The composition and the structure of bacterioferritin of *Escherichia coli*

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(Received 31 December 1980/Accepted 9 March 1981)

Bacterioferritin isolated from *Escherichia coli* is of two kinds: a protein containing a polynuclear iron compound, the bacterioferritin proper and a protein free of the polynuclear iron compound, the apo-bacterioferritin. Bacterioferritin of both kinds is characterized by absorption maxima at 417, 530 and 560 nm, contributed by protohaem IX. Single crystals of bacterioferritin of the space group *I*432 suggest that the molecule is made up of 24 identical subunits related by a cubic point symmetry. The molecular weight of the protein subunit, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, is 15 000. In the electron microscope the bacterioferritin molecule appears to be a sphere of 9.5 nm (95 Å) diameter composed of a negatively staining outer shell and an inner electron-dense core of 6 nm (60 Å) diameter.

The study of the metabolism of iron in bacteria, and in *Escherichia coli* in particular, has given much information on the mechanism of iron uptake by the cells, but not on the physical state and the chemical nature of the iron in the cell (Rosenberg & Young, 1974). Ferritin, the iron-storage protein of animals and plants, has not been found in bacteria and in yeasts (Harrison & Hoy, 1973), except for the recent discovery of a ferritin-like protein in *Azotobacter vinelandii* (Stiefel & Watt, 1979). Both the protein of *E. coli*, whose isolation and properties we describe here, and the ferritin-like protein of *A. vinelandii* are haemoproteins and are thus different from the ferritins of animals and plants, in which no organic chromophores were found. This is why we suggest calling the ferritin-like haemoproteins of bacteria by the generic name 'bacterioferritin'.

We describe here the spectral properties and the structural characteristics of bacterioferritin as revealed by electron microscopy and by X-ray diffraction from single crystals. Bacterioferritin was isolated in crystal form from a soluble fraction obtained from *E. coli* grown on a medium rich in iron. Apo-bacterioferritin was isolated from a soluble fraction of *E. coli* by precipitation with antibody and separation from antibody by gel filtration in an acid-urea solution. The properties of the polynuclear iron compound of the bacterioferritin of *E. coli* were described previously (Bauminger *et al.*, 1980).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and methods

Bacterioferritin

Bacterioferritin was isolated from a β -galactosidase-less strain of *E. coli* K12, W2244. Bacteria were grown in a fermenter in a volume of 300 litres, with stirring and aeration (200 litres/min) and were harvested in the mid-exponential phase. The growth medium of pH 7.0 contained, per litre: 13.6 g of KH_2PO_4 and 2.0 g of $(\text{NH}_4)_2\text{SO}_4$, which were sterilized *in situ*, and 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g of glycerol, 10 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 mg of thiamin, which were sterilized separately, and added shortly before the inoculum. Thiamin is not required for growth of strain W2244 and its inclusion in the growth medium is an accident that was repeated for the sake of reproducibility. However, the high concentration of the iron salt is no accident; in fact, it was not possible to obtain the dense crystals of bacterioferritin by the procedure described here from bacteria grown on this medium when it contained less than 5 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /litre.

The bacteria were harvested in a Sharples centrifuge (3200 g) and washed with cold Tris/acetate buffer, pH 6.9, containing 0.05 M-Tris, 0.002 M- MgCl_2 and 0.1 M- NaCl . In a typical preparation, 300 g of packed cells were suspended in 600 ml of ice-cold buffer and broken by few passages through a French press. Cell debris was removed by centrifugation in a Sorvall refrigerated centrifuge (20 000 g/30 min) and the supernatant cleared in a Spinco ultracentrifuge at 100 000 g for 90 min. The

supernatant was fractionated by precipitation with $(\text{NH}_4)_2\text{SO}_4$. The fraction that precipitated between 33–55% saturation was dissolved in 90 ml of buffer and heated for 10 min at 60°C on a water bath and then chilled in ice. Coagulated protein was removed by centrifugation (20000 g/10 min) in a Sorvall centrifuge. To the supernatant, optical-grade CsCl was added to a concentration of 40% (w/w) and the resulting clear solution was transferred into screw-cap polycarbonate cells, which were centrifuged in a Spinco ultracentrifuge (Ti 60 rotor) at 30°C for at least 30 h at 30000 rev./min ($r_{\text{av.}} = 6.3$ cm). Red crystals of bacterioferritin were found at the bottom of the centrifuge tubes and were quite separate from the less-dense soluble and insoluble material at the top. They were transferred by aspiration to a 55% (w/w) CsCl solution in Tris/acetate buffer, pH 6.9 (see above) in which they were practically insoluble and stable over prolonged time at room temperature. Usually 300 g of bacteria yielded 1 mg of crystalline bacterioferritin. The crystals of bacterioferritin were isotropic in polarized light. Their buoyant density, determined in CsCl solutions, was 1.75 g/ml. A typical preparation was characterized by a protein/Fe ratio of 8:1.

Crystals of bacterioferritin were readily soluble in Tris/acetate buffer, pH 6.9, or in 0.1 M NH_4HCO_3 . Electrophoresis of bacterioferritin on cellulose acetate strips at pH 6.0, 7.0 and 8.5, and staining with Nigrosine, revealed only one protein band, which migrated to the anode. Electrophoresis on an SDS-containing polyacrylamide slab gel, with proteins of known molecular weights as markers, gave 15000 as the molecular weight of the major band, which accounted for more than 95% of the material in the slab.

Apo-bacterioferritin

Apo-bacterioferritin was originally isolated from the same bacteria from which bacterioferritin was isolated. However, we describe here the isolation of apo-bacterioferritin from bacteria grown on standard media. In both cases, apo-bacterioferritin was isolated in the form of an immune precipitate. Whereas in the first case the immune precipitate contained both apo-bacterioferritin and bacterioferritin, in the latter case the precipitate contained solely apo-bacterioferritin.

Apo-bacterioferritin was isolated from a β -galactosidase-constitutive strain of *E. coli* K12, 3300. Bacteria were grown, harvested and broken as described above, except that the growth medium now contained per litre: 3 g of KH_2PO_4 , 6 g of Na_2HPO_4 , 2 g of $(\text{NH}_4)_2\text{SO}_4$, 50 mg of MgSO_4 , 5 mg of FeSO_4 , 3 g of Difco casamino acids, 20 ml of glycerol and 0.5 mg of thiamin. Both freshly harvested bacteria and bacteria stored at -20°C were used for the isolation of apo-bacterioferritin.

A batch (500 g) of broken bacteria in a volume of approx. 1.5 litres were centrifuged (20000 g/30 min) in a refrigerated Sorvall centrifuge to remove cell debris, and the supernatant was left to age at 4°C for 24–48 h and then again cleared in a Sorvall centrifuge (20000 g/30 min). Apo-bacterioferritin was precipitated from the crude supernatant by the addition of 20 mg of anti-bacterioferritin antibody, either in the form of a globulin concentrate or as whole antiserum. (Titration of extract with antibody established the optimal concentration of antibody for precipitation of apo-bacterioferritin, whose content in the precipitate was measured from A_{417} .) The mixture was kept at 4°C for 24 h with occasional stirring and the red precipitate was collected by centrifugation. The precipitate was washed in Tris/acetate buffer, pH 6.9, and dissolved by prolonged contact with 5–10 ml of an acid-urea solution. (The solution was prepared by dissolving urea to a concentration of 8 M in Tris/acetate buffer and adding acetic acid to a concentration of 2%.) When all the red colour in the precipitate had dissolved, the solution was cleared by centrifugation (6000 g/10 min) and the clear supernatant was fractionated by passage through a 200 ml column of Sepharose-4B in the acid-urea solution. First to appear was the antibody, after 2/3 column volume had been eluted, followed by apo-bacterioferritin, which could be recognized by its red colour. After extensive dialysis in Tris/acetate buffer, pH 6.9, the apo-bacterioferritin was cleared of a white precipitate, of undetermined nature, by centrifugation (6000 g/10 min) and sterilized by filtration. In this form it was stored at 4°C for prolonged periods without evidence of deterioration; 500 g of bacteria yielded 5–10 mg of apo-bacterioferritin.

Apo-bacterioferritin gave only one protein band on electrophoresis (see above) and was indistinguishable from bacterioferritin. It was also free of any contamination by rabbit γ -globulin or β -galactosidase (determined by the Ouchterlony double-diffusion technique with respective antibodies). Apo-bacterioferritin does not cross-react with antibody to horse spleen ferritin, neither does it precipitate with normal rabbit serum.

Anti-bacterioferritin antibody

Bacterioferritin in Tris/acetate buffer, pH 6.9, was used to immunize rabbits. Each rabbit was injected intradermally with 100 μ g of bacterioferritin dispersed in a homogenate made of 1.5 ml of full Freund's adjuvant and 1 ml of saline (0.9% NaCl). This was followed 2 weeks later by a booster of 100 μ g of bacterioferritin in saline administered intramuscularly. A week later rabbits were bled and antisera were tested for antibody by precipitation in a capillary. All the antisera were active, and weekly bleedings of the rabbits were continued for many

weeks until the titre of antibody in the antisera declined. Antisera were prepared by keeping the blood in plastic containers overnight at 4°C. The sera were separated, cleared by centrifugation (6000 g/10 min) and stored frozen at -20°C. The globulin fraction was prepared by precipitation with 33%-satd. $(\text{NH}_4)_2\text{SO}_4$, washing the precipitate with 33%-satd. $(\text{NH}_4)_2\text{SO}_4$, dissolution of the precipitate in phosphate buffer, pH 6.8 (0.01 M-sodium phosphate/0.1 M-NaCl), extensive dialysis in the same buffer, clearing by centrifugation (20 000 g/10 min) and filtration. The globulin fraction was stored frozen at -20°C.

The antibody content in the antisera and the enriched fraction was most effectively determined by dissolving, in an acid-urea solution, precipitates obtained at various apo-bacterioferritin concentrations and recording their u.v. and visible spectra. Antibody in precipitate is determined from A_{280} after correcting for apo-bacterioferritin in precipitate from its A_{417} .

Results

Visible and u.v. spectroscopy

Absorption spectra were measured with a Cary 219 spectrophotometer. The absorption spectrum of bacterioferritin of *E. coli* reproduced in Fig. 1(a) is very similar to the spectrum of bacterioferritin of *A. vinelandii* (Bulen *et al.*, 1973). Both spectra are dominated by absorption reminiscent of band-edge absorption of polycrystalline solid semiconductors. (The optical-band-gap energies derivable from the spectra do not, however, fit with the known values for iron oxide hydroxides.) In this, bacterioferritin spectra are similar to the spectra of ferritin (Granick, 1946; David & Easterbrook, 1971). However, the wavelength-dependence of the absorption of bacterioferritin in the u.v. range is steeper than that of ferritin and suggests a higher degree of crystallinity for the iron compound of bacterioferritin. The spectra of bacterioferritin are also characterized by absorption maxima at 560, 530 and 417 nm that are absent in the spectra of ferritin. It was shown by Bulen *et al.* (1973) that the bacterioferritin of *A. vinelandii* contains protohaem IX. On reduction the α -, β - and Soret bands of the bacterioferritin of *A. vinelandii* shifted to 557.5, 527 and 425 nm respectively (Stiefel & Watt, 1979).

The absorption spectrum of what we, for lack of a better term, call 'apo-bacterioferritin' of *E. coli* is characterized by maxima at 560, 530, 417 and 278 nm (Fig. 1b). The spectrum of reduced apo-bacterioferritin in the visible range was determined by measuring the absorption spectrum of the same sample after addition of a small amount of solid $\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 1b). Reduced apo-bacterioferritin is

Fig. 1. Absorption spectra of (a) bacterioferritin and (b) apo-bacterioferritin

Continuous lines give the absorbance of the native proteins in Tris/acetate buffer, pH 6.9, of the following composition: 0.05 M-Tris, 0.002 M-MgCl₂ and 0.1 M-NaCl. The broken line is the absorbance of apo-bacterioferritin immediately after a few milligrams of $\text{Na}_2\text{S}_2\text{O}_4$ were dissolved in the sample that gave the absorption spectrum represented by the solid line.

characterized by maxima at 557, 527 and 425 nm. The ratio $\epsilon_{557}/\epsilon_{425}$ is 0.202.

Additional evidence that apo-bacterioferritin contains a small and finite number of binding sites for protohaem IX was provided by spectral analysis and a binding experiment. However, owing to uncertainty in the weight of protein, we cannot, at this stage, know the stoichiometry of haem to protein.

Haem content of apo-bacterioferritin was determined as the pyridine haemochromogen as described by Appleby & Morton (1959). By using 191 500 litre·mol⁻¹·cm⁻¹ for the Soret-band absorption coefficient (ϵ) of pyridine haemochrome of protohaem IX (Falk, 1964), the ϵ_{417} per haem group of apo-bacterioferritin was calculated to be 137 000 litre·mol⁻¹·cm⁻¹.

Binding of protohaemin IX to apo-bacterioferritin was performed in a double-sector cell. One compartment contained a solution of 5 µg/ml haemin (twice-crystallized; Schwarz & Mann, Orangeburg, NY, U.S.A.), the other a solution of apo-bacterioferritin (both in 0.1 M-sodium acetate, pH 4.7). Spectra before and after mixing are reproduced in Fig. 2. The experiment demonstrates that in apo-bacterioferritin, as prepared by us, the occupancy of the haem-binding sites is not complete and that on binding of protohaemin IX at this site the characteristic 417 nm absorption of apo-bacterioferritin is generated.

X-ray diffraction from single crystals

Crystals of *E. coli* bacterioferritin in a CsCl solution [55% CsCl (w/w) in 0.1 M-Tris/acetate, pH 6.9] and cross-linked crystals of bacterioferritin in a buffer solution were analysed. Crystals suspended in a CsCl solution were cross-linked by treatment with 1% glutaraldehyde for 10 min and

then soaked in a 0.1 M-Tris/acetate buffer, pH 6.9. They were mounted in thin-walled quartz capillaries and exposed to a high-intensity X-ray beam. Native crystals in the CsCl solution produced no Bragg reflections when exposed for up to 36 h. Cross-linked crystals gave Bragg reflections to a spacing of approx. 1 nm (10 Å). Laue photographs and 2°C unscreened precession photographs (Plate 1) were used to find the major crystallographic axes. From the symmetry of these and upper-level photographs and the systematic absences, the space group is *I*432, with cell dimensions of 14.6 nm (146 Å). The crystallographic properties of the cross-linked crystal are listed in Table 1.

Electron microscopy

Grids were prepared from bacterioferritin and apo-bacterioferritin of *E. coli* in 0.1 M-Tris/acetate buffer, pH 6.9, and negatively stained with 1% uranyl acetate. Electron micrographs of bacterioferritin are shown in Plate 2. Most projections are circular, about 9.5 nm (95 Å) in diameter, and suggest that the structure that gives this projection is spherical. The circular projections show a negatively stained outer shell about 1.5 nm (15 Å) thick and an inner electron-dense core of about 6 nm (60 Å) diameter. The dimensions of the particles and their regularity suggest that they represent single bacterioferritin molecules. Obviously, such a molecule can accommodate many 15 000-dalton subunits and is, therefore, a multimeric structure. By analogy with ferritin, it is tempting to visualize the protein subunits as forming the negatively stained outer shell, which encloses an electron-dense non-protein material of the core. The morphological similarity to ferritin is perhaps most evident in Plate 2(b), where electron-dense bodies can be seen in a non-stained region of the grid. There are, however, features discernible in some views of the core (Plate 2d) in which the electron-dense material has granular appearance interrupted by negatively staining structures. In Plate 2(c) a regular mode of packing of the particles can be discerned. The projection has the appearance as of slightly overlapping spheres or of an end-on view of overlapping cylinders. Electron micrographs of apo-bacterioferritin are of similar appearance.

Fig. 2. Binding of protohaemin IX to apo-bacterioferritin

The continuous line is the combined absorbance of 1.0 ml of haemin solution and of 1.0 ml of apo-bacterioferritin solution, each kept in one sector of a double-sector cell (optical path 2×0.437 cm). The broken line is the absorbance after a thorough mixing of the solutions in the two sectors.

Table 1. Crystallographic properties of cross-linked bacterioferritin

Crystals were cross-linked by treatment with glutaraldehyde and then soaked in 0.1 M-Tris/HCl buffer, pH 6.9. Crystal density was measured in a water/saturated xylene/carbon tetrachloride gradient tube. The estimate of crystal density as 1.40 ± 0.05 g/ml is an average value for three of the larger crystals (maximum dimensions ~ 0.1 mm).

Unit cell dimensions	$a = 14.6$ nm (146 Å)
Reflections observed only for	$h + k + l = 2n$; $h00 : h = 2n$
Space group	<i>I</i> 432
Number of asymmetric units/unit cell	48
Density of crystal	1.40 ± 0.05 g/ml

EXPLANATION OF PLATE 1

Single crystals of bacterioferritin and their 2° X-ray precession photographs

The upper photograph is of bacterioferritin crystals in 55% CsCl solution as described in the Materials and methods section. The photograph was taken with a low-magnification microscope. The soft red colour of the crystals is best described as peach red. 2° unscreened precession photographs of the principal zones of the cross-linked bacterioferritin crystals were taken with unfiltered radiation from an Elliot rotating Cu anode fitted with a 200 μ m microfocus cap, running at 29 mA/40 kV, with exposure times of approx. 3 h. X-ray photographs (below): (a) projection down the [001] axis (the [100] and [010] axes are diagonal lines in the picture); (b) projection down the [111] axis; (c) projection down the [011] axis.

EXPLANATION OF PLATE 2

Electron micrograph of bacterioferritin
Grids were prepared from protein in Tris/acetate buffer, pH 6.9, and negatively stained with 1% uranyl acetate. Part of (a), (c) and (d) were photographed over a hole in the carbon-coated grid. (b) is of an unstained area. Magnification is $\times 132\,000$ (a) and $\times 255\,000$ (b, c and d). Arrows indicate in (b) regular packing of the particles, and in (d) non-contiguity of the electron-dense material in the core and the presence of interstitial negatively stained structures.

Discussion

Bacterioferritin of *E. coli* is a red protein containing two chromophores. One of the chromophores is a polynuclear iron compound of unknown structure, but distinct from the polynuclear iron compound of ferritin (Bauminger *et al.*, 1980). The other chromophore is protohaem IX, which is present in the protein in its oxidized form. The presence in the molecule of two chromophores, both containing iron, complicates terminology. Because structurally it resembles ferritin, we call it 'bacterioferritin'. By analogy with ferritin, we call the form devoid of the polynuclear iron compound 'apo-bacterioferritin'. Apo-bacterioferritin is, however, a holoprotein in its own right, since it can be resolved into a colourless protein and protohaem by extraction with butan-2-one (methyl ethyl ketone) as described by Teale (1959). The shifts in the visible spectrum on reduction establish that it is a cytochrome *b*-type protein hitherto not found in *E. coli* (cf. Hagihara *et al.*, 1975). Similar proteins have, however, been isolated from *A. vinelandii*, as mentioned above, and from *Rhodospirillum rubrum* (Bartsch *et al.*, 1971), suggesting that it is a protein of wide occurrence in bacteria.

Bacterioferritin is normally present in *E. coli* in its apo form. Only growth under conditions of iron excess leads to the central-cavity filling with the iron storage compound. However, the iron stored in bacterioferritin is but a minute fraction of the total iron in the cell, and the protein is but a small fraction of cell protein, as was discussed previously (Bauminger *et al.*, 1980). Therefore it could well be that, in contrast with ferritin, the function of bacterioferritin centres on the haem rather than on the iron storage compound.

Whatever is the physiological function of bacterioferritin in *E. coli*, the remarkable morphological similarity between this protein and ferritin becomes apparent from comparison of the two structures. A casual comparison of the electron micrographs of bacterioferritin in Plate 2 with any of the many published micrographs of ferritin immediately establishes the similarity. In both cases the structures can be described as giving circular projections made of an outer, negatively staining, layer and an inner dense core. Even the dimensions are similar: the diameter of the whole structure approximates to 10 nm (100 Å) and that of the core approximates to 6 nm (60 Å). It is indeed fortunate that ferritin and bacterioferritin are so distinctive in their electron-microscope images, since they are rather unorthodox structures whose shape and dimensions are difficult to assess from hydrodynamic measurements (cf. Fischbach & Anderegg, 1965). For a bacterioferritin

molecule we calculate the molecular mass of protein from the dimensions suggested by electron microscopy [a shell 1.75 nm (17.5 Å) thick of a sphere of 9.5 nm (95 Å) diameter] and partial specific volume of 0.75 to be 269 000 daltons. With all the uncertainty involved in the above calculation, by providing the order of magnitude for the molecular weight, it strengthens the picture of the protein molecule as an assembly of 24 identical subunits suggested by the X-ray-diffraction data. In the body-centred cubic space group *I*432, the 48 asymmetric units are arranged in two clusters of 24 asymmetric units, one centred at the origin and the other at the body centre of the unit cell. Such a molecule is of a quaternary structure strikingly similar to that of ferritin (cf. Banyard *et al.*, 1978).

We would like to acknowledge the advice given to us by Dr. D. Cahen (spectroscopy), by Dr. Z. Eshhar (immunological procedures) and by Dr. J. Janin and Dr. J. Moulton (X-ray diffraction), and thank them for their interest. We thank Mr. I. Segal for his help with preparation of bacterioferritin and Mr. M. Rotman for his help with preparation of antibody. E. R. B., S. G. C. and S. O. thank 'Stiftung Volkswagenwerk' for their support.

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