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Reversible heat inactivation of copper sites precedes thermal unfolding of molluscan (*Rapana thomasiana*) hemocyanin

Krassimira Idakieva [a,*], Filip Meersman [b], Constant Gielens [c]

[a] Institute of Organic Chemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 9, Sofia 1113, Bulgaria
[b] Department of Chemistry, Christopher Ingold Laboratories, University College London, 20 Gordon Street, London WC1H 0AJ, UK
[c] Division of Biochemistry, Molecular and Structural Biology, Department of Chemistry, KU Leuven, Celestijnenlaan 200 G, B-3001 Leuven, Belgium

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ABSTRACT

Hemocyanin (Hc) is a type-3 copper protein, containing dioxygen-binding active sites consisting of paired copper atoms. In the present study the thermal unfolding of the Hc from the marine mollusc *Rapana thomasiana* (RtH) has been investigated by combining differential scanning calorimetry, Fourier transform infrared (FTIR) and UV–vis absorption spectroscopy. Two important stages in the unfolding pathway of the Hc molecule were discerned. A first event, with nonmeasurable heat absorption, occurring around 60 °C, lowers the binding of dioxygen to the type-3 copper groups. This pretransition is reversible and is ascribed to a slight change in the tertiary structure. In a second stage, with midpoint around 80 °C, the protein irreversibly unfolds with a loss of secondary structure and formation of amorphous aggregates. Experiments with the monomeric structural subunits, RtH1 and RtH2, indicated that the heterogeneity in the process of thermal denaturation can be attributed to the presence of multiple 50 kDa functional units with different stability. In accordance, the irreversible unfolding of a purified functional unit (RtH2-e) occurred at a single transition temperature. At slightly alkaline pH (Tris buffer) the C-terminal β-sheet rich domain of the functional unit starts to unfold before the α-helix-rich N-terminal (copper containing) domain, triggering the collapse of the global protein structure. Even around 90 °C some secondary structure is preserved as shown by the FTIR spectra of all investigated samples, confirming the high thermostability of molluscan Hc.

1. Introduction

Hemocyanins (Hcs) are complex respiratory proteins found in the hemolymph of many arthropods and molluscs [1,2]. The interest in Hcs is due not only to their important biological function, connected with oxygen transport, but also to their potential application as immunomodulators [3]. Recently, we have shown that the Hc isolated from the marine gastropod *Rapana thomasiana* and/or its subunits can be used as adjuvants or as protein-carriers of small non-immunogenic molecules for increasing their immunogenicity [4].

Hcs have been classified as metalloproteins containing binuclear "type 3" Cu active sites [5]. The type 3 sites are also found in phenoloxidases, tyrosinases and catecholoxidases and in the multicopper oxidases (MCOs) although geometric and electronic structure differences in comparison with Hc active sites have been established [6–8].

R. thomasiana hemocyanin (RtH) is an oligomer of twenty subunits organized in a hollow cylindrical structure consisting of a wall and two collars, one at each end of the cylinder [9]. Two types of subunits, RtH1 and RtH2, with same carbohydrate content (2.6%, w/w) and very similar monosaccharide composition, can be discerned on the basis of biochemical and immunological characteristics [10]. Each subunit in turn consists of 8 covalently linked globular entities with a molecular mass of approximately 50 kDa, referred to as functional units (FUs), termed abcdefgh [11]. FUs abcdef make up the wall of the Hc cylinder, while FUs g and h form the internal collar complex. FUs in the wall stabilize each other by associating interactions with their neighbors when assembled into the cylindrical quaternary structure. Although the distinction between RtH1 and RtH2 reflects differences between FUs of both subunits, the FUs share a high sequence homology and it is assumed that their tertiary structures are very similar. The crystal structure of FU RtH2-e, isolated from subunit RtH2, has been determined at 3.38 Å resolution [12]. The protein consists of an N-terminal domain which is mainly α-helical and a smaller C-terminal domain that contains mainly β-sheet structure. The oxygen binding site, containing two copper atoms, is located in the N-terminal domain.

Our studies, by means of differential scanning calorimetry (DSC), have shown that gastropodan Hcs possess considerable thermal stability. Apparent transition temperatures (T_m) in the range from 83

Abbreviations: RtH, hemocyanin of *Rapana thomasiana*; FTIR, Fourier transform infrared; DSC, differential scanning calorimetry; Tris–HCl, tris (hydroxymethyl) amino–methane hydrochloride; MES, 2-(*N*-morpholino) ethanesulfonic acid

* Corresponding author. Tel.: +359 2 9606 190; fax: +359 2 8700 225.
E-mail address: idakieva@orgchm.bas.bg (K. Idakieva).

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to 90 °C were observed for Hcs from gastropods *R. thomasiana* [13], *Helix pomatia* [14] and *Concholepas concholepas* [15]. In another DSC study, Sterner et al. have reported a high thermostability of the Hc from tarantula *Eurypelma californicum* (T_m 90 °C) [16]. By contrast a recent investigation of *Rapana venosa* (an alternative name for *R. thomasiana*) Hc by circular dichroism (CD) spectroscopy suggested a much lower thermal stability (T_m ~ 55 °C at pH 7) [17,18] although this finding seemingly contradicts earlier reports based on CD spectroscopy [19,20].

In the present work, the structural subunits RtH1 and RtH2 and a representative functional unit (RtH2-e) are studied in addition to didecameric RtH. The thermal stability is investigated not only by DSC, but also by Fourier transform infrared (FTIR) and UV–vis spectroscopy. These spectroscopic techniques have the advantage over DSC that they enable us to monitor the temperature-induced conformational changes. Our results show that on increasing temperature a reversible change in the tertiary structure, affecting the binding of dioxygen to the copper active sites, precedes the irreversible unfolding of the secondary structure elements.

2. Experimental procedures

2.1. Sample preparation

The holo-forms of RtH, its structural subunits RtH1 and RtH2, and FU RtH2-e were obtained as described previously [10,21]. The apo-forms (copper-deprived Hcs) were prepared by dialysis against 50 mM Tris–HCl, containing 25 mM KCN, pH 7.2, for 48 h, at 4 °C, according to Beltramini et al. [22]. Protein concentration was determined spectrophotometrically using the absorption coefficient $A_{278}^{0.1\%} = 1.36\ \mathrm{mg^{-1}\ ml\ cm^{-1}}$ (20 °C) [10]. DSC measurements with the holo and apo-forms of Hcs were carried out in 100 mM MES, pH 6.5 (20 °C), using four different protein concentrations (2.0, 3.5, 5.0 and 7.5 mg $\mathrm{ml^{-1}}$). The Hc samples, prepared for FTIR experiments, were dialysed against deuterated MES buffer (100 mM, pD 6.5) or against Tris–DCl buffer (50 mM, pD 8.2; 20 °C) at a protein concentration of 20–30 mg $\mathrm{ml^{-1}}$. In order to remove any insoluble material the solutions were centrifuged at 12 000 *g* for 2 min before use. The absorption spectra of holo RtH were recorded using a protein sample dialyzed against 100 mM MES, pH 6.5. The protein concentration was 0.9 mg $\mathrm{ml^{-1}}$.

Due to the temperature dependence of the pK_a value of the buffers, the pH value of the samples in MES buffer drops with 0.011/°C and that of the samples in Tris buffer with 0.028/°C during the heating experiments.

2.2. Differential scanning calorimetry

DSC experiments were performed on a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with a sensitivity > 0.017 mJ $\mathrm{K^{-1}}$ and a noise level < ± 0.05 μW. A constant pressure of 2 atm was maintained during all DSC experiments to prevent possible degassing of the solution on heating. Three different scan rates (0.5, 1.0 and 2.0 K $\mathrm{min^{-1}}$) were used. The protein solution in the calorimetric cell was reheated after the cooling from the first run to estimate the reversibility of the thermally induced transitions. In all cases, the thermal unfolding was found to be irreversible. Therefore the thermogram corresponding to the reheating run was used as the instrument baseline. The transitions were corrected for the difference in heat capacity between the initial and final state by using a linear chemical baseline. The calorimetric data were evaluated using the ORIGIN (MicroCal Software) program package. The temperature at the maximum of the excess heat capacity curve was taken as the transition temperature (T_m).

2.3. Fourier transform infrared spectroscopy

Infrared spectra were recorded on a Bruker IFS66 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a liquid nitrogen cooled mercury cadmium telluride detector. 256 interferograms were co-added after registration at a resolution of 2 $\mathrm{cm^{-1}}$. The sample compartment was continuously purged with dry air. The heat unfolding was followed using a temperature cell with CaF_2 windows separated by a 50 μm teflon spacer. The cell was placed in a heating jacket controlled by a Graseby Specac Automatic Temperature Controller (Specac Ltd, Orpington, UK). The temperature increment was 0.2 K $\mathrm{min^{-1}}$.

The spectra were treated by Fourier self-deconvolution using the Bruker software (OS/2 version). The assumed lineshape was Lorentzian. The half-bandwidth was assumed to be 21 $\mathrm{cm^{-1}}$ and an enhancement factor *k* of 1.7 was used. Finally, a linear baseline was assumed in the amide I′ region (1600–1700 $\mathrm{cm^{-1}}$). The prime denotes the deuterated band. The T_m-values were obtained by a non-linear, least-squares fit to the data using the following equation:

$$y = \frac{(y_f + m_f T) + (y_u + m_u T)\left(\exp\left(\frac{\Delta H}{RT}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right)\right)}{1 + \left(\exp\left(\frac{\Delta H}{RT}(T_m - T)\right)\right)}$$

where y_f and m_f, and y_u and m_u are the intercepts and the slopes of the pre- and post-transition baselines. The equation is derived analogously to that for chemical unfolding [23], making use of the van't Hoff relationship between the apparent equilibrium constant *K* and the enthalpy change Δ*H*.

2.4. UV–vis absorption spectroscopy

Absorption spectra were recorded with UV–vis spectrophotometer model Evolution 300, Thermo Electron Corporation, equipped with a Peltier temperature control accessory. A sample temperature probe accessory was used to monitor the temperature of the protein solution in the cell (optical pathlength 10 mm). The heating rate was 1.0 K $\mathrm{min^{-1}}$.

3. Results and discussion

3.1. DSC reveals heterogeneity in the process of thermal unfolding of RtH and its subunits

DSC measurements of the holo forms of RtH, its structural subunits RtH1 and RtH2, and the FU RtH2-e were performed in 100 mM MES buffer, pH 6.5 (20 °C). In all cases the thermal unfolding was found to be calorimetrically irreversible, as no thermal effect was observed in a second heating of the protein solutions. The scan rate dependence of the calorimetric traces indicates that the thermal unfolding of the investigated proteins is kinetically controlled [13,24]. The T_m and calorimetric enthalpy (ΔH_{cal}) values for the thermal unfolding of RtH were found to be independent of the protein concentration (data not shown), indicating that the dissociation of the protein into monomers does not take place before the rate-determining step of the process of thermal unfolding [13].

Consistent with our previous study, one main transition and a shoulder with apparent transition temperatures (T_m) at 82.4 °C and 90 °C, were detected in the thermogram of holo RtH (Fig. 1a). Likewise, two transitions with comparable T_m values were detected in the thermograms of related gastropodan Hcs, namely the β-Hc from the terrestrial snail *H. pomatia* (β-HpH) and *C. concholepas* Hc (CCH) [14,15]. A calorimetric behavior showing more than one transition has also been observed for Hcs from other species, including *E. californicum* (tarantula) and *Palinurus vulgaris* (lobster) [16,24].