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Evidence that electron-dense bodies in *Cyanidium caldarium* have an iron-storage role

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Abstract

The acidophilic and thermophilic unicellular red alga, *Cyanidium caldarium* (Tilden) Geitler, is widely distributed in acidic hot springs. Observation by transmission electron microscopy (TEM) showed that algae grown in Allen's medium contained electron-dense bodies with diameters from 100 to 200 nm. Electron dispersive x-ray analysis indicated that the electron-dense bodies contained high levels of iron, phosphorous, and oxygen; P/Fe ratios were from 1.3 to 2.0. The electron spin resonance (ESR) spectrum of the intact *C. caldarium* cells showed an isotropic signal at a g value of 2.00. Density-gradient centrifugation of the cell lysate yielded a fraction that included substances showing the isotropic ESR signal. EDTA treatment of this fraction reduced the ESR signal intensity, whereas it increased a signal that is typical of Fe(III)-EDTA. The fact that the isotropic signal dominates the ESR spectrum, together with a previous finding that iron is confined to the electron-dense bodies, led us to conclude that iron in the electron-dense bodies accounts for the isotropic ESR signal. Since the intensity of the ESR signal depends on the amount of iron in the cells, the electron-dense bodies are probably iron storage sites.

Abbreviations: EDX – energy dispersive x-ray; ESR – electron spin resonance; TEM – transmission electron microscope.

Introduction

Cyanidium caldarium is an acidophilic and thermophilic unicellular alga that is widely distributed in acidic hot springs. Because this alga has one nucleus, one mitochondrion, and one chloroplast, it is considered an ancestor of eukaryotes (Seckbach 1994). Ultrastructural studies of algal cells indicate the presence of electron-dense bodies in the cytoplasm near the nucleus (Nagashima & Fukuda 1981). Electron dispersive x-ray (EDX) analysis of the electron-dense bodies indicates high levels of Fe, P, and O (Nagasaka *et al.* 2002). In addition, elemental mapping of *C. caldarium* cells demonstrates that these elements are confined exclusively to the electron-dense bodies (Nagasaka *et al.* 2002).

Some cellular components, such as ferritin, siderosome, and globoid, exhibit high electron density when observed with an electron microscope (Buttrose 1978; Seckbach 1982; Lu *et al.* 1995). All of these components concentrate metals and play a role in their storage and homeostasis. Ferritin is the major iron storage system in animals, plants, and bacteria (Theil 1994). Ferritin synthesis is regulated at the transcriptional level in plants (van der Mark 1983) but at the translational level in animals: synthesis is me-

diated by a system of iron-responsive elements and iron-responsive protein (Klausner *et al.* 1993; Theil 1994).

The high iron levels of the electron-dense bodies of *C. caldarium* strongly suggest their implication in iron homeostasis. In this study, the electron-dense bodies were analyzed by both EDX and ESR spectrometry. We present evidence that clearly supports the hypothesis that the bodies store iron.

Materials and methods

Culture conditions of C. caldarium

C. caldarium strain R-11 was obtained from the Institute of Molecular and Cellular Biology, the University of Tokyo, and was cultured autotrophically in Allen's medium (Allen 1959) containing 71 μM Fe. The pH of the medium was adjusted to 2.0 with 0.5 M H_2SO_4. The algae were grown in shaking flasks at 50 °C under continuous light (36 μmol photons m^{-2} s^{-1}) for three weeks, and were then analyzed by EDX. In preparation for ESR spectrometry, the algal cells were precultured in Allen's medium for two weeks, inoculated to Allen's medium or a modified Allen's medium containing 0, 7.1, or 710 μM Fe, and cultured for two more weeks.

EDX analysis

The algal cells were frozen using a high-pressure freezing apparatus (HPM 010, BAL-TEC, Liechtenstein). For the ultrastructural study, the frozen cells were incubated at −80 °C with anhydrous acetone containing 4% (W/V) osmium tetroxide to displace water. The cells were embedded in a mixture of Epon 812 and Araldite resins (TAAB, Berkshire, England). Ultra-thin sections were double-stained with 3% uranyl acetate and lead citrate and were then observed with an electron microscope (JEOL 1010, Tokyo, Japan). For EDX analysis, the frozen cells were incubated at −80 °C with anhydrous acetone alone to displace water. The cells were embedded in the two resins without being stained. Ultra-thin sections were analyzed using an electron microscope with an x-ray detector (JEOL 2010-FX, Tokyo, Japan). The relative abundances of elements were calculated using the SMTF computer program (JEOL, Tokyo, Japan).

Density gradient centrifugation

The *C. caldarium* cells cultured in Allen's medium were washed with diluted H_2SO_4 (pH 2.0) and resuspended in buffer A (50 mM HEPES, protease inhibitor mix [complete^{TM}-EDTA free (Sigma, St Louis, MO, USA), 1 tablet/ 50 ml] [pH 7.0]). The cells were disrupted by vortexing with glass beads and the cell debris was removed by centrifugation at 2500 rpm for 5 min. The supernatant (20 ml) was added to 20 ml Percoll solution (80% Percoll, 50 mM HEPES [pH 7.0]) in a 50-ml centrifugation tube. After centrifugation at 8000 rpm for 20 min at 4 °C, the pellet (0.5 ml) was collected. Half of the pellet was resuspended in buffer A and the ESR spectrum was measured. The other half was resuspended in 100 mM EDTA in buffer A, left for 2 h at 25 °C, and then the ESR spectrum was measured.

ESR spectrum study

ESR spectra were recorded on a JSE-FA 100 spectrometer (JEOL, Tokyo, Japan). The algal cells grown in Allen's media with varying iron concentrations were washed three times with diluted H_2SO_4 (pH 2.0). The cells were then harvested by centrifugation at 2500 rpm for 5 min. After the cell pellet had been resuspended in 1 ml diluted H_2SO_4, 0.5 ml of the suspension was put into quartz ESR tubes (5 × 270 mm). The suspension was then frozen in liquid nitrogen, and ESR spectra were measured. The ESR spectra for Fe(III)-EDTA, $FePO_4$, Fe(III)-polyphosphate (Fe-polyP), and ferritin were also measured, at 77 K. 50 mM EDTA (pH 8.0) and 10 mM $FeCl_3$ were mixed and diluted by deionized H_2O to a final concentration of 1 mM and 0.5 mM, respectively. 0.5 ml of the solution was placed into an ESR tube for ESR measurement. Solid $FePO_4$ was mixed with a 100-fold amount of glucose and ground to a fine powder with a mortar and pestle. Precipitate of Fe-polyP was obtained by centrifuging a mixture of 5 ml $FeCl_3$ (1 mM) and 5 ml polyphosphate (polyP) solution containing 10 mM P (Wako, Osaka, Japan) at 10,000 rpm for 5 min. and was resuspended in 0.5 ml deionized H_2O. The suspension was placed into an ESR tube for ESR measurement, along with an aliquot of 0.3 ml horse spleen ferritin (Sigma, St Louis, MO, USA) containing 30 mg apoprotein.

All spectra were recorded at the following instrumental settings: frequency 9.13 GHz; modulation frequency 100 kHz; microwave power 1 mW; ampli-

tude 100; time constant 1 second; scan time 4 min; scan range 400 mT.

Determination of iron concentration in C. caldarium *cells*

The cell suspension (0.5 ml) prepared for the ESR spectrum measurement was dried, weighed, and digested with concentrated HNO_3 at 150 °C for 5 h. Iron concentration was determined by inductive coupled plasma emission spectrometry using a spectrometer (Seiko SPS1200 VA, Tokyo, Japan).

Results

Figure 1A shows a TEM image of a *C. caldarium* cell, in which the nucleus, mitochondrion, and chloroplast are identified. In addition to these organelles, an electron-dense body can be seen in the cytoplasm near the nucleus. Almost all cells contained one or two electron-dense bodies with diameters from 100 to 200 nm. Bodies were also observed in unstained specimens (Figure 1B & C). Three endospores were observed in another specimen, which indicates that the second endospore division had already occurred; there was at least one electron-dense body in each endospore (Figure 1C).

Table 1 summarizes the molar percentage of elements in the *C. caldarium* electron-dense bodies. Almost all the elements found were Fe, P, and O, which had near-constant levels. The P/Fe ratio was 1.3 to 2.0. Low levels of K were also detected in all the electron-dense bodies; some bodies also contained low levels of S, Mg, or Zn.

The ESR spectrum of intact *C. caldarium* cells cultured in medium containing 71 μM Fe is shown in Figure 2A. The sharp signal at a g value of 2.00 is attributable to organic radicals; the one at a g value of 4.15 could be caused by high-spin ferric iron in a tetragonal symmetry of the crystal field. Six signals that appeared at a magnetic field from 301.89 to 349.37 mT (shown by arrowheads) are characteristic of Mn(II) ions. In addition to these signals, a broad isotropic signal was observed at a g value of 2.00. Density-gradient centrifugation yielded a precipitate that showed only a single ESR signal at a g value of 2.00 (Figure 2B). This spectrum is almost identical to the broad isotropic signal observed for intact *C. caldarium* cells. Adding EDTA to the precipitate depressed the broad isotropic signal and revealed a

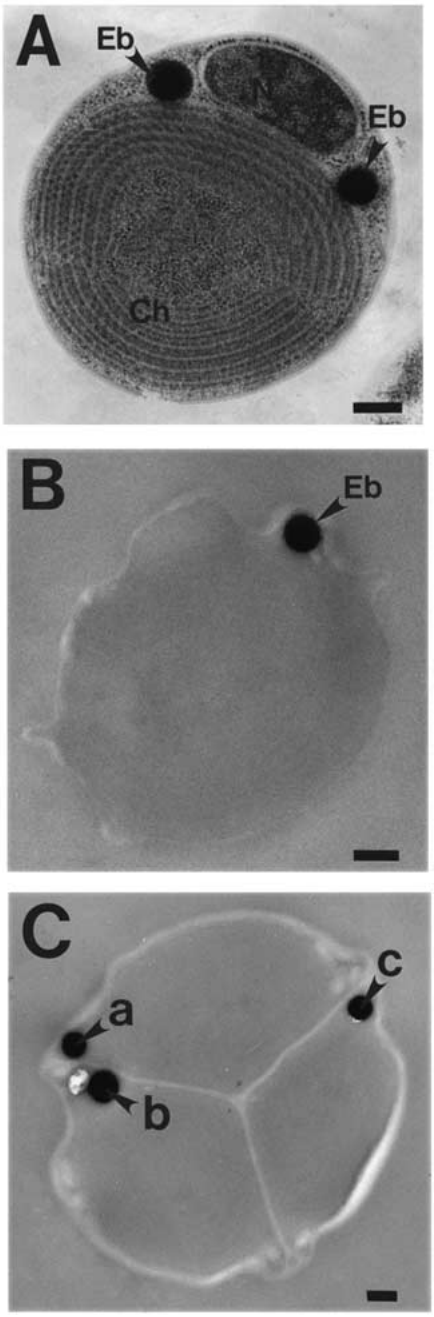

Fig. 1. Electron micrographs of ultra-thin sections of a *C. caldarium* cell with or without staining. A: TEM image of a stained *C. caldarium* cell. A mature *C. caldarium* cell has one nucleus (N), one mitochondrion, and one well-developed chloroplast (Ch). Two electron-dense bodies (Eb) are visible in the cytoplasm near the nucleus. B: TEM image of a non-stained *C. caldarium* cell. The electron-dense body (Eb) has a higher electron density than the other cell components. C: TEM image of *C. caldarium* cells containing three endospores. All endospores contained electron-dense bodies. Sites of EDX analysis are indicated by arrowheads (a–c). Scale bars represent 200 nm.

Table 1. Elemental components of electron-dense bodies.

Specimen No.[a]	Mole percentage							
	O	P	Fe	K	S	Mg	Zn	P/Fe[b]
1	68.7	16.8	12.6	1.1	0.8	–	–	1.3
2	67.2	17.4	12.2	0.8	2.4	–	–	1.4
3-a	68.0	18.6	10.9	1.2	1.3	–	–	1.7
3-b	72.9	14.1	10.7	1.1	1.2	–	–	1.3
4-a	62.3	21.7	10.9	2.1	–[c]	2.1	0.9	2.0
4-b	65.2	20.7	11.8	2.3	–	–	–	1.8
4-c	61.1	21.8	11.7	2.4	–	3.0	–	1.9

[a]Specimens 1 and 2 contained a single electron-dense body; specimen 3 contained two bodies. Specimen 4 consisted of cells just after the second endospore division; each endospore contained a single electron-dense body.
[b] Ratio of P to Fe.
[c]No peak was detected in the EDX spectrum.

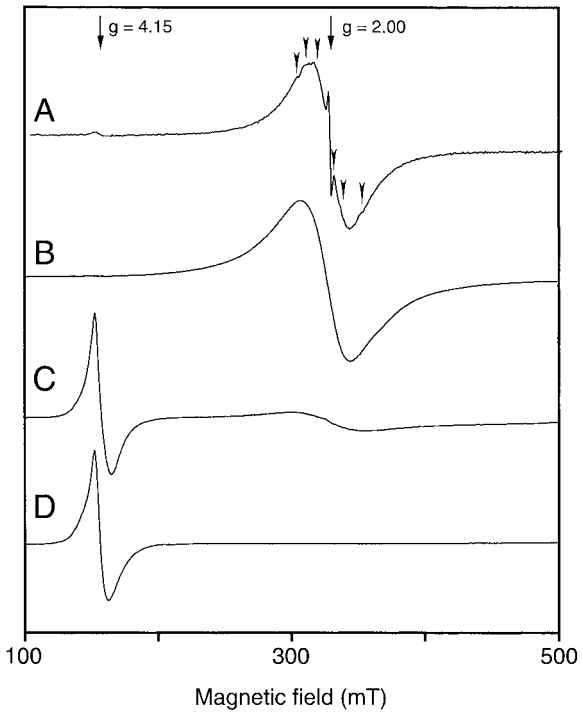

Fig. 2. ESR spectra of the intact *C. caldarium* cells cultured in Allen's medium (A) and the precipitate of density-gradient centrifugation (B). In ESR spectra of the intact cell, six signals that were characteristic of Mn(II) ion were observed (arrowhead). The precipitate was treated for 2 hours with 100 mM EDTA and then the ESR spectrum was measured (C). The spectrum for Fe(III)-EDTA solution is shown for reference (D).

signal due to the Fe(III)-EDTA complex at g = 4.15 (Figures 2B, C & D). These results suggest that the broad isotropic signal is derived from Fe(III) ions in *C. caldarium* cells.

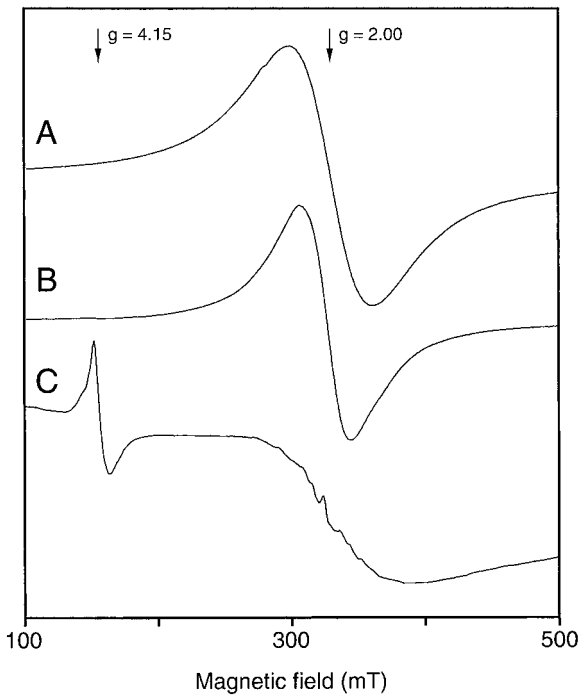

Fig. 3. ESR spectra of substrates containing iron and phosphorous. A: Fe-polyP; B: $FePO_4$; C: horse spleen ferritin.

The ESR spectra were measured for Fe-polyP precipitate (Figure 3A), $FePO_4$ (Figure 3B), and ferritin (Figure 3C). The spectra of the Fe-polyP precipitate and $FePO_4$ demonstrated a broad isotropic signal at a g value of 2.00, which, except for the width of the line, closely resembled the spectrum of the precipitate from the density-gradient centrifugation (Figure 2B). These spectra contrasted, however, with the ferritin spectrum, which exhibited a much broader signal that was

Table 2. Iron concentration and isotropic signal intensity of *C. caldarium* cells cultured with varying concentrations of iron.

Iron concentration in medium (μM)[a]	Cellular iron concentration (μg/mg cell dry weight)	Amount of iron used for ESR measurement (μg)	Signal area of the broad isotropic signal[b]
0	0.11	1.74	–[c]
7.1	0.15	3.43	–
71	0.96	15.5	100
710	1.79	36.6	200

[a]All media were Allen's medium that varied only in iron concentration.
[b]Signal area relative to that of the spectrum for the cells cultured in Allen's medium.
[c]The isotropic signal was low in intensity and could not be distinguished from the other signals.

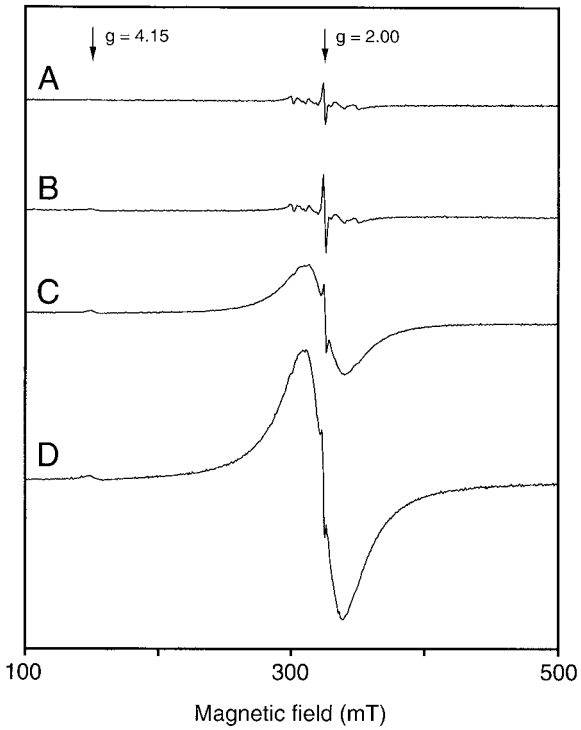

Fig. 4. ESR spectra of *C. caldarium* cells cultured in Allen's medium (C, 71 μM Fe) or modified Allen's medium (A, 0 μM Fe; B, 7.1 μM Fe; D, 710 μM Fe).

centered at g = 2.00 and peaked sharply at g = 4.15. The ratio of one-half the width of the integrated spectrum to the peak-to-peak width of the spectrum of precipitate from the density-gradient centrifugation (Figure 2B) was 1.71 for the broad isotropic peak; this value is close to 1.732, the representative value of a Lorentzian line (Weil *et al.* 1994)

Figure 4 shows the ESR spectra of the *C. caldarium* cells cultured in Allen's media of varying iron concentration. The intensity of the broad isotropic signal at g = 2.00 increased with iron concentration in the media, whereas the signal from organic radicals was constant. The signal of high-spin Fe(III) at g = 4.15 increased slightly with iron concentration. The iron concentration in the algal cells, the amount of iron used for ESR measurement, and the signal area of the broad isotropic peak are shown in Table 2. The iron concentration in the algal cells increased with the concentration of iron in the medium. Also, the signal area of the broad isotropic signal paralleled the amount of iron used for ESR measurement.

Discussion

Iron is an essential micronutrient for living organisms, including algae, which is involved in many important cellular processes. However, excess free iron ions in the cytoplasm are potentially toxic, mainly because the ions produce reactive hydroxyl radicals via the Fenton reaction. Hence, free iron in cytoplasm should be kept at a minimum.

C. caldarium cells contained electron-dense bodies that consisted mainly of Fe, P, and O (Table 1). This peculiar elemental composition resembles that of ferritin, which is the most common iron storage system of organisms. When examined using electron microscopy, animal ferritin and bacterial ferritin (bacterioferritin) exhibit a high electron density, which is derived from an iron core containing Fe, P, and O. For example, the iron core in rat liver ferritin is comprised of ferrihydrate and phosphate (Mann *et al.*1986). Although ferritins have a P/Fe molar ratio that is specific to organisms and also to organelles in the same organism, the ratio is maintained between 0.1 and 1 (Mann *et al.* 1986; Watt *et al.* 1986; Treffry *et al.* 1987; Wade *et al.* 1993). In contrast, the P/Fe ratio in electron-dense bodies of *C. caldarium* was greater, ranging

from 1.3 to 2.0; this range of values differs from any reported ferritin iron core P/Fe ratio.

The accumulation of Fe, P, and O is confined to electron-dense bodies (Nagasaka *et al.* 2002). Our ESR study identified two Fe(III) species in the cells: the broad isotropic peak was a major component and the high-spin Fe(III) ions were a minor component. These results show that the Fe(III) ions in the electron-dense bodies are responsible for the isotropic ESR signal. The Lorentzian-line shape of the signal indicates that Fe(III) ions reside near one another to facilitate the exchange of electrons between them. Electron diffraction analysis indicates that the electron-dense bodies have no crystal structure (data not shown) and are thus amorphous. The ESR spectra of $FePO_4$ and the Fe-polyP precipitate resemble the spectrum of the electron-dense bodies. These results suggest that iron ions in an electron-dense body bind to orthophosphate or a phosphate moiety of polyphosphates or organic phosphates. Binding of polyP with various metal ions is common. In many organisms, polyP is found in polyP granules and it chelates with divalent cations, mainly Mg^{2+} and Ca^{2+} (Archibald & Fridovich 1982; van Veen *et al.* 1993); this polyP acts as a Ca^{2+} sink in yeast (Dunn *et al.* 1994). Polyphosphate also binds heavy metals such as Ni and Mn (Archibald & Fridovich 1982; Gonzalez & Jensen 1998), although polyphosphate is not known to store iron.

The intensity of the ESR isotropic signal measured in this study paralleled the iron content of the algal cells (Table 2). Thus, the synthesis of electron-dense bodies is probably regulated by the amount of iron in a cell (Figure 3). The intensity of the isotropic signal also decreased after culturing in medium without iron, demonstrating that iron could be released from the electron-dense bodies for intracellular use. The minimal change in signal intensity of high-spin Fe(III) reveals that the amount of iron used inside the cell was virtually constant. Thus, the electron-dense bodies may be important for iron homeostasis in *C. caldarium* cells and may play a major role in storage of iron.

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