Purification, characterization and function of bacterioferritin from the cyanobacterium *Synechocystis* P.C.C. 6803

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Storage and buffering of iron is achieved by a class of proteins, the ferritins, widely distributed throughout the living kingdoms. All ferritins have in common their three-dimensional structure and their ability to store large amounts of iron in their central cavity. However, eukaryotic ferritins from plants and animals and bacterioferritins have no sequence similarity, and besides non-haem iron bacterioferritins contain haem residues whereas eukaryotic ferritins do not. In this paper we report the first purification and characterization of a bacterioferritin from a cyanobacterium. It has a molecular mass of 400 kDa and is built up from 19 kDa subunits. Its N-terminal sequence shows 73 % identity with that of the *Escherichia coli* bacterioferritin subunit. It contains 2300 atoms of iron and 1500 molecules of phosphate per ferritin molecule and 0.25 haem residue per subunit; the α -peak of the cytochrome has its maximum at 559 nm. In contrast with what is known for eukaryotic ferritins, we found that bacterioferritin from *Synechocystis* is not inducible by iron under the conditions that we have tested and that it has a constant concentration whatever the iron status of the cells, even at very low iron concentration. Bacterioferritin from *Synechocystis* P.C.C. 6803 is fully assembled *in vivo* and it is shown by labelling with ^{59}Fe that it is able to load iron *in vitro* as well as *in vivo*. Bacterioferritin from *Synechocystis* is shown to have an iron-buffering function while the bulk of cellular iron is found associated with a pool of low-molecular-mass electronegative molecules. The role of *Synechocystis* bacterioferritin in iron metabolism is discussed.

INTRODUCTION

Iron plays an essential role in biological systems. Respiration and photosynthesis are two examples that illustrate the importance of the co-ordination of this metal to proteins involved in electron transport. In higher plants, chloroplasts are the subcellular compartment within which photosynthesis operates. These organelles are thought to have evolved from cyanobacteria, and this origin of plastids, known as endosymbiotic origin, is well documented (Weeden, 1981; Zurawski & Clegg, 1987). Therefore chloroplast biochemistry can gain a lot of information from studies on cyanobacteria and vice versa. So far, iron metabolism in plants, and particularly in chloroplasts, has received little attention, at least in molecular terms (Laulhère & Mache, 1978; Spiller *et al.*, 1987), despite the fundamental role it plays as mentioned above. For example, it is not known how iron uptake by chloroplasts is achieved (Lobreaux & Briat, 1991). On the other hand iron storage in plastids is better documented. It is performed by ferritins, a class of multimeric proteins (24-mers) that is widely distributed throughout the living kingdoms. Ferritin molecules are each able to store up to 4500 iron atoms in their central cavity (Theil, 1987; Harrison *et al.*, 1989). Plant ferritins are known to be distributed in chloroplasts and in non-green plastids of various organs (for review see Seckbach, 1982). Structure and function of plant ferritins have been mainly studied from seeds of the Leguminosae family (Crichton *et al.*, 1978; van der Marck *et al.*, 1981; Sczekan & Joshi, 1987; Laulhère *et al.*, 1988, 1989; Briat *et al.*, 1989). They are nuclear-encoded and plastid-located, and we have reported that they have a eukaryotic origin, that is sharing a strong sequence similarity with animal ferritins except for a 28-

amino acid-residue extension and a transit peptide at the N-terminal extremity (Ragland *et al.*, 1990). Nevertheless, how ferritin iron is exchanged with other iron proteins (such as ferredoxin for example) during chloroplast differentiation remains unknown.

On the other hand, the effect of iron starvation on the regulation of the expression of genes involved in photosynthesis in cyanobacteria is extensively documented (Guikema & Sherman, 1984; Laudenbach *et al.*, 1988; Laudenbach & Straus, 1988; Riethman & Sherman, 1988). However, very little is known about iron uptake and nothing about the storage of iron and its exchange with iron protein involved in photosynthesis, although iron uptake in other bacterial systems, especially in *Escherichia coli*, has been extensively studied this last decade (for review see Bagg & Neilands, 1987). It is important to note that the three-dimensional structure of *E. coli* bacterioferritin is closely related to those of eukaryotic ferritins (Smith *et al.*, 1989); however, the complete sequence of the *E. coli* bacterioferritin (Andrews *et al.*, 1989) and the partial sequence of the *Nitrobacter winogradskyi* and *Azotobacter vinelandii* bacterioferritins (Kurokawa *et al.*, 1989; Andrews *et al.*, 1991) revealed no sequence similarity to eukaryotic ferritins. Another peculiarity of all the bacterioferritins purified so far (Stiefel & Watt, 1979; Yariv *et al.*, 1981; Chen & Crichton, 1982; Moore *et al.*, 1986; Kurokawa *et al.*, 1989) is the presence of haem in these molecules. Indeed, a molecule previously isolated from *E. coli* and named cytochrome b_1 has been shown to be identical with bacterioferritin (Smith *et al.*, 1988).

Since we are interested in knowledge of the molecular mechanisms that control iron uptake and storage in plants we have initiated a study of these aspects in cyanobacteria. In the

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present paper we report the purification and characterization of the bacterioferritin from *Synechocystis* P.C.C. 6803. We show it resembles other bacterioferritins and therefore it is different from its counterpart in plant plastids. Furthermore, for the first time, we show by labelling with ^{59}Fe *in vivo* that bacterioferritin is indeed an iron-buffering protein and that the bulk of cellular iron is found associated with low-molecular-mass molecules. Finally, use of antibodies raised against the subunit of *Synechocystis* P.C.C. 6803 bacterioferritin allowed us to address the question of the accumulation of this protein subunit in relation to iron nutrition.

MATERIALS AND METHODS

Cultures

Synechocystis P.C.C. 6803 was obtained from the Pasteur Institute (Paris) collection. It was cultivated autotrophically in the BG-11 medium of Rippka *et al.* (1979) supplemented with 0.2 g of $\text{Na}_2\text{CO}_3/\text{l}$. Cultures (2 litres) in Roux bottles were agitated and aerated with the air-lift system using air supplemented with 1% CO_2 at 30 °C. Light intensity was adjusted to the cell density by maintaining 5–15 $\mu\text{Einsteins}$ of unabsorbed light/ m^2 per cm passing through the fermenter. Iron was added as 30 μM -Fe as ferric ammonium citrate. When the effects of iron overload were to be investigated, the iron concentration was raised to 166 μM , in the early exponential phase, 18 h before harvesting for ferritin extraction. Cultures for iron nutrition experiments and for protein extraction for ferritin immunodetection were performed either in 300 ml Erlenmeyer flasks containing 60 ml cell suspensions or in 12-well plates (Corning cell wells; Corning, New York, NY, U.S.A.) containing 12 $\times$ 3 ml cell suspensions. Flasks and well plates contained a compartment independent of the one containing the cell suspensions but sharing the same gas phase; this independent compartment contained a carbonate/bicarbonate buffer (25% 2 M- K_2CO_3 , 75% 2 M- KHCO_3) allowing maintenance of a constant CO_2 concentration of about 2%.

Ferritin purification

A 10 ml sample of packed cells from a 2-litre cell culture was suspended in 10 ml of 50 mM-Tris/maleate buffer, pH 7.5, frozen at -20 °C and disrupted in an X press (AB-Bio X-Jarfallä, Stockholm, Sweden). During the thawing 300 μl of 1% *o*-phenanthroline and 3 mg of phenylmethanesulphonyl fluoride were added. Then the slurry was adjusted to 0.1% Triton X-100 and to 1.1 g/ml density by addition of 10% (w/v) caesium trifluoroacetate (Pharmacia, Uppsala, Sweden). The mixture, of a final volume of 32 ml, was agitated and centrifuged at 10000 rev./min in a JA20 Beckman rotor for 5 min at 15 °C. An 8 ml portion of the supernatant was deposited on top of a layer of 4 ml of caesium trifluoroacetate (density 1.25 g/ml) in 0.1% Triton X-100/20 mM-Tris/maleate buffer, pH 7.5. After centrifugation at 50000 rev./min in a Beckman 50 Ti rotor for 3 h at 15 °C, the soluble brown part of a brown and green pellet was carefully dispersed in 20 mM-Tris/maleate buffer, pH 7.5. The green insoluble material was eliminated by centrifugation at 12000 rev./min in Eppendorf tubes for 2 min. The supernatant was then applied on an Ultrogel AcA 22 (I.B.F., Villeneuve la Garenne, France) column (1.5 cm $\times$ 47 cm) and eluted with 50 mM-Tris/maleate buffer, pH 7.5, at a flow rate of 12 ml/h. Fractions (1.1 ml) were collected for protein and iron concentration measurements. Ferritin was concentrated by ultracentrifugation at 100000 rev./min in a TLA100-2 rotor (Beckman) for 30 min at 4 °C.

Preparation of native and SDS-denatured proteins

Thawed cell pellets were suspended in 0.5 ml of lysis solution [20 mM-Tris/maleate buffer, pH 7.5, containing 10 mM-EDTA, 10% (w/v) sucrose, 0.3 mg of phenylmethanesulphonyl fluoride/ml and 10 mg of lysozyme/ml] and left at 37 °C for 30 min with occasional shearing of the suspension by passing through a syringe needle. After centrifugation at 12000 rev./min in Eppendorf tubes for 3 min, pelleted cells were subjected to an osmotic shock for 15 min in 300 μl of aq. 0.1% Triton X-100 before sonication for 10 s at 50 W with the microprobe of an Ultrasons Annemasse sonifier (Annemasse, France). For analysis of total native protein, the resulting suspension was loaded directly on to 5–20%-polyacrylamide-gradient gels after determination of the protein concentration. For analysis of total denatured protein, the suspension was adjusted to 1% SDS and 2% (v/v) 2-mercaptoethanol before sonication. The resulting sonicated suspension was used to determine protein concentration. Portions were loaded on to denaturing SDS/15%-polyacrylamide gels after boiling them for 10 min.

Protein analysis

Determination of protein concentration, h.p.l.c., *N*-terminal sequencing and native and denaturing PAGE were carried out as previously described (Laulhère *et al.*, 1988, 1989; Briat *et al.*, 1989). Spectrophotometric analysis of purified bacterioferritin was done with a Hitachi U2000 spectrophotometer with 1 cm-path microcuvettes. Reduction of the iron core of bacterioferritin was achieved with a large excess of ascorbate (100 mM) in a cuvette containing 0.3 ml of bacterioferritin at a concentration of 0.7 mg/ml in 25 mM-Tris/maleate buffer, pH 7.35. Reduction of non-haem iron was monitored kinetically by scanning between 350 and 650 nm at 6 min intervals until no further changes in absorbance occurred. Then the haem iron was reduced by including an excess of dithionite in the same cuvette, and the spectrum of this reduced state was recorded between 350 and 650 nm.

Production of antibodies and immunodetection of *Synechocystis* bacterioferritin

Portions (80 μg) of h.p.l.c.-purified bacterioferritin were injected with Freund's adjuvant into a rabbit on four occasions at 10-day intervals. The antibody titre was determined after the

Fig. 1. Elution profile of bacterioferritin from *Synechocystis* P.C.C. 6803 on an Ultrogel AcA 22 gel-filtration column

Proteins from the caesium trifluoroacetate purification step (see the Materials and methods section) were solubilized in 2 ml of Tris/maleate buffer and loaded on to an Ultrogel AcA 22 column (1.5 cm $\times$ 47 cm). Fractions (1.1 ml) were collected and analysed at 280 nm (—). Iron was measured, after reduction of a sample of each fraction with thioglycolic acid in the presence of Ferrozine, by absorbance at 562 nm (◆—◆) of the Fe^{2+} -Ferrozine complex.

Fig. 2. Characterization of bacterioferritin from *Synechocystis* P.C.C. 6803 by ^{59}Fe labelling *in vitro* and non-denaturing PAGE

(a) Coomassie Blue R-250 staining of a 5–20% polyacrylamide-gradient gel loaded with: lane 1, 6 μg of horse spleen ferritin; lane 2, 6 μg of pea seed ferritin; lane 3, 4 μg of *Synechocystis* bacterioferritin (from peak B in Fig. 1). Molecular masses indicated correspond to dimer of horse spleen ferritin (900 kDa), horse spleen ferritin (450 kDa), L-alanine dehydrogenase (230 kDa) and lactate dehydrogenase (140 kDa). (b) Autoradiogram of the same gel as in (a) loaded with horse spleen ferritin (lane 1), pea seed ferritin (lane 2) and *Synechocystis* ferritin (lane 3) that had been labelled *in vitro* for 3 h at room temperature with 35 μM - ^{59}Fe /700 μM -citrate in the presence of 3 mM-ascorbate as described by Lahlère *et al.* (1990). Electrophoresis was for 15 h at 10 V/cm.

third injection before a final bleed at 10 days after the fourth injection.

Immunodetection of bacterioferritin was achieved after electroblotting of protein samples that had been run on a denaturing SDS/15% polyacrylamide gel by using the peroxidase method that we have already described (Lahlère *et al.*, 1988). Antisera raised against *Synechocystis* bacterioferritin were used at a dilution of 1:1200. The second antibody was sheep anti-(rabbit IgG) immunoglobulins coupled with peroxidase (Biosys, Compiègne, France) and used at a dilution of 1:600.

Iron and phosphate measurements

After reduction of iron with thioglycolic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), iron concentrations were determined spectrophotometrically at 510 nm in the presence of *o*-phenanthroline or at 562 nm in the presence of Ferrozine [3-(pyrid-2-yl)-5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine]

(Sigma Chemical Co.) as previously described (Lahlère *et al.*, 1990).

Labelling of bacterioferritin *in vitro* with ^{59}Fe (57 μg of Fe/ml, 9 mBq/ml, IFS1; Amersham International, Amersham, Bucks., U.K.) in the presence of ascorbate was achieved as previously reported for pea seed ferritin (Lahlère *et al.*, 1990). After labelling with ^{59}Fe *in vivo*, native proteins were separated by PAGE (Lahlère *et al.*, 1988). Gels were dried between cellophan sheets and autoradiographed at -70°C . Total iron and [^{59}Fe]iron contents were measured from gel slices as already described (Lahlère *et al.*, 1990), allowing the calculation of the specific radioactivity of iron incorporated in the different molecules separated by electrophoresis.

The phosphate content of purified ferritin was measured by the method of Ames (1966).

RESULTS

Purification of bacterioferritin from *Synechocystis* P.C.C. 6803

Ultracentrifugation of a crude extract of *Synechocystis* cells grown in the presence of 166 μM -ferric ammonium citrate in a dense caesium trifluoroacetate solution as described in the Materials and methods section gave a dark-brown pellet. When proteins contained in this pellet were fractionated by filtration through an Ultrogel AcA 22 column, iron and proteins were found in the exclusion volume (peak A in Fig. 1) and corresponding fractions were yellow and not brown. Peak B in Fig. 1 is composed of brown fractions and contains protein and a large amount of iron. Peak C contains a blue pigment. In peak B a single protein is found on native gradient-gel electrophoresis (Fig. 2a) of apparent molecular mass 400 kDa (Fig. 2a and Table 1). This protein can be labelled *in vitro* with ^{59}Fe from [^{59}Fe]ferric citrate in the presence of ascorbate, in the same way as pea seed and horse spleen ferritins can be labelled with radioiron (Fig. 2b). Furthermore, this protein is iron- and phosphate-rich since, on the basis of a molecular mass of 400 kDa, it contains 2300 atoms of iron and 1500 molecules of phosphate per molecule of protein (Table 1); therefore the Fe/ P_i ratio of this protein is 1.53 and is in the range of what is known for bacterioferritins (Mann *et al.*, 1987). No proteins have these characteristics in peak A. We therefore concluded that a protein present in peak B illustrated in Fig. 1 has the properties of the iron-storage proteins known as ferritins (Theil, 1987; Harrison *et al.*, 1989).

Table 1. Properties of bacterioferritins of different bacteria

Property	<i>Synechocystis</i> 6803 bacterioferritin	<i>A. vinelandii</i> bacterioferritin*	<i>E. coli</i> bacterioferritin†
Molecular mass in solution (Da)	400 000	660 000	452 000
Molecular mass of subunit (Da)	19 000	17 000	18 495
No. of haem groups/subunit	0.25	0.5	0.5
No. of iron atoms/ferritin molecule	2300 $\pm$ 100‡	1600	1100
No. of phosphate molecules/ferritin molecule	1500 $\pm$ 200‡	800	500
α -Peak (nm)	559	557.5	557

* Data from Stiefel & Watt (1979) and Mann *et al.* (1987).

† Data from Andrews *et al.* (1989) and Yariv *et al.* (1981).

‡ Mean value calculated from six repeats.

Fig. 3. Characterization of the *Synechocystis* P.C.C. 6803 bacterioferritin subunit by using reverse-phase h.p.l.c. and SDS/PAGE

(a) *Synechocystis* bacterioferritin purified by Ultrogel AcA 22 gel filtration (50 μ g) was analysed on a 30 nm-pore-size C3 silica column (Ultrapore RPSC; Beckman) as previously described (Laulhère *et al.*, 1988). (b) Coomassie Blue R-250 staining of an SDS/15%-polyacrylamide gel loaded with 4 μ g of marker (lane M), with total protein contained in fractions 1, 2 and 3 illustrated in (a) (lanes 1, 2 and 3) or with 2 μ g of bacterioferritin subunit contained in fraction 4 (lane 4).

Fig. 4. Immunoblot analysis of an SDS/15%-polyacrylamide gel loaded with different ferritin subunits

A 0.25 μ g portion of pea seed ferritin subunit (lane 1) and 0.25 μ g of *Synechocystis* bacterioferritin subunit (lane 2) were loaded in duplicate on an SDS/15%-polyacrylamide gel. After electrophoresis, the proteins in the gel were electrotransferred on to nitrocellulose and detected either with pea seed ferritin antibodies diluted at 1:500 (panel a) or with *Synechocystis* bacterioferritin antibodies diluted at 1:1200 (panel b). The second antibody was sheep anti-(rabbit IgG) immunoglobulins (diluted at 1:600) coupled with peroxidase.

Characterization of bacterioferritin from *Synechocystis* P.C.C. 6803

The bacterioferritin subunit from *Synechocystis* is eluted at 48% (v/v) acetonitrile when subjected to reverse-phase h.p.l.c., indicating that it is very hydrophobic (Fig. 3a, fraction 4). A large peak in the absorbance at 214 nm is observable at a concentration higher than 50% (v/v) acetonitrile (fractions 1, 2 and 3 in Fig. 3a). These fractions contain a low concentration of protein, which is represented by a small amount of bacterioferritin subunit (Fig. 3b, tracks 1, 2 and 3). Therefore the absorbance in fractions 1, 2 and 3 is mainly due to an unknown non-protein compound. The bacterioferritin subunit has a molecular mass of 19 kDa as determined by denaturing SDS/PAGE (Fig. 3b, track 4). The h.p.l.c.-purified bacterioferritin subunit (fraction 4 in Fig. 3a) has been used to raise rabbit antibodies and to determine its

N-terminal sequence. The cyanobacterial bacterioferritin shares neither immunological determinants (Fig. 4) nor, within the first 54 amino acid residues, amino acid sequence similarity (Fig. 5) to plant ferritin. Indeed, the first 54 amino acid residues of the N-terminal sequence of the bacterioferritin subunit of *Synechocystis* are 50% identical and 73% similar to their *E. coli* counterpart and 40% identical and 60% similar to their *N. winogradskyi* counterpart (Fig. 5). Similarity of sequence was determined according to the procedure of Feng *et al.* (1985). It must be borne in mind that this sequence similarity is found in the analysed region, i.e. one-third of the bacterioferritin sequence.

Bacterioferritin from *Synechocystis* P.C.C. 6803 is a b-type haemoprotein

Bacterioferritins have been purified and characterized from a small number of bacteria. All of them contain protoporphyrin IX (Stiefel & Watt, 1979; Yariv *et al.*, 1981; Chen & Crichton, 1982; Moore *et al.*, 1986; Kurokawa *et al.*, 1989). The relation between their ability to store non-haem iron and the presence of their haem is not understood. As illustrated in Fig. 6, the bacterioferritin from *Synechocystis* P.C.C. 6803 absorbs in the Soret region near 420 nm. The absorption spectrum of the cyanobacterial ferritin is very similar to those of the bacterioferritins of *E. coli* (Yariv *et al.*, 1981) and *A. vinelandii* (Stiefel & Watt, 1979) and different from the spectrum of pea seed ferritin (Fig. 7a), which shows no evidence of a Soret band at 420 nm. Even though the redox potentials of the core will be less than that of ascorbate, a large excess of ascorbate (100 mM) enables reduction of the non-haem iron (Fig. 6b). Therefore ascorbate does permit the preparation of apo-bacterioferritin from *Synechocystis*. In its oxidized state, α - and β -bands are not detected (Fig. 6c). After reduction by addition of an excess of dithionite, α - and β -bands are detectable with maxima respectively at 559 nm and 527 nm; the Soret band is shifted to 425 nm (Fig. 6c). On the basis of 19 kDa as the molecular mass of the *Synechocystis* bacterioferritin subunit and using 24800 M⁻¹·cm⁻¹ as the molar absorption coefficient per haem group of the α -peak at 559 nm (Kurokawa *et al.*, 1989) we found a ratio of 0.25 mol of haem/mol of *Synechocystis* bacterioferritin subunit (Table 1).

Analysis of bacterioferritin from *Synechocystis* cultured under various iron concentration conditions

The function of bacterioferritin has not been studied *in vivo*; it is known to contain iron but its function in iron metabolism is

1	10	20	30	40	50	
MKGKPAVLAQLHKLRLGELAARDQYFTHSRMYQDWGLEKLYSRIDHEMQDET.HA	<i>Synechocystis</i>					
---DTK-INY-N---GN--V-IN---L-A--FKN---KR-NDVEY--SI--MK--	<i>E. coli</i>					
---D-K-IDY-N-A--H--T-IN--WL-Y-LLDN--IKD-AKKWRA-SIE	<i>N. winogradskyi</i>					
---DKI-IQH-N-I-GN--I-IN---L-A---E-----GKHEY--SI--MK--	<i>A. vinelandii</i>					

Fig. 5. Comparison of the *N*-terminal sequences of bacterioferritins from *Synechocystis* P.C.C. 6803, *E. coli*, *N. winogradskyi* and *A. vinelandii*

The *N*-terminal sequence of *Synechocystis* bacterioferritin was determined on h.p.l.c.-purified protein (fraction 4 in Fig. 3) by automatic Edman degradation with the use of an Applied Biosystems 477A apparatus with an on-line model 120 A phenylthiohydantoin analyser. A 1.5 nmol portion of polypeptide was sequenced with an average repetitive yield of 95.3%. The *E. coli* bacterioferritin sequence is from Andrews *et al.* (1989), the *N. winogradskyi* bacterioferritin sequence is from Kurokawa *et al.* (1989) and the *A. vinelandii* bacterioferritin sequence is from Andrews *et al.* (1991). Dashes (–) indicate identity of amino acid residues in the different sequences and the point (.) in the *Synechocystis* bacterioferritin sequence introduces a gap in order to align the following H and A residues.

Fig. 6. Absorption spectra of *Synechocystis* P.C.C. 6803 bacterioferritin

(a) Spectra of pea seed ferritin (P) and of *Synechocystis* P.C.C. 6803 bacterioferritin (S) in Tris/maleate buffer, pH 7.35. (b) Production of apo-bacterioferritin by reduction of non-haem iron in the presence of 100 mM-ascorbate; reduction is monitored kinetically by recording spectra each 6 min. (c) Spectra of apo-bacterioferritin in its oxidized state (O) and in its reduced state (R) after addition of a few milligrams of Na₂S₂O₄.

unknown. In order to investigate the fate of bacterioferritin *in vivo* we have cultured *Synechocystis* cells under different iron concentration conditions: 3 μ M-iron/citrate (iron-poor conditions), 30 μ M-iron/citrate (iron-sufficient conditions) and 300 μ M-iron/citrate (iron-overloading conditions). These three conditions do not introduce significant changes in the growth rate of the cells (Fig. 7a). This is not the case when iron is absent from the culture medium or when its concentration is higher than 300 μ M-iron/citrate; in the first case cells do not grow, and in the second case they die. The maximum iron concentration is found within the cells grown at 300 μ M-iron/citrate (Fig. 7b).

Synthesis of plant and animal ferritins is known to be induced

by iron when its intracellular concentration increases (Theil, 1987); in these eukaryotic organisms, iron loading leads to the synthesis of apo-ferritin and accumulation of iron-loaded ferritin. In cyanobacteria, recovery from iron starvation also induces synthesis of specific proteins (Guikema & Sherman, 1984). Indeed, when *Synechocystis*, after 48 h of preculture without iron, is inoculated for cultures at 3 μ M- and 30 μ M-iron/citrate, one can see in Fig. 8(a) that some polypeptides accumulate. However, none of these conditions induces accumulation of the bacterioferritin subunit as determined by immunoblots. Bacterioferritin is present even before iron/citrate addition, when cells have been starved for iron for 2 days (Fig. 8b, lane 0h). Therefore the bacterioferritin subunit has a constant concentration whatever the iron status of the cells. This observation raises the question of the function of bacterioferritin *in vivo*. By analogy with animals, ferritin is expected to behave as an iron-storage protein *in vivo*, but it could also be one of the electron carriers in the reverse electron-transport system from cytochrome *c*-522 to NADP⁺ (Kurokawa *et al.*, 1989). The possibility of finding haemobacterioferritin subunits associated with electron-transfer protein complexes other than its native multimeric structure has been tested. Native proteins were prepared from cultures as described in the Materials and methods section and separated on Ultrogel AcA 22 filtration gels. Each fraction was probed with antibodies raised against the bacterioferritin subunit from *Synechocystis*. A positive reaction was found only in the fractions of the elution profile (Fig. 1) known to contain the multimeric protein (results not shown).

In order to specify the function of *Synechocystis* bacterioferritin we have followed the fate of ⁵⁹Fe after its uptake and measured its distribution among different proteins including bacterioferritin. After labelling with ⁵⁹Fe *in vivo* cells were lysed in a non-denaturing medium. Proteins and electronegative compounds were then separated according to their size on non-denaturing polyacrylamide-gradient gel. A ⁵⁹Fe autoradiogram of gels from iron-poor, iron-sufficient and iron-overloaded *Synechocystis* cells labelled for 3, 6, 12 and 24 h is shown in Fig. 9. It is important to note that this experiment can be considered as a pulse-labelling since the bulk of radioiron (1.7 μ M-⁵⁹Fe as the only iron source during the labelling period) is completely absorbed by cells after 3 h (results not shown). ⁵⁹Fe is found in four types of associations: firstly, in the wells of the gel, where it probably corresponds to cellular debris and unlysed cells (results not shown); secondly, in bacterioferritin, and this is the first time that ⁵⁹Fe is shown to label this protein *in vivo* as well as *in vitro* [BFR in Figs. 9(a)(i) and (a)(ii)]; thirdly, with iron proteins of lower molecular mass than bacterioferritin [NBP in Fig. 9(a)(ii)]; fourthly in low-molecular-mass molecules that migrate at the front and have a molecular mass lower than 10 kDa [LMMM in Fig. 9(a)(ii)].

It is noteworthy that bacterioferritin iron is quickly and strongly labelled in iron-poor cells [Fig. 9(a)(ii)]. Quantification

Fig. 7. Effect of iron concentration on growth rate and iron loading of *Synechocystis* P.C.C. 6803

(a) Influence of iron concentration in the culture medium on *Synechocystis* growth rate. Colonies from Petri dishes were used to inoculate 3 ml cultures in Corning cell wells containing respectively 0 μM - (□), 3 μM - (◇), 30 μM - (▲), 300 μM - (△) and 1000 μM -iron (■) in BG-11 medium (Rippka *et al.*, 1979). Growth rates were estimated by measuring turbidity of the cell suspensions at different time with a Uniskan II spectrophotometer (Labsystems, Les Ullis, France) at 600 nm. Data presented correspond to the mean values of three independent experiments. (b) Influence of iron concentration in the culture medium on iron uptake and iron concentration in *Synechocystis* cells. Cells were grown as in (a) but for each iron concentration (0 μM -, 3 μM -, 10 μM -, 30 μM -, 300 μM - and 1000 μM -iron/citrate) in BG-11 medium $1.7 \mu\text{M}$ - ^{59}Fe ($1 \mu\text{Ci}$) was added. After 24 h (A_{600} 0.5) cells were pelleted, washed with 20 mM-KCl/5 mM-EDTA and re-pelleted in order to eliminate non-specific binding of iron/citrate on the cell surface. Radioactivities of pellets (▲) were counted directly in a Beckman scintillation counter, then cells were lysed, as described in the Materials and methods for protein preparation, and intracellular iron concentrations (△) were measured spectrophotometrically after reduction, with the use of Ferrozine as ferrous chelate. Data presented correspond to the mean values of three independent experiments.

Fig. 8. Effect of iron nutrition conditions on *Synechocystis* P.C.C. 6803 bacterioferritin subunit concentration

(a) Coomassie Blue R-250 staining of an SDS/15%-polyacrylamide gel loaded with total protein (15 μg in each lane) extracted from cultures grown for 2 days without iron (time 0 h) and then supplemented with 3 μM - and 30 μM -iron/citrate for 2, 4, 8, 12 and 24 h; M is for molecular-mass markers; stars (★) show polypeptides that accumulate during recovery from iron starvation (Guikema & Sherman, 1984). (b) Immunoblot of proteins (15 μg in each lane) that were electrotransferred on to nitrocellulose after electrophoresis and probed with *Synechocystis* bacterioferritin antibodies diluted at 1:1200. The second antibody was sheep anti-(rabbit IgG) immunoglobulins (diluted at 1:600) coupled with peroxidase. Proteins were extracted from cultures grown for 2 days without iron (0 h) and the supplemented with 3 μM -, 30 μM - and 300 μM -iron/citrate for 6, 12 and 24 h; lanes 1 and 2 were loaded respectively with 15 and 30 ng of purified *Synechocystis* bacterioferritin.

of total iron and radioiron throughout this polyacrylamide gel allows the calculation of specific radioactivities of two of the different associations mentioned above, namely BFR and LMMM in Fig. 9(b). In iron-poor cells the highest measurable specific radioactivity is found in bacterioferritin iron, with a maximum at 6 h followed by a decrease; in contrast, the specific radioactivity of iron in the low-molecular-mass molecules increases constantly suggesting a transfer of iron from bacterioferritins. In overloaded cells, ferritin iron represents a very small proportion of the total and labelled iron content. Bacterioferritins are important intermediates in the pathway of iron in deficient cells, and almost not involved in this pathway in case of iron overload. This is the opposite of what would be expected from iron-loading experiments in animals. Iron in non-bacterioferritin proteins [NBP in Fig. 9(a)(ii)] is progressively

labelled in iron-poor cells. The label is evenly distributed with the same kinetics. In contrast, non-ferritin proteins are not labelled in overload conditions.

Low-molecular-mass molecules (LMMM in Fig. 9) contain the bulk of cellular iron and the bulk of radioiron in the case of overload. Saturable low-molecular-mass molecules appear to be the sink in case of iron excess.

DISCUSSION

In prokaryotes, the intracellular concentration of iron is mainly controlled at the uptake level by highly specific genetic systems that have been extensively studied (Bagg & Neilands, 1987). In contrast, the different forms and content of iron in bacteria are not very extensively documented. Among these forms, ferritin-

Fig. 9. Distribution *in vivo* of iron within different molecules according to the iron status of *Synechocystis* P.C.C. 6803 cells

(a)(i) Autoradiogram of a 5–20% polyacrylamide-gradient gel loaded with 4 μg of pure *Synechocystis* bacterioferritin that had been labelled *in vitro* for 3 h at room temperature with 35 μM - ^{59}Fe /700 μM -citrate in the presence of 3 mM-ascorbate (lanes BFR) and loaded with non-denatured extracts containing 20 μg of protein prepared as described in the Materials and methods section from cultures grown for 2 days without iron and then supplemented for 3 days with 30 μM -iron/citrate. After this time, cells were centrifuged and resuspended in a culture medium containing 1.7 μM - ^{59}Fe (1 μCi) for 3, 6, 12 and 24 h as the only iron source; note the labelling *in vivo* of bacterioferritin by ^{59}Fe . (a)(ii) Autoradiogram of a 5–20% polyacrylamide-gradient gel loaded with non-denatured extracts containing 20 μg of protein prepared as described in the Materials and methods section from cultures grown for 2 days without iron and then supplemented for 3 days with 3 μM -, 30 μM - and 300 μM -iron/citrate. After this time, cells were centrifuged and resuspended in a culture medium containing 1.7 μM - ^{59}Fe (1 μCi) for 3, 6, 12 and 24 h as the only iron source. BFR, NBP and LMMM are respectively for bacterioferritin, non-bacterioferritin proteins and low-molecular-mass molecules. Before the preparation of the crude extracts cells were pelleted and washed with 20 mM-KCl/5 mM-EDTA until no radioactivity was detected in the supernatant. (b) Comparison of iron specific radioactivity associated with bacterioferritin (■) and with low-molecular-mass molecules (□) after 3, 6, 12 and 24 h of growth in the presence of 3 μM -, 30 μM - and 300 μM -iron. After autoradiography, slices of gel corresponding to bacterioferritin and low-molecular-mass molecules were cut out and incubated for 48 h at room temperature in 200 μl of 0.75 M-sodium acetate buffer, pH 4.8, containing 10% thioglycolic acid and 0.1% Ferrozine. Radioactivity of samples was measured with the use of the Ready Safe liquid-scintillation cocktail from Beckman. Iron concentration was determined spectrophotometrically at 562 nm with a Uniskan II spectrophotometer.

like molecules have been purified from *A. vinelandii* (Stiefel & Watt, 1979) and *E. coli* (Yariv *et al.*, 1981) as well as from *Pseudomonas aeruginosa* (Moore *et al.*, 1986), *Azotobacter chroococcum* (Chen & Crichton, 1982) and *N. winogradskyi* (Kurokawa *et al.*, 1989). They have an electron-dense iron-containing core with a protein coat that resembles mammalian ferritin by its predicted secondary structure (Andrews *et al.*, 1989) and molecular symmetry (Smith *et al.*, 1989; Harrison *et al.*, 1989). However, they differ from their eukaryote analogues in three respects. Firstly, the Fe/P_i ratio of their mineral core is much higher (1:1 to 1:2) than that of animal ferritins (1:10 to 1:20) (Mann *et al.*, 1987). Secondly, they contain haem residues with properties similar to those in *b*-type cytochromes. Thirdly, the only bacterioferritin complete sequence reported so far

diverges completely from the primary structure of known eukaryotic ferritin sequences (Andrews *et al.*, 1989). The occurrence of ferritin in cyanobacteria has not been reported hitherto, although plant plastids contain ferritin with some sequence similarity to animal ferritins (Ragland *et al.*, 1990).

By using a combination of centrifugation through dense solutions of caesium trifluoroacetate and of gel filtration, we have purified the bacterioferritin from *Synechocystis* P.C.C. 6803. It is important to note that we have successfully purified maize and pea seed ferritins by using this method (J.-P. Laulhère, O. Massenet & J.-F. Briat, unpublished work). The bacterioferritin from *Synechocystis* P.C.C. 6803 has the same spectrophotometric characteristics as other bacterioferritins analysed so far (Kurokawa *et al.*, 1989; Stiefel & Watt, 1979; Yariv *et al.*,

1981; Moore *et al.*, 1986; Chen & Crichton, 1982), its Fe/P_i ratio is 1.53 (Table 1) and fits well the Fe/P_i ratios of other bacterioferritins (Mann *et al.*, 1987) and its N-terminal sequence shows strong similarity to those bacterioferritins for which sequence data have been reported (Kurokawa *et al.*, 1989; Andrews *et al.*, 1989). This latter observation proves that plant ferritins, which are located in plastids and have a high degree of sequence similarity to animal ferritins (Ragland *et al.*, 1990), have a eukaryotic ancestor and do not derive from cyanobacterial bacterioferritin.

Bacterioferritin from *Synechocystis* P.C.C. 6803 shares with all known ferritins certain important functional features: it is capable of iron uptake *in vivo* as well as *in vitro*, it has a high iron content per molecule, and it is found fully assembled *in vivo*. Nevertheless, a major difference between plant and animal ferritins and the bacterioferritin from *Synechocystis* P.C.C. 6803 concerns the regulation of its synthesis, since its subunit concentration is constant whatever the iron status of the cells (Fig. 8). In other words, *Synechocystis* bacterioferritin does not accumulate in response to iron as do the ferritins of eukaryotes (Theil, 1987). This conclusion contrasts with the report by Yariv *et al.* (1981), who were not able to isolate bacterioferritin from *E. coli* cells grown in iron-poor medium. However, their isolation procedure was also based on centrifugation through high-density gradients. Therefore it is conceivable that only iron-rich bacterioferritins are purified by this method; indeed, Reid *et al.* (1990) have shown by Mössbauer spectroscopy that growth conditions can affect the form of the iron cores from *Ps. aeruginosa* bacterioferritin. Only the use of an antibody could address the question of bacterioferritin subunit concentrations under different growth conditions.

The position of bacterioferritin in the metabolic pathway of iron as revealed by labelling experiments *in vivo* also deserves discussion. Under conditions of iron deficiency, after 2 days of iron starvation followed by 3 days of iron nutrition at only 3 µM, newly imported iron is distributed between *Synechocystis* bacterioferritin and low-molecular-mass molecules, and bacterioferritin appears to load radioiron faster than low-molecular-mass molecules (see iron specific radioactivities at 3 µM in Fig. 9b); this situation is reversed after 6 h. At least under these conditions, *Synechocystis* bacterioferritin acts as a temporary depository for iron, playing a role as a buffering system. In case of iron overload, low-molecular-mass molecules carry the bulk of the cellular iron content, and fulfil the storage function. Therefore it is unlikely that bacterioferritin plays the major role in iron storage, in the strict sense, in *Synechocystis*. This conclusion is in perfect agreement with the report by Bauminger *et al.* (1980) and more recently that by Matzanke *et al.* (1989). These authors have shown that in *E. coli* bacterioferritin iron represents no more than 1% of the total cellular iron and that the bulk of iron in the iron-rich cells is in the form of aggregates (Bauminger *et al.*, 1980), which could be associated with a novel form of iron-protein (Matzanke *et al.*, 1989). At this point we do not know if some proteins are involved in the pool of low-molecular-mass molecules that we have observed in *Synechocystis*; nevertheless these low-molecular-mass molecules have a molecular mass lower than 10 kDa. We could hypothesize that the abundance of saturable low-molecular-mass molecules, which could vary according to the cellular iron content (Bauminger *et al.*, 1980), may control the fate of newly imported iron. In case of iron overload, the small-molecules storage pool would take care of excess iron and bacterioferritin would be little involved. On the other hand, in case of iron deficiency, a rapid overflow of iron from the saturable low-molecular-mass molecules is conceivable and bacterioferritin would buffer this release of iron before its use for metabolic requirements. In this

context, it may be noted that in *E. coli* under normal iron concentrations (i.e. not iron-rich conditions) non-bacterioferritin iron proteins are detected by Mössbauer spectroscopy in addition to bacterioferritin and iron aggregates (Bauminger *et al.*, 1980), as we observed under poor and normal iron concentration conditions in *Synechocystis* cells [see NBP in Fig. 9(a)(ii)].

From an evolutionary point of view, our results raise two questions: (i) why did bacterioferritin disappear from plastids after the endosymbiont event by which cyanobacteria invaded eukaryotic cells to give chloroplasts (Weeden, 1981), and (ii) why has it been replaced by its eukaryotic counterpart in a prokaryotic type organelle? The answer may be related to the difference of cytological compartment in which ferritins are found. In cyanobacteria they are located in the same place as low-molecular-mass molecules that assume the storage function (Fig. 9), and the regulation of their synthesis in response to iron is not necessary (Fig. 8). In plant cells, iron transporters are low-molecular-mass molecules such as citric acid and other organic acids and are mainly stored within vacuoles that could be the main iron storage site in plant cells in case of iron loading (Lescure *et al.*, 1990); in contrast, ferritins are found in chloroplasts and other plastids, where they assume also an iron-storage function, which has to be related to the fact that plastids must be protected against iron overflow because of the abundance in these organelles of O₂ and reducers able to induce Fenton reactions (Laulhère *et al.*, 1989, 1990). Plant ferritins are regulated at the transcriptional level by iron (Proudhon *et al.*, 1989; Lescure *et al.*, 1991). Replacement of a prokaryotic gene that is not regulated by iron by a eukaryotic gene whose expression is regulated by iron might have had an economical and functional advantage for plants. In animals, ferritins are located in the cytoplasm and among their different functions they detoxify iron for the whole cell and not only for a subcellular compartment as in plants; it could explain why the control of ferritin synthesis in response to iron in animals occurs mainly at the translational level by recruiting pre-existing ferritin mRNAs (Theil, 1987), leading to a quicker response.

We thank Professor P. Harrison and Dr. S. Andrews for critical reading of this manuscript and for helpful advice. The help of S. Lobreaux for computer drawing of some Figures is greatly appreciated.

REFERENCES

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Andrews, S. C., Smith, J. M. A., Guest, J. R. & Harrison, P. M. (1989) *Biochem. Biophys. Res. Commun.* **158**, 489–496
- Andrews, S. C., Findlay, J. B. C., Guest, J. R., Harrison, P. M., Keen, J. N. & Smith, J. M. A. (1991) *Biochim. Biophys. Acta*, in the press
- Bagg, A. & Neilands, J. B. (1987) *Microbiol. Rev.* **51**, 509–518
- Bauminger, E. R., Cohen, S. G., Dickson, D. P. E., Levy, A., Ofer, S. & Yariv, J. (1980) *Biochim. Biophys. Acta* **623**, 237–242
- Briat, J. F., Massenet, O. & Laulhère, J.-P. (1989) *J. Biol. Chem.* **264**, 11550–11553
- Chen, M. & Crichton, R. R. (1982) *Biochim. Biophys. Acta* **707**, 1–6
- Crichton, R. R., Ponce-Ortiz, Y., Koch, M. H. J., Parfait, R. & Stuhmann, H. B. (1978) *Biochem. J.* **171**, 349–356
- Feng, D. H., Jonhson, H. S. & Doolittle, R. F. (1985) *J. Mol. Evol.* **21**, 112–125
- Guikema, J. A. & Sherman, L. A. (1984) *Plant Physiol.* **74**, 90–95
- Harrison, P. M., Artymuk, P. J., Ford, G. C., Lawson, D. M., Smith, J. M. A., Treffry, A. & White, J. L. (1989) in *Biomineralization: Chemical and Biochemical Perspectives* (Mann, S., Webb, J. & Williams, R. J. P., eds.), pp. 257–294, VCH, Weinheim
- Kurokawa, T., Fukumori, Y. & Yamanaka, T. (1989) *Biochim. Biophys. Acta* **976**, 135–139
- Laudenbach, D. E. & Straus, N. A. (1988) *J. Bacteriol.* **170**, 5018–5026

- Laudenbach, D. E., Reith, M. E. & Straus, N. A. (1988) *J. Bacteriol.* **170**, 258–265
- Laulhère, J.-P. & Mache, R. (1978) *Physiol. Vég.* **16**, 643–656
- Laulhère, J.-P., Lescure, A. M. & Briat, J. F. (1988) *J. Biol. Chem.* **263**, 10289–10294
- Laulhère, J.-P., Labouré, A. M. & Briat, J. F. (1989) *J. Biol. Chem.* **264**, 3629–3635
- Laulhère, J.-P., Labouré, A. M. & Briat, J. F. (1990) *Biochem. J.* **269**, 79–84
- Lescure, A. M., Massenet, O. & Briat, J. F. (1990) *Biochem. J.* **272**, 147–150
- Lescure, A. M., Proudhon, D., Pesey, H., Ragland, M., Theil, E. C. & Briat, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8222–8226
- Lobreaux, S. & Briat, J. F. (1991) *Biochem. J.* **274**, 601–606
- Mann, S., Williams, J. M., Treffry, A. & Harrison, P. M. (1987) *J. Mol. Biol.* **198**, 405–416
- Matzanke, B. F., Müller, G. I., Bill, E. & Trautwein, A. X. (1989) *Eur. J. Biochem.* **183**, 371–379
- Moore, G. R., Mann, S. & Bannister, J. V. (1986) *J. Inorg. Chem.* **28**, 329–336
- Proudhon, D., Briat, J. F. & Lescure, A. M. (1989) *Plant Physiol.* **90**, 586–590
- Ragland, M., Briat, J. F., Gagnon, J., Laulhère, J.-P., Massenet, O. & Theil, E. C. (1990) *J. Biol. Chem.* **265**, 18339–18344
- Reid, N. M. K., Dickson, D. P. E., Greenwood, C., Thompson, A., Kadir, F. H. A. & Moore, G. R. (1990) *Biochem. J.* **272**, 263–264
- Riethman, H. C. & Sherman, L. A. (1988) *Biochim. Biophys. Acta* **935**, 141–151
- Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979) *J. Gen. Microbiol.* **111**, 1–61
- Szcekan, S. R. & Joshi, J. G. (1987) *J. Biol. Chem.* **262**, 13780–13788
- Seckbach, S. (1982) *J. Plant. Nutr.* **5**, 369–394
- Smith, J. M. A., Quirk, A. V., Plank, R. W. H., Diffin, F. M., Ford, G. C. & Harrison, P. M. (1988) *Biochem. J.* **255**, 737–740
- Smith, J. M. A., Ford, G. C., Harrison, P. M., Yariv, J. & Kalb, A. J. (1989) *J. Mol. Biol.* **205**, 465–467
- Spiller, S. C., Kaufman, L. S., Thompson, W. F. & Briggs, W. R. (1987) *Plant Physiol.* **84**, 409–414
- Stiefel, E. I. & Watt, G. D. (1979) *Nature (London)* **279**, 81–83
- Theil, E. C. (1987) *Annu. Rev. Biochem.* **56**, 289–315
- van der Marck, F., de Lange, T. & Bienfait, H. F. (1981) *Planta* **153**, 338–342
- Weeden, N. F. (1981) *J. Mol. Evol.* **17**, 133–139
- Yariv, J., Kalb, A. J., Sperling, R., Bauminger, E. R., Cohen, S. G. & Ofer, S. (1981) *Biochem. J.* **197**, 171–175
- Zurawski, G. & Clegg, M. T. (1987) *Annu. Rev. Plant Physiol.* **38**, 391–418

Received 22 April 1991/21 June 1991; accepted 3 July 1991