Isolation and Selected Properties of a Molluscan Ferritin with a Low P/Fe Ratio

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Abstract

Ferritin has been isolated and characterized from the hemolymph of the limpet *Patella laticostata*. The intact protein was found to have a M_r of approx. 480 000 and be composed of a single subunit with an approx. M_r of 26 000. In addition, it possessed a pI of 6.6–6.8 despite displaying a low number of basic residues on amino acid determination. Chemical analysis revealed an iron to protein ratio of approx. 2000:1, while electron microscopy showed a core diameter of approx. 8 nm. Of particular interest is the extremely low phosphate to iron ratio which varied between 1:45 and 1:35 and which is far less than that reported for mammalian ferritins.

Introduction

As part of our continuing investigations of the biological mineralization of iron, we have reported the chemical composition and ultrastructure of biominerals present in the radula teeth of two groups of marine molluscs, chitons and limpets [1–5]. In these animals, the iron-binding protein, ferritin, in addition to its commonly considered role as an iron-storage protein [6], also appears to function as a high capacity iron-transport protein [7–9].

We have recently characterized ferritin from the chiton *Acanthopleura hirtosa* (formerly known as *Clavarizona hirtosa* [10]). In comparison to horse spleen ferritin *A. hirtosa* ferritin has a high M_r (530 000 *versus* 467 000) and is composed of 2 subunits rather than one. Furthermore, on isoelectric focussing it exhibits microheterogeneity in the pI range 5.3–6.0. We report here the isolation and characterization of a ferritin from the limpet *Patella laticostata* that has a simpler macromolecular structure and hence is a promising candidate for crystallographic and related studies of the molecular basis for mineralization in ferritin. Moreover, this ferritin has a comparatively low P to Fe ratio in its core and hence its study would contribute to the resolution of the vexed question of the role of phosphate in the structure and function of ferritin.

Experimental

Specimens of *P. laticostata* were collected from off-shore reefs near Port Gregory, Western Australia (28 °S, 114 °E). Shell dimensions were, in general, 8 cm × 5 cm, earning it the common name 'giant limpet'. Hemolymph (~12 ml per specimen) was collected as described previously for other limpet species [11, 12] and frozen immediately at –20 °C. For isolation of hemolymph ferritin, the general procedures employed for isolation of ferritin from the hemolymph of *A. hirtosa* were used [9] *viz.*, heat denaturation, precipitation with $(NH_4)_2SO_4$, ultracentrifugation and gel chromatography on Sephacryl S-300 (Pharmacia) in the presence of sodium borate buffer (0.025 M, pH 8.6) containing 0.15 M NaCl. The column was calibrated using MW marker proteins *viz.*, thyroglobulin (M_r 669 000), horse spleen ferritin (476 000), IgG (158 000) and bovine serum albumin (68 000).

Electrophoresis in polyacrylamide gels (7.5%) was carried out following the method of Davis [13] while isoelectric focussing was performed in polyacrylamide gels (5%) containing 2% ampholines in the pH range 4–7 (LKB). Electrophoresis under dissociating conditions (2% sodium dodecylsulfate, 5% mercaptoethanol) followed the method of Laemmli [14] using commercially available marker proteins (Sigma Chemical Co.). Samples were treated prior to electrophoresis (90 °C, 5 min) to aid dissociation. Gels were stained for protein with Coomassie Brilliant Blue R-250 and for iron using $K_4Fe(CN)_6$ (2%) and HCl (2%) mixed (1:1, *v*/*v*) immediately before use.

Immunodiffusion experiments were performed in agarose gels (1%). Hemolymph ferritin from *A. hirtosa* and its antiserum (raised in rabbits) were

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prepared as described elsewhere [9]. Apoferritin was prepared by reduction of native ferritin with thioglycolate (1%, pH 5.0).

Samples of purified ferritin were analysed for protein by the Lowry method [15] using bovine serum albumin as the standard and for iron and phosphorous by inductively coupled plasma (ICP) spectrophotometry. Iron determinations were also carried out by atomic absorption spectrophotometry. Spectra in the UV–Vis region were recorded on a Hewlett-Packard HP8450 instrument. Following hydrolysis in 6 M HCl, 110 °C overnight, the amino acid content was determined using Varian 5560 HPLC instrumentation. Ferritin was visualized for electron microscopy by negative staining with 2% phosphotungstic acid, pH 6.0 and examined on Formvar coated grids using a Philips 301 electron microscope.

Results and Discussion

Longitudinal incisions into the foot and gills readily yielded a brown–grey coloured hemolymph from which ferritin could be isolated as a homogeneous protein that gave a single band on electrophoresis in polyacrylamide gels. This band stained positively for both iron and protein. Following chromatography on S-300, the M_r of this ferritin was determined as 480 000, indistinguishable from that of horse spleen ferritin (476 000). Electrophoresis of the apoprotein in dissociating conditions revealed that the oligomeric protein consisted of a single subunit of M_r 26 000 ± 2000 (Fig. 1a). This parallels the observation of only a single subunit in ferritin obtained from another limpet species *Patella vulgata* [11], and contrasts with that obtained from the chiton *A. hirtosa* which is more complex [9]. Although the *P. laticostata* ferritin exhibited a somewhat broadened band in the pH range 6.5–6.8 on isoelectric focussing it did not display the considerable microheterogeneity seen in ferritin from *A. hirtosa* [9]. However, like chiton ferritin this limpet ferritin is clearly appreciably more basic than horse spleen ferritin (Fig. 1b). On amino acid analysis, however, limpet ferritin had a somewhat lower level of basic residues than horse spleen ferritin, (23% *versus* 26%) [16] suggesting that, in limpet ferritin, more of the basic residues are exposed on the outer surface. The observation that, on immunodiffusion, limpet ferritin does not cross-react with antiserum to horse spleen ferritin also suggests that considerable surface differences exist between these two ferritins. No reaction was observed between limpet ferritin and antiserum to chiton ferritin. The antigenic sites on horse spleen ferritin have been identified [17] and it is clear that these differ appreciably from those on the limpet ferritin.

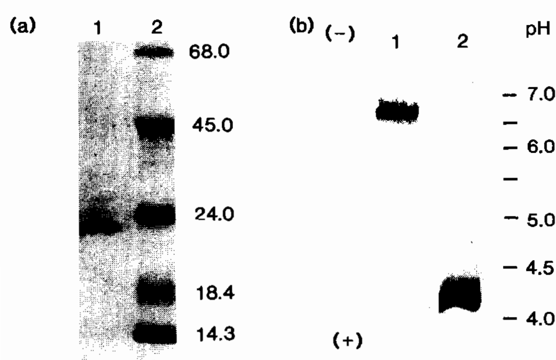

Fig. 1. Electrophoresis of ferritin from the limpet *Patella laticostata*. (a) After dissociation in SDS-CH_3CH_2SH (gel 1); MW standards (gel 2) were bovine serum albumin (68 000), egg albumin (45 000), trypsinogen (24 000), β-lactoglobulin (18 400), lysozyme (14 300). (b) In pH gradient, *i.e.* isoelectric focussing (gel 1); horse spleen ferritin (gel 2).

Under the electron microscope negatively-stained preparations of the protein revealed the iron core of limpet ferritin as an electron dense core of approx. 8 nm diameter surrounded by an annulus or 'halo' of polypeptide. Chemical analysis indicated that the iron content of the protein was approx. 2000 Fe atoms/molecule, rather less than that for mammalian ferritins but appreciably more than the approx. 1000 reported for bacterioferritins [18]. The UV–Vis spectrum consists of a broad absorption increasing through the Vis to the UV region (λ_{max} 280 nm), indicating that no heme groups characteristic of bacterioferritins are present.

A particularly interesting result obtained in this study is that the core contains a rather low amount of phosphate. The P:Fe ratio varied between 1:45 and 1:35 while for mammalian ferritins this ratio has been reported to be 1:4.5 to 1:16.7 [19, 20]. The molecular structure of the core would thus need to accommodate, in a volume that is slightly larger (8 nm) than that of horse spleen ferritin (7 nm), somewhat fewer Fe atoms and many fewer P groups. The structure is presumably a rather open and probably hydrous iron-(III) oxyhydroxy polynuclear cluster. A brief report on some magnetic properties of this core has appeared [21], and a more detailed analysis, based on electron diffraction and variable temperature Mössbauer spectroscopic studies will appear elsewhere [22].

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