Rapana thomasiana grosse (gastropoda) haemocyanin: spectroscopic studies of the structure in solution and the conformational stability of the native protein and its structural subunits

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1. The stability towards pH changes, thermal and chemical (guanidine hydrochloride) denaturation of the oxy- and apo-forms of the native *Rapana thomasiana* haemocyanin and its structural subunits, RHSS1 and RHSS2, has been investigated using fluorescence and CD spectroscopy. The association of the subunits into haemocyanin aggregates increases considerably the melting temperature and the free energy of stabilization in water. The guanidine hydrochloride denaturation of the aggregated oxygen-transporting protein depends slightly on the protein concentration. The denaturation of the individual subunits is concentration-independent. *Rapana* haemocyanin is 5.9–7.5 kJ/mol more stable than the constituent polypeptide chains. 2. Upon excitation of the native haemocyanin and the subunits at 295 or 280 nm the fluorescence emission is determined by tryptophyl residues ‘buried’ deeply in the hydrophobic interior

of the protein globules. This is confirmed by quenching experiments with acrylamide, caesium and iodide ions. The efficiency of the radiationless energy transfer between the phenol (donor) and indole (acceptor) fluorophores in the three species, native haemocyanin, RHSS1 and RHSS2, has been determined. An efficient ‘interchain’ energy transfer between tyrosyl and tryptophyl residues from different polypeptide chains occurs in the non-dissociated form of the haemocyanin. 3. The tryptophan emission of the oxyhaemocyanin, oxy-RHSS1 and oxy-RHSS2 is strongly quenched by the copper–dioxygen complex at the active site and the respective quantum yields of fluorescence of the oxygenated species are 4–7 times lower than those of the apo-forms. Protonated imidazole groups quench the fluorescence of neighbouring excited indole rings, probably by charge-transfer complex formation.

INTRODUCTION

In many invertebrates the circulatory transport of dioxygen to the tissues is ensured by reversible binding of O₂ to the giant respiratory proteins called haemocyanins (Hcs). These extracellular biopolymers, found in the haemolymph of arthropods and molluscs, are comparable in size to ribosomes or small viruses [1]. Arthropodan Hcs exist as molecular aggregates composed of six, or multiples of six, structural subunits (polypeptide chains). The molecular mass of these subunits varies in the region 67–90 kDa [2]. Each polypeptide chain contains a single binuclear dioxygen-binding site [3]. Usually, the aggregates of Hcs from arthropodan organisms contain different types of subunits. The organization and size of the molluscan Hc subunits are quite different and the polypeptide chains with a molecular mass of 330 to 460 kDa contain seven or eight domains. Each domain represents a functional unit of approx. 50 kDa molecular mass and contains one active centre capable of binding reversibly a dioxygen molecule. The molecular architecture of the respiratory proteins from the two phyla is described in reviews [2,3].

The respiratory protein Hc has been successfully applied in medicine. Thus, the keyhole limpet Hc from the gastropod *Megathura crenulata* is widely used in laboratories and clinics as an immune stimulant [4,5] and in the immunotherapy of bladder cancer [6,7] and of renal cell carcinoma [8]. The quaternary structure, subunit and domain composition [9], as well as the role

of the two distinct subunit types in the architecture of this dioxygen carrier, have been investigated [10].

The biological importance of dioxygen transport and the application of Hcs in medicine stimulate studies on their structure in solution and their stability. At present, little information is available in this field, especially as regards the conformational stability of gastropodan Hcs. The thermal denaturation of the arthropodan Hc from *Palinurus vulgaris* has been investigated by differential scanning calorimetry [11]. The urea denaturation of the Hc from another arthropod, *Carcinus maenas*, has also been studied [12].

Several years ago we isolated and characterized a novel Hc from the haemolymph of the marine gastropod *Rapana thomasiana* grosse [13]. This invertebrate was originally living along the coast of Japan, in the Yellow Sea and in the East China Sea. In the middle of this century it was discovered on the west coast of the Black Sea, where it adapted. The salinity of the Black Sea is less than half of that of the Pacific Ocean and the specific ecosystem can affect the physiological properties of *Rapana thomasiana*. In the electron micrographs the associated Hc molecules appear as cylinders with 380 Å height and circles with 300 Å diam. After reassociation of the dissociated material, *Rapana* Hc shows a tendency to form ‘tubular’ structures (i.e. to aggregate further) of various lengths. Two different subunits, termed RHSS1 and RHSS2, with an apparent molecular mass of 450 kDa each, were isolated from the dissociated *Rapana* Hc

Abbreviations used: Hc, haemocyanin; RHSS1 and RHSS2, subunits of the *Rapana* haemocyanin; Ac-Trp-NH₂, *N*-acetyltryptophanamide; GnHCl, guanidine hydrochloride; T_m, melting temperature.

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[13]. The carbohydrate content and monosaccharide composition of the native Hc and its structural subunits were also determined [14].

Here we describe the stabilities to pH, thermal and chemical denaturation of the oxy- and apo-forms of the native (aggregated) *Rapana* Hc and its structural subunits. Considerable stabilization of the conformation in solution of the Hc aggregates was observed in comparison with the constituent polypeptide chains. The structure in solution of these species has also been investigated.

MATERIALS AND METHODS

Isolation of the *Rapana* Hc and its structural subunits: preparation of the apo-forms

Animals were collected from the west coast of the Black Sea, near Varna, and stored in sea water before extraction of the haemolymph. The crude material was filtered on gauze and centrifuged. The rotor was operated for 30 min at 4 °C and 5000 g. Hc was isolated and purified by the procedure given in [13]. The obtained material was stored at -20 °C in the presence of 20 % (w/v) sucrose until used. The two subunits were isolated as described in [15]. The apo-forms of the *Rapana* Hc and its subunits were prepared by dialysis against 0.025 M KCN.

Spectroscopic measurements of thermal and guanidine hydrochloride (GnHCl) denaturation

Fluorescence measurements were performed with a Perkin Elmer model LS 5 spectrofluorimeter equipped with a thermostatically controlled assembly and a Data Station model 3600. The optical absorbance of the solutions was lower than 0.05 at the excitation wavelength to avoid inner filter effects. Excitation at 295 nm was used for measurement of the tryptophyl fluorescence.

Fluorescence quantum yields were determined by the following equation [16]:

$$Q_x = Q_{st}(F_x/A_x)(A_{st}/F_{st})$$

where Q_x , F_x and A_x are the emission quantum yield, the emission intensity at wavelength λ and the absorbance at the excitation wavelength, respectively, for the protein sample, and Q_{st} , F_{st} and A_{st} are the same parameters for the reference standard. *N*-Acetyltryptophanamide (Ac-Trp-NH₂) with a quantum yield of 0.13 [17] was chosen as a standard.

The efficiency, e , of the tyrosine-to-tryptophan energy transfer was calculated using the relationship [18]:

$$Q = Q_{Trp}[f_{Trp}(\lambda) + e \cdot f_{Tyr}(\lambda)]$$

where Q is the fluorescence quantum yield of the protein sample at the respective excitation wavelength λ ; Q_{Trp} is the fluorescence quantum yield of the tryptophyl residues in the protein molecule after excitation at 300 nm; and $f_{Trp}(\lambda)$ and $f_{Tyr}(\lambda)$ are the fractional absorptions of tryptophan and tyrosine, respectively, at the excitation wavelength λ , calculated from their molar ratio in the protein.

The temperature dependence of the tryptophyl fluorescence was determined at pH 7.0 in 0.05 M Tris/HCl buffer. The samples were kept for 10 min at the desired temperature prior to the measurement, to ensure the attainment of thermal equilibration.

The results of the quenching reactions between the excited tryptophyl side chains and acrylamide were analysed according to the Stern-Volmer equation [17]:

$$F_o/F = 1 + K_{sv}[X]$$

where F_o and F are the fluorescence intensities at an appropriate emission wavelength in the absence and presence of quencher;

K_{sv} is the collisional quenching constant; and $[X]$ is the quencher concentration. The inner filter effect due to acrylamide was corrected by the factor $Y = \text{antilog}(d_A + d_E)/2$ [19], where d_A and d_E are the absorbances at the excitation and emission wavelengths respectively. The thermal denaturation of the *Rapana* Hc and its subunits RHSS1 and RHSS2 was also followed by CD measurements in a Jasco J-720 dichrograph equipped with a personal computer IBM PC-AT, PS/2, multiscan monitor CMS-3436 and a Hewlett-Packard colour graphics plotter model HP 7475A. Software DOS version was used for calculations with the CD data. Protein solutions in quartz cuvettes were placed in a cell holder, which was thermostatically controlled using a NESLAB thermostat model RTE-110 connected with a digital programming controller. The temperature inside the cuvette was monitored with a thermocouple. The samples were kept for 10 min at the desired temperature to ensure the attainment of thermal equilibrium, which was confirmed by the constancy of the ellipticity. Each spectrum, used for the further calculations, represents an average of three measurements.

GnHCl denaturation was followed using a Roussel Jouan Dichrographe III instrument. The far-UV CD spectra were recorded between 200 and 250 nm using protein solutions in 0.05 M Tris/HCl buffer, pH 7.0. The attainment of equilibrium at each concentration of the denaturant was confirmed with the constancy of the ellipticity at 221 nm.

Calculation of the free energy of stabilization in water

The free energy of GnHCl denaturation, ΔG_D , was estimated from the equation:

$$\Delta G_D = -RT \ln K$$

where K is the equilibrium constant:

$$K = ([\Theta]_{\text{obs}} - [\Theta]_N)/([\Theta]_D - [\Theta]_{\text{obs}})$$

$[\Theta]_{\text{obs}}$ is the observed ellipticity at 221 nm at different concentrations of the denaturant, and $[\Theta]_N$ and $[\Theta]_D$ are the ellipticities at the same wavelength for the folded and unfolded conformations of the proteins. The free energy of denaturation in water at 25 °C, $\Delta G_D^{\text{H}_2\text{O}}$, was determined from a plot of ΔG_D versus GnHCl concentration.

RESULTS AND DISCUSSION

Fluorescence spectroscopy is a suitable tool for studying the Hc structure and conformation in solution and changes in conformation. The sensitivity of fluorescence emission allows studies to be performed using diluted protein solutions. In this way, problems connected with aggregation and low solubility of the giant *Rapana* Hc subunits can be avoided. Table 1 summarizes fluorescence parameters of the oxy- and apo-forms of the *Rapana* Hc and its structural subunits RHSS1 and RHSS2. After excitation at 280 nm, where phenol and indole groups absorb, or at 295 nm, where the tryptophyl side chains are selectively excited, the fluorescence spectra (results not shown) have maxima at $(329-332) \pm 1$ nm, which are typical for deeply buried tryptophans in a hydrophobic environment. The apo-proteins have fluorescence maxima at $(327-332) \pm 1$ nm, i.e. practically in the same region as the oxygenated forms. It can be concluded that the fluorescence emission of both oxy- and apo-forms of the three species are dominated by deeply buried tryptophyl side chains. This finding was further supported by quenching studies with neutral and ionic quenchers. Fluorescence quenching reactions have been widely used for studying the degree of exposure and electronic environment of aromatic amino acid residues.

Table 1 Fluorescence parameters of *Rapana thomasiana* Hc and its structural subunits

Species	Emission $\lambda_{\max}$ (excitation at 295 nm) (nm)	Emission $\lambda_{\max}$ (excitation at 280 nm) (nm)	Quantum yield (FCCLI Q)	Tyr-to-Trp energy transfer efficiency ϵ (%)	Acrylamide quenching K_{sv} (M^{-1})
Oxy-Hc	329 ± 1	330 ± 1	0.012	—	1.84
Apo-Hc	327 ± 1	328 ± 1	0.074	65	1.90
Oxy-RHSS1	331 ± 1	330 ± 1	0.013	—	2.26
Apo-RHSS1	332 ± 1	332 ± 1	0.052	37	3.40
Oxy-RHSS2	331 ± 1	332 ± 1	0.011	—	2.10
Apo-RHSS2	331 ± 1	330 ± 1	0.082	16	2.72
Ac-Trp-NH ₂	350 ± 1	350 ± 1	0.130*	—	16.33*

* Values taken from [22].

Figure 1 Stern-Volmer plots for fluorescence quenching with acrylamide of the *Rapana* oxy-Hc (▲), oxy-subunit RHSS1 (○) and oxy-subunit RHSS2 (●)

The experiments were performed at 25 °C after excitation of the protein samples at 295 nm.

Acrylamide is an efficient quencher of tryptophan fluorescence and can discriminate between 'buried' and 'exposed' side chains. Its ability to collisionally quench the excited indole rings depends only on their 'exposure' to the quencher [20]. *Rapana* Hc and its structural subunits contain eight tryptophyl residues per functional unit of 50 kDa [13]. For this reason a discrimination among the indole fluorophores was expected. However, the fluorescence quenching with acrylamide of the oxy- and apo-forms of the Hc aggregates and structural subunits followed the classical Stern-Volmer equation (Figure 1). The observed lin-

earity of the Stern-Volmer plots can be explained by the similarity of the individual K_{sv} constants [21]. This means that the tryptophyl residues of these proteins differ only slightly in accessibility. Similar linear plots have been observed also for a number of other multi-tryptophan-containing proteins: pepsin, trypsin and aldolase [20]. The slope of the plots at a low concentration of acrylamide reflects to a large extent the quenching of the more accessible residues and selective quenching can be observed only if the K_{sv} constants differ by a lot [20]. The linearity of the initial part of the curves confirms that in the cases of *Rapana* Hc and its subunits the difference between these constants is not large.

K_{sv} values from 1.84 to 3.40 M^{-1} were calculated with acrylamide as a quencher (Table 1). This means a very low efficiency of quenching because the values are significantly lower than $K_{sv} = 16.33 M^{-1}$ for the model compound Ac-Trp-NH₂, i.e. for tryptophan in aqueous solution. K_{sv} constants in the region 1.0–3.5 M^{-1} have been determined with acrylamide for chymotrypsin, chymotrypsinogen, ribonuclease, trypsin and lysozyme containing 'buried' tryptophyl residues [20]. On the other hand, Stern-Volmer constants of 13.0 and 10.5 M^{-1} were measured for the single 'exposed' tryptophans of adrenocorticotropin and glucagon respectively [20]. These results confirm the conclusion that the indole groups in the oxy- and apo-forms of the three species are located in the interior of protein globules. Quenching of 'buried' tryptophans by acrylamide has been explained in terms of structural fluctuations of the protein molecules that facilitate the inward diffusion of the quencher [21]. The lowered K_{sv} values for the native Hc in comparison with those for the constituent subunits reflect the additional limitations of the accessibility to the tryptophyl side-chains imposed by the quaternary structure of the aggregates. These conclusions are supported also by the results of the quenching experiments with Cs⁺ and I[−] ions. In contrast with acrylamide, which can penetrate the protein matrix, the ionic quenchers are hydrated and cannot diffuse into and through the protein molecule; they can thus quench only surface fluorophores. Thus, the ionic species can discriminate between 'exposed' and 'buried' chromophores. Exposure of oxy- and apo-forms of Hc, RHSS1 and RHSS2 to increasing Cs⁺ or I[−] concentrations had no effect on their fluorescence after excitation at 295 nm. This confirms the conclusion that the tryptophyl side chains are deeply buried in the interior of the investigated proteins.

The tryptophyl emission quantum yields of the *Rapana* oxy-Hc, oxy-RHSS1 and oxy-RHSS2 are very low, 0.011–0.013, in comparison with that for the model compound Ac-Trp-NH₂ (Table 1). The removal of copper from the oxygen-binding sites

Figure 2 Tyrosine-to-tryptophan energy transfer efficiency in the *Rapana* apo-Hc (▲), apo-subunit RHSS1 (○) and apo-subunit RHSS2 (●)

The upper () and the lowest () solid curves are theoretical and are obtained for different values of the transfer efficiency ($e = 1.00$ and 0 respectively).

causes a considerable increase of the emission intensity, and quantum yields of 0.052–0.082 were determined for the apo-forms of the three species (Table 1). This finding can be explained by radiationless energy transfer from the excited indole rings to the copper–dioxygen complex. Theoretical possibilities for such a transfer exist because the emission spectra of the tryptophyl chromophores (donors) overlap the absorption spectrum of the copper–dioxygen complex (acceptor). The observed effect can be explained also by quenching with the bound dioxygen [23] or with ‘heavy atom’ and ‘paramagnetic ion’ effects [24]. The existence of a class of non-fluorescent tryptophans can also be supposed. The removal of copper ions from the dioxygen-binding site ‘demasked’ these chromophores and caused a considerable increase of the quantum yield (Table 1).

Rapana Hc and its constituent subunits contain both tyrosyl and tryptophyl residues. The absence of tyrosyl emission in the fluorescence spectra after excitation at 280 nm, where both phenol and indole groups absorb, can be explained by a singlet–singlet radiationless energy transfer from phenol groups (donors) to indole rings (acceptors) according to Förster’s theory of electronic energy transfer in donor–acceptor systems [25]. The probability of transfer of electronic energy between chromophores depends largely on the mutual orientation in space of the donor and acceptor dipoles [26]. We have determined the efficiency of Tyr-to-Trp transfer of excitation energy in the investigated apo-proteins (Figure 2) and the results are shown in Table 1. Tryptophans in the *Rapana* apo-Hc are acceptors of a considerable part, 65%, of the incident light absorbed by the tyrosyl side chains. Exceptionally lower degrees of Tyr-to-Trp energy transfer, 37 and 16%, were observed in the two subunits. This can be explained by more possibilities of suitable mutual orientation of donor and acceptor groups in the Hc aggregates in comparison with the individual subunits. Evidently, an efficient ‘interchain’ energy transfer between phenol and indole groups from different subunits occurs in the non-dissociated form of Hc. The effect of pH on the tryptophyl fluorescence quantum yield of the *Rapana* Hc and its structural subunits is shown in Figure 3. No changes in the emission of the protein aggregates and the constituent subunits RHSS1 and RHSS2 were observed in the pH region 6.3–9.0. Transitions with midpoints at pH 5.1, 5.2 and 5.3 were evident in the pH profiles of the three species.

Figure 3 pH-dependence of the tryptophyl fluorescence of the *Rapana* apo-Hc (▲), apo-subunit RHSS1 (○) and apo-subunit RHSS2 (●)

The emission was monitored after excitation of the protein samples at 295 nm.

However, these transitions were not connected with conformational changes because the emission maximum positions of the fluorescence spectra were independent of pH between 4 and 8. Moreover, the far-UV CD spectra of the non-dissociated aggregates and of the subunits were practically constant over the pH range 4.5–10, suggesting that the investigated proteins preserve their structural integrity under these conditions. Figure 4 demonstrates the pH-dependence of the ellipticity at 222 nm, connected mainly with the α -helix content. These results are in line with the fluorescence emission maximum position and confirm the conclusion that the structure of the three species is stable over the pH range where transitions in the emission were observed. Most probably, the transition midpoints represent apparent pKs of ionogenic groups located in the immediate vicinity of the fluorophores. The calculated values are characteristic for imidazole groups of histidyl residues. It can be concluded that the emission of tryptophyl residues in the Hc aggregates as well as in

Figure 4 pH-dependence of the ellipticity at 222 nm of the *Rapana* apo-Hc (▲), apo-subunit RHSS1 (○) and apo-subunit RHSS2 (●)

The following buffers were used: (a) 0.05 M sodium citrate (pH 3.0–5.0); (b) 0.05 M sodium phosphate (pH 5.0–7.0); (c) 0.05 M Tris/HCl (pH 7.0–9.0); and (d) 0.05 M carbonate/bicarbonate (pH 9.0–10.5).

Figure 5 Temperature dependence of the tryptophyl emission maximum position of the *Rapana* apo-Hc (▲), apo-subunit RHSS1 (○) and apo-subunit RHSS2 (●)

Fluorescence spectra were recorded after excitation at 295 nm and thermal equilibration.

the dissociated subunits is quenched by nearby protonated imidazole groups, which are capable of forming complexes with indole [27]. Electron transfer can be realized in such a system from the excited indole ring to the imidazolium ion. The decrease of the tryptophyl fluorescence in the alkaline region, at pH values above 9, is probably due to an energy transfer from the excited indole groups to ionized tyrosyl side chains.

Thermostability and stability in the presence of chemical denaturants is an important property of biomolecules, especially regarding their practical application. The conformational stability of the *Rapana* Hc and its subunits RHSS1 and RHSS2 against heat was studied by following the change of fluorescence emission maximum position, a parameter which is very sensitive to the environment, with temperature. Thermal denaturation studies were performed only with the apo-forms of the three species because the increase of temperature caused a loss of oxygen bound to the binuclear copper active site influencing the fluorescence parameters. In Figure 5 we compare the thermal denaturations of apo-Hc, apo-RHSS1 and apo-RHSS2, which were irreversible. Probably, this is a common problem for the giant Hc molecules; the heat denaturation of the arthropodan Hc from *Palinurus vulgaris* has also been shown to be irreversible [11]. For this reason, the thermostability of these proteins is described and compared using the melting temperature, T_m . After excitation at 295 nm the three species showed tryptophan fluorescence with a peak at 327–332 nm, which is characteristic for ‘buried’ indole fluorophores. The increase of temperature caused a bathochromic shift of the emission maximum position to final values of 342–346 nm, typical for ‘exposed’ tryptophans. The T_m values were determined as midpoints of the transition curves. The *Rapana* apo-Hc had a T_m of 70 °C and for the apo-RHSS1 and apo-RHSS2 values of 46 and 57 °C respectively were calculated (Table 2). Evidently, the aggregated Hc is considerably more thermostable than the dissociated subunits.

CD was used as a second method to follow the thermal unfolding of the apo-forms of the *Rapana* Hc and its structural subunits. The spectra below 250 nm, which reflect the backbone conformation of the three species, were recorded in the temperature interval 25–80 °C. A general decrease of the negative ellipticity was observed at temperatures higher than 35 °C. The increase of temperature resulted in a marked reduction in secondary structure. A sigmoidal curve was obtained when the

Table 2 Free energy of stabilization in water and T_m values of *Rapana thomasiana* Hc and its structural subunits RHSS1 and RHSS2

A protein concentration of 0.36 mg/ml was used in the CD experiments. The absorbance of the protein solutions used for the fluorescence measurements was less than 0.05 at 295 nm to avoid inner filter effects.

Species	$\Delta G_D^{H_2O}$ (kJ · mol ⁻¹)	T_m value	
		Fluorescence (°C)	CD (°C)
Oxy-Hc	21.6	—	—
Apo-Hc	18.0	70	65
Oxy-RHSS1	15.7	—	—
Apo-RHSS1	12.3	46	44
Oxy-RHSS2	14.1	—	—
Apo-RHSS2	11.5	57	59

Figure 6 Temperature dependence of the ellipticity at 222 nm of the apo-subunit RHSS1 in 0.05 M Tris/HCl buffer (pH = 7.0)

ellipticity at 222 nm was plotted as a function of temperature (Figure 6). Similar curves were obtained for the other two species. The thermal denaturation of the investigated proteins is irreversible. We have followed the forward reaction and the stability was characterized by the melting temperature, T_m . A very good coincidence of the data obtained by fluorescence spectroscopy and CD is observed (Table 2).

Denatured states of proteins play an important role in the protein folding, transport across membranes and proteolysis [28], which has been the reason for the increasing interest in these states during the past few years. $\Delta G_D^{H_2O}$, the free energy of stabilization in water, is a quantitative measure for the protein stability in water solutions. This is the free energy change for the reaction globular conformation $\rightleftharpoons$ random-coiled conformation in the absence of denaturant. However, the equilibrium between the native and denatured states cannot be studied in the absence of denaturant. GnHCl is a widely used reagent for studying such equilibria. The mechanism of denaturation involves modification of the water structure by GnHCl and interaction of the denaturant with peptide groups [29,30]. In order to characterize the

Figure 7 Denaturation of the *Rapana* apo-Hc (the upper panel) and of the apo-subunit RHSS1 (the lower panel) by GnHCl at three different protein concentrations, measured using CD at 222 nm: ●, 0.18 mg of protein/ml; ○, 0.36 mg of protein/ml; and ▲, 0.54 mg of protein/ml

stability of the *Rapana* Hc and its structural subunits, we have investigated the guanidine denaturation of these species and determined $\Delta G_{D^{H_2O}}$ values for the oxy- and apo-forms. The unfolding reactions were followed by CD spectroscopy. It should be mentioned that the CD spectra of the oxy- and apo-forms of the Hc and the structural subunits were remarkably similar. Removal of GnHCl by dialysis showed a partial refolding and the denaturation was approx. 50% reversible. The recorded spectra can be considered as an approximation of the true equilibrium spectra which would be obtained in the absence of irreversible changes. The $\Delta G_{D^{H_2O}}$ values for the oxy- and apo-forms of the *Rapana* Hc and its subunits, summarized in Table 2, are in the region of 11.5–21.6 kJ/mol. The experiments in the presence of GnHCl indicate that *Rapana* Hc, in addition to the increased thermostability, exhibits also an increased resistance to chemical denaturation compared with its subunits.

The protein concentration effect on the denaturation in the presence of GnHCl of the native *Rapana* Hc and its subunits was also investigated. The denaturation experiments were performed at protein concentrations of 0.18, 0.36 and 0.54 mg/ml. A slight concentration dependence was observed for the denaturation of the Hc aggregates. This effect can be explained with linked unfolding and dissociation of the aggregates. The upper panel of Figure 7 demonstrates the protein concentration effect on the

GnHCl denaturation of the *Rapana* apo-Hc. The 3-fold increase of the Hc concentration from 0.18 to 0.54 mg/ml increased $\Delta G_{D^{H_2O}}$ by only 1.5 kJ/mol. Similar results were obtained for the *Rapana* oxy-Hc. The aggregation stabilizes this Hc with 5.9–7.5 kJ/mol in comparison with the constituent subunits. The denaturation of the subunits RHSS1 and RHSS2 was concentration independent, as expected for proteins in monomeric form. The lower panel of Figure 7 shows the protein concentration dependence of the apo-RHSS1 denaturation in GnHCl at protein concentrations of 0.18, 0.36 and 0.54 mg/ml. Similar results for the apo-RHSS2 were obtained.

In conclusion, the investigations described here permit comparison of the conformational stability of *Rapana* Hc with that of the constituent subunits. Subunit–subunit interactions are important for the stability of the native (aggregated) protein towards thermal and chemical (GnHCl) denaturation. The results are of particular interest in light of the important role of Hc stability in the biochemical and medical application of these respiratory proteins.

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REFERENCES

- Sternier, R. and Decker, H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4835–4839
- Herskovits, T. T. (1988) Comp. Biochem. Physiol. **91B**, 597–611
- Salvato, B. and Beltramini, M. (1990) Life Chem. Rep. **8**, 1–47
- Recker, F. and Rübner, H. (1989) Urol. Int. **44**, 77–80
- Jennemann, R., Gnewuch, C., Bosslet, S., Bauer, B. L. and Wiegandt, H. (1994) J. Biochem. (Tokyo) **115**, 1047–1052
- Jurinic, C. D., Engelmann, U., Gasch, J. and Klippel, K. F. (1988) J. Urol. **139**, 723–726
- Lamm, D. L., DeHaven, J. I., Riggs, D. R., Delgra, C. and Burrell, R. (1993) Urol. Res. **21**, 33–37
- Bichler, K.-H., Kleinknecht, S. and Strohmaier, W. L. (1990) Urol. Int. **45**, 269–283
- Gebauer, W., Harris, J. R., Heid, H., Söling, M., Hillenbrand, R., Söngen, S., Strake, A. W. and Markl, J. (1994) Zoology **98**, 51–68
- Markl, J., Niemann, A. S., Strake, A. W., Söling, M., Schneider, A., Gebauer, W. and Harris, J. R. (1991) Naturwissenschaften **78**, 512–514
- Guzman-Casado, M., Parody-Morreale, A., Mateo, P. L. and Sanchez-Ruiz, J. M. (1990) Eur. J. Biochem. **188**, 181–185
- Ricchelli, F., Filippi, B. and Salvato, B. (1981) In Invertebrate Oxygen-binding Proteins (Lamy, J. and Lamy, J., eds.), pp. 31–39, Marcel Dekker, Inc., New York and Basel
- Boteva, R., Severov, S., Genov, N., Beltramini, M., Filippi, B., Ricchelli, F., Tallandini, L., Pallhuber, M. M., Tognon, G. and Salvato, B. (1991) Comp. Biochem. Physiol. **100B**, 493–501
- Stoeva, S., Rachev, R., Severov, S., Voelter, W. and Genov, N. (1995) Comp. Biochem. Physiol. **108B**, 761–765
- Idakieva, K., Severov, S., Svendsen, I., Genov, N., Stoeva, S., Beltramini, M., Tognon, G., DiMuro, P. and Salvato, B. (1993) Comp. Biochem. Physiol. **106B**, 53–59
- Kirby, E. P. and Steiner, R. F. (1970) J. Biol. Chem. **245**, 3600–3606
- Lehrer, S. S. (1971) Biochemistry **10**, 3254–3263
- Eisinger, J. (1969) Biochemistry **8**, 3902–3907
- Genov, N., Shopova, M., Boteva, R., Jori, G. and Ricchelli, F. (1982) Biochem. J. **207**, 193–200
- Eftink, M. R. and Ghiron, C. A. (1976) Biochemistry **15**, 672–680
- Eftink, M. R. and Ghiron, C. A. (1981) Anal. Biochem. **114**, 199–227
- Ricchelli, F., Jori, G., Shopova, M., Boteva, R. and Genov, N. (1981) Int. J. Peptide Protein Res. **17**, 330–337
- Bannister, W. H. and Wood, E. J. (1971) Comp. Biochem. Physiol. **40B**, 7–18
- Ricchelli, F., Beltramini, M., Flamigni, L. and Salvato, B. (1986) Med. Biol. Environ. **14**, 169–172
- Stryer, L. (1978) Annu. Rev. Biochem. **47**, 819–846
- Haas, E., Katchalski-Katzir, E. and Steinberg, I. (1978) Biochemistry **17**, 5064–5070
- Burstein, E. and Bussel, E. (1971) Spektroskopie **30**, 69–73
- Dill, K. A. and Shortle, D. (1991) Annu. Rev. Biochem. **60**, 795–825
- Prakash, V., Loucheux, C., Scheufele, S., Gorbunoff, M. and Timasheff, S. (1981) Arch. Biochem. Biophys. **210**, 455–464
- Nandi, P. K. and Robinson, D. R. (1984) J. Am. Chem. Soc. **106**, 6661–6668