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Biomineralization of Iron: Isolation of Ferritin from the Hemolymph of the Limpet *Patella vulgata*

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Abstract

Ferritin has been isolated from the hemolymph of the limpet *Patella vulgata.* The protein was homogeneous by electrophoretic criteria and appeared under the electron microscope as a central electron dense core surrounded by a polypeptide shell. The iron content of the protein was 22.5%, present in the central core which had a median diameter of 70–75 Å and was poorly crystalline. The polypeptide shell consisted of a single subunit of apparent M_r 25 000.

Introduction

The teeth of the tongue-like radula of limpets, common marine molluscs of global distribution, contain biological deposits of the iron(III) oxyhydroxide mineral goethite, α-FeOOH [1–3]. Amorphous silica, SiO_2, is also present with goethite in the organic matrix of the teeth [4, 5]. We have initiated recently an extensive study of the composition, structure and biosynthesis of these biomineralized goethite deposits. Analysis using the scanning proton microprobe has revealed a complex sequence of compositional changes within the teeth at various stages of maturity and mineralization [6, 7]. These changes have been followed also by electron microscopy [8] and ^{57}Fe Mössbauer spectroscopy [9] which indicate that iron is present as both microcrystalline goethite and a poorly crystalline component whose relative concentration in the tooth decreases as mineralization progresses. This latter form of iron is found in the basal regions of the radula teeth.

Deposition of iron as goethite in the teeth is a continuing process [1, 2] and iron concentrations of 3000 μg/100 ml have been reported [10] in limpet hemolymph (blood). In this paper we report the isolation and definitive identification of the iron-binding protein ferritin from the hemolymph of the limpet *Patella vulgata.* Ferritin is a metalloprotein of considerable interest for bioinorganic chemistry since it can be considered as a giant clathrate compound with a metal–protein stoichiometry that, although variable, is the largest yet reported. The best characterized ferritin, that from horse spleen, consists of a protein shell of 24 identical subunits surrounding a 70 Å diameter particle of hydrous iron(III) oxide that contains also some phosphate. The central iron core has a maximum capacity of 4500 atoms of iron, with an average of about 3000. The three-dimensional molecular structure of the protein shell is being revealed by X-ray structure analysis [11, 12].

As shown below, *Patella vulgata* hemolymph ferritin contains an electron dense iron core surrounded by an outer protein shell, as expected for ferritins of non-bacterial origin, but it also differs in some significant respects from these ferritins. Further differences are evident when these ferritins are compared with bacterial ferritins, as discussed in detail elsewhere [13–15]. Recently, ferritin has been identified in the hemolymph of two other limpet species, *Patelloida alticostata* and *Patella peronii* [10].

Experimental

Specimens of *Patella vulgata* were collected from the inter-tidal and shallow water region at both the Gower peninsula and Plymouth, U.K., where they are locally abundant. Hemolymph was obtained

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from a series of shallow incisions in the muscular foot and from the blood vessels that lie behind the gills.

Isolation of ferritin from the hemolymph was achieved by a combination of $(NH_4)_2SO_4$ precipitation and sequential centrifugation. Pooled hemolymph was cleared of cell debris and made 65% saturated with solid $(NH_4)_2SO_4$, whereupon the pH dropped to 6.6, and left overnight at 4 °C. Centrifugation (12 000 g) yielded a brown coloured pellet that was resuspended in phosphate buffer (0.1 M, pH 6.8) and dialyzed exhaustively against this buffer. After centrifugation (6000 g) to remove any undissolved material, the clear brown solution was treated by ultracentrifugation (120 000 g, 3 h). The resultant brown pellet was redissolved in the above buffer and the solution frozen overnight. On thawing, ferritin remained in solution but contaminants of hemocyanin, the major hemolymph protein [10] were precipitated and removed by centrifugation (6000 g). Ferritin was further purified to homogeneity by repeated ultracentrifugation.

Electrophoresis in polyacrylamide gels was performed both with [16] and without [17] the denaturing detergent sodium dodecyl sulfate (SDS). Protein bands were localized by staining with Coomassie Brilliant Blue R-250. The iron content of ferritin preparations was determined by atomic absorption spectrophotometry.

Electron microscopic studies of purified ferritin were performed on a JEOL CX-100 microscope. Negative staining with uranyl acetate (2%, pH 7) was used to visualize the polypeptide shell.

Results and Discussion

Hemolymph from the limpet *Patella vulgata* was readily obtained by dissection, each specimen yielding approximately 2 ml of brown coloured fluid whose pH was 7.5. Ferritin purified from this hemolymph was homogeneous, yielding a single band on electrophoresis in polyacrylamide gels, pH 8.6, even when the gels were overloaded with sample in order to detect any minor components. Treatment with SDS and subsequent electrophoresis in the presence of SDS yielded one major band of apparent M_r 25 500 and two minor bands (corresponding to <10% of total protein stained) at 24 500 and 21 000. These minor components are most probably derived from the single major component and the holoprotein can be described as containing only the single subunit of M_r 25 500. In this regard *P. vulgata* hemolymph ferritin resembles horse spleen ferritin [11] which

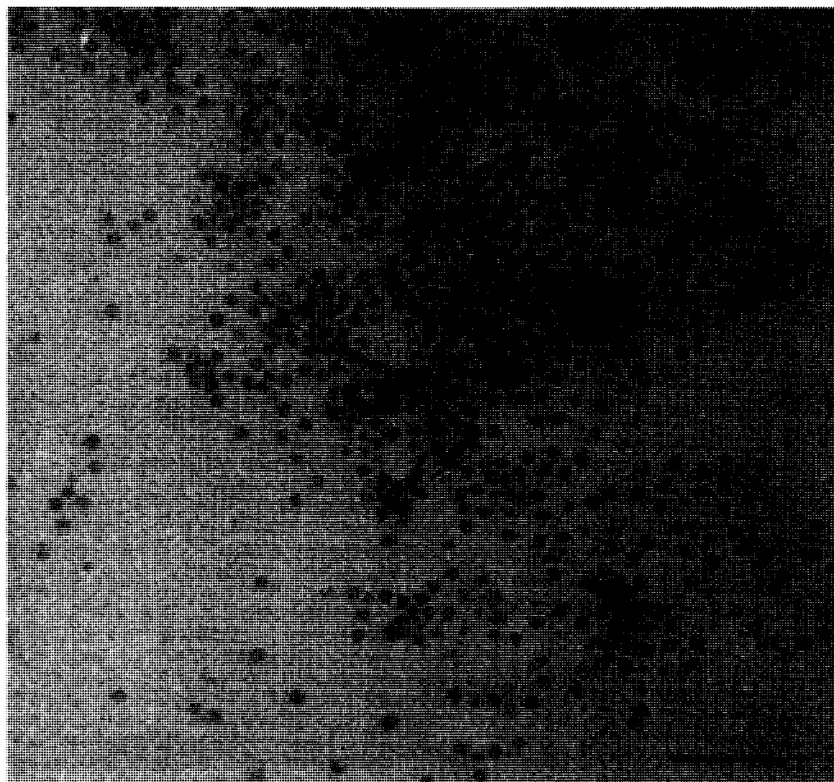

Fig. 1. Electron micrograph of purified *Patella vulgata* hemolymph ferritin. Magnification 294 950×. Bar = 50 nm. Unstained preparation.

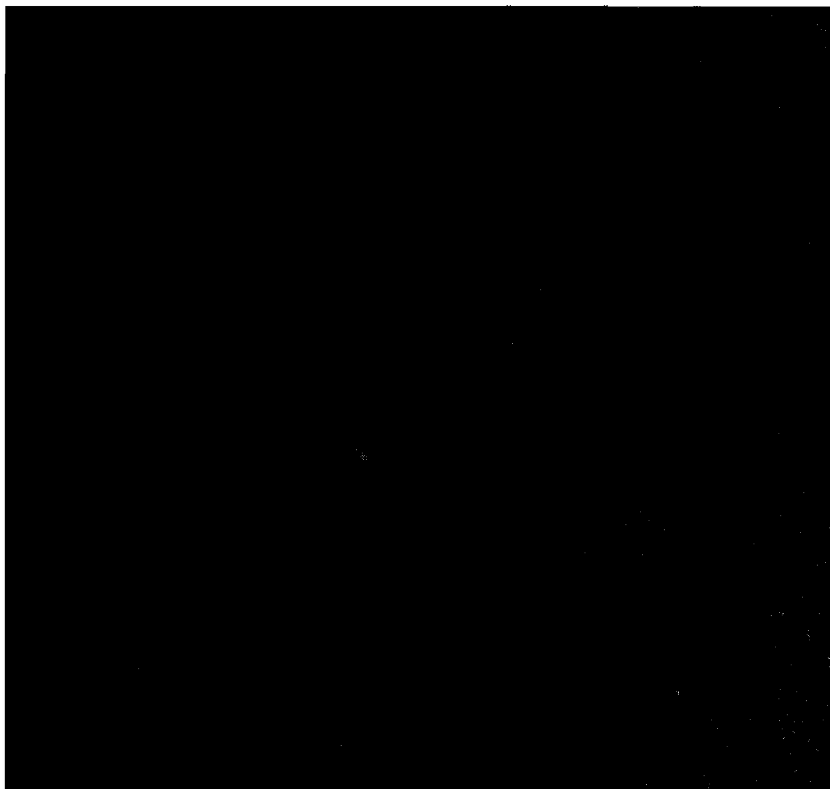

Fig. 2. Electron micrograph of purified *Patella vulgata* hemolymph ferritin. Magnification 294 950×. Bar = 50 mm. Preparation stained with uranyl acetate.

also contains only one subunit which is, however, somewhat smaller (M_r 18 500) than that of limpet ferritin. Limpet hemolymph ferritin would then appear to be a suitable candidate for crystallization and subsequent X-ray structure analysis. Ferritin isolated from the hemolymph of another marine mollusc, the chiton *Clavarizona hirtosa,* contains two types of subunit with M_r 28 000 and 25 000 [18].

Purified hemolymph ferritin from *P. vulgata* contained 22.5 wt.% iron as determined by atomic absorption spectrophotometry. This is essentially the same as that of 23% reported for the ferritin [18, 19] of chitons, inter-tidal molluscs that occur in the same ecological environment as limpets and which contain biomineralized deposits of magnetite, Fe_3O_4 in their radula teeth [1, 2, 18]. However, this iron content is appreciably lower than the 29% iron of human ferritin and very much higher than the 7% iron of ferritin from *Pseudomonas aeroginosa* [13].

Under the electron microscope, *P. vulgata* ferritin appeared as electron dense particles (Fig. 1) consistent with the presence of a compact iron core. With negative staining, the outer polypeptide shell was clearly outlined, as shown in Fig. 2. Taken together, these electron microscopic results unambiguously confirm the identification of this homogeneous protein as ferritin.

The ready availability of this highly purified ferritin has supported comparative molecular studies of ferritins from a variety of biological sources [13, 14, 19]. Under high resolution electron microscopy, the iron cores of *P. vulgata* hemolymph ferritin have a median diameter of 70–75 Å, appreciably greater than the 60–65 Å determined for human spleen ferritin. Furthermore, human spleen ferritin shows extensive lattice fringes derived from the considerable regularity in the solid state structure of ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) in the iron core. In contrast, *P. vulgata* hemolymph ferritin shows little if any crystallinity [13, 19]. This comparatively low iron content, iron density and structural crystallinity in this ferritin have significant implications for the magnetic properties and hence Mössbauer spectroscopic behaviour of its iron core [14].

The room temperature Mössbauer spectrum of *P. vulgata* hemolymph ferritin consists of a quadrupole split doublet whose spectral parameters are consistent with the presence of iron(III) in the core. The doublet persists as the temperature is lowered to 50 K when it begins to collapse into a sextet, as reported elsewhere [14, 19]. This behaviour is

not simply that due to magnetic relaxation of small particles exhibiting superparamagnetism but also involves the paramagnetic phase transition. The blocking temperature of 25–30 K is very much lower than the 50 K observed for the superparamagnetic iron cores of human spleen ferritin. The electron microscopic and Mössbauer spectroscopic data thus indicate that limpet ferritin contains a rather large iron core that is poorly crystalline and hence exhibits a low blocking temperature. An even lower blocking temperature has been observed with ferritin from *P. aeroginosa* [14].

A major biological role for limpet hemolymph ferritin in the transport of iron has been proposed [10]. The high iron content would be consistent with the transport of iron to the continually mineralizing tissues of the radula. In this regard it should be noted that chiton hemolymph ferritin binds ^{59}Fe presented to the hemolymph and furthermore takes up iron much more rapidly than does horse spleen ferritin [18], consistent with this proposed biological function. In these cases, ferritin can be involved in iron transport processes rather than, or in addition to, its more widely reported role of iron storage [11].

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