Contribution of Disulfide Bonds and Calcium to Molluscan Hemocyanin Stability

Dessislava N. Georgieva[a,b,*], Nicolay Genov[b], Markus Perbandt[a,c], Wolfgang Voelter[d], and Christian Betzel[a]

[a] Universitätsklinikum Hamburg-Eppendorf, Zentrum für Experimentelle Medizin, Institut für Biochemie und Molekularbiologie I, c/o DESY, Notkestr. 85, Geb. 22a, D-22603 Hamburg, Germany. Fax: (040) 89984747. E-mail: Georgieva@unisgi1.desy.de
[b] Institute of Organic Chemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev str. Bl. 9, Sofia 1113, Bulgaria
[c] Institut für Biochemie und Lebensmittelchemie, Abteilung für Biochemie und Molekularbiologie, D-20146 Hamburg, Germany
[d] Abteilung für Physikalische Biochemie, Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Straße 4, D-72076 Tübingen, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 281–287 (2004); received November 19/December 18, 2003

Disulfide bonds and calcium ions contribute significantly to the stability of the hemocyanin from the mollusc *Rapana thomasiana* grosse (gastropod). An extremely powerful protective effect of Ca^{2+} at a concentration of 100 mM (100% protection) against the destructive effect of reductants like dithiothreitol was observed. This is important for the practical application of molluscan hemocyanins in experimental biochemistry, immunology and medicine.
The reduction of the disulfide bonds in the *Rapana* hemocyanin leads to a 20% decrease of the α-helical structure. The S-S bonds contribute significantly to the free energy of stabilization in water increasing $\Delta G_D^{H_2O}$ by 6.9 kJ mol$^{-1.}$ The data are related to the X-ray model of the *Rapana* hemocyanin functional unit RtH2e. The results of this study can be of common validity for related respiratory proteins because the cysteine residues are conserved in all sequences of molluscan hemocyanins published so far.

Key words: Hemocyanin, *Rapana thomasiana*, Disulfide

Introduction

We have investigated for a number of years the hemocyanin (Hc) from the prosobranch gastropod *Rapana thomasiana* grosse. This mollusc is a marine snail, originally living along the coast of Japan, in the Yellow Sea and East China Sea. In 1947 this invertebrate was discovered in the Black Sea where it adapted. The salinity of the Black Sea is less than half of that of the Pacific Ocean and this specific ecosystem can influence physiological properties of *Rapana thomasiana.* In previous papers we have described the isolation of the two structural subunits of the *Rapana* Hc (Idakieva *et al.*, 1993), the determination of the complete amino acid sequence of the *N*-terminal functional unit (FU) (Stoeva *et al.*, 1997a) and the arrangement of the FUs within the subunits RHSS1 (Stoeva *et al.*, 1997b) and RHSS2 (Idakieva *et al.*, 2000).

Abbreviations: Hc, hemocyanin; FU, functional unit; DTT, dithiothreitol; SWMC, single wavelength melting curve.

The significance of disulfide bonds for the protein structure and function has always been of great interest because the results of such investigations can be used for the design of new proteins with modified properties (Zhu *et al.*, 1995). In this connection it was shown that disulfide bond reactions, especially those with reductants, are suitable tools for studying protein folding, structure and stability (Wedemeyer *et al.*, 2000). In the case of arthropod Hc from *Limulus polyphemus* it was shown that dithiothreitol is a suitable chemical probe to study structure-function relationships in Hcs (Topham *et al.*, 1998). The giant dioxygen-binding proteins in the hemolymph of molluscs and arthropods form one of the three groups of respiratory proteins: hemoglobins/erythrocruorins, hemerithrins and hemocyanins. However, the information reported to date on the stability of these biomolecules is limited (Georgieva *et al.*, 1998). Here we show that the disulfide bonds in the *Rapana thomasiana* Hc contribute significantly to the conformational stability of this protein increasing the free energy of stabilization in water $\Delta G_D^{H_2O}$.

0939–5075/2004/0300–0281 $ 06.00

We have observed an extremely powerful protective effect of Ca^{2+} at a concentration of 100 mM (100% protection) against the destructive action of the reductant dithiothreitol (DTT) on the physiologically active dioxygen-binding site of the investigated molluscan Hc. No such considerable effect has been reported for arthropod Hcs so far. In our opinion, this result has also practical significance, because molluscan Hcs, in contrast to their counterparts from arthropods, found application in experimental biochemistry, immunology, and medicine. Recently, the X-ray structure of the *Rapana* Hc functional unit RtH2e was determined by our group (Perbandt *et al.*, 2003) which allowed explanation of the present results by the 3-D model. Most probably, molluscan Hc FUs have a similar tertiary folding and the results, described here, can be valid for all molluscan Hcs.

Materials and Methods

Preparation of the Rapana thomasiana hemocyanin and separation of the subunits

Living marine snails, *Rapana thomasiana* grosse, were caught at the Bulgarian coast of the Black Sea in the region of Golden Sands and stored in sea water before collection of the hemolymph. The isolation of the hemocyanin was performed as described previously (Boteva *et al.*, 1991). Blue coloured samples of the purified respiratory protein in oxy-form were stored at – 20 °C in 20% solution of sucrose (w/v) until used.

Native oxy-*Rapana* Hc was dissociated by dialysis against 50 mM Tris-(hydroxymethyl)aminomethane/HCl buffer, containing 10 mM EDTA, pH 9.0. The two subunits, oxy-RHSS1 and oxy-RHSS2, were isolated by chromatography on a DEAE-Sepharose CL-6B (37 × 3.0 cm) column, equilibrated with 50 mM Tris-(hydroxymethyl)aminomethane/HCl buffer, containing 10 mM EDTA, 2 M urea, pH 9.0, and eluted under a linear 0.2–0.4 M gradient of NaCl.

Reaction of the oxy-Rapana thomasiana hemocyanin and its separated subunits with dithiothreitol

The reaction of the oxy-*Rapana* Hc and its subunits, oxy-RHSS1 and oxy-RHSS2, was followed spectrophotometrically by changes of the absorbance at 340 nm, the band characteristic for the oxy-Hc (Topham *et al.*, 1998). 25 μl of a 1 M solution of DTT were added to a 2.5 ml solution of the Hc or its structural subunit in 50 mM Tris/HCl buffer, containing 10 mM EDTA, pH 9.0, *i.e.* the reaction mixtures were approximately 10 mM as regards DTT. Experiments with added $CaCl_2$ were performed in the absence of EDTA. The absorbance of the solution at 340 nm was 1.0.

Determination of free sulfhydryl groups after disulfide bond cleavage

The cysteine content of the Hc samples before and after reduction was determined after separation of DTT from the protein by gel filtration and using the method of Ellmann (Ellman, 1959). According to this method, the thiol concentration was determined spectrophotometrically using 5,5′-dithiobis (2-nitrobenzoic acid).

Spectroscopic measurements

Absorption spectroscopy studies were performed using a Shimadzu recording spectrophotometer model 3000, equipped with a thermostated cell holder. Circular dichroism spectra of the native and reduced *Rapana* Hc and its subunits were recorded by 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. A software of the company Jasco was used for calculations of the CD data. The thermal denaturation of Hc samples in oxy- or reduced forms was followed by CD measurements on the same instrument. Protein solutions were placed in a cell holder which was thermostatically controlled using a NESLAB thermostat model RTE-110, connected with a digital programming controller. The samples were kept for 10 min at the desired temperature to ensure the attainment of thermal equilibrium, confirmed by the constancy of the ellipticity. Each spectrum presented is an average of three measurements.

Calculation of the free energy of stabilization in water

The free energy of denaturation in water, ΔG_D, of native and reduced *Rapana* Hc, was determined after denaturation in the presence of guanidine hydrochloride, using the equation:

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

where K is the equilibrium constant, calculated by the following equation:

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

$[\Theta]_{obs}$ 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 protein. The free energy of stabilization in water at 25 °C, ΔG_D^{H2O}, was calculated from a plot of ΔG_D *versus* guanidine hydrochloride concentration.

Computer graphic studies

Computer graphic studies of the *Rapana* Hc FU RtH2e three-dimensional (3-D) structure were carried out using our own coordinates of the refined RtH2e structure (Perbandt *et al.*, 2003). The program TURBO Frodo (Roussel and Cambilau, 1991) was applied.

Results and Discussion

Reaction of the Rapana thomasiana hemocyanin and its subunits with dithiothreitol

The reaction of oxy-*Rapana* hemocyanin and its subunits, oxy-RHSS1 and oxy-RHSS2, with DTT was monitored spectrophotometrically in the near UV region. Addition of the reagent to the solutions of dissociated Hc and the two separated subunits resulted in disappearance of the band at 340 nm (Fig. 1) which is characteristic for the copper-dioxygen system at the binuclear active site. This is a spectroscopic evidence for the disruption of the Hc active sites and loss of dioxygen-binding capacity (Topham *et al.*, 1998). Quantitative analysis of the free sulfhydryl groups in the samples after completion of the reaction (usually after 1 h) showed that the three disulfide bonds per FU were reduced. Evidently, cystines are important for the maintenance of the physiologically active conformation of the *Rapana* Hc whose structural subunits contain eight FUs per subunit. Reduction of disulfides led to changes in the three-dimensional protein structure and disruption of the functionally important active site geometry. As a result, the modified protein can not perform its biological function – transportation of dioxygen to the tissues.

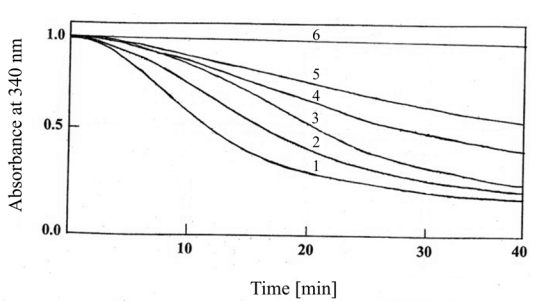

Fig. 1. Kinetics of the active site destruction in: oxy-*Rapana* hemocyanin subunit RHSS2 (1); oxy-*Rapana* hemocyanin subunit RHSS1 (2); oxy-*Rapana* hemocyanin (3); oxy-RHSS2 + 100 mM Ca^{2+} (4); oxy-RHSS1 + 100 mM Ca^{2+} (5) and oxy-*Rapana* hemocyanin + 100 mM Ca^{2+} (6).

Dissociated oxy-*Rapana* Hc samples were prepared in order to increase the accessibility of disulfides to the reducing agent. Dissociation of the native aggregates has no effect on the secondary and tertiary structure of the subunits and the CD spectra of solutions containing undissociated or dissociated Hc are identical (Boteva *et al.*, 1991).

Different kinetics of the active site destruction were observed for the oxy-*Rapana* Hc, oxy-RHSS1 and oxy-RHSS2. In the absence of bound Ca^{2+}, at pH 9.0, the protein aggregates exist as mixtures of monomeric polypeptide chains. The mixture of the two types of subunits, obtained after dissociation of the native Hc (Fig. 1, curve 3), is more resistant to the action of the reducing agent in comparison to the samples containing only one type of polypeptide chains. RHSS1 (Fig. 1, curve 2) is more stable than RHSS2 (Fig. 1, curve 1). In previous studies we have observed a considerable instability of RHSS2 during the purification procedures after the dissociation of the native *Rapana* Hc aggregates (Idakieva *et al.*, 1993). The reason for the different rates of the loss of dioxygen-binding capacity could be differences in the accessibility of disulfide bonds to DTT and in the stability of the respective subunits.

Samples of the reduced *Rapana* Hc and its separated subunits were dialized in order to investigate the possibility for a recovery of the native protein structure and the capability of the active site to bind dioxygen. Only partial recovery (15–20%) of these properties was observed. The absence of reversibility can be explained by burying of reactive groups in the tertiary structure which would prevent their rearrangement in S-S bridges. Another reason can be the formation of nonnative intermolecular cross-links with sulfhydryl groups from neighbouring subunits. Evidently, it is difficult for the huge, 450 kDa polypeptide chains to restore their native conformation.

Effect of Ca^{2+} on the rate of destruction of the active sites

Fig. 1 shows that Ca^{2+} in concentration of 100 mM prevents the active site destruction. The most important result is that under these conditions the native oxy-*Rapana* Hc preserves approximately 100% of the dioxygen-binding capacity after the treatment with DTT (Fig. 1, curve 6). In the presence of added Ca^{2+}, oxy-*Rapana* Hc exists in its native form: cylindrical aggregates, each composed of 20 structural subunits. Most probably, calcium ions impede the reduction of disulfide bridges stabilizing the quaternary structure which makes difficult the penetration of the reducing agent in the interior of the Hc aggregates. Also, Ca^{2+} stabilizes the three-dimensional structure of the subunits and in this way reduces the destructive effect of disulfide bond reduction. It can be supposed that calcium ion binding is connected with conformational changes adding more rigidity and stability to the Hc molecule. The Ca^{2+}-induced stability will be better explained after crystallization and X-ray structure determination of a molluscan Hc FU containing bound Ca^{2+} as the crystal structure determination of the FU Odg from the *Octopus dofleini* (cephalopod, mollusc) Hc (Cuff *et al.*, 1998) and our X-ray investigations on the *Rapana* Hc FU RtH2e (Perbandt *et al.*, 2003) have been performed in the absence of added calcium ions. The protective effect of Ca^{2+} in the case of the separated units oxy-RHSS1 (Fig. 1, curves 2 and 5, respectively) and oxy-RHSS2 (Fig. 1, curves 1 and 4, respectively) can be explained, except with the stabilization of the tertiary structure, with the formation of homo-aggregates hindering the penetration of DTT in the internal regions. It is known (Brenowitz *et al.*, 1984) that calcium ions cause the Hc subunits to undergo self-association and formation of quaternary structures can be expected. 0.1 M Ca^{2+} is not a physiological concentration of this ion. However, such a concentration of $CaCl_2$ can be used for storage of hemocyanin solutions to protect them against undesirable reduction.

Effect of disulfide bond reduction on the secondary structure of the Rapana thomasiana Hc and its subunits

Fig. 2 A shows circular dichroism spectra of the dissociated oxy-*Rapana* Hc (curve 2), apo-*Rapana* Hc (curve 1) and reduced *Rapana* Hc (curve 3).

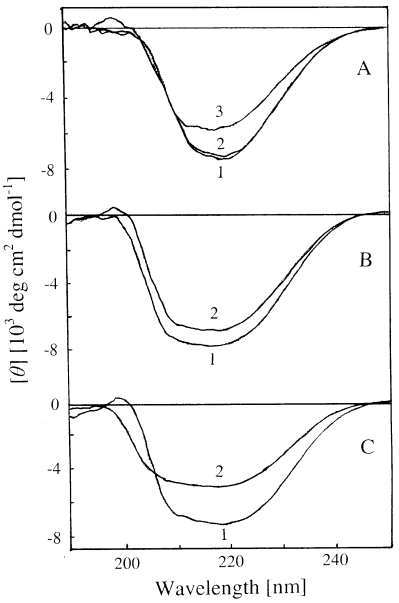

Fig. 2. Far ultraviolet circular dichroism spectra of A: apo-*Rapana* hemocyanin (1), oxy-*Rapana* hemocyanin (2) and reduced *Rapana* hemocyanin (3); B: oxy- (1) and reduced (2) *Rapana* hemocyanin subunit RHSS1; C: oxy- (1) and reduced (2) *Rapana* hemocyanin subunit RHSS2. (dmol = 10^{-1} mol).

Reduction of the disulfide bridges caused changes in the hemocyanin far UV CD spectrum and the intensity of dichroic bands was reduced. The effect is not due to the destruction of the active site because the CD spectrum of the apo-*Rapana* Hc, in which this site is "removed", is practically identical to that of the oxy-*Rapana* Hc. So, the difference between the spectrum of the oxy- and reduced *Rapana* Hc reflects the contribution of disulfides to the protein secondary structure. 20% decrease of the intensity of the band at 221 nm, which is connected mainly with the α-helix structure, was observed. This means rearrangement of part(s) of the structure leading to partial disruption of the helical structure. The partially unfolded structure of the fully reduced *Rapana* Hc may resemble the transient conformational state formed in the first phase of folding of disulfide-intact protein.

The effect of disulfide bond reduction on the secondary structure of the two separated subunits is considerably more pronounced in the case of oxy-RHSS2 (Fig. 2, C) than in oxy-RHSS1 (Fig. 2, B). This result is in line with the observed higher instability of the first subunit in comparison to the second one.

Contribution of the disulfide bonds to the conformational stability of the Rapana thomasiana hemocyanin

The contribution of the disulfide bonds to the conformational stability of the oxy-*Rapana* Hc was assessed by reducing these bonds and determining the free energy of stabilization in water for the native and reduced form. $\Delta G_D^{H_2O}$ is the free energy change for the reaction globular conformation $\rightleftharpoons$ random-coiled conformation in the absence of denaturant and it is a quantitative measure for the protein stability in water solutions. We have investigated the equilibrium in the presence of different concentrations of guanidine hydrochloride, monitored by circular dichroism, and extrapolated the data to zero concentration of the denaturant. The denaturation was in agreement with an apparent two-state mechanism. The $\Delta G_D^{H_2O}$ value for the native oxy-*Rapana* Hc was determined to be 18.1 kJ mol^{-1}, similar to that of the apo-*Rapana* Hc (Dolashka *et al.*, 1996). This means that the conformational stability is not appreciably perturbed after the destruction of the active sites. However, a considerable decrease in the free energy of stabilization in water was found when the disulfide bonds were split and we have calculated a $\Delta G_D^{H_2O}$ of 11.2 kJ mol^{-1} for the reduced *Rapana* Hc. From these values it can be concluded that the disulfide bonds stabilize additionally the *Rapana* Hc structure by 6.9 kJ mol^{-1}.

Effect of disulfide bond reduction on the thermal stability of the oxy-Rapana thomasiana hemocyanin and its subunits

Circular dichroism was used also to follow the thermal protein unfolding and the far UV spectra were recorded after approximately 5 °C increases in cell temperature. The spectra were dominated by negative bands at 208–209 nm and 220–222 nm, connected mainly with the α-helix structure. A general decrease of the negative ellipticity was observed at temperatures higher than 50 °C. The thermal denaturation was irreversible due probably to aggregation of the huge Hc molecules. The same problem was reported for other Hcs (Guzman-Casado *et al.*, 1990). For this reason we have followed only the forward reaction and the thermostability is described and compared using the transition or "melting" temperature, T_m, the midpoint of the sigmoidal denaturation curves. Representative single wavelength melting curves

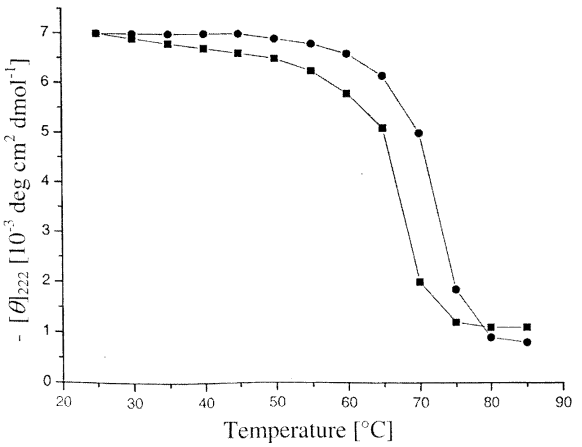

Fig. 3. Single wavelength melting curves for the thermal denaturation of the oxy- (●–●) and reduced (■–■) *Rapana* hemocyanin. T_m values of 73 and 67 °C were calculated for the oxy- and reduced form, respectively.

(SWMC) for the oxy- and reduced dissociated *Rapana* Hc are shown in Fig. 3. Similar SWMCs were obtained for RHSS1 and RHSS2. The midpoints of the temperature transitions of oxy-*Rapana* Hc, oxy-RHSS1 and oxy-RHSS2 are at 73, 64 and 62 °C, respectively, which characterizes these dioxygen carriers as relatively thermostable proteins. The values for the reduced forms of the Hc, RHSS1 and RHSS2 were calculated to be 67, 60 and 56 °C, respectively. It is evident that the reduction of S-S bridges in the *Rapana* Hc and its subunits causes a significant decrease of the melting temperature.

Relation of the data to the X-ray model of the Rapana thomasiana hemocyanin functional unit RtH2e

The primary effect of S-S bonds in a polypeptide chain is to impose distance and angle constraints between the C^β and S^γ atoms of the joined cysteine residues, thus reducing the entropy (Pace *et al.*, 1988). These bridges can also stabilize the folded state enthalpically through favorable local interactions, *e.g.* by stabilizing the packing of a local cluster of hydrophobic residues (Wedemeyer *et al.*, 2000). Thus, the reduction of the disulfides can have a complex effect on the protein three-dimensional structure. We have inspected the *Rapana* Hc FU RtH2e 3-D structure using our own coordinates. The crystallographic model showed that the *N*-terminal (core) domain contains two of the

three disulfide bonds. Cys47–Cys58 anchors two short strands to each other and stabilizes the loop 47–60 which is in the neighbourhood of the active site. Cys171 from the helix α_6 and Cys238, which lies at the beginning of α_8, form the second bridge and connect a loop region with the helical core. The two S-S bonds add stability to the core domain containing the physiologically active dioxygen-binding site. The third disulfide bond, Cys328–Cys334, is located in the *C*-terminal domain and connects strands β_{10} and β_{11}. Cleavage of the bridges destabilizes the structure in the region of the active site leading to changes in its geometry. These facts can explain the destructive effect of disulfide reduction on the dioxygen-binding site.

Conclusions

The X-ray model of the *Rapana* Hc functional unit RtH2e reveals that six of the seven cysteines, which are conserved in all published sequences of molluscan Hc FUs (Miller *et al.*, 1998; Lieb *et al.*, 2000) form three disulfide bridges. These units are highly homologous globular structures and most probably the three S-S bonds are also conserved in all of them. In such a case it seems that the conclusions about the contribution of the disulfide bridges and calcium ions to the stability of the *Rapana thomasiana* Hc will be of common validity for the related molluscan respiratory proteins. The present study demonstrates that disulfide bridges in these giant biomolecules are important elements of their structure and contribute significantly to the free energy of stabilization in water. These bonds are critical for the preservation of the physiological function – reversible transport of dioxygen to the tissues because their reduction leads to destruction of the active sites and loss of dioxigen-binding ability. The destructive effect of reductants such as DTT on the physiologically active geometry of the active site can be completely avoided in the presence of 100 mM $CaCl_2$ which ensures 100% preservation of the biological activity. This is important especially for the storage of molluscan Hcs.

Acknowledgements

D. N. Georgieva thanks the Alexander von Humboldt Foundation, Bonn, Germany for providing a Research Fellowship, IV-BUL/1073481 STP. This work was supported in part by the Bulgarian National Foundation for Scientific Research, grant X-1209.

Boteva R., Severov S., Genov N., Beltramini M., Filippi B., Ricchelli F., Tallandini L., Pallhuber M. M., Tognon G., and Salvato B. (1991), Biochemical and functional characterization of *Rapana thomasiana* hemocyanin. Comp. Biochem. Physiol. **100B**, 493–501.

Brenowitz M., Bonaventura C., and Bonaventura J. (1984), Self-association and oxygen-binding characteristics of the isolated subunits of *Limulus polyphemus* hemocyanin. Arch. Biochem. Biophys. **230**, 238–249.

Cuff M. E., Miller K. I., van Holde K. E., and Hendrickson W. A. (1998), Crystal structure of a functional unit from *Octopus* hemocyanin. J. Mol. Biol. **278**, 855–870.

Dolashka P., Genov N., Parvanova K., Voelter W., Geiger M., and Stoeva S. (1996), *Rapana thomasiana* grosse (gastropod) haemocyanin: spectroscopic studies of the structure in solution and the conformational stability of the native protein and its structural subunits. Biochem. J. **315**, 139–144.

Ellmann G. L. (1959), Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**, 70–76.

Georgieva D., Stoeva S., Ali A., Genov N., and Voelter W. (1998), Circular dichroism study of the hemocyanin thermostability. Spectrochim. Acta A **54**, 765–771.

Guzman-Casado M., Parodi-Morreale A., Mateo P. L., and Sanchez-Ruiz J. M. (1990), Differential scanning calorimetry of lobster haemocyanin. Eur. J. Biochem. **188**, 181–185.

Idakieva K., Severov S., Svendsen I., Genov N., Stoeva S., Beltramini M., Tognon G., Di Muro P., and Salvato B. (1993), Structural properties of *Rapana thomasiana* grosse hemocyanin: isolation, characterization and *N*-terminal amino acid sequence of two different dissociation products. Comp. Biochem. Physiol. **106B**, 53–59.

Idakieva K., Stoeva S., Pervanova K., Genov N., and Voelter W. (2000), Arrangement of functional units within the *Rapana thomasiana* hemocyanin subunit RtH2. Biochim. Biophys. Acta **1479**, 175–184.

Lieb B., Altenhein B., and Markl J. (2000), The sequence of a gastropod hemocyanin (HtH1 from *Haliotis tuberculata*). J. Biol. Chem. **275**, 5675–5681.

Miller K. I., Cuff M. E., Lang W. F., Varga-Weisz P., Field K. G., and van Holde K. E. (1998), Sequence of the *Octopus dofleini* hemocyanin subunit: structural and evolutionary implications. J. Mol. Biol. **278**, 827–842.

Pace C. N., Grimsley G. R., Thomson J. A., and Barnett B. J. (1988), Conformational stability and activity of ribonuclease T_1 with zero, one, and two intact disulfide bonds. J. Biol. Chem. **263**, 11820–11825.

Perbandt M., Guthhörlein E. W., Rypniewski W., Idakieva K., Stoeva S., Voelter W., Genov N., and Betzel Ch. (2003), The structure of a functional unit from the wall of a gastropod hemocyanin offers a possible mechanism for cooperativity. Biochemistry **42**, 6341–6346.

Roussel A. and Cambilau C. (1991), Silicon graphics geometry partners directory. Silicon graphics, Mountain View, CA, p. 86.

Stoeva S., Idakieva K., Genov N., and Voelter W. (1997a), Complete amino acid sequence of dioxygen-binding functional unit of the *Rapana thomasiana* hemocyanin. Biochem. Biophys. Res. Commun. **238**, 403–410.

Stoeva S., Dolashka P., Pervanova K., Genov N., and Voelter W. (1997b), Multidomain structure of the *Rapana thomasiana* (gastropod) hemocyanin structural subunit RHSS1. Comp. Biochem. Physiol. **118B**, 927–934.

Topham R., Tesh S., Cole G., Mercatante D., Westcott A., and Bonaventura C. (1998), Active-site disruption in native *Limulus* hemocyanin and its subunits by disulfide-bond reductants: a chemical probe for the study of structure-function relationships in the hemocyanins. Arch. Biochem. Biophys. **352**, 103–113.

Wedemeyer W. J., Welker E., Narayan M., and Scheraga H. A. (2000), Disulfide bonds and protein folding. Biochemistry **39**, 4207–4216.

Zhu H., Dupureur C. M., Zhang X., and Tsai M.-D. (1995), Phospholipase A2 engineering. The roles of disulfide bonds in structure, conformational stability and catalytic function. Biochemistry **34**, 15307–15314.