

***Viviparus ater* Hemocyanin: Investigation of the Dioxygen-Binding Site and Stability of the Oxy- and Apo-Forms**

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The active site of *Viviparus ater* (mollusc) hemocyanin was investigated using the fact that the binding of dioxygen to the binuclear copper-containing sites of hemocyanins is connected with the appearance of specific dichroic bands which are very sensitive to changes in the structure and polarity of the environment. Oxy-*Viviparus ater* hemocyanin exhibits near UV and visible circular dichroism spectra different from those of other molluscan and arthropodan hemocyanins. These differences are due probably to variations in the geometry or charge distribution in the dioxygen binding sites of the compared proteins.

The thermostability of *Viviparus ater* hemocyanin and the significance of the copper-dioxygen system for the stability were also investigated. "Melting" temperatures, T_m , of 77 °C for the oxy-hemocyanin and 57 °C for the apo-protein were calculated from the denaturation curves which demonstrates the considerable role of the binuclear active site for the thermostability. *Viviparus ater* hemocyanin is more thermostable than other hemocyanins for which data are published.

Introduction

Hemocyanins (Hcs) are copper-containing dioxygen-transporting proteins freely dissolved in the hemolymph of mollusca and arthropoda. These giant biopolymers are the most complex and sophisticated respiratory proteins, comparable in size to ribosomes or small viruses (Sternier and Decker, 1994). Hcs bind dioxygen reversibly at a binuclear active site and ensure its circulatory transport to the tissues. In the hemolymph, Hcs exist as aggregates of subunits (polypeptide chains). Although similar in function, arthropodan and molluscan respiratory proteins differ in the organization and size of the subunits. Arthropodan Hcs are composed of six or multiples of six subunits with a molecular mass of the individual polypeptide chain in the region of 67–90 kDa (Herskovits, 1988). Each structural/functional subunit contains a single binuclear copper-containing dioxygen-binding site. Molluscan Hcs form cylindrical mono- (cephalopods) or didecameric (gastropods) structures. The wall of the decamer is made up of

60 globular functional units (FUs) and an internal „collar“ containing 10 or 20 functional units (Lieb *et al.*, 2000). The subunits are composed of 7 (cephalopods) or 8 (some cephalopods and gastropods) 50 kDa FUs. Each FU contains a single active site (van Holde *et al.*, 1992). During the last decade some Hcs were used for medical purposes. Thus, the Hc from the gastropod *Megathura crenulata* is widely used in laboratories and clinics as an immune stimulant (Jennemann *et al.*, 1994), in the immunotherapy of cancer (Lamm *et al.*, 1993) as well as for the preparation of vaccines (Wang *et al.*, 2000).

The complete amino acid sequences of two molluscan Hc structural subunits, those from the cephalopod *Octopus dofleini* (Miller *et al.*, 1998) and gastropod *Haliotis tuberculata* (Lieb *et al.*, 2000), as well as the complete molecular structure of the arthropod Hc from *Euripelma californicum* (Voit *et al.*, 2000) have been determined. The first crystallographic structure of a FU from molluscan Hc has recently been reported (Cuff *et al.*, 1998). The structure of a molluscan Hc didecamer, HtH1

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from the *H. tuberculata*, has been solved at 1.2 nm resolution by cryoelectron microscopy (Meissner *et al.*, 2000).

The copper-dioxygen system at the Hc active site generates CD spectra in the visible region and near ultraviolet (Ellerton *et al.*, 1983). In this paper we describe studies performed with the aim of characterizing the binuclear active site of the *Viviparus ater* (gastropod, mollusc) Hc and its significance for the protein thermostability.

Materials and Methods

Materials

Viviparus ater hemocyanin was a generous gift from Professor B. Salvato (University of Padova, Italy). The protein in oxy-form was purified by the method described in (Ricchelli *et al.*, 1984). Apo-Hc (hemocyanin in which the copper-dioxygen system is removed) was obtained by dialysis against 25 mM KCN (Salvato *et al.*, 1974). All chemicals and reagents were of analytical grade.

Amino acid analysis

The copper ions were completely removed from the protein samples by precipitation of the apo-protein with trichloroacetic acid and washing the pellet with several portions of 0.1 M HCl. Amino acid composition of the *Viviparus ater* Hc was determined after hydrolysis in 5.7 M HCl in evacuated sealed tubes for 24, 48 and 72 h at 110 °C. For tryptophan determination the samples were hydrolyzed in the presence of 5% thioglycolic acid. An Eppendorf model LC 3000 automatic amino acid analyzer was used. The values are expressed as number of amino acid residues per functional unit of 50 kDa.

Spectroscopic measurements

Circular dichroism spectra were recorded and the thermal denaturation of the oxy- and apo-Hc followed using a Jasco J-720 dichrograph, equipped with a personal computer IBM PC-AT, PS/2, multiscan monitor CMS-3436 and a Hewlett-Packard colour graphics plotter model HP 7475 A. A DOS software was used for calculations with the CD data. Protein solutions in 50 mM Tris-(hydroxymethyl)-aminomethan hydrochloride buffer, pH 7.0, containing 5 mM CaCl₂, were placed in a

cell holder which was thermostatically controlled using a NESLAB thermostat model RTE-110, connected with a digital programming controller. The samples were kept for 10 min at the desired temperature to ensure the attainment of thermal equilibrium, which was confirmed by the constancy of the ellipticity. Each spectrum presented is an average of three measurements. The CD data are expressed in terms of $[\theta]$, i. e. the mean residue molar ellipticity. The protein concentration was determined using $\epsilon_{280} = 7.14 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ per functional unit.

Results and Discussion

Table I shows the amino acid composition of *Viviparus ater* Hc. A relatively high content of acidic amino acids, Asp and Glu, was observed. The amount of these amino acids is 21% of the total content. Comparison with the compositions of other molluscan Hcs (Ghiretti-Magaldi *et al.*, 1966) shows similarities.

Far UV CD spectra of oxy-*Viviparus ater* Hc at different temperatures are shown in Fig. 1. The spectra are dominated by negative bands at 220–222 nm and a shoulder at 210 nm, connected mainly with the α -helix structure. Similar spectra

Table I. Amino acid composition of *Viviparus ater* hemocyanin. The samples are hydrolyzed in 5.7 N HCl or in 5% thioglycolic acid for 24, 48 and 72 h at 110 °C. The values are expressed as number of amino acid residues per functional unit of 50 kDa.

Amino acid	<i>Viviparus ater</i> hemocyanin
Aspartic acid	47
Threonine	22
Serine	27
Glutamic acid	44
Proline	26
Glycine	29
Alanine	30
Half cystine	6
Valine	23
Methionine	8
Isoleucine	16
Leucine	35
Tyrosine	20
Phenylalanine	28
Histidine	29
Lysine	19
Arginine	21
Tryptophan	8
Total	438

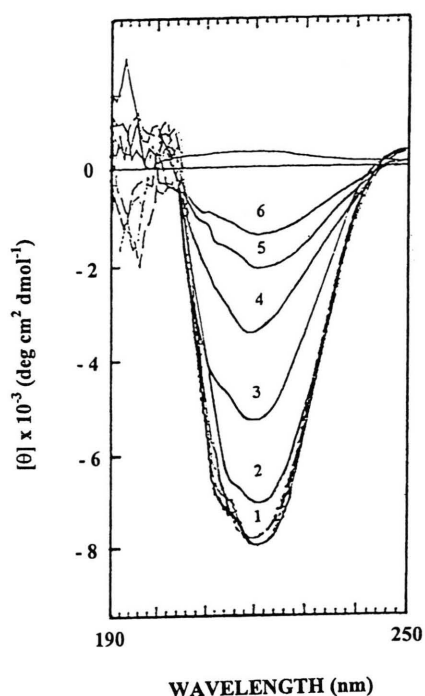


Fig. 1 Characteristic circular dichroism spectra in the far ultraviolet region of oxy-*Viviparus ater* hemocyanin at pH 7.0. The spectra are recorded at the following temperatures: (1), 15–40 °C; (2), 65 °C; (3), 75 °C; (4), 78 °C; (5), 81 °C and (6), 84 °C.

in the peptide region below 250 nm, were observed for other Hcs.

Two main transitions in the CD spectrum of the oxy-Hc were observed between 250 and 300 nm with bands at 254 and 294 nm and small shoulders at 275 and 287 nm (Fig. 2). The spectrum in the near UV region is due to contributions of aromatic residues. The 254 nm band may be due to tyrosine transitions (Tamburro *et al.*, 1976). The presence of this band in the spectrum of apo-Hc suggests that the transition does not arise from a chromophore associated with the active site. The band at 294 nm is mainly from contributions of tryptophyl residues.

The 300–800 nm region in the spectrum of oxy-*V. ater* Hc is characterized by 4 main transitions. The bands in this region are characteristic of the active site copper-dioxygen system and disappear on destruction of the active site (Figs. 2 and 3). This is demonstrated by the CD spectra of the apo-Hc shown in the same figures. The CD spectrum in the 250–500 nm range, reported in

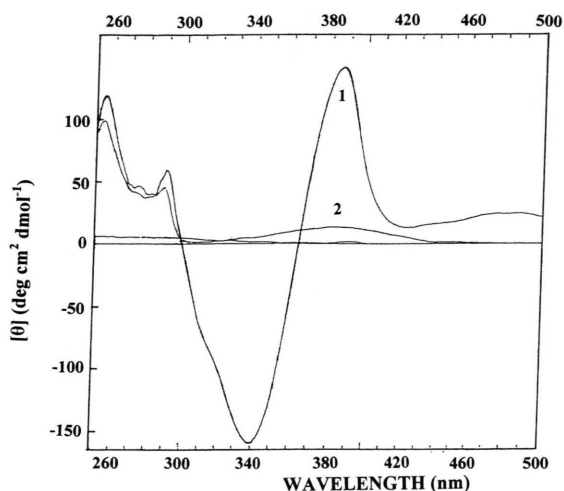


Fig. 2 250–500 nm circular dichroism spectra of oxy-(1) and apo-(2) *Viviparus ater* hemocyanin at pH 7.0.

Fig. 2, is dominated by a strong negative band at 340 nm and a positive band at 387 nm. The negative 340 nm band is common for the Hcs of both *phyla*, Arthropoda and Mollusca, and has been assigned to the peroxide $\pi\sigma^* \rightarrow \text{Cu(II)} d(x^2 - y^2)$ charge transfer transition (Beltramini *et al.*, 1992). The CD bands in the spectra of Hcs above 300 nm should be attributed to the bicupric-peroxo system at the active site (Ellerton *et al.*, 1983).

Oxy-*Viviparus ater* Hc exhibits near UV and visible CD spectra (Figs. 2 and 3), different from those of other molluscan and arthropodan oxy-Hcs. Thus, the strong positive band at 387 nm is absent in the CD spectra of the other Hcs, reported so far (Tamburro *et al.*, 1976; Ellerton *et al.*, 1983; Salvato and Beltramini, 1990; Boteva *et al.*, 1991; Beltramini *et al.*, 1992). Arthropod oxy-Hcs have negative bands at 260–300 and 340 nm and positive bands at 490 and 610 nm. Molluscan oxy-Hcs exhibit negative bands at 340 and 570 nm and positive bands in the region of 260–300 nm, at 450 and 710 nm (Ellerton *et al.*, 1983).

In the visible CD spectrum of oxy-*V. ater* Hc a small positive dichroism is observed in the 450–520 nm region (Fig. 3). Also, negative dichroism is observed between 520 and 720 nm with a shoulder at 610 nm and a strong negative peak at 658 nm (Fig. 3). The CD spectra of other molluscan Hcs in this spectral region, reported in the literature so far, do not exhibit a strong negative band at

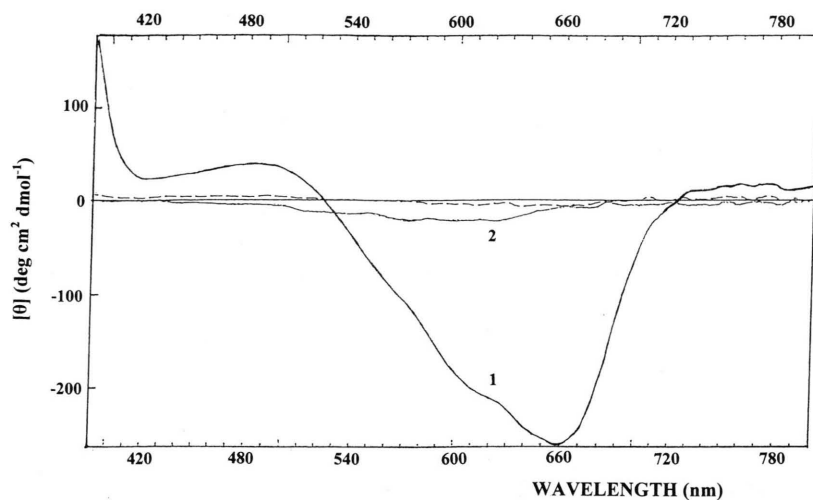


Fig. 3 Circular dichroism spectra of oxy-(1) and apo-(2) *Viviparus ater* hemocyanin in the visible spectral range at pH 7.0.

658 nm; they have a positive maximum around 700 nm and negative bands at 570–580 nm (Tamburro *et al.*, 1976; Ellerton *et al.*, 1983; Boteva *et al.*, 1991). The differences between the CD spectrum above 365 nm of oxy-*V. ater* Hc and the respective spectra, reported for molluscan and arthropodan Hcs, are due probably to geometric variations in the dioxygen binding sites. The bands in this spectral region are absent in the CD spectra of apo-*V. ater* Hc with a destroyed active site (Figs. 2 and 3). Differences in position and surrounding of side chains near to this site and any changes in the charge distribution at the active site can create changes in optical activity caused by the copper-dioxygen system.

In order to investigate the thermostability of *V. ater* Hc and the significance of the copper-dioxygen system at the active site for the stability, we followed changes in the ellipticity of hemocyanin samples at temperatures up to 85 °C (Fig. 1). A decrease of the negative ellipticity was observed at temperatures higher than 60 °C for the oxy-Hc and 40 °C for the apo-Hc. Single wavelength melting curves (SWMCs), measured by CD, are shown in Fig. 4. We have followed only the forward reaction because the thermal denaturation of both, oxy- and apo-form, was irreversible. The irreversibility of the Hc denaturation is probably connected with an aggregation of the giant biomolecules (Guzman-Casado *et al.*, 1990). “Melting”

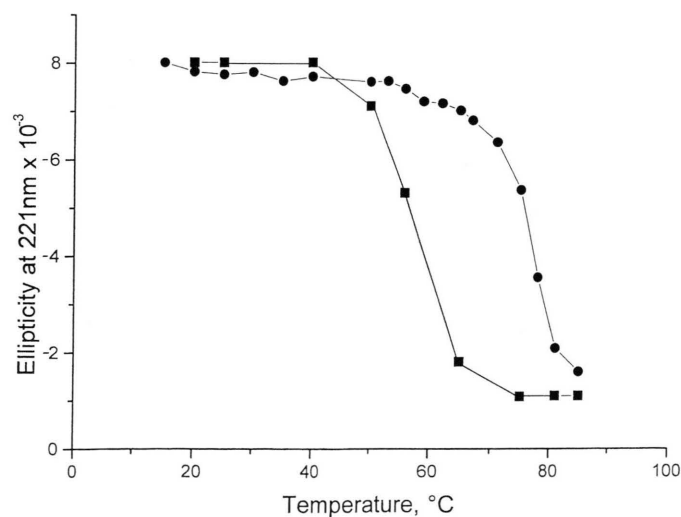


Fig. 4 Single wavelength melting curves, measured by circular dichroism, for the thermal denaturation of oxy- (●—●) and apo- (■—■) *Viviparus ater* hemocyanin at pH 7.0. T_m values of 77 and 57 °C were calculated for the oxy- and apo-form, respectively. The data are expressed in terms of mean residue molar ellipticity.

temperatures, T_m , of 77 °C for the oxy-Hc and 57 °C for the apo-protein were calculated from the midpoints of SWMCs (Fig. 4). This result demonstrates the considerable role of the binuclear active site for the thermostability of hemocyanin molecule. The removal of the copper-dioxygen system from the active site led to a decrease of the "melting" temperature by 20 °C. Comparison with the thermostability of other molluscan Hcs, for

which data are published (Georgieva *et al.*, 1998), shows that the respiratory protein from *Viviparus ater* is more stable by 11–21 °C in its melting point.

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