Fluorescence Spectroscopy of the Tryptophan Microenvironment in *Carcinus aestuarii* Hemocyanin

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The steady-state and time-resolved fluorescence properties of the multitryptophan minimal subunit *Cae*SS2 from *Carcinus aestuarii* hemocyanin have been studied with the aim of probing the environment of the fluorophores within the protein matrix. Subunit *a* of *Panulirus interruptus* hemocyanin, whose X-ray structure is known, has been also studied. The results are compared with those collected with other two monomeric fractions (*Cae*SS1, *Cae*SS3) produced by dissociation of the native, oligomeric protein as well as with those of the hexameric aggregate. Three classes of tryptophan residues can be singled out by a combination of fluorescence quenching and lifetime measurements on the holo-Hc (the copper containing, oxygen binding form) and the apo-Hc (the copper-free derivative). One class of tryptophans is exposed to the protein surface. Some of these residues are proposed to be involved in the intersubunit interactions in *Cae*SS1 and *Cae*SS3 fractions whereas in *Cae*SS2 the protein matrix masks them. This suggests the occurrence of conformational rearrangements after detachment of the subunit from the native aggregate, which could explain the inability of *Cae*SS2 to reassociate. A second class of tryptophan has been correlatively assigned, by comparison with the results obtained with *Panulirus interruptus* hemocyanin, to residues in close proximity to the active site. The third class includes buried, active site-distant, residues.

Introduction

Hemocyanins (Hcs) are giant oligomeric copper-containing proteins. They occur freely dissolved in the hemolymph and are responsible for oxygen transport and/or storage in many molluscs and arthropods (Van Holde and Miller, 1995). Hc binds dioxygen in an active site containing two copper ions coordinated to three histidine residues each. While the structural and physico-chemical properties of Hcs from the two *phyla* are remarkably similar at the active site level, molluscan and arthropod Hcs largely differ for their quaternary structure, subunit composition and domain organization of the various individual polypeptide chains. Molluscan Hcs have the structure of a hollow cylinder whereas arthropod Hcs occur in the hemolymph as oligomeric aggregates of a 16S (about 450 kDa molecular mass) unit. 16S unit is a hexamer of the minimal functional subunit (5S, about 75 kDa). The specific aggregation forms depend on the species (Markl, 1986). The most representative aggregates are referred to as: 24S, 37S and 62S, corresponding, respectively, to about 900, 1800 and 3600 kDa. In the hemolymph of most Crustacea, hexamers (16S) or dodecamers (24S) or both are found. However, the biological significance for the occurrence of such different aggregation forms is still controversial (Markl and Decker, 1992). Dissociation of oligomers to yield the 5S (75 kDa) minimal functional subunits can be accomplished by removing Ca^{2+} ions and increasing the pH above 9.0 (Van Holde and Miller, 1995; Ellerton *et al.*, 1983). Most arthropod Hcs have a heterogeneous subunit composition. In Crustacea, three subunits (*a*, *b* and *c* subunits) are found (Bak and Beintema, 1987; Jekel *et al.*, 1988; Neuteboom *et al.*, 1992). They are differently expressed in the various species (Mangum, 1993) and are necessary to guarantee the specific aggregation state. Usually, the reconstitution of the native aggregates requires the full complements of subunits. However,

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in some instances (i.e. *Carcinus aestuarii* Hc used in the present study) the higher aggregation forms (the 24S dodecamer or above) are scarcely populated in reassociation experiments (Dainese *et al.*, 1998). A characteristic feature of *Carcinus aestuarii* Hc is the inability of one subunit (*Cae*SS2) to reassociate after its separation from the subunits pool. Thus, it retains its monomeric state also at neutral pH and in the presence of Ca^{2+}. It is therefore a convenient system for structural studies on a subunit where the aggregation state is not subjected to changes as a function of the experimental conditions and without the interference due to the presence of other subunits. Furthermore, a hexameric form stable also in the absence of calcium ions can be isolated (Dainese *et al.*, 1998).

Fluorescence studies on Hcs are complicated by the multitryptophan nature of these proteins. Moreover, the heterogeneity of subunit composition adds further complication since the number and the distribution of tryptophan (Trp) residues within the subunits might be different. Nevertheless, the Trp-correlated emission properties of Hcs have been shown useful to elucidate structural aspects of these proteins, in particular some conformational features of both the active site-containing hydrophobic core and the intersubunit contact areas (Shaklai *et al.*, 1978; Bannister and Wood, 1971; Ricchelli *et al.*, 1980, 1984, 1987; Boteva *et al.*, 1993). Moreover, since the fluorescence emission of Hc is strongly affected by oxygen binding in the active site, this parameter has been succesfully used for the determination of the oxygen binding constants (Loewe, 1978).

In this paper we have addressed the characterization of the fluorescence properties of the multitryptophan subunit *Cae*SS2 of *Carcinus aestuarii* Hc, a simplified system as compared with the previously studied hexameric and dodecameric Hc (Ricchelli *et al.*, 1980). On these latter protein forms, by acrylamide fluorescence quenching experiments, three classes of tryptophan residues were proposed. One class of residues freely exposed to the solvent, a second class in a hydrophobic environment but accessible to the solvent, a third class deeply buried and accessible to acrylamide only when the protein structure is modified. In contrast, with molluscan Hc (from *Octopus vulgaris*) only one class of buried tryptophans has been reported (Ricchelli *et al.*, 1984) in the case of the native aggregate. However, upon dissociation, a class of solvent exposed residues become evident, thus suggesting that they are involved in the subunit-subunit contact areas (Ricchelli *et al.*, 1987).

Mapping of the fluorescence emission properties of tryptophans in *Cae*SS2 subunit was performed by steady-state and time resolved fluorescence studies combined with fluorescence quenching. Furthermore this approach was extended also to the subunit *a* of *Panulirus interruptus* Hc whose sequence and X-ray crystal structure are available (Volbeda and Hol, 1989). In this way we were able to assign some fluorescence characteristics to defined tryptophan residues of the protein, as successfully done in the case of *Eurypelma californicum* Hc (Boteva *et al.*, 1993), and to infer, correlatively, on the tryptophan distribution in *Carcinus Cae*SS2 subunit.

Materials and Methods

Carcinus aestuarii hemocyanin was purified from the hemolymph of living animals as described by Bubacco *et al.* (1992). Protein concentration was determined spectrophotometrically by using the absorption coefficient $E_{278}^{O.1\%} = 1.21\ mg^{-1}\ ml\ cm^{-1}$ in 10 mM Tris-HCl buffer, pH 7.5 (Bubacco *et al.*, 1992). No differences were observed between the proteins in native form and isolated 75 kDa subunits. The oxygen binding form, referred to as holo-Hc, either as native oligomers or dissociated monomers, shows an absorption ratio $A_{337}/A_{278} = 0.21$. The absorption spectra were recorded with a diode-array Hewlett Packard HP 8452A and double beam Perkin Elmer Lambda 16 spectrophotometers.

The dissociation of native Hc was obtained by dialyzing the protein in Tris(hydroxymethyl)methylamine/HCl (Tris/HCl) 50 mM pH 9.2 containing 10 mM EDTA and changing the buffer solution three times in 48 h. The separation of subunits was obtained by a combination of gel filtration and ion-exchange chromatography using a Pharmacia FPLC apparatus. Gel filtration was carried out with a Superdex 200 10/30 analytical column and with a Superdex 200 26/60 preparative column. Ion-exchange chromatography was carried out with a Mono Q HR 5/5 analytical column and with a Sepharose Q 10/26 preparative column using a

0–1.0 M NaCl gradient. The 16S (450 kDa) hexamer and the 75 kDa monomeric fractions *Cae*SS1, *Cae*SS2 and *Cae*SS3 were obtained according to Dainese *et al.* (1998).

Subunit *a* from *Panulirus interruptus* Hc was a generous gift of Prof. H. Decker, University of Mainz, Germany.

The apo-form (copper-deprived) of native protein and dissociation products was obtained by exhaustive dialysis against 25 mM KCN in 100 mM Tris/HCl, pH 8 buffer at 4 °C for 48 h; then the proteins were dialyzed against the same buffer without KCN containing EDTA 10 mM and finally against Tris/HCl 100 mM, pH 7.0 buffer.

Steady-state fluorescence measurements

The fluorescence spectra were recorded with a Perkin Elmer LS 50B spectrophotofluorimeter equipped with a thermostatic cell holder. The relative quantum yields (Φ) were measured by comparing the integrated corrected fluorescence emission spectra of Hcs with those of N-acetyltryptophanamide, normalized to the same absorbance at the excitation wavelength (295 nm). The quantum yield of the standard was 0.13 at 21 °C (Lehrer, 1971).

Fluorescence quenching experiments were performed with acrylamide and I^- (sodium salt) as external quenchers, in the concentration range: 0–0.8 M. When I^- was used, the ionic strength of the solution was kept constant by addition of suitable amounts of KCl. The fluorescence quenching data were analyzed according to the Stern-Volmer relationship (Lehrer, 1971):

$$F°/F = 1 + K_Q\,[Q] \qquad (1)$$

where F° and F are the fluorescence intensities in the absence and in the presence of the quencher (either acrylamide or I-, see above and Fig. 1), [Q] is the quencher concentration and K_Q is the quenching constant. Since in a multitryptophan protein, with an heterogeneous distribution of fluorescing groups, each fluorophor may have different quenching constants, the measured decrease in fluorescence emission can be a non linear function of the concentration of the quenching agent. In this case, the quenching effect is better described by a modified Stern-Volmer equation:

$$F°/(F°-F) = (1/[Q])(1/\Sigma f_i K_Q i) + \Sigma K_{Qi}\,/\Sigma f_i K_{Qi} \qquad (2)$$

where F°, F and [Q] are as defined above, and f_i and K_{Qi} represent, respectively, the fractional fluorescence and the quenching constant relative to the i-th tryptophan residue. Plotting F°/(F°–F) vs. 1/[Q]) , the ratio intercept/slope is $\Sigma K_{Qi} = (K_Q)_{eff}$, the effective quenching constant, and 1/intercept gives $\Sigma f_i K_{Qi}/\Sigma K_{Qi} = (f_a)_{eff}$, the effective fraction of fluorescence accessible to the quencher (Lehrer, 1971).

Time-resolved fluorescence measurements

Fluorescence decays were recorded by a nanosecond single photon counting spectrofluorimeter System PRA 2000. A thyratron-gated flash lamp filled with N_2 was used for excitation (at 297 nm), the emission was recorded at 340 nm; the time resolution was 0.1 ns/channel, 512 channels were used for the deconvolution analysis.

Decay profiles were analyzed by the method of iterative reconvolution employing a nonlinear least-squares procedure. The decays were fitted by a two-or three-exponential decay function. The resulting residuals were regularly distributed along the time axis, and the reduced χ^2 values were ≤ 1.2. Each reported lifetime, τ, was obtained from the average of three measurements, the scatter being < 10%. The percent contribution (F_i) of each decay component to the total fluorescence was calculated as:

$$F_i = A_i\tau_i/\tau A_i\tau_i$$

where A_i is the weight to decay of the every particular lifetime τ_i.

Results and Discussion

Upon dissociation of the whole molecule at moderately alkaline pH (9.2) and subsequent separation by FPLC techniques, three minimal molecular weight fractions (MW ~ 75 kDa) could be isolated from *C. aestuarii* Hc (Dainese *et al.*, 1998). They have been conventionally named *Cae*SS1, *Cae*SS2, *Cae*SS3. The latter two are predominant whereas the former contributes only about 10% to the total amount. Only fraction *Cae*SS2 was found to be homogeneous by N-terminal sequencing (data not shown) whereas fractions SS1 and SS3 contain more than one polypeptide chain, proba-

bly reflecting microheterogeneity. Under appropriate experimental conditions, the fractions *Cae*SS1 and *Cae*SS3 are able to individually reassociate to the hexameric form. On the contrary, *Cae*SS2 is incapable to form the homohexamer, even if the incubation medium is supplemented with Ca^{2+} (Dainese *et al.*, 1998).

We first performed sets of comparative experiments by following the fluorescence quenching properties of the three monomeric fractions and the 16S native hexameric aggregate. These experiments can provide useful information on the accessibility of the fluorophores to the external medium, the presence of adjacent charged groups and, when several emitters are present, the homogeneity of their environments (Ricchelli, 1990). These experiments were followed by a more detailed characterization of the pure *Cae*SS2 subunit by time-resolved fluorescence measurements.

Steady-state fluorescence measurements

The fluorescence quenching experiments by acrylamide were performed for the three Hc monomeric fractions before and after homologous reassociation and for the native 16S heterologous aggregate in the oxygenated state (holo-forms). The effect of copper removal was also studied (apo-forms). For subunit *Cae*SS2, which is not able to reassociate, the effect of pH (7.5 and 9.2) and Ca^{2+} on the monomeric structure could be studied. Iodide (NaI) and caesium (CsCl) were both used to selectively quench the emission of exposed fluorophores. Actually, ionic species cannot penetrate the protein internal regions, due to their high hydration sphere. No quenching was observed with Cs^{+} up to 0.8 M in all the experimental conditions used.

As an example, in Fig. 1 the Stern-Volmer plots obtained by applying Equ. (1) and describing the fluorescence quenching of *Cae*SS1, *Cae*SS2, *Cae*SS3 and 16 S hexamer in the oxygenated state by either acrylamide and iodide are reported. For those cases showing heterogeneous emission (non linear Stern-Volmer plots), the quenching data were treated according to the modified Stern-Volmer equation (Equ. 2) (data not shown).

Table I summarizes the values of the parameters K_Q and f_a obtained for all the Hc samples tested. For the experiments with iodide, only the fractions of fluorescence accessible to the quencher are reported.

Slight differences between Trp environments in *Cae*SS1 and *Cae*SS2 can be observed, as deduced from the quenching parameters at pH 9.2, where monomeric forms are present. Such high pH has

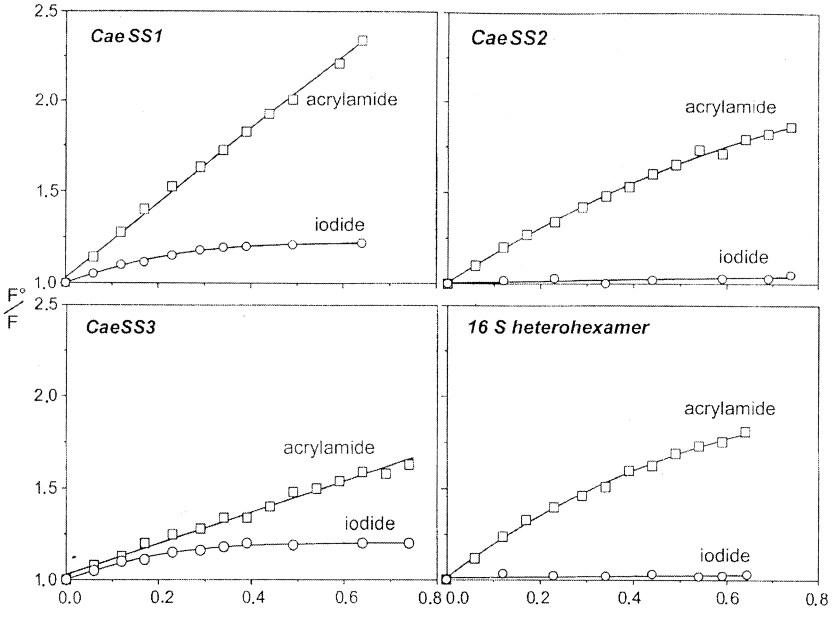

Fig. 1. Fluorescence quenching of *Carcinus aestuarii* Hc. Stern-Volmer plots (Equ. 1) describing the quenching of oxygenated *Cae*SS1, *Cae*SS2, *Cae*SS3 monomeric fractions and 16 S native heterohexamer by acrylamide and iodide. The monomers were dissolved in 100 mM Tris/HCl buffer, pH 9.2. The 16 S hexamer was dissolved in the same buffer at pH 7.5.

Sample	Acrylamide			Iodide		
	K_Q(holo) [M^{-1}]	f_a (oxy-)	K_Q (apo) [M^{-1}]	f_a (apo)	f_a (oxy-)	f_a (apo)
*Cae*SS1						
Monomeric[a]	2.2	1.0	4.0	1.0	0.21	0.20
Homohexameric[b]	1.5	1.0	2.6	1.0	0.00	0.25
*Cae*SS2						
pH 9.2	2.1	0.74	4.0	1.0	0.00	0.00
pH 7.5	2.4	0.73	3.5	1.0	0.00	0.00
pH 7.5 + Ca^{2+}	1.7	1.0	2.4	1.0	0.00	0.00
CaeSS3						
Monomeric[a]	0.9	1.0	10.0	1.0	0.16	0.30
Homohexameric[b]	1.1	1.0	2.8	1.0	0.00	0.20
Heterohexamer[c]						
without Ca^{2+}	4.3	0.58	2.6	1.0	0.00	0.22
with Ca^{2+}	1.4	1.0	2.8	1.0	0.00	0.25

Table I. Quenching constants (K_Q) and fractional accessible fluorescence (f_a) of monomeric *Cae*SS1, *Cae*SS2, *Cae*SS3 and 16 S hexamers by using acrylamide and iodide as external fluorescence quenchers (concentration range: 0–0.8 M).

[a] at pH 9.2; [b] at pH 7.5 + 20 mM Ca^{2+}; [c] at pH 7.5 without Ca^{2+}. The precision of the data (SD) is within ± 5%.

no influence on the Hc conformation as it can be observed by a comparison with the results obtained at pH 7.5 for *Cae*SS2. Conversely, *Cae*SS3 shows quite relevant differences as compared to the other fractions exhibiting much lower accessibility (lower K_Q value) of Trp residues to the bulk medium in the holo-form but higher accessibility after copper removal. This anomalous behaviour is still matter of investigation since *Cae*SS3 is characterized by a marked heterogeneity being composed of at least two components, as evidenced by ion-exchange chromatography (Dainese *et al.*, 1998). A striking difference between the three fractions examined is the lack, in *Cae*SS2, of a part of fluorescence sensible to the I^- action (Table I). This fluorescence is probably associated, in *Cae*SS1 and *Cae*SS3, to Trp located in close proximity to some positively charged amino acid residues (Lys, Arg) (Ricchelli, 1990) since the nonpenetrant species Cs^+ is fully inefficient as a quencher. These fluorophores become shielded by the quaternary structure upon aggregation (at pH 7.5 plus 20 mM $CaCl_2$) to form the corresponding homohexamers or the native heterohexamer thus suggesting a possible involvement in the intersubunit contact interactions. Since a possible role of calcium in masking the I^--sensitive, surface fluorophores cannot be excluded, we repeated these experiments on the native hetero-16 S in the absence of calcium (pH 7.5 in Table I). Actually, Ca^{2+} is known to normalize all the Trp environments by cross-linking to negatively charged protein groups and immobilization of the protein structure (Ricchelli *et al.*, 1987) as it can be easily checked by the homogenization of the Trp emission and the similarity in the quenching parameters between the homohexamers, the heterohexamer (with Ca^{2+}) as well as the monomeric *Cae*SS2 after addition of the ion. In spite of the Ca^{2+} absence, no I^--quenchable fluorescence could be evidenced in the native hetero-16 S, thus confirming that the surface-exposed Trp become inaccessible upon formation of the quaternary structure. However, this class of Trp can interact with iodide in the aggregated apoforms, probably as a consequence of increased protein flexibility after copper removal.

Time-resolved fluorescence measurements

Subunit *Cae*SS2 was investigated in more detail by time-resolved fluorescence studies and a comparison was made with subunit *a* from *Panulirus interruptus* Hc, whose aminoacid sequence and X-ray structure are already well defined (Volbeda and Hol, 1989).

In Fig. 2 is reported a typical decay profile for the apoform of *Cae*SS2, obtained by three-exponential analysis.

Table II reports the lifetimes (τ) and the percent contributions (F) to the total fluorescence of the

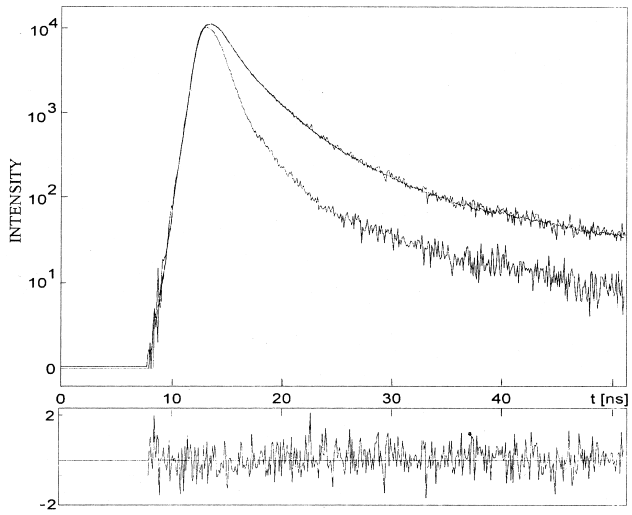

Fig. 2. Fluorescence decay of the apo-form of subunit *Cae*SS2 of *Carcinus aestuarii* Hc; excitation = 297 nm, emission = 340 nm. The results from the deconvolution analysis are given in Table II. Curve (a) – instrumental response function; (b) – superposition of the observed decay and fitted curve; (c) – distribution of the residuals, $\chi^2 = 1.18$.

Table II. Fluorescence quantum yields (Φ), lifetimes (τ) and the percent contributions (F) to the total fluorescence for the subunit *Cae*SS2 and subunit *a* from *P. interruptus* Hc[a].

Sample	τ_1 (ns)	F_1 (%)	τ_2	F_2 (%)	τ_3	F_3 (%)	Φ
*Cae*SS2							
oxy	0.15	71.0	–	–	3.1	29.0	0.04
apo[b]	0.11	11.0	0.33	49.0	3.5	40.0	0.21
apo[c]	–	–	0.30	58.0	3.2	42.0	0.21
subunit a							
P. interruptus							
s	0.16	63.0	–	–	2.5	37.0	0.03
oxy	–	–	0.31	68.0	2.5	32.0	0.17
apo							

[a] The confidence limit of the fitted lifetimes are ± 10%. The decay parameters have been calculated by the emission τ of 340 nm ($\tau_e$xc = 297 nm). [b, c] Lifetimes were determined after three-exponential (b) or bi-exponential (c) analysis of the decay curves.

various decay components for *Cae*SS2 and subunit *a* of *Panulirus* Hc, in both holo- and apo-forms, as well as the fluorescence quantum yields (Φ) determined correlatively with reference to the standard N-acetyltryptophanamide.

Analysis of the fluorescence decay reveals that Trp residues of *Cae*SS2 subunit can be classified into three classes with fluorescence lifetimes around 0.11–0.15, 0.33 and 3.1–3.5 ns, respectively. The short-lived component is mostly responsible for the decay in the holo-form. His contribution to the overall fluorescence (around 70%) corresponds to that calculated as the most acrylamide-accessible fluorescence (see Table I). The class of Trp with intermediate lifetime value (~ 0.33) is present only after copper removal; hence, it can be identified with residues localized in the close surrounding of the active site whose fluorescence is fully quenched by copper-related heavy-atom and paramagnetic ion effects in the oxygenated form (Ricchelli *et al.*, 1987; Boteva *et al.*, 1993). Due to masking of this species with longer τ, the short-lived component is not, or very partially, evident in the apoform (Table II). The slowly decaying species (τ = 3.0–3.5 ns) is present in both Hc forms and probably relates to buried, active site-distant, Trp residues.

Closely similar results were obtained after analysis of the decay parameters of subunit *a* from *P. interruptus* Hc which slightly differs from

*Cae*SS2 in the lifetime value of the long-lived component and in the individual contributions to the total fluorescence. Moreover, the fluorescence quantum yields of the two subunits are very similar before and after copper depletion. Based on these similarities and owing to the strict structural homology between arthropodan Hcs, considerations on *Panulirus* Hc can be reasonably extrapolated to *Carcinus* Hc, whose primary and tertiary structures are still unknown. This is particularly valid for those Trp which are part of the highly conserved regions around the active site.

Table III shows the Trp distribution around the copper ions of the active site for subunit *a* of *Panulirus* Hc, based on the atomic coordinates of the X-ray structure (Volbeda and Hol, 1989). The Trp positions, together with the decay parameters, can give useful information on the fluorescence properties of individual residues. Actually, fluorescence quenching of indole luminescence by copper ions (see above) are very short range processes, the quenching interaction extending within chromophore-quencher separations of about 1.4 nm (Strambini and Gabellieri, 1991). On these basis, Trp-197, -204, -222 and, probably, Trp-248 should constitute the class of fluorophores, characterized by a τ value of about 0.3, which becomes fluorimetrically active only after copper removal. Most of these residues which are distributed in close proximity of the active site are preserved also in Hcs from other arthropods such as *Tachypleus tridentatus* and *Limulus polyphemus* (Linzen *et al.*, 1985). Based on the same value of lifetime and quantum yields, we postulate a closely similar distribution for this class of Trp in *Carcinus* Hc. This conclusion is also supported by the peculiar behaviour of the Hc from tarantula *Eurypelma californicum.* In this Hc, only the position –197 is maintained as compared to *Panulirus* whereas Trps-204, -222 and -248 are substituted by other hydrophobic aminoacids (Boteva *et al.*, 1993). At variance with most arthropodan Hcs, where only 50% of Trp are located in the vicinity of the metal centres (Linzen *et al.*, 1985 and Table III) most of the indole residues of *Eurypelma* are located within a short distance (less than 1,1 nm) from copper A and/or B, which explains the exceptionally strong fluorescence quenching in the oxy-form. Actually, Φ_{oxy}:Φ_{apo} = 1:15 (Boteva *et al.*, 1993), in comparison with a ~ 1:4, 1:5 ratio found for other Hcs (Table II) (Ricchelli *et al.*, 1987; Bannister and Wood, 1971; Shaklai *et al.*, 1978). The emission of *Eurypelma* after copper release mainly arises, other than from Trp-197, from residues at the positions -195, -564, -346, -386. The lifetime of this class of fluorophores range between 7.6 and 9 ns. The abnormally high τ value for Trp residues in close proximity to the active centre, as compared to *Panulirus* and *Carcinus* Hc, demonstrate that their position in the sequence is also critical in determining the fluorescence decay parameters which can be quenched or enhanced by the action of Trp-vicinal amino acid side chain groups.

Table III. Distances between tryptophan and copper in subunit *a* from *Panulirus* Hc, calculated from the atom of the indole ring nearest to the metal.

Trp[a]	Cu(A) [nm]	Cu(B) [nm]
71	1.887	1.757
96	2.685	2.955
197	0.574	0.845
204	1.224	1.342
205	1.620	1.811
222	1.252	1.382
239	2.867	2.830
248	1.459	1.145

[a] the nomenclature of Trp is given on the basis of the sequence of subunit *a* (Bak and Beintema, 1987; Volbeda and Hol, 1989).

At variance with subunit SS2 from *Carcinus* Hc but similarly with the fractions SS1 and SS3, subunit *a* from *Panulirus* Hc is able to reassociate to form the homohexamer. Moreover, it exhibits a fraction of fluorescence (30%) sensitive to the iodide quenching (results not shown). It can be postulated that some conformational rearrangements occur on the protein surface after isolation of *Cae*SS2 from the whole molecule. This conformational readjustment leads to changes in the microenvironment of some surface Trp residues and can play a role in the anomalous behaviour of *Cae*SS2 towards the aggregation.

Acknowledgments

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Bak H. J. and Beintema J. J. (1987), *Panulirus interruptus* hemocyanin. The elucidation of the complete amino acid sequence of subunit *a*. Eur. J. Biochem. **169**, 333–348.

Bannister W. H. and Wood E. J. (1971), Ultraviolet fluorescence of *Murex trunculus* hemocyanin in relation to the binding of copper and oxygen. Comp. Biochem. Physiol. **40B**, 7–18.

Boteva R., Ricchelli F., Sartor G. and Decker H. (1993), Fluorescence properties of hemocyanin from tarantula (*Eurypelma californicum*): a comparison between the whole molecule and isolated subunits. J. Photochem. Photobiol. B: Biol. **17**, 145–153.

Bubacco L., Magliozzo R. S., Beltramini M., Salvato B. and Peisach J. (1992), Preparation and spectroscopic characterization of a coupled binuclear center in cobalt(II)-substituted hemocyanin. Biochemistry **31**, 9294–9303.

Dainese E., Di Muro P., Beltramini M., Salvato B. and Decker H. (1998), Subunits composition and allosteric control in *Carcinus aestuarii* hemocyanin. Eur. J. Biochem. **256**, 350–358.

Ellerton H. D., Ellerton N. F. and Robinson A. (1983), Hemocyanin – a current perspective. Prog. Biophys. Molec. Biol. **41**, 143–248.

Jekel P. A., Bak H. J., Soeter N. M., Vereijken J. M. and Beintema J. J. (1988), *Panulirus interruptus* hemocyanin. The amino acid sequence of subunit *b* and anomalous behaviour of subunits *a* and *b* on polyacrylamide gel electrophoresis in the presence of SDS. Eur. J. Biochem. **178**, 403–412.

Lehrer S. S. (1971), Solute perturbation of protein fluorescence. The quenching of the tryptophan fluorescence of model compounds and of lysozyme by iodide ion. Biochemistry **10**, 3254–3263.

Linzen B., Soeter N. M., Riggs A. F., Schneider H.-J., Schartau W., Moore M. D., Yokota E., Behrens P. Q., Nakashima H., Takagi T., Nemoto T., Vereijken J. M., Bak H. J., Beintema J. J., Volbeda A., Gaykema W. P. J. and Hol W. G. J. (1985), The structure of arthropod hemocyanins. Science **229**, 519–524.

Loewe, R. (1978), Hemocyanins in spiders. V. Fluorimetric recording of oxygen binding curves and its application to the analysis of allosteric interactions in *Eurypelma californicum* hemocyanin. J. Comp. Physiol. **128**, 161–168.

Mangum C. P. (1993), Hemocyanin subunit composition and oxygen binding in two species of the lobster *genus Homarus* and their hybrids. Biol. Bull. **184**, 105–113.

Markl J. (1986), Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods. Biol. Bull. **171**, 90–115.

Markl J. and Decker H. (1992), In: Advances in Comparative and Environmental Physiology. Blood and Tissue Oxygen Carriers (Mangum, C. P., ed.), Vol. 13, Springer-Publ., Berlin, pp. 325–376.

Neuteboom B., Jekel P. A. and Beintema J. J. (1992), Primary structure of hemocyanin subunit *c* from *Panulirus interruptus*. Eur. J. Biochem. **206**, 243–249.

Ricchelli F. (1990), An investigation of the electronic environment of tryptophan in proteins: reliability of fluorescence quenching experiments. J. Photochem. Photobiol. B: Biol. **7**, 93–100.

Ricchelli F., Beltramini M., Flamigni L. and Salvato B. (1987), Emission quenching mechanisms in *Octopus vulgaris* hemocyanin: Steady-state and time-resolved fluorescence studies. Biochemistry **26**, 6933–6939.

Ricchelli F., Jori G., Tallandini L., Zatta P., Beltramini M. and Salvato B. (1984), The role of copper and quaternary structure on the conformational properties of *Octopus vulgaris* hemocyanin. Arch. Biochem. Biophys. **235**, 461–469.

Ricchelli F., Salvato B., Filippi B. and Jori G. (1980), Conformational changes of *Carcinus maenas* haemocyanin induced by urea. Arch. Biochem. Biophys. **202**, 277–288.

Shaklai N., Gafni A. and Daniel E. (1978), Proximity relationships of tryptophanyl residues and oxygen binding site in *Levantina hierosolima* hemocyanin. A fluorimetric study. Biochemistry **17**, 4438–4442.

Strambini G. B. and Gabellieri E. (1991), Quenching of indole luminescence by copper ions: A distance dependence study. J. Phys. Chem. **95**, 4347–4352.

Van Holde K. E. and Miller K. I. (1995) Hemocyanins Adv. Protein Chem. **47**, 1–81.

Volbeda A. and Hol W. G. J. (1989) Crystal structure of hexameric hemocyanin from *Panulirus interruptus* refined at 3.2 E resolution. J. Mol. Biol. **209**, 249–279.