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o-Diphenol oxidase activity of molluscan hemocyanins

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Abstract

Diphenoloxidase activities of two molluscan hemocyanins, isolated from the marine snails *Rapana venosa* and garden snails *Helix vulgaris* were studied using *o*-diphenol and L-Dopa as substrates. The dimers of *H. vulgaris* Hc show both, diphenol (K_m = 2.86 mM and K_{cat} = 4.48) and L-Dopa activity due to a more open active sites of the enzyme and better access of the substrates. The K_m value of molluscan *H. vulgaris* Hc is very close to those of *Helix pomatia* and *Sepia officinalis* Hcs, but several times higher compared to those of *Rapana* and *Octopus* Hcs. Also HvH has a very high enzyme activity compared with other molluscan Hcs. Kinetic measurements with native RvH and both structural subunits, RvH1 and RvH2, show that RvH and only one structural subunit, RvH2, exhibited only *o*-diphenol activity, but no L-Dopa oxidizing activity.
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1. Introduction

Type-3 copper proteins have evolved from a dinuclear processor which segregated into hemocyanins (Hcs), tyrosinases (Ty) and phenoloxidases. Molluscan and arthropodan Hcs as well as tyrosinases are copper-containing proteins with similar active sites, but they differ largely with respect to size, primary, tertiary and quaternary structure and their physiological functions (Dolashka et al., 1996; Dolashka-Angelova et al., 2005; Jaenicke and Decker, 2004a,b; Van Holde et al., 2001). While hemocyanins transport dioxygen, phenoloxidase uses one oxygen of the dioxygen molecule for chemical transformations.

Hemocyanins are large multisubunit copper proteins composed of different subunit types and found freely-dissolved in the hemolymph of arthropods and molluscs (Markl and Decker, 1992; Salvato and Beltramini, 1990; Van Holde and Miller 1982; Van Holde and Miller, 1995). Their dioxygen transport and storage function is based on their capability to bind reversibly molecular oxygen at their active sites to which a pair of copper ions is attached (dinuclear-coupled copper site; Van Holde and Miller, 1995).

Phenoloxidases catalyze *o*-hydroxylation of monophenols (cresolases), oxidation of *o*-diphenols to quinones (catecholoxidase) or both substrates (tyrosinases) (Ashida and Brey, 1995; Decker et al., 2000; Decker and Terwilliger, 2000; Hearing and Tsukamoto, 1991; Itoh and Fukuzumi, 2007; Johansson and Söderhäll, 1996; Sanchez-Ferrer et al., 1995; Söderhäll and Cerenius, 1998; Solomon et al., 1996). So far, only one crystal structure of a phenoloxidase is available, that of a catecholoxidase from sweet potato (*Ipomoea batatas*; Klabunde et al., 1998) which is related to a molluscan hemocyanin with respect to sequence similarity (about 25%) and its active site (Klabunde et al., 1998). Two different types of tyrosinases can be classified based on their sequences. One type is more related to molluscan hemocyanins, and the other type, found in arthropods together

Abbreviations: EM, electron microscopy; Hcs, hemocyanins; HvH, *Helix vulgaris* Hc; K_m, Michaelis–Menten constant; RvH, *Rapana venosa* Hc; RvH1 and RvH2, subunit isoforms of *Rapana venosa* Hc; SDS, Sodium dodecyl sulphate; Ty, tyrosinase; V_{max}, maximum velocity.

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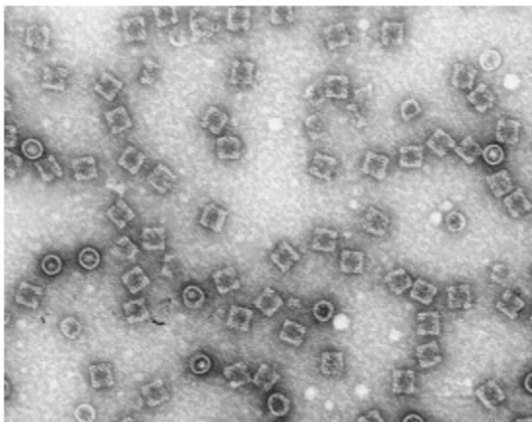

Fig. 1. Electron micrograph of native *Rapana venosa* hemocyanin (RvH) in 50 mM Tris/HCl buffer, pH 7.0, containing 20 mM $CaCl_2$ and 5 mM $MgCl_2$. Staining with 1% uranyl acetate was performed as described in Materials and methods. The scale bar indicates 100 nm.

with hemocyanins is very similar to them (Decker et al., 2001; Nakahara et al., 1983; Salvato and Beltramini, 1990; Salvato et al., 1983; Solomon, 1981; Van Gelder et al., 1997; Wilcox et al., 1985).

Besides exhibiting similar copper oxygen-binding sites, phenoloxidases and hemocyanins have another feature in common: their enzymatic properties can be activated in similar ways by proteolitic cleavage of N- or C-terminal fragments and disturbing their protein structures. Hemocyanins of arthropods and molluscs also exhibit *o*-diphenoloxidase activity after exposure to chaotrophic salts like sodium dodecyl sulphate (Decker et al., 2001; Pless et al., 2003), urea (Morioka et al., 2006) or low pH values (Baird et al., 2007; Salvato et al., 1998; Zlateva et al., 1996).

Several physicochemical properties and functions of Hcs are very similar to those of phenoloxidases (Decker and Rimke, 1998; Decker and Tuczek 2000; Salvato and Beltramini, 1990; Salvato et al., 1998). For example, some Hcs have monophenolase and diphenolase activities (Nakahara et al., 1983), but with much lower efficiency as compared to tyrosinases. Studies on the interaction of *o*-diphenols with the Hc from the molluscs *Octopus vulgaris, Helix pomatia* and *Sepia officinalis* give information on the specific properties of the active site which are relevant for the appearance to catalytic activity (Siddiqui et al., 2006). A reaction mechanism is based on oxy-Hc as active species and on substrate radical formation as a result of substrate interaction.

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. Within Hcs, the proteins from mollusks are more reactive toward exogenous ligands as compared to arthropod Hcs (Nakahara et al., 1983; Nellaiappan and Vinayakam, 1993).

Here we investigated the catalytic activities of oxy-Hcs isolated from the mollusc *Rapana venosa* and *Helix vulgaris* against *o*-diphenol and 3,4-dihydroxy-L-phenylalanine (L-Dopa) as substrates in comparison to those of other Hcs from molluscs and arthropods.

2. Materials and methods

2.1. Purification procedures

R. venosa Hc was isolated from the hemolymph of marine snails living in the west coast of the Black Sea near Varna. The hemolymph was centrifuged at 5000 ×*g* at 4 °C and phenylmethanesulfonyl fluoride (PMSF) was added to inhibit proteases. Hemocyanin was isolated by preparative ultracentrifugation, using a Beckman L-80 ultracentrifuge, equipped with a Ti 45 UZ rotor, at a speed of 24 000 rpm for 4 h at 4 °C. The protein was stored at −20 °C in the presence of 20% sucrose.

The two subunits, RvH1 and RvH2, were isolated by the procedure described in (Dolashka-Angelova et al., 2003). The native Hc was dialyzed in 0.13 M glycine/NaOH buffer, pH 9.6, for 24 h. The dissociated fractions were loaded on an ion exchange chromatogramphy DEAE-Sepharose CL-6B column, equilibrated with 50 mM Tris/HCl buffer, 10 mM EDTA, pH 8.2. The structural subunits were separated by elution with the same buffer and a linear sodium chloride gradient (0.15–0.40 M NaCl) with a flow rate 0.2 mL min^{-1}.

H. vulgaris hemolymph was collected from garden snails from the Sofia area and centrifuged at 5000 ×*g* for 15 min to remove hemocytes. Hc was sedimented in a Beckman L-80

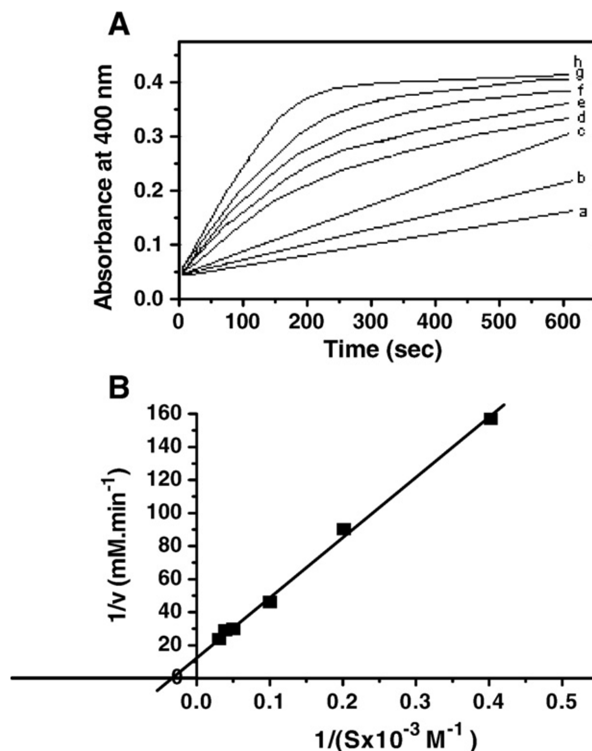

Fig. 2. *o*-Diphenoloxidase activity of native (didecamers) *Rapana venosa* hemocyanin, expressed as initial velocity (ΔA_{475} nm/min) of quinone formation in the standard assay: 20 mM phosphate buffer, pH 6.8, 25 °C. (A) Time courses of quinone formation in the presence of *R. venosa* Hc at different concentrations of *o*-diphenol: (a) 1.25×10^{-3} M, (b) 2.5×10^{-3} M, (c) 5.0×10^{-3} M, (d) 10×10^{-3} M, (e) 15.0×10^{-3} M, (f) 20.0×10^{-3} M, (g) 25.0×10^{-3} M, (h) 30.0×10^{-3} M. (B) The Lineweaver–Burk plot was constructed from data obtained at different concentrations of the substrate *o*-diphenol.

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