Bulgarian Chemical Communications, Volume 49, Special Issue L (pp. 12-16) 2017

Thermal stability of *Helix aspersa* maxima hemocyanin

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Submitted October 6, 2016; Accepted February 1, 2017

The thermal stability of the hemocyanin, isolated from garden snails *Helix aspersa* maxima (HaH), was studied by differential scanning calorimetry. One transition, with an apparent transition temperature (T_m) at 79.8 °C, was detected in the thermogram of HaH in 20 mM HEPES buffer, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 7.06 at 80 °C), using a heating rate of 1.0 °C/min. The thermal denaturation of HaH was an irreversible process. The scan rate dependence of the calorimetric profiles indicated that the thermal unfolding of the investigated Hc is kinetically controlled. T_m and ΔH_{cal} values for the thermal denaturation of HaH were found to be independent of the protein concentration, suggesting that the dissociation of the Hc into subunits does not take place before the rate-determining step of the process of thermal unfolding started. The thermal denaturation of HaH was described by the two-state irreversible model. On the basis of this model, the parameters of the Arrhenius equation were calculated.

Key words: hemocyanin, differential scanning calorimetry, irreversible denaturation

INTRODUCTION

Hemocyanins (Hcs) are respiratory glycoproteins, isolated from the hemolymph of mollusc and arthropod species [1]. Interest in Hcs is determined not only because of their important biological function, but also because of the opportunities for their successful application in medicine. Hcs, isolated from different organisms, are used in preparation of vaccines, as immunomodulators, as well as in the therapy of bladder cancer [2-6]. Recently, a potential anti-cancer effect of Hcs on a murine model of colon carcinoma was demonstrated, suggesting their use for immunotherapy of different types of cancer [7].

The thermal stability is an important characteristic of proteins having potential therapeutic applications. In the present study, the thermal stability of the Hc, isolated from garden snails *Helix aspersa* maxima (HaH), was studied by differential scanning calorimetry (DSC). This protein can be produced in large quantities for biotechnology and medical use of snails bred in special farms under controlled conditions.

EXPERIMENTAL

Isolation and purification of hemocyanin

HaH was isolated according to the procedure described in [8]. Briefly, the native Hc was obtained from the hemolymph, collected from the snails *Helix aspersa* maxima, by ultracentrifugation at 180 000 x *g* (ultracentrifuge Beckman LM-80, rotor Ti 45), for 4 hours, at 4 °C. The pellets were resuspended in 50 mM phosphate buffer, pH 7.2, and the HaH was further purified by gel filtration chromatography. Protein concentration was determined spectrophotometrically using the specific absorption coefficient $A_{278}^{0.1\%}$ = 1.413 $ml.mg^{-1}.cm^{-1}$ for HaH [9].

Differential scanning calorimetry

Calorimetric measurements were performed on a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia). 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. The calorimetric data were evaluated using the ORIGIN (MicroCal Software) program package. Molecular mass of 9 000 000 Da for Hc was used in the calculation of molar quantities.

RESULTS AND DISCUSSION

Irreversibility of the thermal denaturation of HaH

The thermogram of HaH in buffer 20 mM HEPES (pH 7.06 at 80 °C), at a heating rate of 1.0 °C/min, is shown in Fig. 1 (line a). Heat absorption was observed between 70 and 87 °C, with an apparent transition temperature (T_m) at 79.8 °C. Value of 130.5 MJ mol^{-1} was calculated for the calorimetric enthalpy (ΔH_{cal}) by an integration of the heat capacity curve. The thermal denaturation of HaH was irreversible as no thermal effect was

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detected in a reheating scan (Fig. 1, line b). Irreversibility of the thermal denaturation was observed in the DSC measurements of all of the studied Hcs to date [10-13]. Heat-induced aggregation has been considered to be a major reason for the irreversibility of thermal denaturation of proteins. Indeed, our research on the thermal unfolding of Hc from marine gastropod *Rapana thomasiana* (T_m 82.4 °C) by means of Fourier transform infrared (FTIR) spectroscopy clearly demonstrates that above 70 °C the protein irreversibly unfolds with a loss of secondary structure and formation of amorphous aggregates [14].

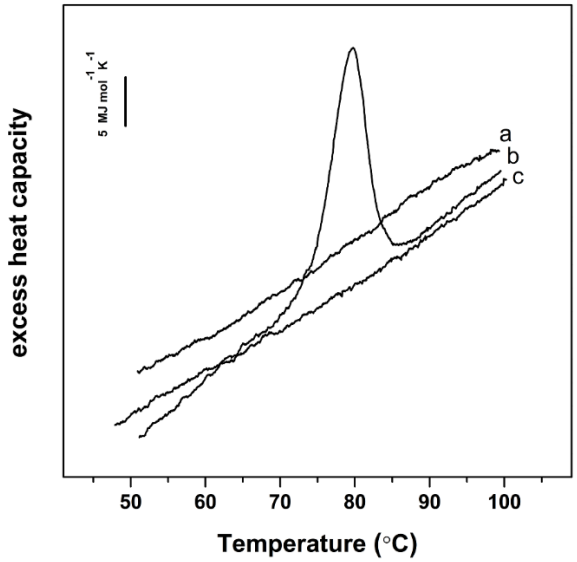


Fig. 1. (a) Experimental *C*p transition curve of HaH in buffer 20 mM HEPES, containing 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ (pH 7.06 at 80 °C), at a heating rate of 1.0 °C/min; (b) Reheating run; (c) Buffer-buffer base lane.

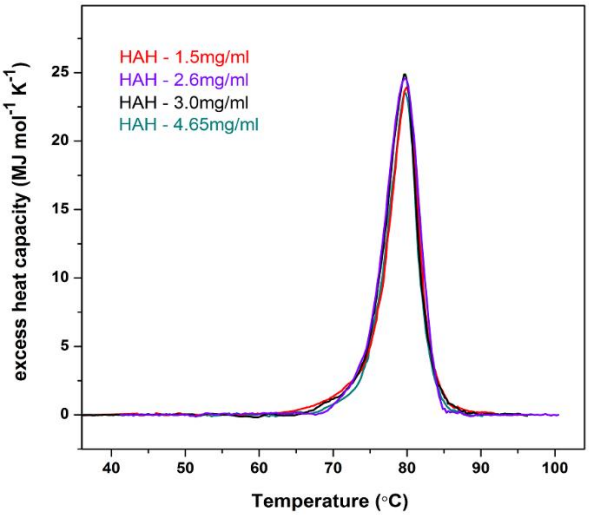


Fig. 2. *C*p transition curves of HaH in buffer 20 mM HEPES, containing 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ (pH 7.06 at 80 °C), recorded at a heating rate of 1 °C/min and different protein concentrations.

Concentration dependence of the temperature of denaturation

The quaternary structure of the molecule of HaH, like in other gastropodan Hcs, represents oligomer of twenty subunits. Dissociation of the Hc molecule to individual subunits may occur during the denaturation process. In order to determine whether this is occurring, DSC traces were collected at varying protein concentrations, at a constant heating rate of 1 °C/min. The T_m and ΔH_{cal} values for the thermal unfolding of HaH were found to be independent of the protein concentration (Fig. 2). Consistent with our previous studies, the oligomeric state of the Hc molecule does not change during denaturation [11-13]. This indicates that the interaction between subunits, constituting the Hc molecule, is strong enough to prevent protein dissociation before the rate-determining step of the process of thermal unfolding.

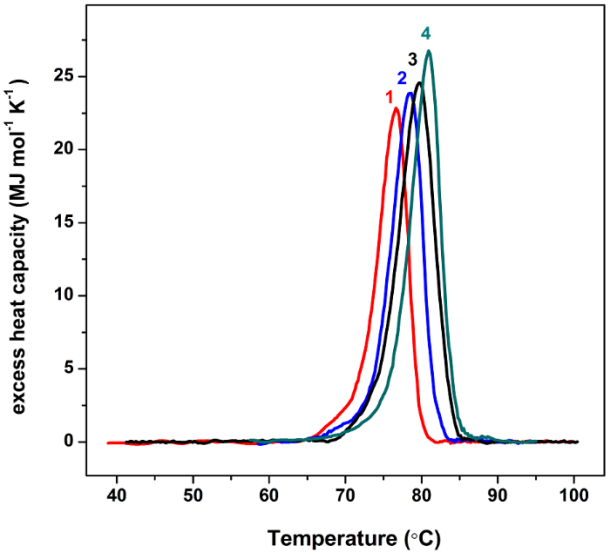


Fig. 3. Dependence of the *C*p transition curves of HaH in buffer 20 mM HEPES (pH 7.06 at 80 °C), with heating rate: (1) 0.2 °C/min; (2) 0.55 °C/min; (3) 1 °C min/min; (4) 1.8 °C/ min. In all cases the protein concentration was 4.65 mg/ml.

Scan rate dependence

Figure 3 shows the excess heat capacity function vs. temperature profiles for HaH at four different heating rates (0.2, 0.55, 1.0 and 1.8 °C/min). The transition temperature of the irreversible thermal denaturation of HaH is scan rate dependent: the maximum of the DSC profiles is shifted toward lower temperatures with a decrease in the heating

rate. The scan rate dependence of the calorimetric traces indicates that the thermal unfolding of the investigated HaH is kinetically controlled [11, 15].

Analysis of the calorimetric data

In most cases, the irreversible thermal denaturation of proteins monitored by DSC or other techniques is described using a simple two-state kinetic model, which is a limiting case of the Lumry-Eyring model [16]:

$$\mathrm{N} \xrightarrow{k} \mathrm{D} \quad (1)$$

This model considers only two significantly populated macroscopic states, the initial or native state (N) and the final or denatured (D) state. In this case, the process is characterized by a first-order rate constant k which follows the Arrhenius equation:

$$k = \exp\left[\frac{E_\mathrm{a}}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)\right] \quad (2),$$

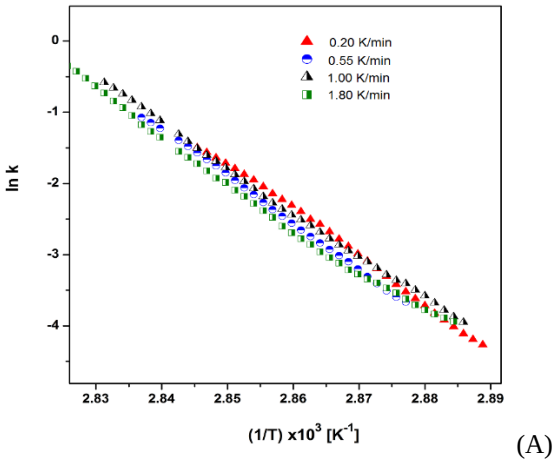
where E_a is the activation energy of the denaturation process, R is the gas constant, and T^* is the temperature at which k is equal to 1 min^{-1}. The two-state irreversible model has been used for the interpretation of the DSC data for the thermal denaturation of Hcs from different species [11-13, 15]. Four different methods have been devised from Sanchez-Ruiz and co-workers [17] for an evaluation of the activation energy from heat capacity curves.

Method A. The rate constant k (inverse seconds) is obtained at each temperature from the following equation:

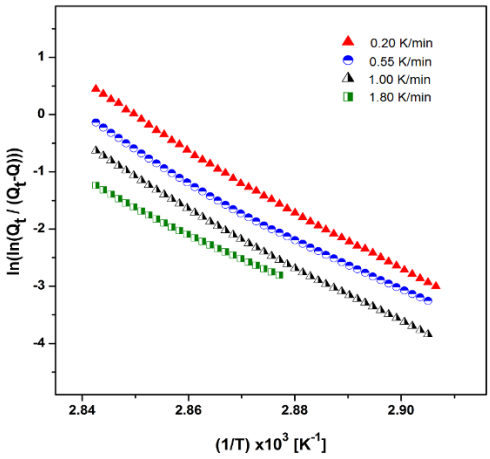
$$k = \frac{\nu C_\mathrm{p}}{\Delta H_\mathrm{cal} - \Delta H} \quad (3),$$

where ν refers to the heating rate in Kelvin per seconds, C_p is the molar heat capacity, ΔH (kJ mol^{-1}) is the corresponding enthalpy at T, and ΔH_cal is the total molar transition enthalpy. For each heating rate, k was evaluated for all data in the interval $(T_\mathrm{m}-2) \leq T \leq (T_\mathrm{m}+2)$. The Arrhenius plots obtained are shown in Fig. 4A. The mean and standard deviation value of the E_a determined from the slopes of the Arrhenius plots are as follows

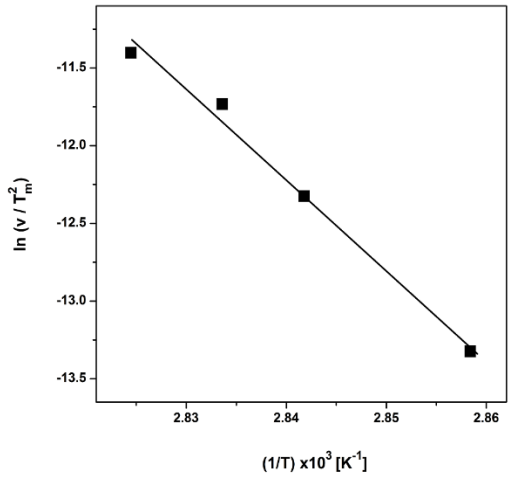
E_a = 545 ± 12 kJ mol^{-1}.



(A)



(B)



(C)

Fig. 4. (A–C) Determination of activation parameters for the HaH denaturation from the experimental heat capacity functions. Methods (A–C) are according to Sanchez-Ruiz et al. [17].

Method B. The variation of the transition temperature, T_m, with a heating rate is given by the following relationship:

$$\frac{v}{{T_m}^2} = \frac{AR}{E_a}\exp\left(-\frac{E_a}{RT_m}\right) \quad (4)$$

If the proposed model is correct a linear dependence of $\ln(v/ T_m^2)$ versus $1/T_m$ should be established with a slope of $-E_a/ R$. The value of E_a determined from the slope of the plot shown in Fig. 4B is as follows

$E_a = 491 \pm 18$ kJ mol^{-1}

Method C. The third estimate of E_a stems from the following relationship:

$$\ln\left[\ln\left(\frac{\Delta H_{cal}}{\Delta H_{cal} - \Delta H}\right)\right] = \frac{E_a}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (5)$$

Plotting the left side of Eq. (7) versus 1/T should yield a straight line with a slope of – E_a /R for each heating rate. The results are shown in Fig. 4C. The average value of the E_a obtained from the four slopes is as follows

$E_a = 418 \pm 38$ kJ mol^{-1}

Method D. Finally, the E_a can be evaluated from the heat capacity at the transition temperature, ${C_p}^m$, using the equation:

$$E_a = \frac{eR{T_m}^2{C_p}^m}{\Delta H_{cal}} \quad (6)$$

Using method D the value obtained of the E_a is as follows

$E_a = 539 \pm 17$ kJ mol^{-1}

The apparent value of the E_a averaged over the results obtained from the four methods is as follows

$E_a = 498 \pm 21$ kJ mol^{-1}

The values of T_m and E_a determined for the denaturation of HaH were found to be within the range of the data obtained up to now for other studied gastropodan Hcs (Table 1).

Table 1. Parameters for a thermal denaturation of the Hc of *Helix aspersa* maxima, obtained by DSC, at a heating rate of 1 °C/min. Comparison with the data obtained for gastropodan Hcs of *Rapana thomasiana* [11]; *Helix pomatia* [12], *Concholepas concholepas* [13] and *Haliotis rubra* [18].

Hemocyanin species	T_m [°C]	ΔH_{cal} [MJ mol^{-1}]	E_a [kJ mol^{-1}]
Helix aspersa maxima	79.8	130 ± 1.0	498 ± 21
Rapana thomasiana	82.4*	195 ± 1.0	597 ± 20
Helix pomatia β-isoform	84.0*	190 ± 1.0	521 ± 7
Concholepas concholepas	78.0*	120 ± 1.0	323 ± 2
Haliotis rubra	78.9*	–	535 ± 65

* Main transition

In conclusion, the results of the present investigation on the HaH, isolated from snails *Helix aspersa* maxima, allow classifying this Hc as a thermostable protein (T_m 79.8 °C). These data will facilitate the further investigation of therapeutic properties of this respiratory protein.

Acknowledgements*: This work was supported by research grant DFNP-152/ 12.05.2016 from Program for career development of young scientists, BAS, Bulgaria.*

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ТЕРМИЧНА СТАБИЛНОСТ НА ХЕМОЦИАНИН ОТ ОХЛЮВИ *Helix aspersa* maxima

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Постъпила на 6 октомври, 2016 г.; приета на 1 февруари, 2017 г.

(Резюме)

В настоящето изследване термичната стабилност на хемоцианин, изолиран от охлюви от вида *Helix aspersa* maxima, беше изследвана с помощта на диференциална сканираща калориметрия. Определена беше температура на топене 79.8 ºC на протеина в буфер 20 mM HEPES, съдържащ 0.1 M NaCl, 5mM $CaCl_2$ и 5 mM $MgCl_2$ (pH 7.06 at 80 ºC), при скорост на нагряване 1.0 ºC/мин. Термичната денатурация на хемоцианина беше необратима. Беше установено, че температурата на топене и калориметричната енталпия зависят от скоростта на нагряване, което показва, че процеса на термична денатурация на хемоцианина има кинетичен характер. Калориметричните данни бяха анализирани съгласно “two-state” модела, описващ необратима денатурация на протеини. Изчислена беше активиращата енергия за процеса на термична денатурация на хемоцианина.