Isolation, purification and characterization of an iron-binding protein from the horseshoe crab (*Limulus polyphemus*)

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The presence of an iron-binding protein in the haemolymph of the horseshoe crab, *Limulus polyphemus*, was detected by gel filtration of ^{59}Fe -labelled haemolymph. Lysis of amoebocytes did not change the amount of iron-binding protein in haemolymph samples. The protein was purified to homogeneity by ion-exchange chromatography. The molecular mass of the purified protein was estimated to be $282\,000 \pm 10\,000$ Da by gel filtration and analytical ultracentrifugation. SDS/polyacrylamide-gel electrophoresis demonstrated that the protein is composed of ten subunits having a molecular mass of $28\,000 \pm 2\,000$ Da. The purified, unlabelled protein efficiently sequestered ^{59}Fe in the absence of haemolymph indicating that no other haemolymph factors are required for the incorporation of iron into the protein. No ^{59}Fe was removed from the purified protein with EDTA or 2,2'-bipyridyl. Partial removal of ^{59}Fe was achieved by dialysis with nitrilotriacetic acid or desferal. Analysis of the iron-loaded protein indicated that each subunit has the capacity to bind two iron atoms with high affinity. The isolation of an iron-binding protein from *L. polyphemus* supports the proposal that such proteins are an ancient evolutionary development not necessarily linked to the appearance of iron proteins which serve as oxygen carriers.

INTRODUCTION

Iron participates in a wide variety of essential biological processes as a result of its ability to exist in two stable oxidation states, Fe^{2+} and Fe^{3+} , in aqueous solutions. The redox properties of iron that make it so useful in biocatalysis and oxygen transport present problems in its metabolism. Fe^{2+} is readily oxidized to Fe^{3+} by O_2 . At the pH, ionic strength and oxygen tension of most physiological fluids the stable oxidation state of iron is Fe^{3+} . The hydrolytic tendency of Fe^{3+} is so great that the equilibrium concentration of free, aqueous Fe^{3+} cannot exceed 10^{-17} M in neutral solution. Thus, throughout the biosphere, organisms have been compelled to evolve specific iron-sequestering molecules to maintain the element in soluble form so that it may be transported and used for the biosynthesis of iron proteins and enzymes.

In vertebrate organisms, iron is sequestered and transported in blood plasma by the protein transferrin. Transferrin binds iron sufficiently tightly to resist formation of insoluble hydrolytic products and delivers it to cells with a specific requirement for this biologically essential element. It has been proposed that transferrin and transferrin-like molecules are relative newcomers to the evolutionary scene, being found only in the phylum Chordata (Aisen & Listowsky, 1980).

Even though iron requirements were small until iron proteins were used for oxygen transport, virtually all organisms that utilize oxygen in the generation of energy have iron-containing cytochromes and, thus, have the need for some mechanism to acquire iron. In recent years specific iron-binding proteins have been identified in the plasma of the tarantula, *Dugesia hentsi* (Lee *et al.*, 1978), in the plasma of the ascidian *Pyura stolonifera* (Martin *et al.*, 1984) and in the haemolymph of the crab,

Cancer magister (Huebers *et al.*, 1982). Although it has not been definitively established that these proteins represent evolutionary precursors to transferrin in vertebrates, they do show some similar properties, and at least two of these proteins donate iron to transferrin receptors of rat reticulocytes.

It has been estimated that the horseshoe crab, *Limulus polyphemus*, has been present for at least 200 million years (Mayr, 1965). It is a very primitive marine arthropod that may be a living link to trilobites. The horseshoe crab contains the copper protein, haemocyanin, as its vehicle for transporting oxygen, and thus its iron requirements should be minimal. The isolation of an iron-binding protein from haemolymph of the horseshoe crab, described in this paper, provides considerable support for the proposal of Huebers *et al.* (1982), that specific iron-binding proteins are an ancient evolutionary development and that the need for such proteins was not necessarily linked to the appearance of iron-containing proteins as a means to accomplish oxygen transport.

MATERIALS AND METHODS

Preparation of haemolymph

Fresh haemolymph was obtained from adult horseshoe crabs collected in the vicinity of Beaufort, NC, U.S.A. Most of the haemolymph samples were prepared under sterile conditions to prevent lysis of the amoebocytes and clotting of the haemolymph. The amoebocytes were immediately removed from these samples by centrifugation. Some haemolymph samples were prepared under non-sterile conditions. These samples were transferred to a glass beaker and were allowed to clot by

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standing at room temperature for 3 h. Most of the clotted material was removed by filtration through cheesecloth. After filtration, these haemolymph samples were centrifuged to remove any remaining clotted material.

Radioactive labelling of haemolymph samples

[^{59}Fe]FeSO₄ (2 μCi in 0.05 M-H₂SO₄) was slowly added to 10 ml of fresh haemolymph. The ^{59}Fe -labelled haemolymph was stored overnight at 4 °C before gel filtration.

Chromatographic columns

A column (2.6 cm $\times$ 85 cm) of Sepharose CL-6B (Pharmacia) for gel filtration of the ^{59}Fe -labelled haemolymph was equilibrated with 0.05 M-Hepes buffer, pH 8.0, containing 2.5 % NaCl and 0.02 % NaN₃. Further purification of the iron-binding protein was achieved with an ion-exchange column (2.6 cm $\times$ 30 cm) of DEAE-Sephacel (Pharmacia) initially equilibrated with 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl and 0.02 % NaN₃.

The molecular mass of the purified iron-binding protein was estimated by gel filtration on a column (1.6 cm $\times$ 90 cm) of Sepharose CL-6B, equilibrated with 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl and 0.02 % NaN₃. Marker proteins (Kit MW-GF-1000; Sigma Chemical Co.) were used to calibrate this column.

A column (1 cm $\times$ 10 cm) of Chelex-100 (Bio-Rad Laboratories) equilibrated with 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl and 0.02 % NaN₃ was used to remove unbound or loosely bound iron from iron-loaded samples of the purified protein.

The elution of protein from these columns was monitored at 280 nm and the fractions specifically containing haemocyanin were identified by measuring the absorbance of individual fractions at 340 nm, where oxyhaemocyanin has a characteristic intense absorption band that results from an O₂²⁻ $\rightarrow$ Cu charge transfer transition.

The elution of ^{59}Fe from these columns was monitored by counting samples of individual fractions with an LKB model 1217 liquid-scintillation counter.

Protein samples obtained from chromatographic procedures were concentrated by ultrafiltration.

Determination of protein concentration

The Bio-Rad protein assay (Bio-Rad Laboratories), which is based on the dye-binding procedure of Bradford (1976), was used to determine protein concentrations in samples containing the iron-binding protein.

Polyacrylamide-gel electrophoresis

The progress of the purification of the iron-binding protein was monitored by polyacrylamide-gel electrophoresis in 4 %, 7 % and gradient (3–10 %) tube gels (5 mm $\times$ 100 mm) with 0.188 M-Tris/glycine buffer, pH 8.9. Gels were stained with Coomassie Blue, a general protein stain, or with Ferene S {3-(2-pyridyl)-5,6-bis-[2-(5-furanesulphonic acid)]-1,2,4-triazine}, a specific iron stain for iron-binding proteins in polyacrylamide gels (Ching-Ming Chung, 1985).

The subunit composition and subunit molecular mass of the iron-binding protein were determined by SDS/polyacrylamide-gel electrophoresis in 10 % tube gels (5 mm $\times$ 100 mm) equilibrated and run with 0.188 M-Tris/glycine buffer, pH 8.9, containing 0.1 % SDS.

Before SDS electrophoresis, samples of the iron-binding protein and marker proteins were treated with 1 % SDS and 0.04 M-dithiothreitol at 65 °C for 30 min. Marker proteins (Kit MW-SDS-70L, Sigma Chemical Co.) were used to calibrate the SDS gels. Gels were fixed with 40 % propan-2-ol/10 % acetic acid, stained with Coomassie Blue, and destained with 10 % acetic acid/10 % propan-2-ol.

Isoelectric focusing

Purified samples of the iron-binding protein and marker proteins were electrofocused on commercially prepared polyacrylamide-gel plates (LKB Ampholine PAG plates, product no. 1804-101) that had a pH range of 3.5–9.5. Marker proteins (Kit IEF-M1, Sigma Chemical Co.) were used to calibrate the electrofocusing gels. The samples were focused at a constant power of 30 W for 1.5 h. The plates were fixed with 11.5 % trichloroacetic acid/3.45 % sulphosalicylic acid, stained with Coomassie Blue, and destained with 10 % acetic acid/25 % ethanol.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed with a Beckman model E analytical ultracentrifuge equipped with schlieren optics. Sedimentation coefficients were calculated from the movement of the maximum ordinate of the peak and were corrected to $s_{20,w}$ by standard procedures. Protein concentrations were 1.4 mg/ml and buffers were 0.05 M-Hepes/0.1 M-NaCl, pH 8.0.

Chelating agents

EDTA (disodium salt), 2,2'-bipyridyl and nitrilotriacetic acid (disodium salt) were purchased from Sigma Chemical Co. Desferal was the generous gift of Ciba-Geigy. Solutions containing a concentration of 1 mM of each of these chelating agents were prepared in 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl and 0.02 % NaN₃.

Preparation of iron-loaded protein samples

Samples (3–5 ml) of the purified iron-binding protein (5–28 μM) were iron loaded either by dialysis against 0.05 M-Hepes buffer (pH 7.4)/0.1 M-NaCl containing a large excess of ferrous ammonium sulphate (1000 μM) or by injection of a freshly prepared solution of ferrous ammonium sulphate to the protein sample to yield a concentration of 1000 μM . In the experiments involving iron loading by dialysis, the dialysis buffer was equilibrated with N₂ before the addition of ferrous ammonium sulphate to retard the auto-oxidation of the ferrous salt that would normally result in the formation of insoluble hydrolysis products of the ferric ion at this pH. All samples of the iron-loaded protein were dialysed with 0.05 M-Hepes buffer (pH 7.4)/0.1 M-NaCl containing 10 mM-EDTA to remove unbound and adventitiously bound iron. The samples were then dialysed with the same buffer but without EDTA to remove iron complexed by EDTA and any excess EDTA. After dialysis, a portion of each sample (approx. 1.5 ml) was passed through a column (1 cm $\times$ 10 cm) of Chelex-100 to ensure complete removal of any iron not bound with high affinity to the protein.

Quantitative iron analyses

The iron contents of the iron-loaded protein samples after EDTA dialysis and after Chelex-100 chromatography were determined by a spectrophotometric method using ferrozine (Davis *et al.*, 1986) and by atomic absorption spectroscopy.

RESULTS

Isolation of the iron-binding protein

Haemolymph prepared under sterile conditions to prevent the lysis of amoebocytes was labelled with ^{59}Fe and subjected to gel filtration on a column of Sepharose CL-6B. As shown in Fig. 1, the first protein peak eluted from the column was haemocyanin, as was evident from its intense blue colour and absorbance at 340 nm. Only a small amount of ^{59}Fe was associated with the large haemocyanin peak. Most of the ^{59}Fe was associated with a small, non-blue, protein peak that eluted immediately following the haemocyanin. A small amount of ^{59}Fe eluted much later from the column. Estimation of the molecular mass of this material suggested that it was unbound iron or a small chelate of iron. The elution profiles of protein and ^{59}Fe from the Sepharose CL-6B column were identical with the samples of haemolymph prepared under non-sterile conditions in which amoebocyte lysis and clotting were permitted to occur.

The fractions (37–47) containing the protein peak with most of the ^{59}Fe were combined and concentrated to 10 ml by ultrafiltration. The concentrated material was placed on a DEAE-Sephacel column that was developed in a stepwise manner with buffers of increasing ionic strength. As illustrated in Fig. 2, the small protein peak eluted with the buffer of lowest ionic strength (0.1 M-

NaCl) contained most of the ^{59}Fe . The large protein peak eluted with 1 M-NaCl was faintly blue indicating that it contained a small amount of haemocyanin. Relatively small amounts of ^{59}Fe (in relation to the amount of protein) were associated with this peak. When buffers of ionic strength intermediate between 0.1 M-NaCl and 1 M-NaCl were employed with this column, some protein peaks were eluted but these peaks had very little ^{59}Fe associated with them.

Progress of the purification of the iron-binding protein was monitored by polyacrylamide-gel electrophoresis of whole haemolymph samples and samples obtained after each chromatographic procedure. After DEAE-Sephacel ion-exchange chromatography, a single band of protein was observed on both 4% and 7% polyacrylamide gels. Electrophoresis of the purified iron-binding protein was also conducted on gradient gels (3–10%). Some of these gels were stained with Ferene S, a specific stain for iron-binding proteins (Ching-Ming Chung, 1985), rather than with the general protein stain, Coomassie Blue. As illustrated in Fig. 3, a single protein band with equal mobility was obtained with both staining procedures.

^{59}Fe labelling of purified unlabelled iron-binding protein

In the plasma of vertebrate organisms, caeruloplasmin, a copper-containing protein, facilitates the oxidative incorporation of iron into transferrin via its ferroxidase activity (Osaki *et al.*, 1966; Roeser *et al.*, 1970). To determine if *Limulus* haemolymph contains a molecular component that promotes the incorporation of iron into *Limulus* iron-binding protein, samples of the iron-binding protein without ^{59}Fe label were prepared. The purified unlabelled samples of iron-binding protein were exposed to ^{59}Fe in a manner identical to whole haemolymph and subjected to gel filtration on Sepharose CL-6B to remove

Fig. 1. Gel filtration of ^{59}Fe -labelled haemolymph

A sample (10 ml) of fresh haemolymph containing $2\ \mu\text{Ci}$ of ^{59}Fe was subjected to gel filtration on a column (2.6 cm $\times$ 85 cm) of Sepharose CL-6B that was equilibrated and developed with 0.05 M-Hepes buffer, pH 8.0, containing 2.5% NaCl. Fractions (6.5 ml) were collected. Elution of protein from the column was monitored continuously at 280 nm. Absorbance of each fraction at 340 nm was also determined. The elution profile of ^{59}Fe from this column was determined by counting a 0.5 ml sample of each fraction. The fractions (37–47) containing the bulk of the ^{59}Fe associated with protein were combined and concentrated to 10 ml by ultrafiltration using an Amicon PM-10 membrane.

Fig. 2. Ion-exchange chromatography of the ^{59}Fe -labelled protein

Concentrated protein solution (10 ml) containing ^{59}Fe obtained after the gel filtration was placed on a DEAE-Sephacel column (2.6 cm $\times$ 30 cm) that had been equilibrated with 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl. This column was initially developed with approx. 500 ml of this buffer and then was stripped of remaining protein with 0.05 M-Hepes buffer, pH 8.0, containing 1.0 M-NaCl. Fractions (6.5 ml) were collected. The elution of protein and ^{59}Fe from this column was determined as described in Fig. 1.

Fig. 3. Comparison of staining of the purified iron-binding protein with Coomassie Blue and Ferene S in gradient polyacrylamide gels

The sample obtained after ion-exchange chromatography on DEAE-Sephacel was subjected to electrophoresis on 3–10% gradient polyacrylamide gels as described in the Materials and methods section. Gels were stained with either Coomassie Blue, a general protein stain, or with Ferene S, a specific stain for iron-binding proteins.

any unbound ^{59}Fe . Specific radioactivity of the purified iron-binding protein labelled in this manner was comparable with the specific radioactivity of the protein isolated from ^{59}Fe -labelled whole haemolymph.

Properties of the purified iron-binding protein

Molecular mass of the purified iron-binding protein, estimated by gel filtration, was 282000 ± 10000 Da (Fig. 4). Consistent with this value of mass was the observation of a single peak with an $s_{20,w}$ of 11.8 S during analytical ultracentrifugation of the purified iron-binding protein.

An isoelectric point of 6.5 was obtained for the iron-binding protein by isoelectric focusing in polyacrylamide gels (Fig. 5).

The subunit composition of the iron-binding protein was investigated by SDS/polyacrylamide-gel electrophoresis. As shown in Fig. 6, a single protein band was obtained with an apparent molecular mass of 28147 ± 2000 Da, suggesting that the native iron-binding protein is composed of ten subunits of identical molecular mass.

Affinity and stoichiometry of iron binding of the purified protein

To estimate the affinity with which iron is bound to the purified protein, samples of the ^{59}Fe -labelled protein were dialysed with an excess of various iron chelating agents (Table 1). EDTA, which chelates both ferrous and ferric iron, and 2,2'-bipyridyl, which specifically chelates ferrous iron, removed very little of the ^{59}Fe bound to the protein. Nitrilotriacetic acid, which chelates both ferrous and ferric iron and readily exchanges iron with vertebrate transferrin, removed approx. 30% of the bound ^{59}Fe . Desferal, a reagent that specifically chelates ferric iron, was most effective in removing bound ^{59}Fe from the protein. However, it still only removed approx. 50% of the ^{59}Fe after extensive dialysis.

To determine the iron-binding capacity of the protein, iron-loaded samples of the purified protein were quantitatively analysed for their content of tightly bound iron after dialysis with EDTA and after Chelex-100 chromatography. Some variability in the iron content of the samples was observed before Chelex-100 chromatography. Chemical and atomic absorption analyses indicated that the protein, before Chelex-100 chromatography, had 3–5 iron atoms bound per subunit, or 30–50 iron atoms bound per molecule of native protein. After Chelex-100 chromatography, the iron-loaded protein consistently had 2 iron atoms bound per subunit, or 20 iron atoms bound per native molecule (Table 2).

Fig. 4. Estimation of molecular mass of the purified iron-binding protein

Samples of marker proteins (2 ml; 3–10 mg/ml) were used to calibrate a column (1.6 cm × 90 cm) of Sepharose CL-6B that was equilibrated and developed with 0.05 M-Hepes buffer, pH 8.0, containing 0.1 M-NaCl. Following calibration, a sample (2 ml) of the purified iron-binding protein was passed through the column. Fractions (2.63 ml) were collected. Protein elution from the column was monitored continuously at 280 nm. Abbreviations: TG, thyroglobulin (8 mg/ml); AF, apoferritin (10 mg/ml); LIBP, *Limulus* iron-binding protein (2.8 mg/ml); AM, β -amylase (4 mg/ml); AD, alcohol dehydrogenase (5 mg/ml); AB, albumin (10 mg/ml); CA carbonic anhydrase (3 mg/ml).

Table 1. Effect of chelating agents on ^{59}Fe bound to be purified protein

Samples (1 ml, 2.8 mg of protein) of the purified ^{59}Fe -labelled protein were dialysed for 24 h with 1 litre of 0.05 M-Hepes buffer (pH 8.0)/0.1 M-NaCl having a concentration of 1 mM of one of the iron chelating agents. These samples were then dialysed for 3 days with frequent changes of 0.05 M-Hepes buffer (pH 8.0)/0.1 M-NaCl to remove ^{59}Fe sequestered by the chelating agent.

Chelating agent	Specific activity (c.p.m./mg of protein)		Decrease (%)
	Before dialysis	After dialysis	
EDTA	43457	44408	0
2,2'-Bipyridyl	49109	45450	7.5
NTA	45371	31160	31.3
Desferal	44331	23096	47.9

Fig. 5. Isoelectric point of the purified, iron-binding protein

Samples of marker proteins and the purified iron-binding protein were electrofocused as described in the Materials and methods section. Approx. 15 μl of each marker protein solution (0.5 mg/ml) and the purified iron-binding protein solution (0.593 mg/ml) were applied to commercially prepared polyacrylamide-gel plates (LKB Ampholine PAG plates) that had a pH range of 3.5–9.5. Abbreviations: AG, amyloglucosidase (pI 3.55); TI, soybean trypsin inhibitor (pI 4.55); LG, bovine milk β -lactoglobulin A (pI 5.13); CAB, bovine erythrocyte carbonic anhydrase B (pI 5.85); LIBP, *Limulus* iron-binding protein; CAH, human erythrocyte carbonic anhydrase B (pI 6.57); MG, horse heart myoglobin (pI 6.76, 7.16); LDH, rabbit muscle L-lactate dehydrogenase (pI 8.30, 8.40, 8.55); T, bovine pancreas trypsinogen (pI 9.3).

Table 2. Analysis of iron content of the iron-loaded protein

Samples of purified iron-binding protein were iron loaded and unbound and loosely bound iron was removed by EDTA dialysis and Chelex-100 chromatography as described under Materials and methods. These samples were analysed for iron content by a chemical method using ferrozine and by atomic absorption as described in the Materials and methods section. Each value in the Table represents the average of the results of three separate determinations.

Sample	Fe content (Fe atoms/subunit)	
	Chemical analysis	Atomic absorption
After EDTA dialysis	4.13 ± 0.50	4.00 ± 0.72
After EDTA dialysis + Chelex	2.03 ± 0.15	2.01 ± 0.11

DISCUSSION

Haemolymph of the horseshoe crab, *L. polyphemus*, contains a protein with a high affinity for iron and a capacity for iron at least equivalent to that of vertebrate transferrin. The fact that the extent of ^{59}Fe labelling of this protein was the same with haemolymph samples in which amoebocyte lysis was prevented, and with haemo-

Fig. 6. Subunit composition and subunit molecular mass of the purified iron-binding protein

Samples of marker proteins and the purified iron-binding protein were treated with SDS and dithiothreitol, subjected to SDS/polyacrylamide-gel electrophoresis, fixed, stained, and destained as described in the Materials and methods section. The relative electrophoretic mobilities of the marker proteins and the purified iron-binding protein were determined by scanning the Coomassie Blue-stained gels at 620 nm with an ISCO model 1310 gel scanning attachment for the ISCO UA-5 absorbance monitor. Abbreviations: BA, bovine albumin; EA, egg albumin; GPD, glyceraldehyde-3-phosphate dehydrogenase; CA, carbonic anhydrase; T, trypsinogen; TI, soybean trypsin inhibitor; LA, α -lactalbumin; LIBP, *Limulus* iron-binding protein.

lymph samples in which amoebocyte lysis was permitted, would strongly suggest that the protein is synthesized in tissues of the crab and does not arise from the amoebocyte.

The *Limulus* iron-binding protein has a molecular mass of 282000 ± 10000 Da compared with the molecular mass of 78000 Da for vertebrate transferrin. The only other clearly demonstrated and carefully characterized iron-binding protein from an arthropod, *C. magister*, was also a large protein with a molecular mass of 150000 ± 10000 Da. Two reasons have been suggested for the large size of the proteins in the haemolymph of these phylogenetically 'older' species that do not have their oxygen transport protein contained in an erythrocyte-type cell (Van Holde & Miller, 1982; Martin *et al.*, 1984). The first of these reasons is that the large size of haemolymph proteins is a contrivance to reduce

colloid osmotic pressure in the haemolymph to a manageable value. The second reason is to prevent inadvertent loss of these proteins during filtration by the excretory gland, the coxal gland in the horseshoe crab (Towle *et al.*, 1982).

The only other carefully characterized invertebrate iron-binding protein was isolated from the plasma of a deuterostome, *P. stolonifera*. In contrast with the large-molecular-mass iron-binding proteins isolated from arthropods, the iron-binding protein of the deuterostome is much smaller with a molecular mass of 41000 ± 2000 Da. This organism, however, has its oxygen-transport protein, haemoglobin, contained in an erythrocyte-type cell and does not have a filtration excretory organ (Martin *et al.*, 1984).

The *Limulus* iron-binding protein and these other well characterized invertebrate iron-binding proteins (Huebers *et al.*, 1982; Martin *et al.*, 1984) all have isoelectric points on the acid side of neutrality, as does vertebrate transferrin (Feeney & Komatsu, 1964). However, in contrast with the iron-binding proteins isolated from *C. magister* and *P. stolonifera* and to vertebrate transferrin, the iron-binding protein of *L. polyphemus* does not appear to be a single polypeptide chain but composed of ten subunits having molecular masses of approx. 28000. The oxygen transport protein, haemocyanin, in the haemolymph of *L. polyphemus* is also an oligomeric protein. In the native state, it contains 48 subunits but only eight immunoelectrophoretically distinguishable subunit types, all of which have molecular masses of approx. 70000 Da (Brenowitz *et al.*, 1981).

Quantitative analyses of the iron content of iron-loaded samples of the purified protein indicate that each subunit has the capacity to bind two iron atoms with high affinity. This stoichiometry is quite interesting in view of the fact that the single polypeptide chain of the iron-binding protein from *C. magister* and the single polypeptide chain of vertebrate transferrin also have the capacity to bind tightly two iron atoms.

The horseshoe crab, *L. polyphemus*, is a very primitive arthropod that should need relatively small amounts of iron as a result of having the copper protein, haemocyanin, as its vehicle for oxygen transport. Hence, the isolation of an iron-binding protein from the haemolymph of this organism provides considerable support to the proposal (Huebers *et al.*, 1982; Martin *et al.*, 1984) that iron-sequestering proteins are not new-comers to the evolutionary scene but are an ancient evolutionary development, and that the need for such proteins did not necessarily arise with the appearance of the iron-containing proteins, haemoglobin and haemerythrin, as a means to accomplish oxygen transport. The need for iron-sequestering proteins may have risen not only to ensure an adequate supply of iron for tissue requirements but also to prevent the toxic accumulation of iron. A comparison of the properties of the iron-binding proteins isolated to date from invertebrate organisms and of those isolated from vertebrate organisms suggests that a variety of such proteins may have evolved to accomplish this physiologically important task, a situation akin to the evolution of several different types of oxygen-carrying proteins.

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