Study of the rhizobacterium *Azospirillum brasilense* Sp245 using Mössbauer spectroscopy with a high velocity resolution: Implication for the analysis of ferritin-like iron cores

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**Highlights**

- Ferritin-like iron cores in *Azospirillum brasilense* Sp245.
- Mössbauer spectroscopy with a high velocity resolution.
- Heterogeneous iron core structure in bacterial ferritin-like proteins.

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**Abstract**

The results of a comparative study of two samples of the rhizobacterium *Azospirillum brasilense* (strain Sp245) prepared in different conditions and of human liver ferritin using Mössbauer spectroscopy with a high velocity resolution demonstrated the presence of ferritin-like iron (i.e. iron similar to that found in ferritin-like proteins) in the bacterium. Mössbauer spectra of these samples were fitted in two ways: as a rough approximation using a one quadrupole doublet fit (the homogeneous iron core model) and using a superposition of quadrupole doublets (the heterogeneous iron core model). Both results demonstrated differences in the Mössbauer parameters for mammalian ferritin and for bacterial ferritin-like iron. Moreover, some differences in the Mössbauer parameters were observed between the two samples of *A. brasilense* Sp245 related to the differences in their preparation conditions.

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**Introduction**

Ferritin superfamily biomolecules perform the functions of iron storage and metabolism in various organisms that range from mammals to bacteria. This protein consists of a nanosized ferric hydroxide oxide core (ca. 8 nm) inside a multisubunit protein shell. An average composition of the iron core was proposed as ferrihydrite with the approximate formula \(\text{(FeOOH)}_8\) or \((5\text{Fe}_2\text{O}_3\cdot9\text{H}_2\text{O})\) including up to about 4500 iron atoms in mammals and a smaller amount in bacteria [1–8]. It is well known that ferritin molecules from different organisms have different amino acid sequences and variations in the iron core structure. Bacteria possess three types of iron-depositing ferritin-like proteins, the archetypical ferritin (bacterial ferritin) similar to that usually found in eukaryotes, bacterioferritin which contains hemes, and the DNA-binding dodecameric ferritin. Bacterial ferritin consists of a 24-subunit shell surrounding a cavity of about 7.9 nm which can hold about 2500 iron atoms. Bacterioferritin consists of 24 protein subunits, which are similar but smaller than mammalian and bacterial ferritin subunits, associated with 12 hemes. Inside the protein shell, there is a cavity of about 6 nm which can hold about 1800 iron atoms. The dodecameric ferritin consists of a 12-subunit protein shell surrounding a cavity of about 4.5 nm which can hold about 500 iron atoms. Although the iron core structure in ferritins has been studied for a long time using various structural techniques (see, for instance, [9–14]), these studies suggested different models for its structure ranging from monocristalline to polycristalline and to polyphas with different degrees of crystallinity.
Mössbauer spectroscopy is a powerful technique to study iron-containing biological molecules including ferritin (for review, see e.g. [15–20]). Mössbauer spectra of various ferritins usually demonstrated a superparamagnetic doublet shape down to low temperatures (below 40 K). Mössbauer parameters obtained for superparamagnetic bacterial ferritins and bacterioferritins at low temperatures in [21–27] were similar to those obtained for mammalian ferritins within the instrumental errors. Superparamagnetic Mössbauer spectra of various ferritins were fitted in three ways: (i) using a model-independent fit with a distribution of quadrupole splitting, (ii) using a one quadrupole doublet fit, and (iii) using a superposition of two or more quadrupole doublets. The fit using one quadrupole doublet should be considered as a rough approximation within the homogeneous iron core model, while the fit using more than one quadrupole doublet may be considered as the fit within the heterogeneous iron core model.

Mössbauer spectroscopy with a high velocity resolution, demonstrating substantially smaller instrumental errors than in the case of conventional spectrometers, was recently used to study the human liver ferritin and ferritin-like iron in chicken and human tissues. It showed the possibility to distinguish Mössbauer hyperfine parameters for the iron cores from different sources within the homogeneous iron core model, as well as to fit Mössbauer spectra much better within the heterogeneous iron core model [28–33]. Following this approach, we commenced a study of the ferritin-like iron in bacteria. In this work, we chose a rhizobacterium of the genus Azospirillum (for which, to the best of our knowledge, no studies on ferritin-like iron-containing components have so far been reported); in particular, the widely studied species Azospirillum brasilense (strain Sp245), which can form associations with roots of various higher plants, promoting their growth and development via phytohormone excretion, N₂ fixation, and other mechanisms [34]. We present here the results of a comparative study of A. brasilense (strain Sp245) biomass samples prepared in two different conditions and human liver ferritin using Mössbauer spectroscopy with a high velocity resolution.

Materials and methods

The bacterium A. brasilense Sp245 (from The Collection of Rhizosphere Microorganisms, [WDCM 1021], Institute of Biochemistry and Physiology of Plants and Microorganisms, RAS, Saratov) was cultivated at 31 °C for 18 h under aeration on a rotary shaker (180 rpm) in a standard phosphate–malate medium with 0.5 g l⁻¹ NH₄Cl as a source of bound nitrogen and 0.070 mM ⁵⁷FeIII–NTA complex (4.0 mg l⁻¹ ⁵⁷FeIII) as a sole source of iron. Then the cells were separated from the culture medium by centrifugation (2370g, 30 min) and washed 3 times with sterile saline solution (aqueous 0.85% NaCl). The resulting cell suspension (after the final centrifugation) was divided into two parts. One part was immediately rapidly frozen in liquid nitrogen and then lyophilized (sample 1). The other part was stored (in the form of the wet nutrient-free dense suspension, closed to prevent evaporation and drying) in the plastic sample holder of the Mössbauer spectrometer (see below) at room temperature for 3 days, then rapidly frozen in liquid nitrogen and lyophilized (sample 2). These two different preparation conditions (i.e., cultivation up to the end of the logarithmic growth phase (for 18 h) for sample 1, and the same with an additional storage for 3 days in a wet dense nutrient-free saline suspension for sample 2) were applied, as the additional stresses of dense culture and starvation (for sample 2) could be expected to induce some metabolic transformations in the living cells, possibly involving the iron-containing components.

A lyophilized normal human liver ferritin containing about 20 wt.% of bound iron (with natural abundance of ⁵⁷Fe) was obtained from the Russian State Medical University, Moscow, Russian Federation (the process of ferritin preparation was described elsewhere [35]). For the present study, lyophilized bacterial biomass and human liver ferritin were used as powders with sample weights of ~50 mg (bacterial sample 1), ~70 mg (bacterial sample 2) and ~100 mg (human liver ferritin). These powders were placed in Plexiglas sample holders with a diameter of 20 mm and a height of 5 mm and pressed with a Plexiglas cover to exclude vibrations of particles. The thicknesses of these samples were about 14–20 μg ⁵⁷Fe/cm² for the bacterial samples and about 5–6 mg Fe/cm² for ferritin.

Transmission electron microscopy (TEM) images of A. brasilense (strain Sp245) were obtained at the Institute of Biochemistry and Physiology of Plants and Microorganisms, RAS (Saratov), using a Libra 120 electron microscope (Carl Zeiss, Germany) at 80 kV. The washed cells were re-suspended in the saline solution up to the initial volume and placed onto nickel grids coated with formvar (1% formvar in dichloroethane).

Mössbauer spectra with a high velocity resolution were measured at the Ural Federal University (Ekaterinburg) using an automated precision Mössbauer spectrometric system built on the base of the SM-2201 spectrometer with a saw-tooth shaped velocity reference signal formed by a digital-analog convertor using quantification with 4096 steps. Details and characteristics of this spectrometer and the system are given elsewhere [36–38]. A ⁵⁷Co(Rh) source of about 1.8 × 10⁹ Bq (Ritverc GmbH, St. Petersburg, Russia) was used at room temperature. The standard absorber of sodium nitroprusside with a thickness of 5 mg Fe/cm² was used for calibration of the velocity scale. The line width of this spectrum was 0.229 ± 0.003 mm/s with the purely Lorentzian line shape.

The Mössbauer spectra of bacterial samples were recorded at 295 K and converted to 2048 channels by consequent summation of two neighboring channels to reach statistics of ~5.2 × 10⁶ counts per channel for sample 1 and ~6.4 × 10⁶ counts per channel for sample 2. The signal-to-noise ratio for the obtained spectra was 27 for sample 1 and 21 for sample 2. The Mössbauer spectrum of human liver ferritin was measured at 295 K in 4096 channels with statistics of ~2.7 × 10⁶ counts per channel and a signal-to-noise ratio of 95. All spectra were computer fitted with the least squares differential spectrum, ½, quadrupole splitting, ΔEQ, line width, I, relative subspectrum area, S, and statistical criterion, x², were evaluated. The criteria for choosing the best fits were differential spectrum, x² and the physical meaning of the spectral parameters. The instrumental (systematic) error
Fig. 2. Mössbauer spectra of the rhizobacterium *A. brasilense* (strain Sp245): samples 1 (a) and 2 (b) in comparison with Mössbauer spectrum of human liver ferritin (c) measured at 295 K with a high velocity resolution and presented in 2048 channels (a and b) and in 4096 channels (c). The indicated components (1–5) are the results of the best fits using the heterogeneous model for the iron core. The results of the rough approximation using a one quadrupole doublet fit for these spectra are shown on the right. The differential spectrum indicating the quality of the fit is given below each spectrum (the red dashed lines indicate the incompleteness of a one quadrupole doublet fit). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
for bacterial ferritin-like iron values for bac-
to 1.2). A typical TEM micrograph of an
logarithmic growth phase (18 h; optical density of the culture up
with a clearly distinguishable polar flagellum.

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shown in Fig. 1. This image represents the size and shape charac-
channels (shown for comparison in Fig. 2c). Considering the Mössbauer
spectrum of human liver ferritin measured at 295 K in 4096 chan-
look like Mössbauer spectra of ferritin-like iron and the Mössbauer
Fig. 2a and b. These spectra represent similar doublet shapes which
measured at 295 K and converted in 2048 channels are shown in

for each spectral point was ±0.5 of a channel (the velocity scale),
the instrumental (systematic) error for the hyperfine parameters
was ±1 channel, while that of the line width was ±2 channels.
The error of \( S \) did not exceed 10%. If an error calculated with the
fitting procedure (fitting error) for these parameters exceeded
the instrumental (systematic) error, we used the larger error
instead. Values of the isomer shifts are given relative to \( \alpha \)-Fe at
295 K.

Results and discussion

Cells of \( A. \) brasilense Sp245 were cultured up to the end of the
logarithmic growth phase (18 h; optical density of the culture up to
1.2). A typical TEM micrograph of an \( A. \) brasilense Sp245 cell is
shown in Fig. 1. This image represents the size and shape charac-
teristic of this bacterium (for the culturing conditions applied),
with a clearly distinguishable polar flagellum.

The Mössbauer spectra of lyophilized bacterial samples 1 and 2
measured at 295 K and converted in 2048 channels are shown in
Fig. 2a and b. These spectra represent similar doublet shapes which
look like Mössbauer spectra of ferritin-like iron and the Mössbauer
spectrum of human liver ferritin measured at 295 K in 4096 chan-
nels (shown for comparison in Fig. 2c). Considering the Mössbauer
spectra of bacterial samples as ferritin-like spectra, all the experi-
mental data were fitted in the frame of the two aforementioned
approaches used for the iron core analysis. Firstly, we used the
rough approximation for fitting the spectra with one quadrupole
doublet that corresponds to the homogeneous iron core model.
This approach allowed small differences in the values of Mössbauer
hyperfine parameters to be revealed in a simple way. The obtained
parameters were similar to the previously published data for bac-
terial ferritin and bacterioferritin (see [21–27]).

The plot of quadrupole splitting and isomer shift for bacterial
samples 1 and 2 and human liver ferritin obtained within the first
approximation is shown in Fig. 3. It was interesting to observe that
hyperfine parameters for both bacterial samples 1 and 2 were
noticeably different from those for human liver ferritin; the hyper-
fine parameters for bacterial samples 1 and 2 also demonstrated
small differences. Within the homogeneous iron core model this
fact indicates that the iron core structures are different for both
bacterial ferritin-like iron cores in samples 1 and 2 and for human
liver ferritin. The larger values of \( \Delta E_Q \) for bacterial ferritin-like iron
may be a result of a more amorphous core structure in comparison
with the iron core in human liver ferritin. It is known that bacterial
ferritin and bacterioferritin iron cores contain a larger amount of
inorganic phosphates than that in mammalian ferritin [2–4]. A
smaller value of \( \delta \) for human liver ferritin may indicate a less close
packing of Fe and O atoms in the core in comparison with bacterial
ferritin-like iron cores related to a slightly larger core size in mam-
mal ferritins. As for the small differences of \( \Delta E_Q \) values for bac-
terial ferritin-like iron in the bacterial samples 1 and 2, this may be
due to the differences in the preparation conditions which could
influence the iron core structure formed.

Then, the heterogeneous iron core model was applied to fit
Mössbauer spectra of the bacterial samples and human liver ferri-
tin, as fitting with one component shown in Fig. 2 (see right-hand
spectra) resulted in non-linear differential spectra. Moreover, it is
well known that Mössbauer spectra of ferric hydroxides have
non-Lorentzian line shape [39] and therefore can be better fitted
using a superposition of several components which may be related
to various regions/layers in the iron core (see [29–33]). The best
fits of the measured spectra were obtained using a new model with
several quadrupole doublets with equal line widths assuming the
absence of any significant structural variations within one certain
region/layer. As a result of this fit applied to the Mössbauer spectra
of bacterial samples 1 and 2, a superposition of 4 quadrupole
doublets related to ferritin-like iron was obtained. It should be noted
that in the spectrum of sample 1, an additional quadrupole doublet
(component 5) was revealed related to a ferrous compound (all the
Mössbauer parameters are given in Table 1). Ferrous components

\[ \begin{align*}
\text{Sample} & \quad \text{No.}^a \quad \delta \text{ (mm/s)} \quad \Delta E_Q \text{ (mm/s)} \quad \Gamma \text{ (mm/s)} \quad S \text{ (%)} \\
\text{Bacterial sample 1} & \quad 1 \quad 0.393 \pm 0.004 \quad 0.433 \pm 0.014 \quad 0.311 \pm 0.008 \quad 29.46 \\
& \quad 2 \quad 0.387 \pm 0.004 \quad 0.731 \pm 0.017 \quad 0.311 \pm 0.008 \quad 33.41 \\
& \quad 3 \quad 0.389 \pm 0.004 \quad 1.068 \pm 0.019 \quad 0.311 \pm 0.008 \quad 30.32 \\
& \quad 4 \quad 0.405 \pm 0.008 \quad 1.454 \pm 0.028 \quad 0.311 \pm 0.008 \quad 20.71 \\
& \quad 5 \quad 0.825 \pm 0.019 \quad 3.067 \pm 0.039 \quad 0.311 \pm 0.008 \quad 2.81 \\
\text{Bacterial sample 2} & \quad 1 \quad 0.394 \pm 0.004 \quad 0.490 \pm 0.011 \quad 0.343 \pm 0.010 \quad 43.29 \\
& \quad 2 \quad 0.385 \pm 0.004 \quad 0.816 \pm 0.014 \quad 0.343 \pm 0.010 \quad 35.19 \\
& \quad 3 \quad 0.399 \pm 0.006 \quad 1.159 \pm 0.051 \quad 0.343 \pm 0.010 \quad 17.03 \\
& \quad 4 \quad 0.385 \pm 0.021 \quad 1.501 \pm 0.102 \quad 0.343 \pm 0.010 \quad 4.49 \\
\text{Human liver ferritin} & \quad 1 \quad 0.373 \pm 0.002 \quad 0.377 \pm 0.002 \quad 0.269 \pm 0.003 \quad 19.45 \\
& \quad 2 \quad 0.373 \pm 0.002 \quad 0.390 \pm 0.002 \quad 0.269 \pm 0.003 \quad 35.33 \\
& \quad 3 \quad 0.367 \pm 0.002 \quad 0.810 \pm 0.002 \quad 0.269 \pm 0.003 \quad 26.53 \\
& \quad 4 \quad 0.362 \pm 0.002 \quad 1.071 \pm 0.005 \quad 0.269 \pm 0.003 \quad 14.45 \\
& \quad 5 \quad 0.360 \pm 0.002 \quad 1.385 \pm 0.008 \quad 0.269 \pm 0.003 \quad 4.25 \\
\end{align*} \]

\* The numbers of components correspond to those given in the Mössbauer spectra in Fig. 2.

Fig. 3. Small differences in Mössbauer hyperfine parameters for human liver ferritin and two samples of the rhizobacterium \( A. \) brasilense (strain Sp245) obtained using a homogeneous iron core model (one quadrupole doublet fit): \( \Box \) sample 1, \( \smallcirc \) sample 2, \( \triangle \) human liver ferritin.
with similar Mössbauer parameters were also observed, for instance, in [23,24,26,27,40–43]. However, the amount of the ferrous compound in sample 1 was significantly smaller than that in the mentioned studies. Those ferrous compounds were related to Fe(II) bound to proteins or to other Fe(II) complexes, which exhibited hyperfine parameters closer to those for component 5 (see sample 1, Table 1). If component 5 can be related to such Fe(II) complexes, the difference in the $\delta$ values for our measurement at 295 K and previous measurements at low temperatures may be a result of the second order Doppler shift.

The result of the best fit of the Mössbauer spectrum of human liver ferritin demonstrated the presence of a superposition of 5 quadrupole doublets (previously, using a consistent model with almost the same subspectral areas for the corresponding components, at each of two different temperatures we obtained a superposition of 4 quadrupole doublets [33]). Five components in the best fit for the Mössbauer spectrum of human liver ferritin can be associated with five regions/layers of the iron core. These regions/layers may vary from the surface shell with the largest $\Delta E_Q$ value, related to the most amorphous ferrihydrite structure, to the most internal ferrihydrite region with the smallest $\Delta E_Q$ value related to the highest degree of crystallinity and atomic packing density. As the number of quadrupole doublets related to the ferritin-like iron was found to be different in the best fits of human liver ferritin and bacterial samples 1 and 2, this fact may be related to different sizes of the iron cores, as well as to a different number of iron atoms in mammalian ferritin and in both bacterial ferritin and bacterioferritin.

It was also of interest to compare Mössbauer parameters for the spectra of bacterial samples 1 and 2. Despite the fact that the number of components was the same in the fitting model, the Mössbauer parameters appeared to be different; for instance, the values of relative areas for identical components shown in Fig. 4 (for sample 1, the relative areas for ferritin-like spectral components were recalculated accounting for the presence of the additional ferrous component in the spectrum). It is well known that in the case of the same Mössbauer effect probability ($f$-factor) for different components, the relative areas of these components are proportional to the number of $^{57}$Fe nuclei in the corresponding components. Therefore, if we suppose the same $f$-factor for all regions/layers in the iron cores of both bacterial ferritin-like iron in samples 1 and 2, the differences in the $S$ values for the corresponding components indicate the different number of iron atoms in the corresponding regions/layers of the iron cores in these samples. It should be also pointed out that the values of $\Delta E_Q$ for the components 1–3 appeared to be slightly different beyond the error.

This fact can be explained as a result of some small variations in the degree of crystallinity in the corresponding regions/layers of the core, while those for component 4 in both bacterial samples 1 and 2 were within the calculated error. The observed differences evidently reflect variations in the processes of the iron core formation related to the different preparation conditions.

**Conclusion**

A comparative study of two biomass samples of the rhizobacterium *A. brasilense* (strain Sp245) prepared under different conditions and human liver ferritin by Mössbauer spectroscopy with a high velocity resolution was carried out at room temperature. The results of this study demonstrated that the bacterial spectra featured the presence of ferritin-like iron in the cells comparable with the ferritin spectrum and with some previously published results of Mössbauer studies of bacterial ferritin and bacterioferritin from other microorganisms. Therefore, the measured Mössbauer spectra were fitted in two ways on the basis of a rough approximation using a one quadrupole doublet fit (the homogeneous iron core model) and a superposition of several quadrupole doublets (the heterogeneous iron core model). In both cases, the differences between bacterial ferritin-like iron and human liver ferritin, as well as between two different bacterial samples were obtained. These differences were ascribed to the difference in the mammalian and bacterial ferritin iron core size and in the number of iron atoms in the core, as well as to the different conditions of the bacterial preparation procedure used, which could influence the iron core formation process in the resulting two bacterial samples.

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