Complete Hemocyanin Subunit Sequences of the Hunting Spider Cupiennius salei

RECENT HEMOCYANIN REMODELING IN ENTELEYNE SPIDERS*

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Hemocyanins are large copper-containing respiratory proteins found in many arthropod species. Scorpions and orthognath spiders possess a highly conserved 4 × 6-mer hemocyanin that consists of at least seven distinct subunit types (termed a to g). However, many “modern” entelegyne spiders such as Cupiennius salei differ from the standard arachnid scheme and have 2 × 6-mer hemocyanins. Here we report the complete primary structure of the 2 × 6-mer hemocyanin of C. salei as deduced from cDNA sequencing, gel electrophoresis, and matrix-assisted laser desorption/ionization spectroscopy. Six distinct subunit types (1 through 6) and three additional allelic sequences were identified. Each 1 × 6-mer half-molecule most likely is composed of subunits 1–6, with subunit 1 linking the two hexamers via a disulfide bridge located in a C-terminal extension. The C. salei hemocyanin subunits all belong to the arachnid g-type, whereas the other six types (a–f) have been lost in evolution. The reconstruction of a complex hemocyanin from a single g-type subunit, which commenced about 190 million years ago and was completed about 90 million years ago, might be explained by physiological and behavioral changes that occurred during the evolution of the entelegyne spiders.

Hemocyanins are large allosteric respiratory proteins that occur freely dissolved in the hemolymph of many arthropod and molluscan species (1, 2). Oxygen binding of hemocyanins is mediated by a pair of copper atoms that are coordinated by six histidine residues (2–4). Arthropod and molluscan hemocyanins differ essentially in structure and sequence and are most likely independent of evolutionary origin (1, 5–8). These proteins have been proven to be an excellent topic of functional, structural, and evolutionary studies (1, 2, 7–9).

Arthropod hemocyanins are hexamers (6-mers) composed of distinct although related subunits in the 75-kDa range that may combine to multimers up to 8 × 6 subunits, depending upon the taxon or the physiological conditions (1, 10). The sequences of various hemocyanin subunits have been determined from euarthropod subphyla, including the Chelicerata, Crustacea, Myriapoda, and Hexapoda (7). Phylogenetic analyses demonstrate that subunit evolution took place independently within each subphylum (5, 7, 10, 11). The estimated rate of amino acid replacement in the chelicerate hemocyanins is about the half of that found in the crustaceans or myriapods (7), which is most likely linked to the conserved structure of these proteins (1, 12). The hemocyanins of orthognath spiders, scorpions, and related Arachnida are 4 × 6-mer protein complexes consisting of two identical 2 × 6-mers (1, 10). The 8 × 6-mer hemocyanin of xiphosurs (Merostomata) such as Limulus polyphemus consists of two identical 4 × 6 halves that correspond structurally to the 4 × 6-mer arachnid hemocyanins. Typically, seven distinct subunit types, termed a–g, are present in a chelicerate hemocyanin, which have been immunologically correlated between L. polyphemus, the scorpion Androctonus australis, and the tarantula Euryypelma californicum (13–15). The sequences of these subunits have been determined in E. californicum (12). In this species, formation of the 4 × 6-mer requires a stoichiometric association of four copies of subunits a, d, e, f, and g and two copies of subunits b and c (16, 17). Each subunit occupies a specific position within the native 4 × 6-mer molecule (17–19). Estimates assuming a molecular clock have led to the conclusion that the diversification of the seven distinct subunits commenced early in evolution, more than 500 million years ago (MYA), and was completed about 420 MYA (5, 12).

4 × 6-mer hemocyanins are also present in a number of labidognath spider families such as Araneidae, Linyphiidae, and Theridionidae (10). However, many other labidognath spider families such as Agelenidae, Salticidae, Thomisidae, Dysderidae, Clubionidae, Lycosidae, and Ctenidae diverge from this standard scheme of chelicerate hemocyanin structure (10). They possess a 2 × 6-mer hemocyanin with only two hemocyanin subunit types identified by immunological means, suggesting a severe rearrangement of the subunits. A carefully studied example is the Central American tetragnath spider, Cupiennius salei (Fig. 1). In the hemolymph of this species, a mixture of 1 × 6- and 2 × 6-mer hemocyanins occurs in an approximate 1:2 ratio (20, 21). Although both hemocyanin forms include five electrophoretically distinct but immunologically identical monomer subunits, an additional subunit dimer is present in the 2 × 6 molecules (21, 22). This subunit dimer is immunologically distinct from the monomers (23) and is linked

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession numbers AJ307903, AJ307904, AJ307905, AJ307906, AJ307907, AJ307908, AJ307909, AJ307910, and AJ307911 (C. salei hemocyanin subunits 1–5, 6, 6′, and 6″, respectively).

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1 The abbreviations used are: MYA, million years ago; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RTA, retrolateral tibial apophysis; PAM, accepted point mutations per site.
by a cysteine-mediated disulfide bridge (21, 22). It is responsible for the formation of the 2 × 6-mer by connecting two hexamers.

To understand the architecture of the C. salei hemocyanin and the evolutionary processes that led to the construction of the hemocyanin multimer, we have cloned and sequenced the cDNAs of all subunits of this hemocyanin. This is the second chelicerate hemocyanin for which the full subunit sequence has been elucidated (12).

**EXPERIMENTAL PROCEDURES**

*Animals*—The Central American ctenid spider, *Cupiennius salei* (Chelicerata, Araneae, Ctenidae; Fig. 1) was obtained from Prof. E.-A. Seyfarth (Institute of Zoology, Frankfurt, Germany). The animals were kept at 28 °C with a 12:12 h light-dark cycle and fed on insects. Specimens used in this study had ~3–3.5-cm body length and 10–15-cm legs span.

**Protein Biochemistry**—Adult spiders were immobilized for 2 h at 4 °C. The hemolymph was withdrawn from the median-dorsal region of the opisthosoma by a syringe and centrifuged for 10 min at 10,000 × g to remove the hemocytes. For some experiments, the hemolymph was dialyzed overnight at 4 °C to remove the hemocytes. For some experiments, the hemolymph was dialyzed against alkaline glycine-NaOH buffer before PAGE; 15 μg of total protein was applied. V, hemocyanin subunit dimer (CsaHc-1); VI, dissociated non-respiratory protein; VII, hemocyanin subunits (see panel C). HL, hemolymph. C, Coomassie-stained gel with subunit bands after dissociation in alkaline buffer as used for MALDI-TOF. The numbers 2–6 refer to the different hemocyanin subunits, CsaHc-2–6.

**MALDI-TOF experiments** were performed by Dr. Christian Hunziger (Proteasys, Mainz, Germany) on proteins that had been separated by native PAGE, stained with Coomassie Brilliant Blue, and digested with trypsin. The MALDI-TOF mass spectra were evaluated using the program PEPTIDE-MASS.²

**Sequence Analysis and Phylogenetic Studies**—Sequence analyses were carried out with the programs provided by software package 9.0 from the Genetics Computer Group (GGC, Wisconsin) and the ExPAsy web server.² Sequences were added by hand to an alignment of the published hemocyanin sequences (12) using GeneDoc Version 2.6.² The alignment is available from the authors upon request. The PHYLIP 3.6b2 software package was used for phylogenetic analyses (27). Distances between pairs of protein sequences were calculated and corrected for multiple changes according to Dayhoff’s empirical PAM 001 matrix (28) with the PROTDIST program. Phylogenetic trees were constructed either by the neighbor-joining method or the maximum parsimony method implemented in the PROTPARS program. The reliability of the trees was tested by bootstrap analysis (29) with 100 replications (SEQBOOT program). To estimate the divergence times, the PAM matrix was imported into the Microsoft Excel 2000 spreadsheet program (30). A linearized tree that corresponds to the phylogeny of the chelicerate hemocyanins was calculated on the basis that Merostomata and Arachnida separated about 450 MYA (5, 31). The confidence limits were estimated using the observed standard deviation of the protein distances.

**RESULTS**

**Subunit Composition of C. salei Hemocyanin**—The hemolymph proteins of six adult individuals of *C. salei* were extracted and subjected to electrophoretic studies (Fig. 2). The total protein content of the hemolymph of these individuals varied between 28 and 65 mg/ml. In the first set of experiments, the proteins were applied to a native PAGE immediately after the bleeding of the animals (Fig. 2A). The bands represent the 2 × 6- and 1 × 6-mer hemocyanins, as well as a non-respiratory protein and a significant amount of a hemocyanin heptamer, which is formed by partial dissociation of the 2 × 6-mer molecule (21). In a second experiment, the hemolymph proteins were dialyzed in an alkaline glycine buffer at pH 9.6 before the PAGE to ensure the dissociation of the 2 × 6- and 1 × 6-mer hemocyanin into subunits (Fig. 2B). In both types of analysis, no detectable variation in the migration of the hemocyanin multimers and subunits was observed, although in spec-

² Available at the ExPasy web server: www.expasy.ch.

² K. B. Nicholas and H. B. Nicholas, Jr. (1997) www.psc.edu/biomed/ gendoc/.
120 kDa, respectively, when Western blot analyses under both reducing and nonreducing conditions revealed a single prominent hemocyanin band in the 70-kDa range (Fig. 3, lanes 1 and 2). The non-respiratory protein of C. salei forms a single band of about 110 kDa under nonreducing condition (lane 2), and a double band of 100 and 120 kDa, respectively, when β-mercaptoethanol was added (lane 1; cf. Ref. 21). An additional band of 140 kDa appears only under nonreducing conditions (lane 2), which was suspected to be the dimeric hemocyanin subunit that is linked by a disulfide bridge (21). This was confirmed by Western blotting using anti-C. salei hemocyanin antibodies, which stain the 70-kDa subunits as well as the 140-kDa dimer (lanes 3 and 4). A minor cross-reaction with the 110-kDa non-respiratory protein was observed, probably due to some contamination in the hemocyanin preparation used for immunization.

Cloning and Sequencing of the C. salei Hemocyanin cDNAs—A cDNA library was constructed according to Kempter (32) from C. salei 10 days after induction of hematopoiesis by bleeding. The library was screened with specific anti-C. salei hemocyanin antibodies (21). A total of 27 positive clones was identified and partially sequenced; 17 of the clones encoded hemocyanin. Comparison of the 5’ and 3’ sequences revealed that they represent a total of nine distinct hemocyanin cDNAs. The complete sequences of these clones were obtained on both strands by primer walking. The full-length cDNAs cover the complete coding regions for the different subunits together with 44 to 67 bp of the respective 5’ untranslated regions and the complete 3’ untranslated regions comprising the standard polyadenylation signals (AATAAA) and the poly(A)-tails of different lengths (Table I). In each case, the presence of 3 purines upstream of the putative initiator codons (ATG) fulfills the minimum criteria for an eukaryotic translation start site (33). Because three of the sequences are almost identical (>99.2%) with other cDNAs at the nucleotide level, and the distances among the others are in about the same range (Table II), we assume that these sequences represent alleles in the C. salei gene pool. Thus we identified a total of six sequences that are expected to encode the individual hemocyanin subunits of C. salei.

The open reading frames translate into five distinct polypeptides of 626 amino acids and a single one of 634 amino acids with calculated molecular masses in the range of 72 kDa (Table I), which agrees well with observations made by SDS-PAGE (Fig. 3; cf. Ref. 21). To assign these cDNA sequences to distinct subunits that have been identified by gel electrophoresis, the Coomassie-stained bands from a native gel (Fig. 2C) were cuted and submitted to MALDI-TOF analyses. Using a theoretical digest of the hemocyanin polypeptides deduced from the cDNAs, between 4 and 14 unique peptides from each band were unambiguously allocated. The hemocyanin subunits were named according to their apparent migration in the native gel, with CsaHc-1 being the subunit dimer, CsaHc-2 the slowest, and CsaHc-6 the fastest migrating subunit.

**Hemocyanin Sequence Comparison and Evolution—**Pairwise sequence comparison (excluding the allelic sequences) of the C. salei hemocyanin subunits revealed that 76.4–86.1% of the nucleotides and 79.2–88.8% of the amino acids are identical (Fig. 4; Table II). The nucleotide and amino acid sequences of the C. salei hemocyanin subunits were added to the previously published alignments of the chelicerate hemocyanins (5, 12). Although the amino acid identity score among the distinct C. salei hemocyanin subunits is higher than 79.2%, the lower scores were obtained with other known hemocyanin sequences. The highest score was found with the E. californicum hemocyanin subunit g (EcaHc-g) (71.2–72.8% identity at the amino acid level), whereas other sequences were lower than 66% (data not shown).

For phylogenetic inference, four selected crustacean hemocyanin sequences were included in the alignment. Crustacean and chelicerate hemocyanins form two distinct branches, which separated at the time of the divergence of the subphyla in the Cambrian or an earlier period (5, 10). Therefore, the crustacean hemocyanins may be used as the out-group to infer the phylogeny of chelicerate proteins. Tree construction was performed using maximum parsimony or by the neighbor-joining method based on a PAM matrix (Fig. 5). In both analyses, the six C. salei hemocyanin subunits form a single, well-supported clad (100% bootstrap support) nested within the other chelicerate hemocyanins. The C. salei branch is associated with the g-subunit of E. californicum (100 and 99% bootstrap support, respectively). A time scale of chelicerate hemocyanin evolution was inferred under the assumption that the L. polyphemus hemocyanin subunit II and E. californicum hemocyanin subunit a are orthologous proteins (5, 12, 15) and that the Merostomata and Arachnida diverged about 450 MYA in the Ordovician period (31). Assuming a PAM substitution matrix, we calculated a mean amino acid replacement rate of 0.65 ± 0.03 × 10⁻⁹ substitutions per site per year, which is good agreement with the previous estimates using fewer sequences (5, 12). Thus the time of divergence of the branches leading to the C. salei hemocyanin subunits and E. californicum subunit g (EcaHc-g) occurred around 279 ± 5 MYA (Fig. 6). CsaHc-1 and the precursor of the other Cupiennius subunits split 186 ± 10 MYA. Subunit CsaHc-4 diverged 127 ± 5 MYA, CsaHc-2 and CsaHc-5 split 97 ± 1.5 MYA, and CsaHc-3 and CsaHc-6 split 93 ± 1.4 MYA.

**DISCUSSION**

The hemocyanin of the ctenid spider C. salei is represented by a 1 × 6 oligomer formed by five distinct subunits and a 2 × 6 multimer with six subunits, one of which forms a dimer that links the two hexamers (20–21). We have cloned and sequenced six distinct hemocyanin cDNAs (plus three allelic sequences). The MALDI-TOF data allow the unambiguous assignment of each of these polypeptides to one of the protein bands observed in gel electrophoresis and also demonstrate that all subunits were covered by the cDNA data. Besides the 4 × 6-mer hemocyanin of the North American tarantula E. californicum, which consists of seven distinct subunits (12), C. salei hemocyanin is the second chelicerate hemocyanin for which subunit sequences have been completely determined. However, the structures of these two respiratory proteins essentially differ. Although the 4 × 6-mer hemocyanin of E. californicum...
represents the standard type of an arachnid hemocyanin, *C. salei* is the representative of another 2 × 6-mer hemocyanin form only found in a distinct group of the Araneae.

**Cupiennius Hemocyanin Structure**—Based on the cross-reactions of the *C. salei* subunits with antibodies raised against the various hemocyanin subunits of *E. californicum*, the monomers were previously assigned to the chelicerate subunit type *f*, whereas the dimer appeared to belong to the *d*-type subunits (10). However, both sequence comparison and phylogenetic analyses clearly show that the dimer as well as the monomers do in fact belong to the chelicerate subunit type *g* (Fig. 5).

The *C. salei* hemocyanin sequences closely resemble those of the other chelicerates. It is, however, noteworthy that in the *C. salei* hemocyanins as well as in EcaHc-g the copper-binding site A carries a conserved HHWYWH motif, whereas in the other hemocyanins it is, however, noteworthy that in the other hemocyanins. Although in general the first domain is the least conserved region among different hemocyanins, we make up a flexible hinge stabilizing the three-dimensional structure of the subunit, as deduced from other hemocyanins (35).

As already observed with various hemocyanin sequences (1, 5, 12), most variations are present in the first and third structural domains. The second domain, which forms the core of the hemocyanin subunit and includes the copper-binding sites, is strikingly conserved within the different *C. salei* hemocyanin subunits (79.7% identical amino acids) and between those and the other hemocyanins. Although in general the first domain is the least conserved domain of the disulfide link between two CsaHc-1 subunits assume that Cys-631 of CsaHc-1 is in fact responsible for the cysteine available at the subunit surface (Fig. 7). Thus we conclude from our data the position of the other subunits in the native 2 × 6-mer hemocyanin. It should be noted, however, that in reassembly experiments the monomeric subunits (CsaHc-2 to-6) were individually able to form hexamers and to combine with the dimeric subunit (CsaHc-1) to form 2 × 6-mers (23).

The **Evolution of the Cupiennius Hemocyanin**—Seven different subunit types (a-g) and a typical 4 × 6-mer hemocyanin are present in most chelicerate orders (10). The subunit types diverged from an ancestral hemocyanin gene as early as 550 MYA (5, 12). By contrast, the hemocyanin of *C. salei* is a rather recent derivative of this ancient chelicerate hemocyanin structure, which emerged only about 90–190 MYA from a single *g*-type subunit (Fig. 6). It can be assumed that the ancestor of the Cupiennius-type hemocyanin was a simple hexamer, which

| Subunit | Accession number | cDNA | Protein | Molecular mass | pI |
|---------|-----------------|------|---------|----------------|----|
| CsaHc-1 | AJ307903 | 2084 | 634 | 72.69 | 6.02 |
| CsaHc-2 | AJ307904 | 2048 | 626 | 71.02 | 5.58 |
| CsaHc-3 | AJ307905 | 2039 | 626 | 71.34 | 5.59 |
| CsaHc-4 | AJ307906 | 2030 | 626 | 71.06 | 5.43 |
| CsaHc-5 | AJ307907 | 2101 | 626 | 71.40 | 5.85 |
| CsaHc-5' | AJ307908 | 2101 | 626 | 71.50 | 5.63 |
| CsaHc-6 | AJ307909 | 2266 | 626 | 71.61 | 5.55 |
| CsaHc-6' | AJ307910 | 2264 | 626 | 71.54 | 5.59 |
| CsaHc-6'' | AJ307911 | 2265 | 626 | 71.58 | 5.59 |

* GenBank™/EBI DNA data accession number.

"Without poly(A)-tail.

"Including the initiator methionine.

"Without initiator methionine.

**Table II**

**Pairwise sequence identities of the C. salei hemocyanin subunits**

| Nucleotide identities (within the coding region) are above and amino acid identities below the diagonal. |
|--------------------------------------------------|
| CsaHc-1 | CsaHc-2 | CsaHc-3 | CsaHc-4 | CsaHc-5 | CsaHc-5' | CsaHc-6 | CsaHc-6' |
|---------|---------|---------|---------|---------|----------|---------|---------|
| CsaHc-1 | 77.2    | 77.0    | 76.4    | 76.7    | 76.7     | 78.0    | 77.7    | 77.8    |
| CsaHc-2 | 81.6    | 79.6    | 78.4    | 83.9    | 83.9     | 78.8    | 79.1    | 79.0    |
| CsaHc-3 | 80.5    | 86.4    | 79.4    | 79.4    | 79.2     | 86.1    | 86.0    | 86.0    |
| CsaHc-4 | 79.7    | 85.6    | 83.7    | 77.8    | 77.8     | 82.1    | 81.6    | 81.8    |
| CsaHc-5 | 80.2    | 87.9    | 85.5    | 85.5    | 99.2     | 80.1    | 80.2    | 80.1    |
| CsaHc-5' | 80.2   | 88.2    | 85.5    | 85.6    | 99.2     | 80.0    | 80.1    | 80.0    |
| CsaHc-6 | 79.9    | 84.7    | 89.0    | 85.8    | 85.8     | 99.3    | 99.4    |
| CsaHc-6' | 79.7    | 84.5    | 88.8    | 85.8    | 85.6     | 99.7    | 99.6    |
| CsaHc-6'' | 79.6   | 84.3    | 88.7    | 85.6    | 85.5     | 99.5    | 99.8    |
contained a single g-like subunit type. The reasons why the other subunits have been lost is essentially unknown, but it might be speculated that morphological changes made the highly complex 4/6-mer hemocyanin unnecessary. It is noteworthy that the Cupiennius type 2/6-mer hemocyanin appears to be restricted to the "higher" entelegyne Araneae of the retrolateral tibial apophysis (RTA) clade (37), which are active hunters with a complex tracheal system. It is conceivable that the loss of the 4/6-mer hemocyanin is linked to the evolution of such respiratory organs. It is also possible that the ancestors of the RTA clade passed a period of dwarfism in which a simple 1/6-mer hemocyanin acted as a high-affinity oxygen storage protein rather than a sophisticated oxygen carrier. Later, an increase in body size rendered simple tracheal respiration inefficient to sustain the metabolic need of an active hunter. By a gene duplication event about 186 MYA they regained a more complex hemocyanin with an additional dimeric subunit, enabling the formation of 2/6-mer hemocyanin. This in turn would allow more sophisticated allosteric regulations as well as a higher oxygen transport capacity while maintaining blood osmolarity and viscosity. The later diversification of the different subunits by gene duplication 90–120 MYA might either be related simply to the need of more hemocyanin polypeptides or may again enhance the regulation capacity of the protein.

**Implications for the Evolution of the Araneae—Hemocyanin sequences have been used successfully to infer a time scale of the evolution of arthropod taxa (5, 7, 8, 11). Given the sparse fossil record (38), the present knowledge of the evolution of the spiders (Araneae) is poor. The Araneae probably emerged in the Devonian period some 400 MYA. Recent cladistic analyses

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**Fig. 4. Amino acid alignment of the amino acid sequences of the C. salei hemocyanin subunits.** Strictly conserved residues are shaded. Above the sequences, the secondary structure elements as deduced from L. polyphemus subunit II (36) are indicated: A, α-helices; B, β-sheets. Other features are given below the sequences: *, copper ligand; c, disulfide bridges. CsaHc-1–6, hemocyanin subunits 1–6 of C. salei (see Table I for accession numbers). Polyomorphism in alleles of subunits 5 and 6 are underlined.
treat the suborders Mygalomorpha (represented here by E. californicum) and Araneomorpha (C. salei) as sister taxa, which are joined as Opisthothelae (37). The first fossils of the Mygalomorpha derive from the early Triassic period (some 240 MYA), whereas the lower bound of the fossil record of the Araneomorpha is about 160 MYA (38). The time of divergence of the C. salei hemocyanins and EcaHc-g most likely coincides with the split of the Mygalomorpha and Araneomorpha. Assuming a molecular clock, we calculated this date to be about 280 MYA, which agrees with the fossil data.

The most successful subgroup within the Araneomorpha are the Entelegynae, which are subdivided in the Orbicularidae and the spiders of the RTA clade (37). So far, only species that belong to the RTA clade possess a Cupiennius-type hemocyanin (10). However, it is uncertain whether it can be considered as a molecular synapomorphy of this taxon, because its occurrence outside of the RTA clade is still possible. If this is the case it would provide an excellent trait for tracing the closest relatives of the RTA clade. On the other hand, various families of the Orbicularidae possess an Eurypelma-type hemocyanin (10). Thus the formation of the 2 × 6-mer hemocyanin must have occurred after the separation of the Orbicularidae and the progenitors of the RTA clade. The fossil records of both taxa are poor and are mainly restricted to specimens from the Tertiary period preserved in Baltic amber (38). We have calculated that the formation of the Cupiennius-type hemocyanin commenced about 190 MYA, which should be considered as the lower boundary for the time of emergence of the RTA clade. The different subunits of the C. salei hemocyanin have existed as distinct genes for at least 90 MYA. Future studies will elucidate the distribution and relationship of hemocyanin subunits in the Entelegynae and other Araneae and will help to infer the evolution of spiders.
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