Magnetite formation via membrane-bound ferritin and an iron(II) species at the cytoplasmic membrane and in magnetosomes of *Magnetospirillum gryphiswaldense*

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Abstract. Growth and cell fractions of the magnetic bacterium *Magnetospirillum gryphiswaldense* were studied by Mössbauer spectroscopy. In isolated magnetosomes only magnetite particles were observed. The membrane fraction of *Magnetospirillum gryphiswaldense* contains a ferritin-like component and a Fe²⁺ species and also magnetite particles smaller than those observed in the magnetosomes fraction. In the cytosol only ferritin was identified.

1. Introduction

Magnetotactic bacteria (MTB) were discovered in 1975 by Blakemore [1] and exhibit a unique cellular compartments referred to as magnetosomes (MS). MTB accomplish biosynthesis of magnetite in MS. *Magnetospirillum gryphiswaldense* biomineralizes single domain crystals of cubooctahedral magnetite (Fe₃O₄) with up to 4% of the total dry weight [2]. MS vesicles originate from invaginations (IV) of the cytoplasmic membrane forming a cavity at periplasmic side of the cytoplasnic membrane. The IV detach individually as vesicles (MS) from the cytoplasmic membrane [3-8]. The assembly of highly ordered MS chains is under genetic control and involves several specific proteins [9]. This structure is essential for MTB-to navigate along the lines of the earth’s magnetic field in order to find growth-favouring microoxic zones in their aquatic environment. MTB were studied intensively in the last three decades [3-5, 10-12]. These MS have recently gathered multidisciplinary interest due to their crystalline and magnetic properties, e.g. as distinct biomarkers for life on Mars [13,14], or as material for biotechnological applications[15]. Following questions were addressed in this study: (i) Which iron metabolites are formed after iron uptake and in which compartments are they detectable? (ii) Is magnetite detectable in the membrane fraction? (ii) Are there any inorganic phases or precursors in addition to magnetite in the magnetosomes?

2. Materials

Cells were grown in an oxystat bioreactor, which allows control of temperature, pressure and oxygen concentration using a modified protocol for large scale cultivation [16]. Throughout all experiments the strain MSR-1 (DSMZ 6361) of *Magnetospirillum gryphiswaldense* was used. Cells in the mid-logarithmic growth phase were transferred to a low carbon medium [11]. Cells kept under these conditions are still viable, however display significant cell growth and allow, therefore, growth-independent experiments. Iron uptake (induction) was achieved via $^{57}$Fe³⁺- dicitrate added to
the cell suspension. For separation of cell fractions, mature cells with fully formed magnetic chains were disrupted and spun down at different g-values. In addition, the soluble fraction was passed through a 100 kDa filter.

For analysis, cells were removed from a bioreactor sequentially at different time periods after $^{57}$Fe induction and were analyzed by magnetic light scattering and bright-field TEM (Zeiss EM10 transmission electron microscope operating at 60kV, data shown elsewhere [11,12]) For Mössbauer spectroscopy, cells were washed, spun down into Delrin Mössbauer sample holders, frozen in liquid nitrogen, and kept at this temperature until measurement in bath and continuous flow cryostats from Oxford Instruments (constant acceleration mode). Spectra were calibrated against $\alpha$-iron foil at room temperature and were analyzed by least-squares fits of Lorentzian line shapes to the experimental data.

3. Results

3.1. Growth analysis

Mössbauer spectra of whole cells are shown in Figure 1. Already 20 min after $^{57}$Fe induction first traces of magnetite, ferritin (see chapter 3.3) and ferrous iron are detectable. In all Mössbauer spectra (taken at 130 K) two magnetically split sextets resembling magnetite sites A and B ($\delta_A=0.38$ mm/s, $B_{hf,A}=49.5$ T; $\delta_B=0.76$ mm/s, $B_{hf,B}=47.4$ T), a high-spin Fe$^{3+}$-doublet ($\delta=0.47$ mm/s, $\Delta E_q=0.77$ mm/s) and a high-spin Fe$^{2+}$-doublet ($\delta=1.27$ mm/s, $\Delta E_q=2.81$ mm/s) were detected. The amount of all components increases until 40 min after $^{57}$Fe induction. At later times after $^{57}$Fe induction only the growth of the magnetite components continues further.

[Figure 1. Mössbauer spectra at different times after $^{57}$Fe induction: From top to bottom: 20 min, 40 min, 60 min, 95 min, 125 min, 155 min, 215 min and 1230 min.]

3.2. Isolated magnetosomes

Mössbauer spectra of isolated magnetosomes (Figure 2) exhibit two magnetically split sextets attributed to sites A and B of magnetite ($\delta_A=0.38$ mm/s, $B_{hf,A}=49.9$ T; $\delta_B=0.76$ mm/s, $B_{hf,B}=47.9$ T). The magnetic hyperfine field of the isolated magnetosomes is slightly higher than that in the whole-cell fraction.

[Figure 2. Mössbauer spectrum of isolated magnetosomes]

3.3. Membrane fraction

A Fe$^{3+}$ species exhibiting parameters similar to bacterioferritin ($\delta=0.47$ mm/s, $\Delta E_q=0.77$ mm/s, see also next paragraph) and a membrane-bound Fe$^{2+}$ species ($\delta=1.26$ mm/s, $\Delta E_q=2.86$ mm/s) were identified (Figure 3). These species were also detected in the cell spectra (Fig. 1) and were found previously in the cytoplasm of other Gram negative bacteria [17]. Surprisingly, the Mössbauer spectrum of the membrane fraction (Figure 3) also exhibits the two sextets of magnetite. However, the
magnetic hyperfine field is significantly smaller than magnetite in isolated MS (\(\delta_A=0.38 \text{ mms}^{-1}, B_{hf,A}=46.8 \text{T}; \delta_B=0.76 \text{ mms}^{-1}, B_{hf,B}=44.8 \text{T}\)). This provides evidence for the presence of very small magnetite crystallites (<6nm) located in IV of the membrane fraction. This indicates in addition that (i) these IV are still well integrated in the cytoplasmic membrane and (ii) initial magnetite formation occurs at these IV.

Figure 3. Mössbauer spectrum of the membrane fraction at T=130 K: The spectrum is dominated by the ferritin (green) component, accompanied by a high-spin Fe\(^{2+}\) species (cyan). Minor amounts of small magnetite particles (red and blue) were also identified at the high-velocity sides of the spectrum.

Figure 4. Mössbauer spectra of the cytoplasmic fraction (molecular mass >100 kDa) containing ferritin as sole iron species at T=4.2 K (top, blue). The spectrum is fitted with a split ferritin sextet and a non-split ferritin doublet. The spectrum recorded at T=77 K (bottom, red) is fitted with one ferritin doublet alone.

3.4. Fraction above 100 kDa
The Mössbauer spectrum of the 100 kDa supernatant obtained from the cytoplasmic fraction was recorded at 77K (Figure 4). It exhibits a single doublet the parameters of which (\(\delta=0.47 \text{ mms}^{-1}, \Delta E_p=0.77 \text{ mms}^{-1}\)) are similar to typical bacterioferritins (e.g. Azotobacter; \(\delta=0.48 \text{ mms}^{-1}, \Delta E_p=0.78 \text{ mms}^{-1}\)). However, at 4.2 K the majority (86.8 %) of the doublet (observed at 77 K) has transformed into a magnetically split sextet typical for superparamagnetic transitions found in ferritins (\(\delta=0.49 \text{ mms}^{-1}, B_{hf}=47.4 \text{T}\)). The remaining 13.2 % are fitted with a doublet (\(\delta=0.49 \text{ mms}^{-1}, \Delta E_p=0.77 \text{ mms}^{-1}\)). Gel electrophoresis (native & SDS-PAGE) of the ferritin verifies the existence of a high-molecular mass protein (> 100 kDa) that carries large amounts of iron and decays under SDS into 20-40 kDa subunits.

4. Conclusion
With the exception of ferritin-like iron mineral precursors of magnetite were not detected in cell fractions. Therefore it is tempting to assume that magnetite formation in *Magnetospirillum gryphiswaldense* proceeds during subsequent steps by fast coprecipitation of ferrous and ferric iron within the invagination pit, which is likely alkaline to enable magnetite thermodynamic stability [18]:

\[
\begin{align*}
(i) \quad & [\text{Fe}^{2+}R_{A}]_0 + 2 [\text{Fe}^{3+}R_{B}]_0 (\text{ferritin}) + (2x + y) \text{H}_2\text{O} \\
\rightarrow & 2 \text{Fe(OH)}_{x+y}^{3-x} + \text{Fe(OH)}_y^{2-y} + (2x + y) \text{H}^+ + R_{A}^{2-} + 2R_{B}^{3-} \\
(ii) \quad & 2 \text{Fe(OH)}_{2}^{3-x} + \text{Fe(OH)}_y^{2-y} \\
\rightarrow & \text{Fe}_2\text{O}_4 (\text{crystal in statu nascendi}) + (2x + y - 4) \text{H}_2\text{O}
\end{align*}
\]
At the CM level Fe$^{2+}$ and Fe$^{3+}$ ions are ligated to organic carriers $R_a$ and $R_b$. These carriers release iron-hydroxo species directly at the membrane-invagination pit interface (step (i)). The presence of ferrous hexaquo and ferric hydroxo complexes in the complete membrane compartment is very unlikely because of their toxic nature (Fenton chemistry) and chemistry. According to this scenario, a mechanism (step (ii)) is assumed in which nucleation of magnetite occurs at the CM/pit interface. by coprecipitation of two equivalents of Fe(OH)$_{3-x}$ and one equivalent of Fe(OH)$_{2-y}$ to form crystalline Fe$_3$O$_4$ and H$_2$O. How magnetite grows after release of the fully formed vesicle from their membrane sites remains yet unclear.

In summary, we propose a mechanism for initial magnetite formation, where the iron required for magnetite biomineralization is released directly at the membrane invagination pit interface without iron flux via the cytoplasm. This might point to distinct transport pathways for magnetite formation and for biochemical utilization iron. Magnetite formation occurs via membrane-associated crystallites, whereas the final step of magnetite crystal growth is possibly spatially separated from the CM.

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