}}{\rightleftharpoons} \mathrm{Hc^\circ CN} \quad (4)$$

$$\mathrm{Hc^\circ CN} + \mathrm{CN^-} \overset{k_2}{\rightleftharpoons} \mathrm{Hc^{\circ\circ}} + [\mathrm{Cu(CN)_2}]^- \quad (5)$$

where Hc, Hc°, Hc°° indicate deoxy-, half-apo-Hc [containing a single Cu(I) ion/active site] and the apoprotein, respectively.

A recent investigation on the interaction of CN^- with the Hc from *Octopus vulgaris* and *Carcinus maenas*, as representative species of Mollusca and Artropoda respectively, showed that the reaction pathway has a general validity (Beltramini et al. 1984a,b). Strong differences in the kinetic behaviour are, however, disclosed between the Hcs from the two phyla and with the same Hc species upon changing the aggregation state of the protein: these can be related to the different accessabilities to the active site controlled by the protein matrix and to the site-site interactions controlled by the protein tertiary and quaternary structure. These results indicate that both the CN—O_2 ligand-ligand exchange and the copper removal reaction are under steric control and, hence, CN^- can be conveniently used to probe the accessibility of the Ty active site with respect to both O_2 displacement and metal removal. Under appropriate experimental conditions, the interaction of CN^- with Hc or Ty can be studied under equilibrium conditions, namely according to Eqs. (1) and (2). It is also possible to control the reaction to yield the removal of a single copper ion (Eqs. 1-3) or the complete metal release by proteins (Eqs. 1-4). The effect of CN^- on Ty at concentrations below 100 μM is a reversible decrease of the intensity of the 345-nm band, indicating that the ligand competitively displaces oxygen. The metal is not lost as indicated by the constancy of specific activity, after CN^- removal, throughout the titration experiment. Ligand titration data are presented in Fig. 1 in the form of the saturation function:

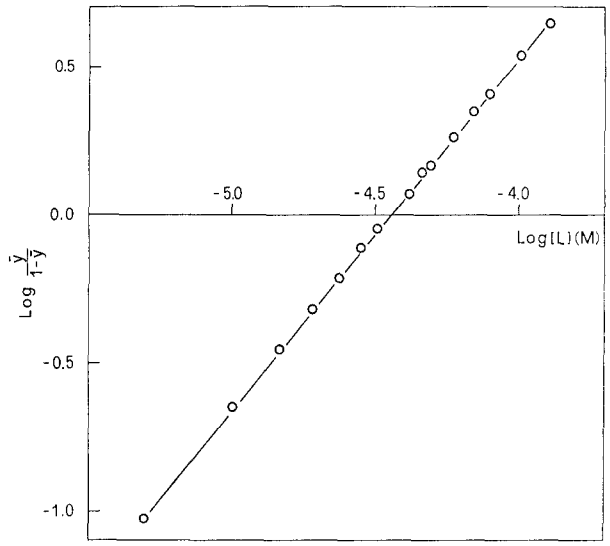

Fig. 1. Equilibrium of *Neurospora* tyrosinase with cyanide. Hill plots (Eq. 6) obtained by titrating in air solutions of the protein in the oxygenated form with increasing amounts of cyanide

$$\mathrm{Log}\left(\frac{\bar{y}}{1-\bar{y}}\right) = \mathrm{p}K(\mathrm{r}) + \log(\mathrm{L}) \quad (6)$$

where $\bar{y}$ is the fractional concentration of Ty-CN complex and $K(\mathrm{r}) = (K_{CN}/K_{O_2}) \cdot [O_2]^{-1}$, the oxygen concentration in the system being constant. The ligand concentration [L] is taken as equal to that of the total cyanide added. K_{CN} (M) and K_{O_2} (M) represent the dissociation constants of Ty for CN^- and O_2, respectively. It is important to consider, however, that $K(\mathrm{r})$ is only formally related to the individual dissociation constants since the population of protein molecules contains species with active sites ligated by either cyanide or dioxygen.

Data points of Fig. 1 can be interpolated by a single straight line with a slope of 0.9, a value very close to unity. The ratio of equilibrium constants for the two competitive ligands K_{CN}/K_{O_2} is equal to 9.6 taking into account $[O_2] = 0.35$ mM at 10° C. In contrast to Ty, the titration data on *Octopus* Hc (Giacometti et al. 1988) can not be interpolated assuming a single equilibrium constant: the shape of the Hill plot can be fully accounted for by negative cooperation due to allosteric interaction linking the different functional units during the CN^- saturation process (Giacometti et al. 1988). The peculiar behaviour of *Octopus* Hc versus Ty is based on its structural organization: this multi-site protein is made up of different functional units, each carrying one active site, in contrast to the monomeric state of Ty. The K_{CN}/K_{O_2} ratio measured for *Octopus* Hc under conditions of high affinity is reported in Table 1.

Table 1. Cyanide reaction on hemocyanins (Hc) and tyrosinase (Ty): comparison of reaction parameters

Protein	K_{CN}/K_{O_2}	k_1 ($M^{-1} s^{-1}$)
Hc *Carcinus*	135[a]	0.8
Hc *Octopus*	37.7[b]	0.5[c]
Ty	13.7 (9.6)[d]	0.6

[a] Recalculated from Beltramini et al. (1984b)
[b] Calculated from Giacometti et al. (1988); value relative to the high-affinity condition in ligand equilibrium experiments
[c] Taken from Beltramini et al. (1984a)
[d] Measured from the kinetic approach (Eq. 7); the value in parentheses is obtained from ligand equilibrium experiments (Eq. 6)

At higher concentrations of CN^-, a time-dependent decrease of specific activity and absorbance at 345 nm is observed. This effect is related to loss of protein-bound copper, as indicated by the concomitant decrease of the metal/protein stoichiometry and by the fact that, upon removing CN^-, the properties of native proteins are not fully restored. This is a pseudo-first-order process for both Ty and *Carcinus* Hc (Fig. 2). In the case of *Octopus* Hc, a continuous decrease of rate constant is observed, again attributable to negative site-site interactions between the functional units (Beltramini et al. 1984a).

The equilibrium and bimolecular constants of reactions 1-3 can be calculated by the following equation (Beltramini et al. 1984b):

$$\frac{[CN^-]}{k_{app}} = \frac{1}{k_1} + \frac{1}{k_1} \cdot \frac{K_{CN}[O_2]}{K_{O_2}[CN^-]} \tag{7}$$

where k_{app} is the pseudo-first-order rate constant of the decrease of concentration of native protein molecules.

In order to correlate the kinetic and equilibrium parameters with the conformational state of the proteins, in a first approach, the kinetics of the CN^- reaction on native Hc (from the arthropod *Carcinus maenas*) is followed under conditions in which the conformation of the protein is modified, namely by the presence of agents capable of inducing swelling (I^-, ClO_4^-, SCN^-)or of increasing compactness (Ca^{2+}) of the molecule. The parameters relative to the displacement of the first copper ion under the different experimental conditions are reported in Table 2 (data at 21° C). The bimolecular rate constant of metal removal is essentially not affected by the perturbants. In contrast, K_{CN}/K_{O_2} decrases in the presence of anions or increases upon Ca^{2+} addition. These results indicate that the accessibility to CN^- increases or decreases respectively (K_{CN}/K_{O_2} is defined as $[HcO_2][CN^-]/[HcCN][O_2]$), in agreement with the expected effects on protein structure and with the expected order of effectiveness of the anions; the dissociation of the HcCN complex is, however, not affected (Beltramini et al. 1988). By applying Eq. (6), it is found that the value of k_1 for Ty (0.5 $M^{-1} s^{-1}$) is quite similar to that obtained with *Carcinus* Hc ($k_1 = 0.8$ $M^{-1} s^{-1}$) (Table 1). The ratio K_{CN}/K_{O_2} is 13.7, quite similar to the value obtained by equilibrium experiments (9.6), indicating the consistency of the two approaches. This value, however, is more than 10-fold lower than that obtained for *Carcinus* Hc (Table 1). The greater reaction efficiency in the case of Ty is attributable to its smaller K_{CN}/K_{O_2}. A difference of one order of magnitude as compared to arthropod Hc is similar to the effect brought about by perturbing the Hc conformation. A much smaller difference exists between Ty and molluscan Hc, although a small underestimation may result from the difference in the temperature at which the measurements were performed (4° C) versus 21° C). In any case, the capability of both molluscan Hc and *Neurospora* Ty to undergo a simple ligand-ligand exchange reaction can be attributed to the accessibility of the active sites being not very different from each other. These results show that, as far as the relative reactivity of the binuclear active site for exogenous ligands is concerned, Ty can be viewed as an Hc with a much more exposed active site, although the evaluation

Table 2. Changes of the ratio of equilibrium constants of *Carcinus* hemocyanin with cyanide and oxygen (K_{CN}/K_{O_2}) and of the bimolecular rate constant of copper removal (k_1) under different experimental conditions

Condition	K_{CN}/K_{O_2}	k_1 ($M^{-1} s^{-1}$)
Pure buffer	50	1.5
I^- (1 M)	69	1.5
ClO_4^- (1 M)	10	1.3
SCN^- (1 M)	1.5	1.5
Ca^{2+}:		
1.9 mM	57	1.5
3.8 mM	71	1.3
5.2 mM	110	1.7
6.1 mM	120	1.8
7.9 mM	150	1.5

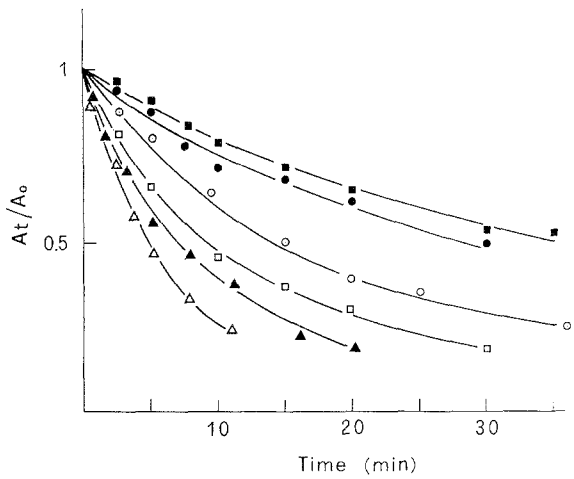

Fig. 2. The reaction of CN^- with tyrosinase. Time dependence of the decrease of the specific activity of *Neurospora* tyrosinase in the presence of CN^- at concentrations of 2.0 mM (■), 2.4 mM (●), 3.8 mM (○), 4.8 mM (□), 5.9 mM (▲), 7.9 mM (△). A_0 and A_t indicate the specific activity at time zero and time t after addition of cyanide

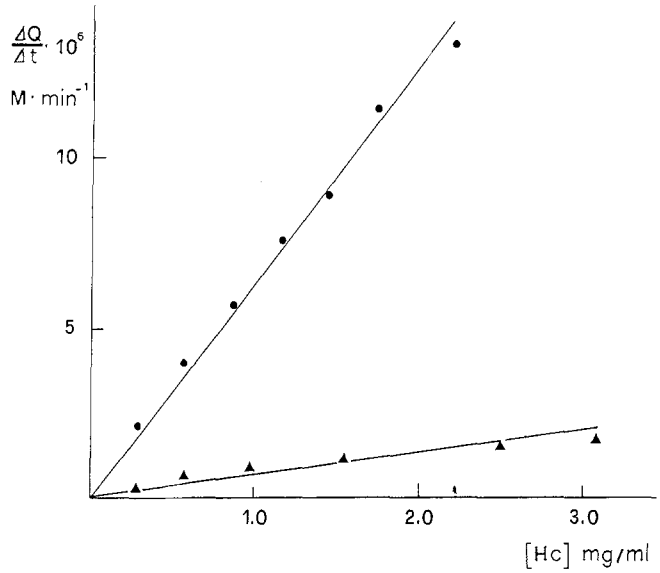

Fig. 3. Dependence of the rate of quinone formation versus protein concentration. Hemocyanins from *Octopus* (Mollusca) (●) and *Carcinus*(Arthropoda) (▲); catechol concentration 0.9 mM in the case of *Octopus* and 1.4 mM in the case of *Carcinus* hemocyanin

of separate equilibrium constants can not be made since K_{O_2} for Ty is still undetermined.

The study of the interaction of Hc with a typical Ty substrate like catechol also provides useful information for comparing the accessibility of active sites. Hcs can oxidize catechol to *o*-quinone, although at a much slower rate than Ty. Furthermore, the oxidation rate (mol quinone formed/min) is found to be higher in *Octopus* (Mollusca) Hc than in *Carcinus* (Arthropoda) Hc (Fig. 3).

In view of the results obtained for the CN^- reaction in the presence of conformation perturbants, the oxidation rate has been measured upon increasing concentrations of perchlorate. As shown in Fig. 4, a five-fold enhancement of reaction efficiency is observed on going to 0.35 mM perchlorate. The trend of data points, furthermore, suggests that a conformational transition may be responsible for the observed increase.

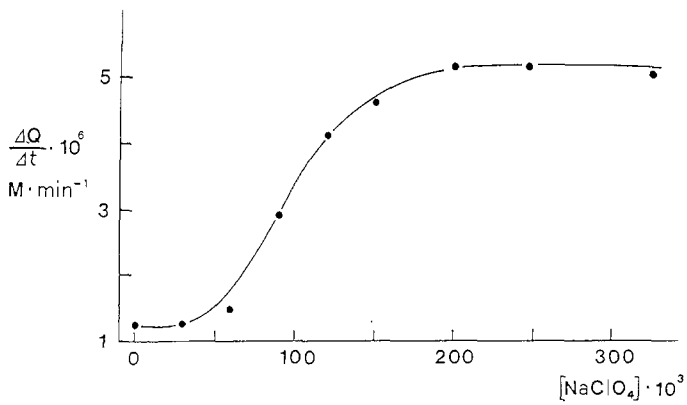

Fig. 4. Dependence of the rate of quinone formation versus $NaClO_4$ concentration. Hemocyanin from *Carcinus* (2.0 mg/ml), catechol concentration 1.43 mM

Molecular oxygen is consumed during the reaction in a stoichiometric ratio of 1.27 mol/2 mol quinone. This value is significantly higher than that found with Ty (1 mol O_2/2 mol quinone), suggesting that the reaction mechanisms are also different. In particular, in the presence of Hc, a semiquinone radical EPR signal can be observed, indicating that the oxidation occurs via a one-electron process involving free-radical formation, in contrast to Ty where the quinone is formed through an internal two-electron redox reaction involving copper and bound peroxide (Wilcox et al. 1985). A free-radical mechanism could result from the interaction of substrate to Hc active site via bound peroxide rather than directly to copper, as has been reported for Ty (Lerch 1981).

In conclusion, it is proposed that the difference in physiological roles of the two proteins is related to a modulation of the protein matrix on the accessibility of the active site: only Ty can readily interact with molecules having a large steric hindrance, whereas for Hc rapid exchange can occur only for molecules as small as dioxygen. Furthermore, the capability of Hc to oxidize diphenols may constitute an interesting indication that the biological role of binuclear copper sites may consist in oxygen activation as a consequence of its binding in the form of peroxide. These results further support the view that the catalytic properties of Ty are related to, although not completely determined by, the greater accessibility of the active site to phenolic substrates as compared to Hc. The active site of the latter protein appears to behave as a strongly asymmetric structure in which the biochemical differences of each metal ion are stressed by the protein moiety (Beltramini et al. 1984a,b; Giacometti et al. 1988; Beltramini et al. 1988). Recent structural analysis of the *Neurospora* tyrosinase gene revealed the presence of a C-terminal elongation corresponding to 200 amino acids. This structure is removed, probably by proteolysis, according to a typical post-transcriptional activation process of the enzyme (Lerch and Germann 1988; Kupper et al. 1989). Removal of this structure in tyrosinase would therefore lead to the activation of the enzyme which becomes capable of binding and oxidizing organic substrates. This extension shows distinct similarity to the C-terminal region of arthropod hemocyanins, corresponding to the so-called domain 3 of the protein. The domain contains a long loop making contact with domain 1, thus shielding the active site present in domain 2, and preventing its interaction with large ligands. Quite interestingly, molluscan hemocyanins are more similar to active *Neurospora* tyrosinase than to arthropod hemocyanin as far as the length of the polypeptide chain in the C-terminal region is concerned. The results of this work correlating the properties of the active site with its exposure towards exogenous ligands further support the current view on the structure/function relationship of Ty and Hc.

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