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A Mössbauer spectroscopy study of cellular acquisition of iron from pyoverdine by *Pseudomonas aeruginosa*

Eugenie V. Mielczarek[1], Paulette W. Royt[2], and Jean Toth-Allen[1,2]

Departments of [1] Physics and [2] Biology, George Mason University, Fairfax, VA 22030, USA

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Summary. Mössbauer spectroscopy was used to investigate the cellular acquisition of iron by *Pseudomonas aeruginosa* which had been incubated with ferripyoverdine for 20, 40, 60, 120 or 360 min. Studies revealed that no ferripyoverdine accumulated in the cells at any of these times and that the amounts and kinds of iron complexes produced by cellular metabolism vary with time. At 20 and 40 min a ferric species, with isomer shift $\delta = 0.38$–0.42 mm/s and quadrupole splitting $\Delta E_Q = 0.94$–0.92 mm/s, was the major iron metabolite comprising approximately 80% of the iron. At later times at least three other ferric species appeared with $\delta = 0.54 \rightarrow 0.72$, $\Delta E_Q = 0.84 \rightarrow 1.07$ mm/s. Ferrous species, $\delta = 1.43 \rightarrow 1.77$ mm/s and $\Delta E_Q = 2.69 \rightarrow 1.82$ mm/s, were also seen at times as early as 20 min and comprised as much as 17% of the total iron at 20 and 40 min. The parameters of all these species identify them as being six-coordinated high-spin complexes. In addition a low-spin species, $\delta = 0.19$ mm/s $\Delta E_Q = 0.67 \rightarrow 0.91$ mm/s, never before reported in cells, appeared at 60, 120, and 360 min as one of the major iron metabolites (50% or more). All isomer shifts are measured with respect to natural iron.

Key words: Mössbauer spectroscopy – Siderophore – Ferripyoverdine – Iron – *Pseudomonas aeruginosa*

Introduction

Siderophores are low-molecular-mass compounds which chelate iron and make it available for cellular requirements (Winkelmann et al. 1987 and references therein). Two of the major questions regarding cellular transport of iron are: does the iron-siderophore complex accumulate in the cell intact, providing an iron reserve for other cellular complexes, or is the iron released at the cell membrane or in the cytoplasm or periplasm? The siderophore pyoverdine is associated with a high-affinity iron-uptake system in pseudomonads (Meyer et al. 1987). This paper reports on Mössbauer spectroscopy studies of *Pseudomonas aeruginosa* which had been incubated with ^{57}Fe-labeled ferripyoverdine for varying lengths of time. Because Mössbauer spectroscopy measures the hyperfine parameters of only the ^{57}Fe nucleus, these studies provide definite answers to many questions. Does ferripyoverdine accumulate in the cell? Is this accumulation time-dependent? If there is metabolism, is the formation of the types of complexes and their oxidation states time-dependent?

Mössbauer spectroscopy has been used to study siderophore-mediated iron uptake and intracellular use of iron in several fungal and bacterial species (Matzanke et al. 1987). Results of these studies have shown that cellular systems differ in their use of siderophore-transported iron. For example, the major extracellular siderophore produced by *Neurospora crassa* is coprogen. Mössbauer spectra of *N. crassa* incubated with [^{57}Fe] ferricoprogen for 1 h show that 85% of the iron in these cells remains as ferricoprogen, that is the ferricoprogen accumulates in the cell. Even 4 h after removal of exogenous ferricoprogen, only 32% of the iron chelated to ferricoprogen has been shunted to other complexes (Matzanke et al. 1987a, b). In other studies with *N. crassa* it was found that intracellular exchange of iron occurred between ferricoprogen and ferricrocin, another ferric siderophore of *N. crassa*, suggesting that ferricrocin may act as an iron-storage complex (Matzanke et al. 1988). Similarly, the iron complexes of the siderophores produced by *Aspergillus ochraceus*, ferrichrysin and ferrirubin, also accumulate in spores and comprise about 74% of the total iron (Matzanke et al. 1987b). *Escherichia coli* K12 RW193 is unable to synthesize enterobactin, the major siderophore of this species. When iron-starved cells were incubated with ferrienterobactin for 20 min, this strain of cells showed an accumulation of ferrienterobactin which amounted to 66% of the cellular iron (Matzanke et al. 1989a). However wild-type cells of *E. coli* which were incubated with ferricrocin did not accumulate ferricrocin (Matzanke et al. 1989b). In studies of the uptake of ferrichrome, an ex-

Offprint requests to: E. V. Mielczarek

tracellular siderophore synthesized by *Ustilago sphaerogena,* a 'reductive iron taxi mechanism' was identified, i.e. the iron was reduced and released at the exterior surface of the cell membrane (Ecker et al. 1982). Our studies in *P. aeruginosa* were undertaken to discover if its extracellular iron-chelated siderophore, ferripyoverdine, accumulates in the cell and to determine the presence of metabolized iron compoounds.

Materials and methods

Sample preparation. ^{57}Fe-labeling and cell-growth procedures have been described (Mielczarek et al. 1990). In these studies cells were grown in casamino acids medium to late-logarithmic phase, harvested, washed, and resuspended in fresh casamino acids medium containing [^{57}Fe]ferripyoverdine (1 mg/l medium). Samples were collected at 20, 40, 60, 120 and 360 min. The doubling time of these cells is approximately 1 h. It is not possible to distinguish precisely between the time during which the cell is metabolizing in an iron-starved condition in contrast to an iron-fed condition. However initially, i.e. especially at 20 min, because the cells were grown in iron-deficient medium they are iron-starved. However at later times, because the cells are growing in the presence of ferripyoverdine, the cells are probably iron-fed.

Mössbauer spectrocsopy studies. After centrifugation, the samples were placed into a high-density linear polypropylene vial sealed with a low-temperature gasket which was compressed by an aluminum ring and quick-frozen in liquid nitrogen. All samples were stored under liquid nitrogen until measurement and then quickly mounted into a liquid-nitrogen-cooled cryostat. The Mössbauer source was 25 mCi ^{57}Co diffused onto a palladium foil (Dupont New England Nuclear). The emitted gamma rays were collimated to an 8-mm-diameter beam.

Results and discussion

Figure 1 shows the Mössbauer spectrum of ferripyoverdine as a function of temperature. This sample was prepared by diluting 19 mg dry [^{57}Fe]ferripyoverdine with 0.1 M pyridine/acetic acid pH 6.5 to a final volume of 0.2 ml and quick-freezing in liquid nitrogen. In addition to these measurements, two other samples were measured: 19 mg dry powder and a sample diluted in 0.1 M Mops pH 7.2. All three spectra were identical. The spectrum is best described as a broad central line with two pairs of outer lines. This type of spectrum is characteristic of many siderophores (Matzanke 1987). Two features of the temperature dependence can be noted. First, as T increases the lines broaden and the resonant intensity of the center of the spectrum increases markedly; second, the line shape of the spectrum at 249 K is not Lorentzian. Ferricoprogen and ferrichrome A exhibit similar spectra. The physical basis for these spectra is understood and in particular the spectrum of ferrichrome A has been extensively investigated by Wickman et al. (1966) and more recently by Hoy et al. (1983). Unpaired electron spins create an internal magnetic field. This would ordinarily lead to a six-line hyperfine spectrum as is seen in the spectra of metallic iron. However, if the fluctuation rate of the spin direction (spin-flip process) is fast compared with

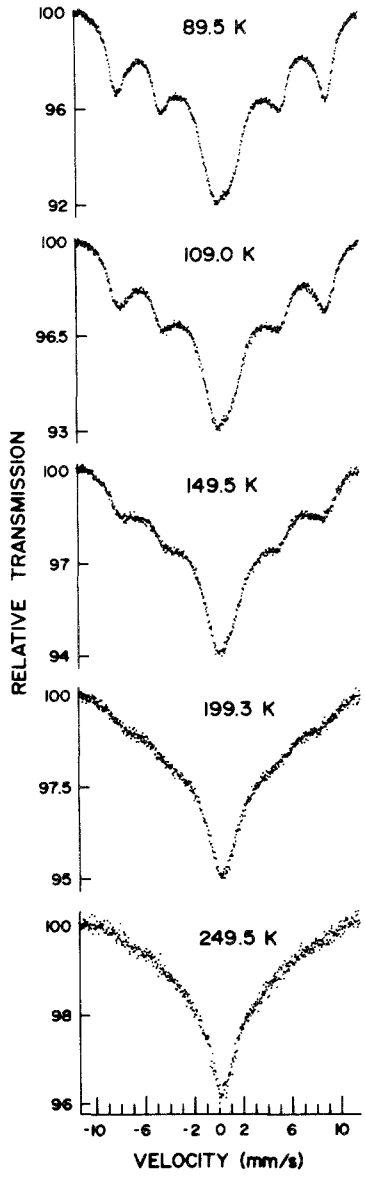

Fig. 1. The Mössbauer spectra of ferripyoverdine as a function of temperature. [Reprinted with permission from Comments on Molecular and Cellular Biophysics (Mielczarek et al. 1989)]

the Larmor precession time of the iron nucleus, the nucleus will sample a magnetic field averaged to zero and no hyperfine field splitting of the spectra will be observed. For ferrichrome A a six-line spectrum can be clearly resolved at 1 K. However at 91 K the spectrum for ferrichrome A has collapsed to a wide non-Lorentzian line. Hoy et al. (1983) calculated a relaxation rate for ferrichrome A of $1.35 \times 10^7\ s^{-1}$. Since the hyperfine spectrum for ferripyoverdine is better resolved than that of ferrichrome A at the same temperature, it is likely that the relaxation time (the reciprocal of the relaxation rate) for the spin-flip processes is longer in ferripyoverdine than in ferrichrome A. The separation of the outermost lines in the spectrum gives a measure of the internal magnetic field. For all siderophores which have been studied to date the value of the internal field is about 55 T (Matzanke 1987). The internal field in fer-

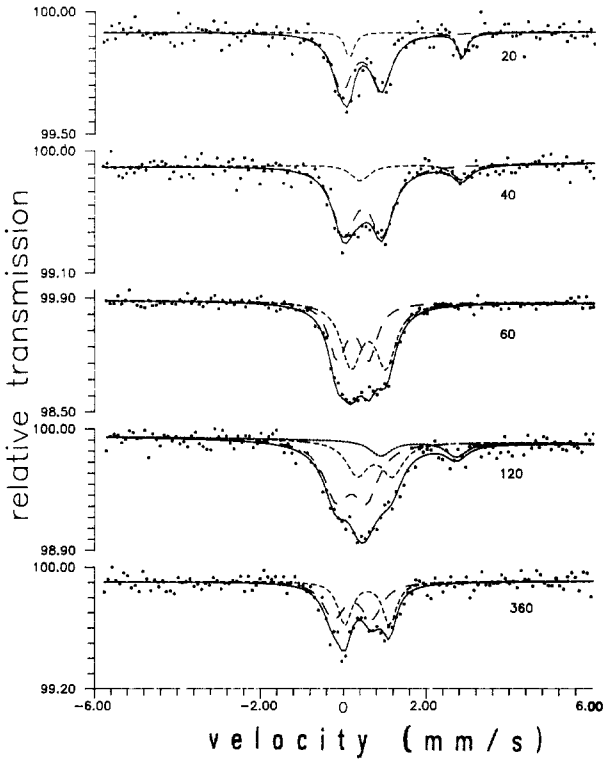

Fig. 2. The Mössbauer spectra of whole cells of *Pseudomonas aeruginosa* at 90 K which had been incubated with ferripyoverdine for 20, 40, 60, 120 and 360 min. Ferrous subspectra can be seen in the 20-min, 40-min, and 120-min graphs. The solid line in each graph is the sum of all the subspectra

ripyoverdine is 52 T. If ferripyoverdine accumulates in the cell, there should be evidence of this wide-line spectrum in whole cells which have been incubated with ferripyoverdine.

Figure 2 shows the Mössbauer spectra of *P. aeruginosa* incubated with ^{57}Fe-labeled ferripyoverdine for varying lengths of time. As described in the Introduction, ferrisiderophores may serve as intracellular iron-storage molecules ensuring that required levels of intracellular iron are maintained. By studying cells which were incubated with ferripyoverdine for times less than, equal to and greater than one generation time, we ensured that the cell population proceeded from iron-starved to iron-fed conditions. It is obvious from Fig. 2 that no ferripyoverdine accumulated in the cell at any of these times. There is no evidence of the wide-line relaxation spectrum which characterizes the ferripyoverdine spectrum such as is shown in Fig. 1. Instead these spectra are characterized by quadrupole split lines with separations not exceeding 2.4 mm/s. Uptake experiments with double-labeled [^{14}C, ^{55}Fe]ferripyoverdine described in the preceding paper provide additional evidence for lack of accumulation of ferripyoverdine (Royt 1990).

The iron complexes contributing to the spectra shown in Fig. 2 have been created by cellular metabolism. Table 1 lists the Mössbauer parameters of these spectra. The information divulged by the isomer shift, δ, is related to the s-electron density at the nucleus of the iron ion. Thus, to a first approximation, a change in the isomer shift reflects small changes in valence state of the iron. The magnitude of the quadrupole splitting, ΔE_Q, reflects the asymmetry of the electric field around the iron. The value of the isomer shift and quadrupole splitting can identify the valence of the iron ion for high-spin species. However the identification of the valence state for low-spin species can be difficult. The general aspects of this data which are interesting are: (a) a high-spin six-coordinated ferric species is found at all times; (b) at 20 and 40 min this species was the major iron metabolite comprising 80% of the total cellular iron; (c) in addition, cells which were measured at incubation times longer than 40 min contain a low-spin species (isomer shift of 0.15→0.19 mm/s); (d) this low-spin species contributed about 50% of the spectrum at each time; (e) ferrous species are resolvable from the 20-min,

Table 1. Mössbauer parameters for whole cells of *Pseudomonas aeruginosa* incubated with ferripyoverdine

Incubation time (min)	Species	δ (mm/s)	ΔE_Q (mm/s)	Γ (mm/s)	Area (% total)
20	I	0.38 (0.02)	0.94 (0.03)	0.57 (0.06)	83
	II[a]	1.43 (0.03)	2.69 (0.06)	0.22 (0.05)	17
40	I	0.43 (0.02)	0.92 (0.02)	0.64 (0.05)	83
	II[a]	1.57 (0.06)	2.43 (0.09)	0.51 (0.08)	17
60	I[b]	0.19 (0.02)	0.76 (0.04)	0.50 (0.06)	43
	II	0.57 (0.01)	0.85 (0.01)	0.58 (0.03)	57
120	I[b]	0.15 (0.03)	0.67 (0.07)	0.71 (0.07)	59
	II	0.72 (0.03)	0.84 (0.01)	0.63 (0.07)	29
	III[a]	1.77 (0.08)	1.82 (0.20)	0.61 (0.02)	11
360	I[b]	0.19 (0.04)	0.91 (0.02)	0.59 (0.10)	57
	II	0.54 (0.02)	1.07 (0.01)	0.39 (0.05)	43

δ, Isomer shift with respect to natural iron; ΔE_Q, quadrupole splitting; Γ, full width at half maximum

[a] Ferrous species

[b] Low-spin species

40-min and 120-min spectra comprising 17% (20 and 40 min) and 11% (120 min) of the total amount of iron present in the cell at these times; (f) the Mössbauer parameters of these ferrous species identifies them as also being high-spin six-coordinated complexes. From Table 1 it can be seen that the low-spin complex which is present at 60, 120 and 360 min is probably the same complex. The isomer shifts of the low-spin complexes at each of these times match to within experimental error; the quadrupole splitting of these complexes also matches to within experimental error for the 60-min and 120-min species. There is a 10% shift in the quadrupole splitting of this complex at 360 min which is probably not significant. This low-spin complex appears to be the most persistent of all the complexes seen in the cell. The high-spin ferric complex seen at 20 min is still present at 40 min. But the fact that the high-spin ferric complexes at 60, 120 and 360 min are different indicates iron metabolism. Another indication of iron metabolism is the fact that all the ferrous complexes seen are different.

A comparison of the parameters of all species with those of previously identified common cellular complexes such as hemes, cytochromes, iron-sulfur proteins and bacterial ferritins yields no matches within experimental error. Matzanke et al. (1987c, 1989a, 1989b) have been forced to draw the same conclusion from their studies of the parameters of complexes found in whole cells of *E. coli* and *N. crassa*. They propose that the two main metabolic iron components which comprise the spectra in their cells are of general cellular importance. In the spectra which we report here only one of the ferric metabolites matches the metabolites designated by Matzanke et al. (1989a) as 'novel components', the ferric species seen at an incubation time of 40 min. The parameters of this species, $\delta = 0.43$ (0.02) mm/s and $\Delta E_Q = 0.92$ (0.02) mm/s, matches that designated by Matzanke et al. as 'novel', $\delta = 0.43$ (0.02) mm/s and $\Delta E_Q = 1.00$ (0.02) mm/s, to within a reasonable error. The appearance of a low-spin complex at all incubation times greater than 40 min has not previously been reported. To date no other published cellular Mössbauer studies have revealed such a complex. A search of the Mössbauer literature of organic complexes produced one match, an iron coordinated to six nitrogens in a Schiff-base ligand (Nelson et al. 1979). Also Fluck (1968; complexes 8 and 12 in Table 4.6) reports values of isomer shift, $\delta = 0.19$–0.23 mm/s, and quadrupole splitting, $\Delta E_Q = 0.76$–0.90 mm/s, for two low-spin planar complexes where the iron is coordinated to oxygens and NO. More recently, St. Pierre and coworkers have seen a low-spin complex in melanoma cells cultured in an iron-rich environment (T. St. Pierre, personal communication).

At the designated times aliquots of cells were also separated into a soluble fraction and inner membranes, and Mössbauer spectra measured (Mielczarek et al. 1990). Two species seen in the whole-cell samples can also be identified in the membrane and soluble fraction samples. The ferric complex seen at 20 min of incubation in the whole-cell sample matches that present in the soluble fraction at 20 min, $\delta = 0.38$ (0.02) mm/s and $\Delta E_Q = 0.94$ (0.03) mm/s, and the low-spin complex seen in these whole cells at 60, 120 and 360 min is found on the inner membrane at an incubation time of 120 min (Mielczarek et al. 1990). However other complexes which are seen in the whole-cell spectra are not seen in the spectra of the soluble fraction and inner membrane samples. There could be several reasons: (a) loss of loosely bound complexes from the membrane during separation of the soluble fraction from the membrane; (b) continued iron metabolism during the separation procedure at times when the cells cannot be held at liquid nitrogen temperature or (c) complexes found on the membrane and in the soluble fraction may be oxidized or reduced forms of compounds seen in the whole cells.

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