Fe₃O₄ PRECIPITATION IN MAGNETOTACTIC BACTERIA

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Using Mössbauer resonance spectroscopy of ⁵⁷Fe, we have determined the nature and distribution of major iron compounds in the magnetotactic bacterium *Aquaspirillum magnetotacticum*. In addition to magnetite (Fe₃O₄), cells contained a low-density hydrous ferric oxide, a high-density hydrous ferric oxide (ferrihydrite), and ferrous iron. Analysis at different temperatures of whole cells harvested early and late in growth, of mutant cells unable to synthesize magnetite, and of cell fractions enriched in ⁵⁷Fe indicated that Fe₃O₄ precipitation resulted from partial reduction of the high-density hydrous ferric oxide precursor.

Introduction

Bacteria that orient and swim along geomagnetic field lines are cosmopolitan in freshwater and marine sediments [1–7]. All species of these magnetotactic bacteria examined by electron microscopy have been found to contain magnetosomes, which are intracellular, enveloped, electron-opaque particles [8]. The magnetosomes of two very different types of magnetotactic bacterium [9,10] have been shown to consist of magnetite (Fe₃O₄). Those of the freshwater magnetotactic spirillum, *Aquaspirillum magnetotacticum* [11] are cuboidal, 40–50 nm in width, and are arranged in a chain that longitudinally traverses the cell (Fig. 1) in close proximity to the inner surface of the cytoplasmic membrane [8]. The number of magnetosomes in the chain is variable, depending upon the culture conditions, but typically averages 20. Magnetosomes are enveloped by electron-transparent and electron-dense layers and each is separated from those adjacent to it by 10-nm regions containing cytoplasmic material free of ribosomes or other particulate elements [8]. The chemical composition of the distinctive region surrounding

bacterial magnetite grains is unknown, but may be important in their formation. It might contain enzymes involved in magnetite synthesis, for instance. Magnetite particles extracted from cells by brief sonication retain an envelope, although their interparticle separation is less than 50% of that separating particles in chains within intact cells [8].

The Fe₃O₄ particles in *A. magnetotacticum* are in the single magnetic domain size range and the chain of magnetosomes imparts a permanent magnetic dipole moment to the cell. The cell magnetic moment lies parallel to its axis of motility and is sufficiently strong to orient it in the geomagnetic field [12]. Thus, magnetotactic bacteria swim along magnetic field lines.

A. magnetotacticum is a microaerophilic chemoheterotroph. Cells obtain carbon and energy by dissimilating succinic, tartaric or other organic acids [13]. Nitrate ion was the principal electron acceptor for cells growing microaerobically on tartrate, although a trace of O₂ was also consumed [14]. Cells of this species synthesize Fe₃O₄ only under microaerobic conditions and when provided adequate (roughly 1 mg/l) Fe in their medium. Under these conditions, they accumulate Fe 20 000-

Fig. 1. Electron micrograph of *A. magnetotacticum* enlarged 17500 times.

to 40 000-fold over its extracellular concentration. At relatively high O_2 tension (6 kPa), cells grow to final yields as high as $3 \cdot 10^8$ cells/ml, but do not synthesize Fe_3O_4 [13].

A. magnetotacticum cells contain *a*-, *b*- and *c*-type cytochromes, although their total cytochrome iron content is a small fraction of the total cellular iron (O'Brien, W. and Blakmore, R., unpublished data). In a previous Mössbauer study of intracellular iron in this species [9], we detected magnetite and an additional ferric-iron component which we speculated might be a magnetite precursor. We are investigating physiological condi-

tions affecting bacterial magnetite synthesis with the intent of elucidating the biochemical pathway of magnetite synthesis. In the present study, we report results of Mössbauer spectroscopic analyses conducted at a variety of temperatures using packed, wet cells of this organism (and a mutant of it unable to synthesize Fe_3O_4) in addition to lyophilized cells and cell fractions after isotopic enrichment with ^{57}Fe .

Materials and Methods

Organism and culture conditions

A. magnetotacticum strain MS-1 was used throughout [11]. Two types of nonmagnetotactic (spirillum) cell obtained from this strain were also used. One type (type MS-1R) had a reddish appearance when concentrated by centrifugation, and a second (strain NM-1) yielded cell pellets having a creamy white color. Type MS-1R cells did not constitute a separate strain. They were usually obtained by harvesting strain MS-1 cells prior to the mid-exponential growth phase. Because their occurrence was unpredictable, culture conditions leading to a preponderance of the reddish cell type are still under investigation. If allowed to continue their growth, reddish cells developed into magnetotactic cells having characteristically dark brown to black color. Cells of strain NM-1, on the other hand, never gave rise to magnetotactic cells, even though they were routinely cultured under conditions optimal for growth of the latter. They are presumed to be a genetically distinct (mutant) substrain of MS-1 cells. A culture of strain NM-1 was obtained by plating $1 \cdot 10^7$ cells of strain MS-1 on solid media, incubating aerobically and selecting one of two white colonies consisting of aerotolerant spirilla lacking magnetosomes (Blakmore, N., unpublished data).

All three cell types were cultured microaerobically on a chemically defined medium as described previously [13]. The medium contained tartaric and succinic acids as carbon sources, ferric quinate or ferrous ascorbate as the principal iron source and sodium nitrate, ammonium sulfate, or a combination as the nitrogen source. Cells were grown in 10-l glass carboys to late logarithmic or early stationary phase (10–14 d; $2.0 \cdot 10^8$ cells/ml), at 30°C. They were harvested by continuous flow

centrifugation in a CEPA model LE electrically driven centrifuge equipped with water cooling. The cells were washed twice by centrifugation at 5°C in 10–20 ml cold 50 mM potassium phosphate buffer (pH 6.97) and Mössbauer spectra were collected either immediately using the wet, packed cell material, or after resuspending cells in 50 mM potassium phosphate buffer (pH 6.97) and lyophilizing them.

$^{57}\text{FeCl}_3$ was prepared by first dissolving 80 mg Fe_2O_3 , isotopically enriched to 90% ^{57}Fe , in 4.5 ml analytical grade concentrated HCl. Excess HCl was then boiled off to concentrate the resulting FeCl_3 to a sample volume of 1 ml. The concentrate was diluted with 5–10 ml distilled H_2O and again concentrated by boiling. This procedure was repeated two more times and the $^{57}\text{FeCl}_3$ was finally taken to near dryness. The salt was then dissolved in 100 ml distilled H_2O and combined with 0.19 g D-quinic acid (Sigma Chemical Co.) to chelate the metal. 20 ml of this 0.01 M stock of ferric quinate sterilized by autoclaving were aseptically added to each 10 l of medium prior to inoculation.

Cell fractionation

Magnetic cells ($3 \cdot 10^{12}$ cells from 60 l culture) were harvested, washed twice by centrifugation, and resuspended in 15 ml 50 mM potassium phosphate buffer (pH 6.79). The resuspended cells were then divided into two portions, each 7.5 ml (Fig. 2). One portion (fraction WC) consisting of 450 mg dry weight, was lyophilized. The second was sonicated for 15 min at room temperature using a Heat Systems Ultrasonics Cell Disruptor model W-375, operating at 130 W. Cell disruption was evaluated using phase contrast microscopy. The magnetosomes were magnetically separated from cell debris by placing the suspension between the pole pieces of a strong (2 kOe) horseshoe-shaped magnet and allowing them to collect on the sides of the vessel close to the poles. They were recovered after aspirating off the suspended cell debris, and were then lyophilized (fraction M). The suspended cell debris (aspirant) was centrifuged at $3000 \times g$ for 15 min at 5°C. The resulting supernatant fluid fraction (fraction SF) was decanted and lyophilized. The pellet containing cell debris (fraction CD) was resuspended and also

Fig. 2. Cell fractionation diagram.

lyophilized. Freeze-dried samples of whole cells (fraction WC), magnetosomes (fraction M), cell debris (fraction CD) and supernatant fluid resulting from centrifugation of cell debris (fraction SF) were each analyzed by Mössbauer resonance spectroscopy which is non-destructive to the sample. After their spectra had been recorded, the whole cells were resuspended in 7.5 ml distilled H₂O and sonicated. The suspension was then digested with 1.5 ml 5 M NaOH for 12 h, and washed in distilled H₂O by centrifugation. The pellet fraction was saved and repeatedly washed in distilled water until the supernatant fluid was clear. The magnetosomes were collected from the washed digest in a strong magnetic field gradient as before. The NaOH and washing fluids removed by aspiration were pooled and centrifuged ($12\,000 \times g$, 10 min) to remove any residual magnetite particles.

The two magnetite fractions were combined and washed twice in 20 ml distilled H₂O using magnetic separation. The resulting magnetite particles (fraction MP) were free of adherent cell debris and lacked the enveloping layers characteristic of intact magnetosomes as evidenced by transmission electron microscopy (Fig. 3). They

were lyophilized and analyzed by the Mössbauer technique.

Mössbauer spectroscopy

Mössbauer measurements were made using a constant acceleration spectrometer with a ⁵⁷Co (in rhodium metal) source. The source was kept at room temperature. Each sample was packed in a plastic container and mounted on a copper block inside a Janis-Supervaritemp dewar. Temperatures between 1.5 and 300 K were maintained and measured with a calibrated Si diode and a Lakeshore Cryotronics temperature controller. Spectra were analyzed by a least-squares fitting program developed by J. Tillet and F. Varret. Parameters obtained from the line fitting procedure are presented in Table I.

Electron microscopy

The spatial distribution of Fe atoms in whole cells of *A. magnetotacticum* and its extracted magnetosomes was determined by Fe X-ray pulse mapping. Unstained cells on carbon-coated grids were examined with a VG Microscopes HB 5 scanning transmission electron microscope fitted

Fig. 3. Electron micrograph of partially purified magnetosomes (fraction MP). Particle size is approx. 45 nm.

TABLE I
MÖSSBAUER PARAMETERS

Sample	$T(K)$	$\delta(mm/s)^a$	$\Delta E_Q(mm/s)^b$	$H_{hf}(kOe)^c$	Comments
Magnetotactic cells (MS-1)					
A ₁	80	0.44	0.65	500 } 470 }	Fe ₃ O ₄ (80%) Fe ³⁺ } 20% Fe ²⁺ }
A ₂		0.74			
B		0.47			
C		1.32			
Nonmagnetotactic Cells (MS-1R)					
A ₁	80	0.40	0.68	495 } 480 }	Fe ₃ O ₄ (14%) Fe ³⁺ (86%)
A ₂		0.65			
B		0.47			
B		4.2			
Nonmagnetotactic Cells (NM-1)					
B	80	0.51	0.65		Fe ³⁺ (84%)
C		1.35	3.04		Fe ²⁺ (16%)
Ferritin	80	0.48	0.73		

^a Isomer shift relative to iron metal at 295 K.

^b Quadrupole splitting.

^c Magnetic hyperfine field.

with a field emission electron source and an energy dispersive X-ray analyzer. The sample was continuously scanned by the electron beam and the instantaneous intensity of the iron K _{α} X-ray at 6.4 keV was used to modulate the brightness of the cathode ray tube image which was recorded on Polaroid film.

Results

Mössbauer spectra of intact MS-1 cells

Mössbauer spectra at 200, 80 and 4.2 K of wet, packed, strain MS-1 cells enriched in ⁵⁷Fe are shown in Figs. 4 and 5. The spectrum obtained at 200 K (Fig. 4) is complex, reflecting several Fe species present within the bacteria. It consists of a superposition of spectra (A₁ and A₂) corresponding to magnetite [15], a broadened quadrupole doublet (B) with parameters characteristic of ferric iron, and a weak quadrupole doublet (C) with parameters corresponding to ferrous iron [16]. The ferrimagnetic mineral magnetite has an inverse spinel structure with iron in both octahedral and tetrahedral crystal lattice sites [17]. Absorption lines denoted by A₁ correspond to Fe³⁺ present in tetrahedral lattice sites, whereas spectral

lines denoted by A₂ correspond to Fe³⁺ and Fe²⁺ within octahedral sites. At temperatures above the Verwey transition (a structural and electronic phase transition in magnetite which occurs at about 120 K), electron exchange between Fe²⁺ and Fe³⁺ in the octahedral lattice sites is rapid compared to the ⁵⁷Fe Larmor precession time [15]. For this

Fig. 4. Mössbauer spectrum of wet, packed magnetotactic bacteria (MS-1) at 200 K. Subspectra A₁ and A₂ are due to Fe₃O₄; spectrum B is a ferric doublet; spectrum C is a ferrous doublet. The solid line is a theoretical least-squares fit to the data.

Fig. 5. Mössbauer spectra of MS-1 at (a) 80 and (b) 4.2 K. Note the reduction in the intensity of spectrum B at 4.2 K. (c) MS-1 at 4.2 K after anaerobic incubation above freezing temperature for 24 hours. Note enhancement of spectrum C at the expense of B.

reason, A_2 in Fig. 4 is a single, averaged six-line subspectrum corresponding to multivalent Fe in this site. The highest velocity lines of the A_1 and A_2 subspectra are fortuitously superimposed at room temperature. Hence, only ten of the twelve possible lines in the magnetite spectrum ($A_1 + A_2$) are resolved at this temperature. Moreover, since two Fe atoms are present in octahedral sites for every Fe atom in a tetrahedral site, the relative intensities of lines A_1 and A_2 should be 1:2. This ratio was, in fact, observed with wet, packed, magnetotactic cells. The ratio of these intensities using lyophilized cells was usually somewhat less than 1:2 [9] and variable from sample to sample. These results are consistent with the possibility of surface oxidation of some of the magnetic particles to gamma- Fe_2O_3 (maghemite), perhaps during or after freeze-drying.

At 80 and 4.2 K, spectra typical of magnetite

below the Verwey transition were obtained (Fig. 5). Although the magnetic structure of Fe_3O_4 below this transition is complex, the Mössbauer spectrum can be resolved into two apparent six-line spectra corresponding to Fe^{2+} in octahedral sites (Fig. 5a; lines A_2) and Fe^{3+} in either octahedral sites, or tetrahedral lattice sites (Fig. 5a; lines A_1). The Fe^{2+} spectrum (A_2), with half as much intensity, is shifted toward higher velocities relative to the Fe^{3+} spectral lines. Spectra obtained at temperatures between 110 and 130 K showed the changes expected of magnetite passing through the Verwey transition (data not shown). Spectral lines B of Figs. 4 and 5a displayed isomer shift and quadrupole splitting parameters typical of high-spin Fe^{3+} compounds and very similar to those of Fe in the Fe storage protein, ferritin [18], or in the mineral ferrihydrite (Ref. 19; Table I). The relative intensities of B spectral lines to those of $A_1 + A_2$ were somewhat variable from sample to sample and depended upon bacterial growth conditions. At 80 K (Fig. 5a) the isomer shift and quadrupole splitting parameters of spectrum B and the relative intensity of B to $A_1 + A_2$ were relatively unchanged compared to those obtained at 200 K (Fig. 4). Between 80 and 4.2 K, however (Figs. 5a, 5b), the intensity of B decreased with decreasing temperature and at 4.2 K only a residual absorption doublet remained. A similar temperature dependence for spectrum B was also obtained with lyophilized cells (data not shown).

The isomer shift and quadrupole splitting parameters of spectrum C correspond to high-spin ferrous iron in coordination with oxygen or nitrogen (Table I). This spectrum was not observed with lyophilized cells, possibly as a result of oxidation during sample preparation. Wet, packed cells of strain MS-1 kept unfrozen under anaerobic conditions contained increased amounts of material responsible for spectrum C and correspondingly less material with spectral characteristics B. Thawing and aeration of these frozen cells resulted in intensity increases in B spectral lines and concomitant intensity decreases in C spectral lines. This indicates that the Fe atoms responsible for spectrum C came from reduction of the Fe atoms giving spectrum B. Unlike that of spectrum B, the intensity of spectrum C did not decrease at low temperature (Fig. 5c).

The decrease in the intensity of spectrum B between 80 and 4.2 K can be explained by the onset of magnetic hyperfine interactions at low temperature resulting in a concomitant decrease in the intensity of the central absorption doublet. This phenomenon has been observed in Mössbauer spectra of purified mammalian ferritin [18,19] and other small magnetic particles. However, in spectra obtained from the bacteria, the magnetic hyperfine lines are obscured by the magnetite spectral lines (A_1 and A_2). To resolve further the nature of the material responsible for spectrum B, we studied the temperature-dependent Mössbauer spectra of nonmagnetotactic cells of strain MS-1 which lacked the interfering magnetite.

Mössbauer spectra of intact MS-1R cells

The 250 K spectrum of lyophilized MS-1R cells (Fig. 6) consisted primarily of the quadrupole doublet characteristic of ferric iron as denoted by spectrum B in Figs. 4 and 5. In addition, a very low intensity spectrum due to Fe_3O_4 (spectral lines $A_1 + A_2$ in Figs. 4 and 5) was observed. These latter spectral lines might be due to a small fraction of magnetotactic cells (MS-1) in the MS-1R sample or to trace amounts of magnetite possibly present in MS-1R type cells. Spectrum B remained unchanged except for some broadening of the lines as the temperature was lowered to about 100 K (Fig. 7a). Below 100 K (Figs. 7b, 7c), the intensity of the quadrupole doublet decreased with decreasing temperature, while the intensity of a six-line spectrum flanking the doublet increased. Thus, the total absorption area remained constant. Most of

Fig. 6. Mössbauer spectrum of lyophilized MS-1R cells at 295 K. There is some residual magnetite in the sample.

Fig. 7. Mössbauer spectra of MS-1R at (a) 100 K; (b) 40 K; (c) 4.2 K.

the change from quadrupole doublet to six-line spectrum occurred as the temperature was reduced from 60 to 10 K. At 4.2 K (Fig. 7c) the spectrum consisted primarily of the six broadened magnetic hyperfine lines, with a small residual doublet in the center. Spectral lines $A_1 + A_2$ were then obscured by the six-line spectrum. Application of a longitudinal magnetic field of 60 kOe to this sample at 4.2 K produced broadening of the six-line spectrum but no appreciable shifts in the line positions and no change in the relative intensities of the absorption lines (data not shown). These spectral characteristics are indicative of small particles of hydrous ferric oxide with antiferromagnetic exchange interactions similar to those of the ferric iron within ferritin micelles [18,20], or in hemosiderin [21]. The Fe atoms in these latter substances are antiferromagnetically ordered. However, at temperatures between 10 and 60 K, thermally activated transitions of the magnetic sublattices between energetically equivalent easy axes of magnetization within the crystal produce a collapse of the magnetic hyperfine spectrum to a quadrupole doublet. Thus, the high temperature ($T > 60$ K) spectrum (Fig. 7a) showed a broadened

quadrupole doublet with parameters characteristic of high spin Fe^{3+} , whereas the low-temperature ($T < 10$ K) spectrum (Fig. 7c) was a magnetic hyperfine sextet with magnetic splitting corresponding to 500 kOe at the Fe nucleus. The magnetic sublattice transition time τ , is an exponential function of the temperature, T , the magnetic anisotropy, K , and volume, V , of the particles:

$$\tau = \tau_0 \exp[KV/2kT]$$

where k is Boltzmann's constant and τ_0 is a constant of the order of 10^{-9} s. When $\tau = \tau_L$, the Larmor precession time of the ^{57}Fe nucleus [16], the six-line spectrum collapses to the doublet. If all the particles had precisely the same volume, this condition would occur at a definite temperature. For a distribution of particle volumes, the collapse of the six-line spectrum will occur over a finite temperature interval, as is the case not only for the material under study, but also for ferritin [18,20]. In principle, analysis of the temperature variation of the relative doublet and sextet intensities can yield the distribution of particle sizes [22]. However, in noncrystalline materials, variation in their anisotropy constant and volume complicates this analysis. If we use values of K and τ_0 appropriate to ferritin [23], the experimental results indicate that hydrous ferric oxide particles in type MS-1R cells are of the order of 100 Å in diameter, or less.

With mammalian ferritin, only the intensities of the sextet spectral lines change with temperature. The line positions and widths remain relatively invariant. In the case of the bacteria (Fig. 7), lines of the sextet broadened and the line splittings as well as the line intensities decreased somewhat with increasing temperature. This may reflect less crystallographic order in the material under study than exists in the micelles of ferritin. The intensity changes in type B spectral lines of MS-1 cells (Fig. 5) occur over the same temperature range as those in spectra of MS-1R cells, but with a slightly different temperature dependence. This indicates that spectrum B in MS-1 is due to the same hydrous ferric oxide as is present within cells of MS-1R, but with a slightly different distribution of particle volumes. On the basis of the similarity of the parameters of spectrum B to those reported for ferritin [18] and for ferrihydrite [19], and because

of the similarity of Fe in ferritin and in ferrihydrite [24,25], we identify the hydrous ferric oxide in MS-1 and MS-1R cells as ferrihydrite. Unlike ferritin (ferrihydrite), however, there was a residual quadrupole doublet in the 4.2 K spectra of cells of MS-1 and MS-1R (Fig. 7c). The intensity of this residual doublet varied somewhat from sample to sample, but its presence suggests another high-spin ferric iron material, perhaps a hydrous ferric oxide in which the Fe atoms are less densely packed than in ferrihydrite. Consequently, magnetic exchange interactions between them are weaker and magnetic splitting of the spectrum at 4.2 K is not observed.

Mössbauer spectra of intact cells of mutant NM-1

The Mössbauer spectrum of wet, packed cells of the nonmagnetotactic strain (NM-1), consisted of a quadrupole absorption doublet at 200 K (Fig. 8a) which persisted at 80 (not shown) and 4.2 K (Fig. 9a). The spectral parameters obtained at 200 and 80 K were similar to those of spectrum B in Figs. 4 and 5 (MS-1) and 6 and 7 (MS-1R), indicative of a high-spin ferric iron material. At 1.6 K (a temperature achieved by decreasing the pressure over the liquid helium bath used in cooling the sample during Mössbauer analysis), the spectrum (not shown) consisted of a quadrupole doublet superimposed upon a broad magnetic hyperfine spectrum. Application of an external 60

Fig. 8. Mössbauer spectra of NM-1 at (a) 200 K; (b) 200 K following incubation above freezing temperature for 24 h.

Fig. 9. Mössbauer spectra of NM-1 at (a) 4.2 K and (b) 4.2 K in a longitudinal magnetic field of 60 kOe.

kOe magnetic field at 4.2 K also resulted in spectra with a broad distribution of hyperfine fields (Fig. 9b). These spectral characteristics indicated the presence of high-spin Fe^{3+} in a hydrous oxide with magnetic exchange interactions of the order of 2–3 K, that is, with Fe atoms less densely packed than in ferrihydrite. This material had spectral characteristics similar to those of the low density ferric oxide material in cells of strain MS-1 and type MS-1R, that produced the residual quadrupole splitting observed in spectrum B at 4.2 K (Fig. 7c). An iron-storage material with similar spectral characteristics has been detected in *Escherichia coli* and in other prokaryotes grown in a high-iron environment [26]. Wet, packed cells of strain NM-1 held above 275 K in an anaerobic environment displayed a ferrous spectrum similar to spectrum C observed with MS-1 cells as well as the ferric-iron doublet (Fig. 8b). This indicates that the hydrous ferric oxide in cells of strain NM-1 became reduced to ferrous iron under these conditions as with cells of strain MS-1.

Mössbauer spectroscopy of MS-1 cell fractions

Room-temperature Mössbauer spectra of lyophilized MS-1 cell fractions are shown in Fig. 10. MS-1 cells grown with the usual Fe supply (not enriched with ^{57}Fe) were lysed and cell fractions prepared as described in the Materials and Methods section.

Fig. 10. Mössbauer spectra of lyophilized MS-1 cell fractions at 295 K: (a) whole cells (fraction WC); (b) magnetic fraction (M); (c) magnetic fraction following NaOH treatment (MP); (d) nonmagnetic fraction (CD).

The Mössbauer spectrum of whole cells (fraction WC; Fig. 10a) consisted of subspectra A_1 , A_2 and B corresponding to those of Fe_3O_4 and ferrihydrite as discussed previously. This sample had a relatively higher fraction of ferrihydrite (spectrum B) than that used to obtain spectra in Figs. 4 and 5. The spectrum obtained using partially purified magnetosomes (fraction M; Fig. 10b) also consisted of these subspectra. Moreover, comparison of the relative absorption areas in the two sample types showed that the ratio of ferrihydrite to magnetite was only slightly lower in the partially purified magnetic fraction than in whole cells. The spectrum obtained with nonmagnetic cell debris (fraction CD; Fig. 10d) was weak, showing that most of the cellular iron was associated with the magnetosomes. The spectrum appeared to overlap

that of spectrum B. The spectrum of purified bacterial magnetite particles (fraction MP; Fig. 10c) corresponded to that of magnetite with a small amount of ferrihydrite also present. Temperature-dependent spectral changes in fractions WC and M were similar to those described previously for intact cells of strain MS-1, indicating that material giving spectral characteristics denoted by lines B in these fractions was ferrihydrite and hydrous ferric oxide. Thus, the results show that ferrihydrite is associated with the bacterial magnetosomes.

Similar results were obtained with cell fractions of strain MS-1 enriched in ^{57}Fe for which whole cell spectra are shown in Figs. 4 and 5. Fig. 11 shows the spectra obtained at 80 and 4.2 K using the wet, packed, cell fraction purified with the use of a magnetic field. Spectra of the magnetically unresponsive cell fractions at these temperatures are shown in Fig. 12. Residual magnetite lines in the latter spectra indicate that the recovery of the magnetosomes in the magnetic fraction was incomplete. Nevertheless, the large enhancement of the spectrum B lines compared to those of $A_1 + A_2$, showed that Fe in the nonmagnetic fraction was primarily present as ferrihydrite or hydrous ferric oxide. B-type spectral lines almost vanished in the 4.2 K spectra of the magnetite-containing fraction but not in the nonmagnetic fraction. This means

Fig. 11. Mössbauer spectra of partially purified magnetosomes (fraction M) from ^{57}Fe -enriched MS-1 cells: (a) 80 K; (b) 4.2 K.

Fig. 12. Mössbauer spectra of nonmagnetic fraction (CD) of ^{57}Fe -enriched MS-1 cells at (a) 80 K; (b) 4.2 K.

that the magnetic fraction contained more ferrihydrite, whereas the nonmagnetic fraction was more enriched in the low-density hydrous ferric oxide.

The concentration of cellular Fe in the magnetosomes is confirmed by the Fe X-ray pulse maps. Fig. 13 compares the total electron density of part of a whole cell with its Fe density. Visual inspection shows that the Fe is concentrated in the electron-dense magnetosomes. Iron density in the cell regions not occupied by magnetosomes is indistinguishable from the background Fe concentration outside the cell.

In summary, intact magnetotactic cells of strain MS-1 contained a low-density hydrous ferric oxide, a high-density hydrous ferric oxide (ferrihydrite), and Fe_3O_4 . Cellular Fe was concentrated in the magnetosomes. In wet, packed cells, ferrous iron was also detected. The ferrous iron concentration in cells kept anaerobically at temperatures above freezing gradually increased and this increase appeared to be at the expense of the hydrous ferric oxides. Ferrihydrite and Fe_3O_4 were both associated with the magnetosome chain. Cells physiologically blocked in the production of magnetite (MS-1R cells) contained the low-density hydrous iron oxide in addition to ferrihydrite. Cells presumed to be mutants genetically blocked in magnetite production (strain NM-1) contained only

Fig. 13. Transmission electron micrograph of MS-1 cells (top) and Fe X-ray map of the same region (bottom), showing the location of iron in the cell.

the low-density hydrous ferric oxide. Ferrous iron was also detected in cells of strain NM-1.

Discussion

From the forgoing results, we propose that cells of *A. magnetotacticum* strain MS-1 precipitate Fe_3O_4 in the sequence (i) Fe^{3+} -quinat_{cell exterior} $\rightarrow \text{Fe}^{2+}$ _{cell interior}, (ii) low-density hydrous ferric oxide, (iii) ferrihydrite, (iv) Fe_3O_4 . Analysis of cell fractions indicated that the deposition of ferrihydrite and its subsequent reduction and precipitation to magnetite occurs in the magnetosomes. It is plausible that the magnetosome envelope develops first, conceivably as an invagination of the cytoplasmic membrane. Alternatively, the magnetosome envelope may develop after the low-density hydrous iron oxide is deposited – perhaps at or near sites of Fe transport into the cell. Participation of the cytoplasmic membrane in magnetosome formation is implicated because these structures always appear to be located adjacent to it within the cell [8].

Iron uptake from Fe quinate, proposed step (i)

in our study system, probably occurred via non-specific transport, because sufficient Fe (20 μM) was added to the culture medium to repress siderophore synthesis [27]. Three microbial Fe transport mechanisms are presently known [27–30]. In one, the Fe^{3+} chelate is transported into the cell where the Fe^{3+} is released from the chelator by reduction to Fe^{2+} and the free chelator is excreted. In another, the Fe^{3+} is passed from its chelator at the cell surface to membrane-bound chelators which shuttle it into the cell, where it is released by reduction to Fe^{2+} . In the third, the Fe^{3+} chelate is transported into the cell where the Fe is released by destruction of the chelator and subsequently reduced to Fe^{2+} . In each case, Fe exists, however briefly, as Fe^{2+} as a result of the transport process. Thus, the small amount of Fe^{2+} seen in wet packed samples of *A. magnetotacticum* may be Fe in transport and would consequently appear as a transient in the step (i) to (ii). It might then be rapidly reoxidized and deposited intracellularly as the low-density hydrous ferric oxide.

The deposition of the hydrous ferric oxide (ferrihydrite) micellar cores in mammalian ferritin occurs following oxidation of Fe^{2+} [31,32]. The Fe enters the protein-enclosed cavity, is chelated and is subsequently oxidized – possibly by dioxygen to form a hydrous ferric oxide. Molecular oxygen is required for bacterial magnetite synthesis (Bazylin-ski, D.A. and Blakemore, R., unpublished results). Thus, by analogy to the oxidation of Fe^{2+} and deposition of ferrihydrite in the core of mammalian ferritin, we speculate that the involvement of O_2 in bacterial magnetite synthesis might be in the formation of the low density hydrous ferric oxide precursor.

The change from (ii) to (iii) presumably involves dehydration, but no change in oxidation state. The magnetosome envelope could play an active role in this process by providing a hydrophobic barrier promoting unidirectional water loss from the crystal to the cell cytoplasm.

The final step in bacterial magnetite synthesis, (iii) to (iv), occurs at a site of Fe reduction within the cell because it involves reduction of one-third of the Fe ions and further dehydration of ferrihydrite to magnetite. Thus, the magnetosome envelope may be the repository of cellular electron carriers and dehydratases involved in electron or

proton flow at such local precipitation sites. The fact that reduction of iron in mammalian ferritin [31] or bacterioferritin [33] does not result in magnetite precipitation suggests specific involvement of the magnetosome envelope in the final precipitation events. Just how the cell precipitates the inverse spinel crystal structure of magnetite as opposed to the hexagonal close-packed structure of hematite, (α -Fe₂O₃) is unknown. According to the thermodynamic equilibrium diagrams of Garrels and Christ [34], Fe₃O₄ is the stable phase in the iron-oxygen-water system at low E_h and high pH. At a pH of about 7, the mineral is stable only below an E_h of about -100 mV, the approximate potential of the Fe(OH)₃/Fe(OH)₂ couple. In fact, colloidal suspensions of magnetite can be prepared in vitro by adding NaOH to aqueous solutions containing Fe²⁺ and Fe³⁺ in molar ratios of 1:2 [35,36]. Thus, control of proton flow may be important in the precipitation of magnetite following reduction of ferrihydrite.

There are probably several enzymes with 'iron reductase' activity in the cell [37]. Several processes involving Fe reduction have been identified, including heme synthesis [38], removal of Fe from siderophores [39,40,41] or terminal electron transport [42]. In addition to the possible involvement of indirect mechanisms of Fe reduction in magnetite synthesis, specific Fe reducing enzymes might also participate. Our results suggest that the magnetosome envelope could be the locus of such enzyme activities.

Reduction of a ferrihydrite precursor to magnetite also occurs in the marine chiton, (subclass *Polyplacophora*). In this organism the radular teeth undergo a progressive mineralization process resulting in a surface coating of Fe₃O₄ [43,44]. Iron is transported to the superior epithelial cells of the radula as ferritin. It is then transferred to a pre-formed organic matrix on the tooth surface as ferrihydrite. Finally, the ferrihydrite is reduced to Fe₃O₄. Molluscan magnetite particles have dimensions of the order of 0.1 μ m [45]. It is possible that the bacterial magnetosome envelope may be similar functionally and structurally to the micellar boundary layer observed in the mineralizing chiton radular denticle.

In conclusion, we have identified ferrous ions, a low-density hydrous ferric oxide, and a high-den-

sity hydrous ferric oxide (ferrihydrite) and Fe₃O₄ in cells of *A. magnetotacticum*. These Fe species appear to be magnetite precursors and the precipitation of bacterial magnetite occurs in a sequence involving Fe reduction, reoxidation, dehydration of the resulting hydrous ferric oxide, and final reduction.

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