Penaeus monodon (Tiger Shrimp) Hemocyanin: Subunit Composition and Thermostability

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Arthropod Hemocyanin, Functional Unit, Stability

Penaeus monodon (class Crustacea, order Decapoda) is one of the largest shrimps of the *Penaeidea* family from the Indo – West Pacific region. The dioxygen-transporting protein hemocyanin, isolated from the hemolymph of this invertebrate, is composed of three 75–76 kDa structural/functional subunits designated as Pm1, Pm2 and Pm3. The N-terminal sequences of the chains were determined and compared with those of other decapodan hemocyanin subunits. Pm2 and Pm3 are highly homologous and electrophoretically undistinguishable polypeptides. In comparison to Pm1, they have an extension of six residues. Pm1 is closely related to the subunit Pv2 of the *Penaeus vannamei* hemocyanin. Probably, subunits like Pm1 and Pv2 are family-specific for the *Penaeidea* hemocyanins and the other subunits are species-specific. Comparison of N-terminal sequences of respiratory proteins from the sub-orders Natantia and Reptantia demonstrated family- and sub-order-specific sequences.

A melting point of 69 °C, lower than those for the di-hexameric decapodan hemocyanins, was determined from the temperature dependence of ellipticity of the mono-hexameric *Penaeus monodon* hemocyanin. Thermostability of decapodan hemocyanins depends on their aggregation state.

Introduction

Hemocyanins (Hcs) are oligomeric dioxygen-transporting proteins, found in the hemolymph of arthropods and molluscs. They are large aggregates with molecular masses in the range 4.5×10^5 Da to 43.4×10^6 Da (Herskovits and Hamilton, 1991). The aggregates are composed of different structural/functional subunits. Arthropodan and molluscan Hcs perform the same physiological function connected with the circulatory transport of dioxygen to the tissues. However, the molecular architecture of aggregates as well as the size of the structural and functional units are quite different in the dioxygen carriers from the two *phyla*. Arthropodan Hcs are hexamers (1×6) or multiples of hexamers (2×6, 4×6, 6×6 or 8×6) of appr. 75 kDa structural subunits. The basic hexamer is built of six bean-shaped subunits located in the corners of a trigonal antiprism. Models of multi-hexameric Hcs were prepared using computer-processed electron micrographs (Markl and Decker, 1992). Each subunit is a single polypeptide chain containing a dinuclear dioxygen-binding site. The active site consists of two non-equivalent copper atoms (Cu_A and Cu_B), each coordinated by three imidazole groups of histidyl residues (Volbeda and Hol, 1989). The three-dimensional organization of the *Panulirus interruptus* deoxy-hemocynin was determined at 3.2 Å resolution by X-ray crystallography (Gaykema *et al.*, 1984). The crystals used for these studies contained subunits **a** and **b** in equal amounts. The model revealed that each subunit is folded into three domains, and that the second domain contains the dinuclear copper site. Also, the crystal structures of deoxygenated and oxygenated *Limulus polyphemus* hemocyanin subunit II were solved at 2.18 and 2.40 Å, respectively (Hazes *et al.,* 1993; Magnus *et al.*, 1994). Analysis of the two forms showed that their tertiary and quaternary structures are quite similar. However, the copper-copper distance in the oxygenated Hc is 1 Å less than that in the deoxygenated subunit II (Magnus *et al.*, 1994). Markl (1986) classified the subunits of arthropodan hemocyanins

into immunologically discernible types. Alpha subunits are immunologically similar and are "phylogenetically conservative". The beta type polypeptide chains differ immunologically among the species and are "phylogenetically variable". Gamma subunits probably have evolved from alpha (Markl, 1986).

Molluscan Hcs form cylindrical structures, 30–35 nm in diameter and 15–18 nm in hight (van Holde *et al.*, 1992; Miller *et al.*, 1998). They are composed of 350–450 kDa structural subunits. Each subunit has 7 or 8 50–55 kDa functional units containing a single dioxygen binding site per unit. Recently, the first complete sequence of a molluscan Hc structural subunit, that of the *Octopus dofleini*, has been published (Miller *et al.*, 1998). Also, the crystallographic structure of the functional unit Odg from the same respiratory protein has been determined at 2.3 Å resolution (Cuff *et al.*, 1998).

The tiger shrimp *Penaeus monodon* (Crustacea, Decapoda) is one of the largest invertebrates of the *Penaeidea* family. This organism lives in areas with tropical and subtropical conditions and is widely distributed throughout the Indo-Pacific region. *P. monodon* is a marine inhabitant of commercial importance. It grows rapidly and tolerates changes in the environmental salinity and temperature (Ellerton and Anderson, 1981; Chen *et al.*, 1994*a*). Although the structure and function of crustacean Hcs have been intensively investigated (Markl and Decker, 1992), the structural information about *Penaeidea* Hcs is quite limited. Studies on the changes of the proteins and free amino acids in the hemolymph of *Penaeus monodon* (Chen *et al.*, 1994*a*) and *Penaeus japonicus* (Chen *et al.*, 1994*b*), as well as on the *in vivo* and *in vitro* synthesis of Hc in *Penaeus semisulcatus* (Khayat *et al.*, 1995) have been published. Hcs were isolated from the hemolymph of *Penaeus monodon* (Ellerton and Anderson, 1981) and *Penaeus vannamei* (White shrimp) (Figueroa-Soto *et al.*, 1997) and characterized. The sequence of a hemocyanin cDNA from the hepatopancreas of the last *Penaeidea* shrimp was determined (Sellos *et al.*, 1997). The oxygen-binding properties of the *Penaeus setiferus* Hc have been studied by different allosteric effectors (Brouwer *et al.*, 1978).

In this paper, we describe the isolation, amino acid composition and N-terminal sequences of three different functional subunits from the *P. monodon* Hc. We also report on the thermostability of the native Hc aggregates. According to our knowledge, no sequence data conserning the *P. monodon* subunits have been published so far.

Materials and Methods

Materials

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Tris (hydroxymethyl)-aminomethane hydrochloride was purchased from Merck (Darmstadt, Germany). Reagents used for sequencing were from Applied Biosystems (Weiterstadt, Germany). *P. monodon* Hc was a generous gift from Prof. B. Salvato (University of Padova, Italy). All chemicals and reagents were of analytical grade.

Purification of the Penaeus monodon hemocyanin and isolation of subunits

The crude material was dialyzed against 50 mM Tris (hydroxymethyl)-aminomethane hydrochloride buffer, pH 7.5, containing 5 mM $CaCl_2$ and purified by ultracentrifugation and gel-filtration on a Sepharose 6B column (62 × 2.6 cm), equilibrated and eluted with the same buffer. For dissociation, the hemocyanin solution was dialyzed overnight against 100 mM $NaHCO_3$ buffer, pH 10.0, containing 20 mM EDTA. Samples were desalted by reverse phase HPLC on an Aquapore RP–300 column using a trifluoroacetic acid (TFA)/acetonitrile/water solvent system. Subunits were separated by HPLC on a Nucleosil $7C_{18}$ (250 × 10 mm) reverse phase column. The following conditions were used: eluent A, 0.05 M TFA; eluent B, 80% acetonitrile in A; gradient program: 10 - 100% B for 45 min at a flow rate of 2.5 ml/min.

Amino acid analysis

The amino acid composition of the copper-free hemocyanin was determined after hydrolysis in 5.7 M HCl in evaquated sealed tubes for 24, 48 and 72 h at 110 °C. Protein samples were made free of copper by precipitating the hemocyanin with trichloroacetic acid and washing the pellet with several portions of 0.1 M HCl. Automatic amino acid analyzers models 6001 and LC–3000 (BIO-

TRONIK/Eppendorf, Hamburg, Germany) were used.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970), using a 10% gel.

Automatic amino acid analysis

Amino acid sequence analysis was performed using an Applied Biosystems sequencer model 473 A (Weiterstadt, Germany) with on-line analysis of the phenylthiohydantoin derivatives. Approximately 50–150 pmol of proteins were applied on the cartridge filter previously treated with polybrene.

Circular dichroism measurements

Thermal denaturation of the *P. monodon* Hc was followed by CD measurements in a Jasco J-720 dichrograph, equipped with a personal computer IBM PC-AT, PS/2, multiscan CMS-3436 and a Hewlett-Packard colour graphics plotter model HP 7475 A. A DOS software was used for calculations with the CD data. Protein solutions in 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, 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.

Results

Penaeus monodon Hc was isolated as a homogeneous material by preparative ultracentrifugation and gel chromatography. It elutes as a single symmetrical peak after chromatography on a Sepharose 6 B column. The amino acid composition of the copper-free protein is shown in Table I and compared with those of other decapodan Hcs. The integral number of amino acid residues was calculated for a molecular mass of 75 kDa per structural/functional subunit. Aspartic and glutamic acid, which are partially in the amide forms in the native protein, constitute 25% of all residues. The amino acid composition of the *P. monodon* Hc is similar to that of other decapodan Hcs (Table I).

Table I. Amino acid compositions of decapodan hemocyanins.

Amino acid	*P. monodon*	*C. sapidus**	*P. vulgaris**	*H. vulgaris**
Aspartic acid	*89*	*88*	*94*	*79*
Threonine	30	33	31	37
Serine	31	30	25	26
Glutamic acid	72	72	70	68
Proline	33	31	30	28
Glycine	46	43	39	36
Alanine	46	45	35	36
Valine	41	44	40	40
Methionine	16	16	19	14
Isoleucine	27	32	32	30
Leucine	47	48	45	45
Tyrosine	26	26	21	21
Phenylalanine	40	36	35	34
Histidine	38	38	42	40
Lysine	36	30	28	29
Arginine	29	31	31	29

* Data from Ghiretti-Magaldi *et al.*, 1966.

The SDS – polyacrylamide gel electrophoresis of the dissociated *P. monodon* Hc (not shown) gave two closely located bands (practically one "double" band) corresponding to molecular masses of 75–76 kDa. The dissociated material was subjected to RP HPLC on a Nucleosil $7C_{18}$ reverse phase column. Application of a linear acetonitrile gradient allowed to fractionate it into three well-resolved symmetrical peaks (not shown), each containing one structural/functional subunit, which were collected and further characterized. The results demonstrated that the native Hc aggregates are built of three different polypeptide chains, designated Pm1, Pm2 and Pm3. Two of these chains, Pm2 and Pm3, were electrophoretically undistinguishable.

The N-terminal sequences of the three *P. monodon* Hc subunits are shown in Table II and aligned with those of other decapodan Hcs. The alignment was done manually and revealed a high sequence similarity between *Penaeidea* Hcs. In comparison to Pm1, the other two almost identical *P. monodon* Hc subunits Pm2 and Pm3, with an amino-terminal sequence identity of 92%, have an extension of six residues. Similar extension was found in the subunit Pv1 of the *Penaeus vannamei* Hc (Sellos *et al.*, 1997). The calculated per cent identity scores are shown in Table III.

Table II. N-terminal sequences of decapodan hemocyanin structural/functional subunits. Pm, *Penaeus monodon*; Pv, *Penaeus vannamei* (Sellos *et al.*, 1997); Pi, *Panulirus interruptus* (Bak and Beintema, 1987; Neuteboom *et al.*, 1992); Pv', *Palinurus vulgaris* (Jekel *et al.*, 1996); Cd, *Cherax destructor* (Neuteboom *et al.*, 1989); Al, *Astacus leptodactylus* (Schneider *et al.*, 1986); Cm, *Carcinus maenas* (Neuteboom *et al.*, 1989); Cp, *Cancer pagurus* (Neuteboom *et al.*, 1989), Cmag, *Cancer magister* (Durstewitz and Terwilliger, 1997).

	1 10 20
Phyla: Arthropod; **order:** Decapoda	
Sub-order: Natantia	
Family: *Penaeidea*	
Pm1	**DVQKQK DVLFLL EKIYGD** IQDG – – **L**LA
Pm2	KAGGAS-**DAQKQH DVNFLLHKIYGN** IRN SN – **L**K
Pm3	AVGGAS-**DAQKQH DVNFLLHKIYGN** I
Pv1	FQVASA**DVQQQK DVLYLLNKIYGD** IQDGD – –**L**LA
Pv2	**DVQQQK DVLYLLNKIYGD** IQDGD – –**L**LA
Sub-order: Reptantia	
Family: Palinura	
Pia	DALGTGNAQK**Q**QDI **N**HLLDKI **Y**E P TKYPD- – **L**K
Pic	ADCQAGDSADKLLAQK**Q**HDV**N**YLVYKL**Y**GD IRDDH- – **L**K
Pv'b	DVHS SDNAHK**Q**HDV**N**HLLDKI **Y**E – IKDEK- – **L**K
Family: Astacidea	
CdM1	DGSGGSDAQK**Q**HDV**N**YLLFKV **Y**EDVNDENSP
CdM3	GVPGDVHDEQK**Q**HD I **N**FLLFKV **Y**EVLX DI X – – **L**K
Alb	DASGAT LAKR**Q**QVV**N**HLLEH I **Y**DHTH FTD – – **L**
Family: Brachyura	
Cmagx	MADSAGAPDAH K**Q**HDV**N**SVLWKV**Y**EDI QDPH – – **L** I
Cmag6	TAGGAFDAQ K**Q**HDV**N**SALWKV**Y**EDI QDPH – – **L** I
Cm2	TCLAH K**Q**QAV**N**RLL YR I **Y**SP I XXXF
Cp4	ADLAH R**Q**QS V**N**RLLY KI **Y** SPI S SAYAE**L**K

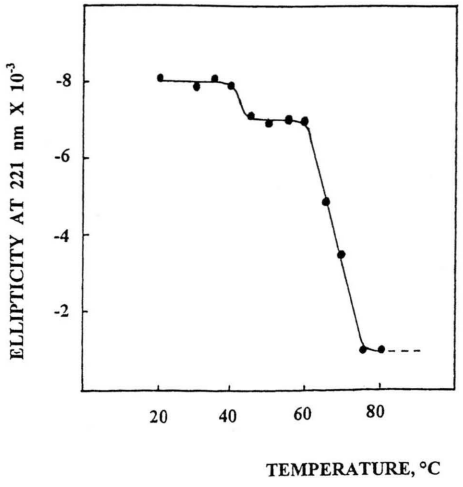

Fig. 1. Single-wavelength melting curve for the thermal denaturation of the oxy-*Penaeus monodon* hemocyanin at pH 7.5. A T_m value of 69 °C was calculated from the curve.

Thermostability of the undissociated *P. monodon* Hc was investigated by following changes in ellipticity at 221 nm with temperature (Fig. 1). The denaturation was irreversible and the thermostability was characterized by T_m, the melting point which is the midpoint of the transition curve. A T_m value of 69 °C was determined from the denaturation curve.

Discussion

The N-terminal sequence is the most variable part of the arthropodan Hc polypeptide chains (Neuteboom *et al.*, 1989; Durstewitz and Terwilliger, 1997). The sequence identity of these regions is a suitable parameter for comparison of structural/functional subunits. A good correlation between the sequence identity scores of the N-terminal parts and those of the whole subunits was observed, although the degree of N-terminal identity is always less (Neuteboom *et al.*, 1989). Table III demonstrates that the structural/functional subunit Pm1 is definitely more closely related to the subunit Pv2 of the *P. vannamei* Hc than the other two chains, Pm2 and Pm3. There are only three substitutions, one of them conservative, in the N-terminal segments of 26 residues of the two

Table III. N-terminal per cent identity scores between structural/functional subunits of decapodan hemocyanins. Amino-terminal extensions are counted as one different position. Abbreviations as in Table II.

Natantia								Reptantia							
Penaeidea					*Palinura*			*Astacidea*			*Brachyura*				
Pm1	Pm2	Pm3	Pv1	Pv2	Pia	Pic	Pv'b	CdM 1	CdM 3	Alb	Cmag x	Cmag 6	Cm2	Cp4	
–	58	70	85	89	42	50	46	46	46	36	50	54	33	32	Pm1
		92	47	50	38	45	47	47	47	29	39	47	38	40	Pm2
			54	60	42	48	54	54	54	31	48	54	45	45	Pm3
				97	32	41	39	39	31	27	37	42	27	30	Pv1
					38	50	42	46	38	32	46	50	29	33	Pv2
						34	55	42	41	50	41	45	42	40	Pia
							44	47	39	29	50	47	38	40	Pic
								48	37	37	50	45	42	43	Pv'b
									47	33	53	55	31	29	CdM1
										19	39	47	23	27	CdM3
											32	32	38	45	Alb
												78	35	37	Cmag x
													31	33	Cmag 6
														64	Cm2

subunits Pm1 and Pv2 building the native aggregates of the *P. monodon* and *P. vannamei* Hcs, respectively. On the other hand, Pm2 and Pm3 have considerably lower homology to the second *P. vannamei* Hc polypeptide chain Pv1. Probably, subunits like Pm1 and Pv2 are family-specific for the *Penaeidea* Hcs and the other subunits are species-specific.

Comparison of N-terminal sequences of respiratory proteins from the sub-orders Natantia and Reptantia demonstrate family- and sub-oder- specific sequences. Invariant motifs: **DV, LL** and **KIYG** are present in positions 7–8, 11–12 and 14–17 in all five *Penaeidea* Hc subunits. Also, in all cases, positions 1, 3, 5 and 26 are occupied by aspartic acid, glutamine and leucine, respectively. Conservation of residues in positions 5 (glutamine), 16 (tyrosine) and 26 (leucine) was observed in all Hcs of the two sub-orders listed in Table II. Except Gln 5 and Tyr 16, Asn 9 is also conserved in the subunits of the Hcs from the sub-order Reptantia. Comparison of the N-terminal per cent identity scores between the members of one family showed that the average degree of homology between the *Penaeidea* Hc subunits is considerably higher, 70%, than that between the Hc subunits of the *Palinura, Astacidea* and *Brachyura* families, 44, 33 and 46%, respectively. Most probably, the polypeptide chains, building the aggregates of the *Penaeidea* dioxygen-transporting proteins, are encoded by the members of the same hemocyanin gene family.

Table IV summarizes melting points of monohexameric (*P. monodon)* and di-hexameric (*C. maenas, C. sapidus* and *M. squinado*) crustacean Hcs. It is evident that respiratory proteins with

Table IV. Melting temperatures of decapodan hemocyanins, determined by CD measurements.

Hemocyanin	T_m of the oxy-Hcs °C
Penaeus monodon	69
*Carcinus maenas**	72
*Callinectes sapidus**	76
*Maia squinado**	76

* *Data from Georgieva et al.*, 1998.

more complex quaternary structure are more stable towards heat denaturation.

In conclusion, the investigations described in the present paper allowed a comparison of respiratory proteins from the sub-orders Natantia and Reptantia. This revealed structural peculiarities, characteristic for decapodan Hc families. In the case of *Penaeidea* Hcs, the subunit heterogeneity is less than that in the other crustacean respiratory proteins, where up to eight immunologically different subunits were described (Markl, 1986). *Penaeideas* are the most primitive crustacean with hexamers as predominant structural form of their dioxygen-transporting proteins (Brouwer *et al.*, 1978; Sellos *et al.*, 1997). This can explain the lower melting point of the *P. monodon* Hc in comparison to those of other decapodan respiratory proteins with di-hexameric quaternary structure. Evidently, the stability of decapodan Hcs depends on their aggregation state. In the hemolymph of invertebrates from other crustacean families, multi-hexameric aggregates with a complicated molecular architecture were found (Herskovits, 1988). In these aggregates, specific subunits link the "building blocks" into multimeric structures.

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