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Influence of limited proteolysis, detergent treatment and lyophilization on the phenoloxidase activity of *Rapana thomasiana* hemocyanin

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ABSTRACT

The intrinsic and inducible phenoloxidase (PO) activity of *Rapana thomasiana* hemocyanin (RtH) and its substructures were studied. With catechol as substrate, a weak *o*-diPO activity was measured for the didecameric RtH and its subunits. Some activation of the *o*-diPO activity of RtH was achieved by limited treatment with subtilisin and by incubation of RtH with 2.9 mM sodium dodecyl sulphate (SDS), suggesting an enhanced substrate access to the active sites. The highest artificial induction of *o*-diPO activity in RtH, however, was obtained by lyophilization of the protein. This is ascribed to conformational changes during the lyophilization process of the didecameric RtH molecules, affecting the accessibility of the active sites. These conformational changes must be very small, since Fourier-transform infrared and circular dichroism spectroscopies did not reveal any changes in secondary structure of lyophilized RtH. The difference in accessibility of the copper containing active site for substrates between catechol oxidase and functional unit RtH2-e was demonstrated by molecular modeling and surface area accessibility calculations. The low level of intrinsic PO activity in the investigated hemocyanin is related to the inaccessibility of the binuclear copper active sites to the substrates.

1. Introduction

Hemocyanin (Hc) is a member of the type-3 copper protein family, which further includes tyrosinases (Tys) (EC 1.14.18.1) and catechol oxidases (COs) (EC 1.10.3.1). Type-3 copper proteins contain a dinuclear copper active site that is comprised of two closely spaced copper atoms each coordinated by 3 histidine nitrogen atoms of the polypeptide chain. Oxygen is reversibly bound to the active site as μ-η^2:η^2 peroxide [1,2]. Although the dinuclear site is highly conserved as witnessed by its characteristic spectroscopic properties, sequence homology and the available crystal structures of Hcs [3–6], CO [7], and Ty [8], the functionality of these proteins is different. Hcs function as oxygen carriers and oxygen storage proteins in several species of arthropods and molluscs, Tys catalyze both the *o*-hydroxylation of monophenols to *o*-diphenols (tyrosinase or monophenoloxidase activity) and subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase or diphenoloxidase activity) and COs catalyze only the second reaction.

It is generally accepted that one of the main reasons for the absence or low level of phenoloxidase activity in Hcs is related to the inaccessibility of the type-3 center to potential substrates [9]. It has been shown that the oxygen-binding function of Hc can be converted to phenoloxidase (PO) activity and furthermore that PO activity can be induced in Hcs by *in vivo* and *in vitro* activation [10]. Compared to the numerous studies of arthropodan Hcs, the enzymatic activity of molluscan Hcs has been less investigated. Hcs isolated from the cephalopods *Octopus vulgaris* and *Sepia officinalis* as well as from the gastropods *Helix pomatia* and *Rapana venosa* (synonym of *Rapana thomasiana*) have been demonstrated to exhibit *o*-diphenoloxidase activity (*o*-diPO) [11–13]. The functional units (FUs) mainly responsible for both the intrinsic PO activity and the enhanced PO activity after induction by limited proteolysis have been recently identified for the first time for *S. officinalis* and *H. pomatia* Hc [12].

In this study we further investigate the intrinsic and inducible PO activity of *R. thomasiana* hemocyanin (RtH) and its substructures to gain further insight into the enzymatic properties of molluscan Hcs. The influence of limited proteolysis and of sodium dodecyl sulphate (SDS), known as an artificial activator of phenoloxidases and of phenoloxidase activity in arthropodan Hcs [14], is studied. Also the effect of lyophilization of the protein on the enzyme activity is investigated.

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2. Materials and methods

2.1. Isolation of Rapana thomasiana hemocyanin

Living marine snails *R. thomasiana* were caught near the Bulgarian coast of the Black Sea (Varna) and stored in sea water. The hemolymph was collected by bleeding through several diagonal slits made on the foot of the mollusc and filtered through gauze. Phenylmethanesulphonyl fluoride (PMSF) at a final concentration of 1 mM was added to the crude material to avoid possible proteolysis of the hemolymph. Hemocytes and other cells were removed by centrifugation at 5000 × *g* for 30 min at 4 °C. Native RtH was isolated from freshly obtained hemolymph by ultracentrifugation at 180,000 × *g* for 4 h and stored in the presence of 20% sucrose (w/v) at −20 °C until used.

RtH was further purified by gel filtration chromatography on a Sepharose 4B column, equilibrated and eluted with 50 mM PBS, pH 7.2. The purity of the isolated Hc was controlled by SDS-PAGE and native PAGE as previously described [15,16].

2.2. Isolation of Rapana hemocyanin structural subunits and functional unit RtH2-e

Purified RtH was dissociated into individual subunits by dialysis against 130 mM glycine–NaOH buffer, pH 9.6, containing 10 mM EDTA, and overnight at 4 °C. Both structural subunits, RtH1 and RtH2, were isolated by anion-exchange chromatography of dissociated RtH on DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) as described elsewhere [15].

Functional unit RtH2-e was isolated after treatment of the structural subunit RtH2 with plasmin, separation of the products, and subsequent limited trypsinolysis of the fragment containing RtH2-e, as described in [17]. RtH2-e was purified to homogeneity by FPL chromatography on a Mono Q (HR 10/10) column (Amersham Biosciences, Freiburg, Germany).

The purity of the isolated RtH substructures was controlled by SDS-PAGE. Protein concentration was determined spectrophotometrically using the specific absorption coefficient $a_{278\,nm}$ = 1.36 mL mg^{-1} cm^{-1} [15].

2.3. Assay of phenoloxidase activity

In the standard procedure, the PO activity was determined at 25 °C in sodium phosphate buffer, pH 7.4, *I* 50 mM, at a final concentration of protein (RtH, subunits or FU RtH2-e) of 1 mg/mL. Catechol (Merck) and L-Dopa (Aldrich) were used as substrates for *o*-diPO activity at a final concentration of 6.0 and 3.4 mM, respectively. Tyramine (BDH) at a final concentration of 3.8 mM was used for the assay of mono-PO activity. The enzyme reaction was followed by absorbance measurement of the *o*-quinone or dopachrome, formed as a result of substrate oxidation, at 400 nm (for substrate catechol) or 475 nm (for substrate L-Dopa), for 10–40 min in a Shimadzu UV-2100 double-beam spectrophotometer. The absorbance value was corrected for the contribution due to the auto-oxidation of the substrate by running the same assay in the absence of protein. The reaction rate was measured from the initial quasi-linear portion of the curves (usually 0–3 min). The kinetic parameters [K_M (mM), V_{max} (nmol min^{-1} mg^{-1}) and k_{cat} (min^{-1}; expressed per copper pair)] were derived from non-linear regression data analysis of the dependence of the initial rates on the substrate concentration using HYPER software, taking into account the molar absorption coefficient of *o*-quinone 1417 M^{-1} cm^{-1} [11] or of dopachrome 3600 M^{-1} cm^{-1} [14] and the molecular mass of a FU (entity with one active site; 50 kDa).

2.4. Induction of phenoloxidase activity

2.4.1. Induction by limited proteolysis of Hc

Samples of RtH were dialyzed against Tris–HCl buffer, pH 8.2, *I* 50 mM, and then subjected to limited proteolysis at 25 °C using subtilisin Carlsberg (Novo Industri) at an enzyme to substrate ratio of 1/500 (w/w) and 1/200 (w/w) for 48 h in order to induce PO activity.

2.4.2. Induction by SDS

SDS, at a final concentration of 2.4–3.3 mM, was added to RtH (1 mg/mL final concentration) at 25 °C in borax–HCl buffer, pH 8.2, *I* 50 mM. Absorption spectra of RtH samples in the same buffer were recorded over the range 240–420 nm after incubation for 2 min with different concentrations of SDS.

2.4.3. Induction by lyophilization

Samples of RtH, its subunits and FU RtH2-e were dialyzed against distilled water, frozen in an ethanol bath at −30 °C and then dried under vacuum using a freeze-dryer Lyovac GT2, equipped with a vacuum pump (Leybold AG, Germany).

2.5. Atomic absorption spectrophotometry

Copper in the Hc sample was measured with a Perkin-Elmer 372 Atomic absorption spectrophotometer (Überlingen, Germany) at 324.7 nm. Prior to Cu measurement, the Hc sample was briefly treated with EDTA at 10 mM final concentration to complex loosely bound Cu. EDTA and complexed Cu were removed by passing the sample through a PD-10 desalting column (GE Healthcare) which was saturated with the buffer in the Hc sample. The absorbance readings from the atomic absorption spectrophotometer were converted to the corresponding concentration, expressed in μg/mL, from a standard curve of absorbance versus known concentration of copper solutions (0.1–3.0 μg/mL).

2.6. Fourier transform infrared spectroscopy

The infrared spectrum of lyophilized RtH (20 mg/mL in deuterated MES buffer, pH 6.5, *I* 100 mM) was recorded on a Bruker IFS66 FTIR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector at a nominal resolution of 2 cm^{-1}. The final spectrum is the average of 256 interferograms. The sample compartment was continuously purged with dry air to minimize the spectral contribution of atmospheric water. A baseline correction was performed in the amide I' region (1600–1700 cm^{-1}) assuming a linear baseline. In order to enhance the component peaks contributing to the amide I' band, the spectra were treated by Fourier self-deconvolution using the Bruker software (OS/2 version). The line shape was assumed to be Lorentzian with a half-bandwidth of 21 cm^{-1} and an enhancement factor *k* of 1.7 was used.

2.7. Circular dichroism

Far-UV circular dichroism measurements were performed at 25 °C with a Jasco J-810 spectropolarimeter in the 190–250 nm region, using a cell with 0.1 cm pathlength. The protein was dissolved in a borax–HCl buffer (pH 8.2, *I* 50 mM) at concentration of 0.1 mg mL^{-1}. Spectra were acquired at a scan speed of 20 nm min^{-1}, with a 1 nm bandwidth and a 1 s integration time.

2.8. Molecular modelling

The modelling program Brugel [18] was used to create the superimposed catechol oxidase (pdb code 1bt3) and hemocyanin (pdb code 1lnl) structures. The following elements were used to fit the