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FLUORESCENCE PROPERTIES AND CONFORMATIONAL STABILITY OF HEMOCYANIN ISOLATED FROM SNAILS *HELIX ASPERSA* MAXIMA

Yuliana Raynova, Ivan Angelov, Krassimira Idakieva

Institute of Organic Chemistry with Centre of Phytochemistry
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 9, Sofia 1113, Bulgaria
E-mail: idakieva@orgchm.bas.bg

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ABSTRACT

The structure in a solution and the conformational stability of hemocyanin isolated from snails Helix aspersa maxima (HaH) and its isoforms, β-HaH and α_{D+N}-HaH, are studied using fluorescence spectroscopy. The emission upon excitation of the proteins' fluorescence at 295 nm is determined by tryptophyl residues located in the hydrophobic interior of the molecules. This is confirmed by the fluorescence quenching experiments with acrylamide and potassium iodide. The pH range of 6.5 - 8.0, characterized by an absence of evident pH-dependent fluorescence changes, is defined as the proper pH range for HaH physico-chemical characterization. The scattering experiments show that HaH molecules are predominantly in a monomeric state in Tris-HCl buffer of pH of 8.0.

Keywords: hemocyanin, Helix aspersa maxima, fluorescence spectroscopy, conformational stability, static light scattering.

INTRODUCTION

Hemocyanins (Hcs) are oligomeric copper-containing respiratory proteins, freely dissolved in the hemolymph of many arthropod and mollusc species [1]. The Hc molecule in molluscs has the shape of a hollow cylinder (35 nm in diameter) and is constituted of ten (cephalopods) or twenty (gastropods) subunits of a molecular mass of 350 kDa - 450 kDa. The subunits themselves are folded into seven or eight globular substructures, the socalled "functional units" (FUs). Each FU has a molecular mass of approx. 50 kDa and contains one binuclear copper active site, reversibly binding one dioxygen molecule. Hc molecules carry a substantial negative charge on their surface determining its hydration and high solubility in the hemolymph. Under non-physiological conditions (e. g. in case of pH > 9 and a treatment with EDTA) Hcs loose their quaternary structure and dissociate into subunits [1]. An expression of functionally distinct Hc isoforms has been observed [2] in certain species of mollusc. In addition to their important biological function, molluscan Hcs have shown promising properties in the development of various medicinal products including antiviral agents, conjugate vaccines and immunotherapy of cancer [3 - 8].

Recently, Hc of garden snails *Helix aspersa* maxima (HaH) has been isolated and characterized [9, 10]. A model of biosensor for quantitative determination of phenols in aqueous solutions has been developed [9] based on o-diphenoloxidase activity of HaH. Studies by differential scanning calorimetry (DSC) have shown that HaH and its isoforms, β-HaH and α_{D+N}-HaH, possess a considerable thermal stability [11, 12]. An antiproliferative effect of the total molecule HaH and structural subunit β-HaH, against bladder cancer cell lines, has been observed [13]. Moreover, strong antimicrobial activity has been reported for the structural subunit β-HaH [14].

The aim of the present study is to obtain new data on the structure in a solution and the conformational stability of HaH and its isoforms by means of fluorescence spectroscopy and static light scattering. The comparison of the obtained data with those referring

to other molluscan Hcs will shed additional light on the structural stability of the oxygen-transport proteins from invertebrates.

EXPERIMENTAL

Isolation and purification of hemocyanins

Native HaH was isolated according to the procedure described in ref. [8]. Briefly, 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 h at 4°C. The pellets were resuspended in 50 mM phosphate buffer of pH of 7.2 and HaH was further purified by gel filtration chromatography on a Sepharose 4B column (90 x 2.4 cm). Further, HaH was separated into its isoforms (β-HaH and α_{D+N}-HaH) by ion exchange chromatography on a DEAE-Sepharose CL-6B column (32 x 1.2 cm) equilibrated and eluted with buffer 50 mM Tris-HCl of pH of 8.0 using a linear gradient 0.1-0.45 M NaCl. The specific absorption coefficient a_{278} nm = 1.413 ml mg^{-1} cm^{-1} referring to HaH [15] was used for determining the protein concentration. All Hc solutions were filtered through Millipore 0.22 μm disposal filters prior to the measurements.

Steady-state fluorescence measurements

The fluorescence spectra of HaH and its isoforms were recorded by means of a spectrofluorimeter Perkin Elmer model LS55 at an excitation wavelength of 295 nm. The optical density of the solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. The relative fluorescence quantum yields were calculated using the equation [16]:

$$Q_x = Q_{st} + (F_x/A_x)/(A_{st}/F_{st}) \quad (1)$$

where Q_x, F_x and A_x were the emission quantum yield, the area of the spectrum and the optical density at the excitation wavelength, respectively, for the protein sample, while Q_{st}, F_{st} and A_{st} were the corresponding parameters of the reference standard. N-acetyltryptophanamide (Ac-Trp-NH_2) of a quantum yield of 0.13 [16] was used as a standard.

The results of the quenching reactions between the excited tryptophyl side chains and acrylamide, CsCl or KI, were analyzed on the ground of the Stern–Volmer equation [17]:

$$I_o/I = 1 + K_{SV}[X] \quad (2)$$

where I_o and I were the fluorescence intensities in absence and presence of the quencher, respectively, K_{SV} was the collisional quenching constant, while $[X]$ was the quencher concentration. A small amount of $Na_2S_2O_3$ was added to the iodide solutions to prevent I_3^- formation. The inner filter effect due to the acrylamide was corrected by the factor:

$$Y = antilog\,(d_A + d_B)/2 \quad (3)$$

where d_A and d_B were the optical densities at the excitation and emission wavelength, respectively.

Each fluorophore in multitryptophan proteins with a heterogeneous distribution of fluorescing groups would have different quenching constants and which is why the measured decrease of the fluorescence emission could be a non-linear function of the quenching agent concentration. In this case, the quenching effect was better described by the modified Stern–Volmer equation:

$$I_o/(I_o - I) = (1/[Q]$$

$$1/\sum f_i\ K_{Qi}) + \sum K_{Qi}\ /\sum f_i\ K_{Qi} \quad (4)$$

where I_o, I and $[Q]$ were as defined above, while f_i and K_{Qi} represented the fractional fluorescence and the quenching constant relative to the *i*-th tryptophan residue, respectively . The intercept/slope ratio of $I_o/(I_o - I)$ vs $1/[Q]$ was equal to the effective quenching constant $\sum K_{Qi} = (K_{Qi})_{\text{eff}}$, while 1/intercept expressed the effective fraction of the fluorescence accessible to the quencher $\sum f_i K_{Qi}\ /\sum K_{Qi} = (f_A)_{\text{eff}}$ [16].

Scattering experiments

The scattering measurements of native HaH were performed on a fiber optics specifically elaborated spectrophotometer on the basis of Ocean Optics QE 65000 spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) with Spectra Suite Software (Ocean Optics Inc., Dunedin, USA) set-up as described in ref. [18] with excitation at 660 nm. The data was analyzed and graphically represented by the computer programme Origin 8.0 (Microcal Software, Inc., Northampton, MA. USA).

RESULTS AND DISCUSSION

Steady-state fluorescence measurements

Fluorescence spectroscopy is one of the most sensitive methods for studying the protein structure and conformation in a solution. The fluorescence parameters of the native total HaH and its isoforms, β-HaH and α_{D+N}-HaH, are summarized in Table 1. After excitation at 295 nm, where the tryptophan side chains are selectively excited, the fluorescence spectra of the investigated Hcs show an emission maximum position, λ_{max}, in the region of 335 nm - 337 nm. It is typical for "buried" tryptophyl side chains in a hydrophobic environment within the protein [19]. The fluorescence emission quantum yields of HaH and both isoforms are low, 0.011 - 0.019, in comparison with that for the model compound Ac-Trp-NH_2 (Table 1). This can be explained with a quenching effect of the specific microenvironment of the emitting tryptophans. Erker et al. present [20] a detailed study verifying that Förster transfer is responsible for oxygen-dependent quenching of the tryptophan fluorescence in tarantula (arthropod) Hc. The tryptophans transfer their excitation energy to the oxygenated active sites.

The absence of tyrosine emission in the fluorescence spectra of the investigated Hcs after excitation at 280 nm can be explained by a singlet-singlet radiationless energy transfer from phenol groups (donors) to indole rings (acceptors) according to Förster's theory for electronic energy transfer in donor-acceptor systems [21]. A similar explanation has been given [22] in respect to the fluorescence measurements of Hc of the related gastropod *Helix pomatia* (β-HpH).

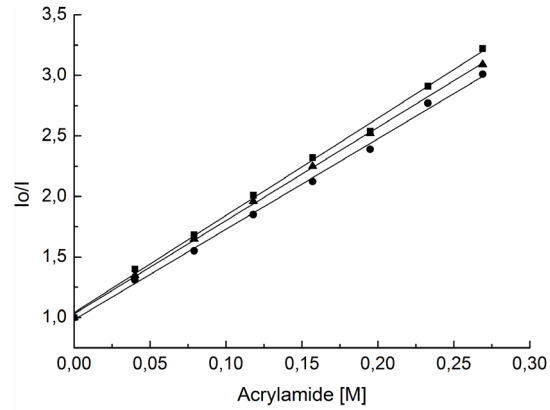

Fig. 1. Stern–Volmer plots for the quenching of the tryptophyl fluorescence of native HaH (●–●) and its isoforms β-HaH (▲–▲) and α_{D+N}-HaH (■–■), by acrylamide according to Eq. 2.

Acrylamide- and I^- fluorescence quenching studies

The fluorescence quenching reactions have been widely used for studying the degree of expousure and the electronic environment of aromatic amino acid residues, mainly tryptophan, in proteins. Acrylamide is an efficient neutral quencher of tryptophan fluorescence and provides topographical information about the emitting chromophores [23]. It can discriminate between "exposed" and "buried" tryptophan side chains, while the results are not influenced by the charge of the chromophore microenvironment. The ability to quench collisionally the excited indole rings depends on its ability to penetrate the protein matrix. The fluorescence quenching with acrylamide of HaH and its isoforms, β-HaH and α_{D+N}-HaH, follows the classical Stern–Volmer Eq. 2 (Fig. 1). The observed linearity of the Stern–Volmer plots can be explained by

Table 1. Steady-state fluorescence parameters of the native Hc of snails *Helix aspersa* maxima and its isoforms, β-HaH and α_{D+N} HaH, at an excitation wavelength of 295 nm.

Hemocyanin	Emission λ_{max}	Relative quantum yield	Acrylamide quenching	KI quenching	
	[nm]		K_{SV} [M^{-1}]	K_{SV} [M^{-1}]	f_a
Native HaH	337.5 ± 1	0.012	7.467	-	-
Isoform β-HaH	337.0 ± 1	0.019	7.699	14.63	0.335
Isoforms α_{D+N} HaH	335.5 ± 1	0.011	8.028	-	-
Ac-Trp-NH_2	350.5 ± 1	0.130*	16.33*	-	-

**Values taken from [25]*

the similarity of the individual K_{SV} constants of the tryptophan fluorophors [24]. This means that the protein tryptophan residues differ only slightly in their accessibility, although a discrimination among the indole fluorophores could be expected as HaH is a multitryptophan protein. K_{SV} values of 7.47, 8.03 and 7.7 M^{-1} are calculated for HaH, α_{D+N}-HaH and β-HaH, respectively (Table 1). These constants are lower than that of Ac-Trp-NH_2, i.e. for tryptophan in an aqueous solution [25] and reflect the limitations of the quencher molecules accessibility to the emitting tryptophans imposed by the protein quaternary structure. The determined K_{SV} values are higher than those obtained for other Hcs of gastropodan organisms [22, 26, 27], indicating a relatively higher accessibility to the quencher acrylamide of HaH molecules emitting fluorophores.

Quenching experiments are also performed with ionic quenchers like potassium iodide (KI) and cesium chloride (CsCl). The ionic quenchers (I^- and Cs^+) are charged and hydrated. In contrast with acrylamide, which can penetrate the protein matrix, they are able to quench only the surface fluorophores and are effective in revealing charge effects. The exposure of neither total HaH nor isoforms to increasing Cs^+ concentrations [0.02 M - 0.4 M] influences their fluorescence after excitation at 295 nm. This confirms the conclusion that the tryptophyl side chains are "buried" in the interior of Hc molecules. The ionic quencher KI [0.02 M - 0.4 M] also has no effect on the fluorescence of HaH and α_{D+N}-HaH isoforms. Given the charged nature of the ionic quenchers, the quenching will depend not only on the tryptophan residues exposure but also on their surrounding charges. Thus, the low quenching efficiency of I^- can be explained by electrostatic repulsion due to the negative charge of the emitting tryptophans microenvironment. It is expected that this effect should decreases in the presence of an increasing medium ionic strength (KCl) because of diminished electrostatic attraction and anions (Cl^-) occupation of the iodide binding sites.

The fluorescence measurements show that the fluorophores in molecules of Hc isoform β-HaH are more accessible to the ionic quencher I^-. The quenching plot of β-HaH demonstrates the tryptophan emission heterogeneity visialized by the downward curvature of the plot (Fig. 2(A)). The modified Stern–Volmer plot (Fig. 2(B)) provides the evaluation of the fraction and K_{SV} value of the most quencher-accessible fluorescence (Table 1). As shown 33.5 % of the total fluorescence emission of β-HaH isoform is accessible to the quenching action of I^- ions.

The effect of pH on the tryptophyl fluorescence quantum yield and the emission maximum position of the native HaH is shown in Fig. 3. One of the most useful parameters of the protein fluorescence spectrum – its maximum position (λ_{max}), which reflects the degree of the chromophores accessibility to the solvent molecules, remains constant (variation within limits of ± 1.0 nm) for a pH-range from about 4.5 to 8.0. This implies that the accessibility of Hc tryptophan side chains to water molecules remains essentially invariant in this pH range. At the same time, no change in the emission of native HaH is observed in the pH region of 6.5 - 8.0 (Fig. 3) indicating the absence of changes of the fluorescence quenching properties of the tryptophan environment in this pH-range. Changes in the microenvironment

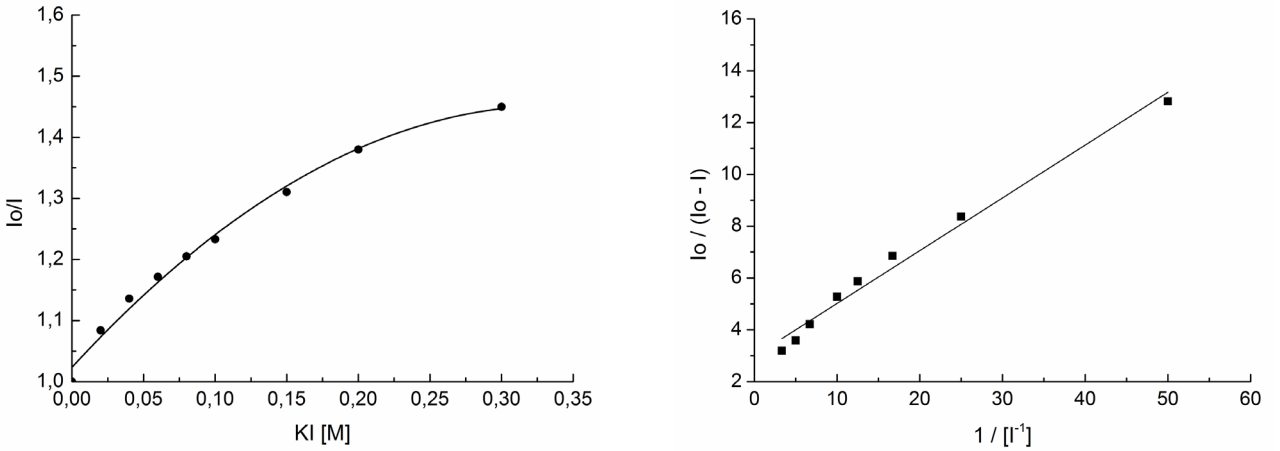

Fig. 2. A-B. Stern–Volmer plots for the quenching of the fluorescence of the β-HaH by KI, according to Eq. 2 (A) and modified Stern-Volmer Eq. 4 (B).

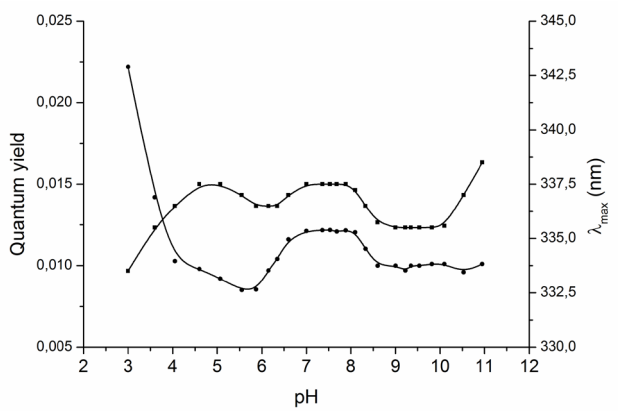

Fig. 3. pH-dependence of the tryptophyl fluorescence quantum yield (●–●) and emission maximum position (■–■) of native HaH. The following buffers were used: 50 mM sodium citrate (pH 3.0 – 7.0); 50 mM Tris-HCl (pH 7.0 – 9.0); 50 mM carbonate/bicarbonate (pH 9.0 - 11.0).

of the emitting tryptophans leading to a decrease of the quantum yield occur at pH values lower or higher than those of this pH range. The transition at pH values higher than 8 is usually attributed to the titration of an ionizable group of an apparent pKa of 8.1, which is within the ionization region of the α-amino group [22, 27]. Alternatively, the quenching process may involve an electron transfer from the excited tryptopan residues to the α-amino group or to an ε-amino group of an abnormal pK, which can occur over an appreciable distance. The transition with a midpoint around pH of 6.3 most probably represents the apparent pKa of imidazole groups of histidyl residues. The tryptophan residues emission is thus most likely quenched by nearby protonated imidazole groups, which can form complexes with indole. The increase of the fluorescence quantum yield of HaH at pH lower than 4.0 has been proven [22, 27, 28] to result from a destruction of the copper–dioxygen system at the active site, which quenches the tryptophan fluorescence. Changes of the emission maximum position at pH values < 4.5 and > 10 indicate significant conformational changes in the protein (Fig. 3).

Static light scattering

Static light scattering (SLS) experiments are performed aiming to evaluate the effect of pH on the oligomeric state of native HaH. According to the literature data and that shown in ref. [29] the protein size and shape can be determined by static light scattering measurements on the ground of the scattered intensities of the investigated protein and its products during the investigated process obtained under identical conditions. The dependence of the scattered intensity I_{scat} due to N molecules in a scattered volume (V) of an environment provides to determine the equivalent radius of a sphere at a scattering angle $\theta = 90^o$. This is done on the ground of:

$$R_x = R_0 \sqrt{I_{Sx}/I_{So}}\ \sqrt{K} \qquad (5)$$

where R_0 and I_{So} are the effective radius of the known initial compound and the measured intensity of the scattered light in the solution containing this compound, while R_x, I_{Sx} are the respective terms referring to the product obtained in the corresponding process. The term K in Eq. (5) depends on the scatter (Q_{scat}) and the absorption (Q_{abs}) efficacy, which in turn depend on many parameters inherent for light scattering process. According to the definition of Q_{scat} and Q_{abs} [29 - 32] it can be expected that the value of the square root of K will be near 1 for wavelengths far from the absorption maximum. The assumed shape of HaH is calculated accepting a value of $\sqrt{K}$ equal to 0.9 and 1. The middle value is taken in view of the corresponding deviations. The space parameters (a radius, a hight, a volume and a specific shape) of Hc of the related gastropod *Rapana tomasiana* (RtH) are determined at pH 7.0 [33]. However, a fast decrease of the scattered intensities is observed during the measurements of HaH in 50 mM Tris-HCl buffer in the pH range of 7.0 - 8.0. Since no conformational changes in Hcs occur in this pH range, the observed effect is most likely due to the interaction of the protein with the buffer compounds. Tris buffer, by virtue of its –OH and –NH_2 groups, can interact with the peptide backbone. Thus, it is reported [34] that Tris buffer alters the charge and the size of the bovine serum albumin mainly through a hydrogen-bonding. HaH molecules in Tris-HCl buffer at pH of 8.0 are most probably in a monomeric state. The intensity of the scattered light is increased the pH range of 4.5 - 7.0 due to the formation of Hc molecules aggregates. Fig. 4 shows the changes of the effective scattered radius of HaH depending on pH of the buffer solution. The results referring to the intesity of the scattered light at diferent pH values of the solution are compared with the intensity obtained at pH of 8.0 for which it is accepted that HaH has an effective scattered radius $R_0 = 1$. The reduction of the

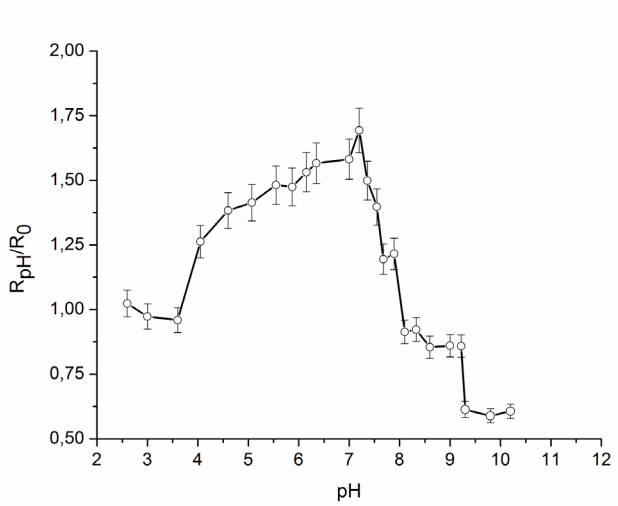

Fig. 4. SLS experiments with native HaH in the following buffers: 50 mM sodium citrate (pH 3.0 – 7.0); 50 mM Tris-HCl (pH 7.0 – 9.0); 50 mM carbonate/bicarbonate (pH 9.0 - 11.0).

scattered intensities at pH values > 9 indicates the beginning of dissociation of Hc molecule. It is shown [35] by transmission electron microscopy that RtH splits into decamers after dialysis against Tris buffer of pH of 9.5. Hc molecule dissociates completely [35] into subunits at higher pH values in presence of EDTA.

CONCLUSIONS

The present fluorescence spectroscopy investigations provide the physicochemical characterization of Hc isolated from snails *Helix aspersa* maxima and its isoforms, β-HaH and α_{D+N}-HaH. The quenching experiments show that the tryptophyl residues buried in the hydrophobic interior of the protein molecules determine the fluorescence emission. Differences are found in the microenvironment of the emmiting fluorophores in the molecules of HaH isoforms. The pH range of 6.5 to 8.0 characterized by an absence of evident pH-dependent fluorescence changes seems to be the proper pH range of choice for the physicochemical characterization of HaH. For the first time it is shown that HaH molecules exist primarily in a monomeric state in Tris-HCl buffer of pH of 8.0.

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