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Role of Siderophores in Iron Storage in Spores of *Neurospora crassa* and *Aspergillus ochraceus*

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Spores of *Neurospora crassa* 74A are lacking in ferritinlike iron pools, as demonstrated by Mössbauer spectroscopic analysis. The cyclic hexapeptide siderophore ferricrocin constituted 47% of the total iron content in spores. After germination and growth, the ferricrocin iron pool disappeared, indicating that the metal was utilized. In spores of *Aspergillus ochraceus*, 74% of the total iron content was bound by ferrichrome-type siderophores. Siderophores may function as iron storage forms in fungal systems.

With few exceptions (1, 10), iron is an essential growth factor for virtually all organisms. Under low-iron-stress growth conditions, microorganisms excrete iron-complexing agents with low molecular masses (500 to 1,500 daltons), called siderophores, which sequester this biologically precious metal (16). Inside the cell, metabolically inactive iron is generally transferred to storage compounds. The main iron storage proteins in animals and plants are ferritins and phytoferritins, respectively (6, 7). Ferritin-type structures have also been observed in various fungal systems like *Mortierella alpina* (5) and *Phycomyces blakesleeanus* (8). In contrast, different structural features are found in bacterial iron storage proteins (2–4, 9, 18). Mössbauer spectroscopic investigations of the iron metabolism in *Neurospora crassa arg-5 ota aga* indicate a lack of ferritinlike structures in this organism (14, 15). However, a component has been detected at a relatively late state of growth which probably represents an iron storage protein exhibiting Mössbauer spectroscopic features similar to those of bacterial iron storage compounds (15). Moreover, it could be demonstrated that siderophores can function as intermediate intracellular storage compounds in mycelia (14, 15).

Little is known about iron storage in fungal spores. Spores of *P. blakesleeanus* possess ferritinlike iron pools, as could be shown by Mössbauer spectroscopy (17). In *N. crassa*, siderophores have been shown to be essential for spore germination (11). In this study, we applied in vivo Mössbauer spectroscopy to obtain information on both the role of siderophores and the existence of iron pools in spores of *N. crassa* and *Aspergillus ochraceus*. This investigation provides evidence for an iron storage function of siderophores in fungal spore systems.

Preparation of ^{57}Fe complexes. Isolation, purification, and preparation of desferri-ferricrocin were done as described elsewhere (19). Ferrichrysin and ferrirubin were isolated from *A. ochraceus* (13). The ^{57}Fe(III) stock solution was obtained by dissolving ^{57}Fe purchased from Rohstoffeinfuhr, Düsseldorf, Federal Republic of Germany (^{57}Fe enrichment, 95%), in a small volume of HNO_3-HCl (1:2 [vol/vol]). The pH of the solution was adjusted to 1.0 with KOH. Synthesis of [^{57}Fe]ferricrocin was achieved by mixing equimolar solutions of ^{57}Fe(III) and aqueous desferri-ferricrocin. The pH was quickly adjusted to 6. The mixture was stirred overnight to complete the reaction. The red-brown reaction product was passed through an XAD-2 column (Serva, Heidelberg, Federal Republic of Germany), and the purity was checked by thin-layer chromatography on silica gel by using a 4-day-old solution of solvent system 1 (chloroform-methanol-water [35:12:2]) and a solution of solvent system 2 (*n*-butanol-acetic acid-water [4:1:1]). R_f values were 0.45, 0.43, and 0.32 for ferricrocin, ferrichrysin, and ferrirubin for solvent system 1, respectively. The R_f values for solvent system 2 were 0.09, 0.11, and 0.25 for ferricrocin, ferrichrysin, and ferrirubin, respectively. (For comparison, see reference 13.) A 10 mM [^{57}Fe]ferric citrate stock solution was prepared from a mixture of ^{57}Fe(III) and citric acid (molar ratio, 1:10).

Strains and media. *N. crassa arg-5 ota aga*, which can be grown siderophore-free if ornithine is omitted from the growth medium, and the corresponding wild-type strain *N. crassa* 74A were a gift from R. H. Davis, Irvine, Calif., and *A. ochraceus* gold was generously supplied by D. van der Helm, Norman, Okla. Strains were maintained on YMG agar containing 4 g of yeast extract (Difco Laboratories, Detroit, Mich.), 10 g of malt extract (Oxoid Ltd., London, England), and 4 g of glucose per liter. Harvesting of conidiospores has been described elsewhere (19). ^{57}Fe-enriched conidiospores were harvested from YMG agar cultures with 50 μM ^{57}Fe added as the citrate complex. The composition of chemically defined low-iron agar was the same as for the liquid cultures (12, 19). ^{57}Fe-enriched spores (2×10^7) were used to inoculate 100 ml of chemically defined low-iron medium (19). The spores were incubated at 27°C in a rotary shaker at 120 rpm for 12 h.

Extraction of siderophores from spores. Conidia were washed with saline and passed through a glass-wool filter to remove hyphal fragments. The spores were suspended in chloroform-methanol-water (10:20:1) and stirred at 40°C on a water bath. The supernatant fluid was concentrated to dryness and redissolved in methanol. This procedure was repeated four times to remove proteins. Then the soluble material was redissolved in water and chromatographed on XAD-2. The compounds were identified by thin-layer chromatography with solvent systems 1 and 2, as described above.

Sample preparations and Mössbauer measurements. ^{57}Fe-containing spores and young mycelia were washed and

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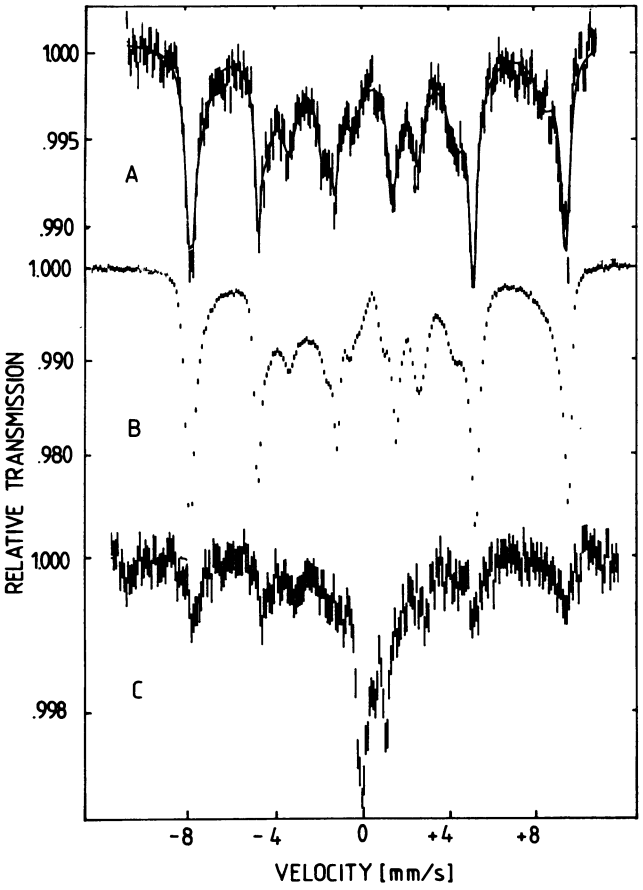

FIG. 1. Mössbauer spectra of ^{57}Fe-siderophores (A and B) and spores of *N. crassa* 74A (C) enriched with ^{57}Fe. Spectra in panels A and B represent frozen aqueous solutions of 2 mM ferricrocin and 3.5 mM ferrichrysin, respectively, diluted with 200 mg of bovine serum albumin per ml. The solid line in the spectrum in panel A results from least-square fits with 14 Lorentzians to obtain a theoretical envelope spectrum which was later stripped from the measured spore spectra. After 2 weeks of growth, the spores were harvested from a complex agar medium containing 50 μM ^{57}Fe as a citrate complex (citrate at 10-fold excess). The measurements were performed at 4.2 K in a magnetic field in which H_{app} = 20 mT perpendicular to the γ ray.

transferred to Delrin (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) Mössbauer sample holders. All sample volumes were about 0.75 ml. Sample thickness did not exceed 9 mm. The containers were quickly frozen in liquid nitrogen and kept in a liquid nitrogen storage vessel until measurement was done. Samples of [^{57}Fe]ferricrocin were prepared as lyophilized powder and as an aqueous solution diluted with bovine serum albumin (200 mg/ml). Samples of frozen aqueous solutions of [^{57}Fe]ferrichrysin and [^{57}Fe]ferrirubin were similarily prepared. Mössbauer spectra were recorded at 4.2 K in the horizontal transmission geometry with a constant acceleration spectrometer operated in conjunction with a 512-channel analyzer in the time scale mode. The source was at room temperature and consisted of 50 mCi of ^{57}Co diffused in Rh foil. A typical run of ^{57}Fe-enriched spores and cells lasted about 80 h. Isomer shifts were given relative to α-iron at room temperature. The calibration spectra exhibited a typical line width of 0.24 mm s^{-1}. The siderophore subspectra were stripped from the measured spectra by using the absorption pattern of the bovine serum albumin-diluted aqueous solution of ferricrocin. To keep the statistical scattering of stripped spectra as low as possible, we avoided using the experimental ferricrocin spectrum in the stripping procedure. Rather, we employed theoretical envelopes of the siderophore spectrum obtained by a nonphysical fit with 14 individual Lorentzians (15).

Growth on low-iron minimal medium agar. To prevent accumulation of Mössbauer-silent ^{56}Fe, we initially attempted to obtain conidiospores from low-iron cultures. However, poor conidiation of *N. crassa arg-5 ota aga* was observed in chemically defined agar cultures. Addition of ferric citrate, siderophores, or multivitamin solutions did not stimulate sporulation. Unknown nutritional factors probably contributed to the induction of fungal sporulation. Conidiation of wild-type *N. crassa* 74A and of *A. ochraceus* was very poor under the same conditions. Therefore, we harvested ^{57}Fe-enriched spores from agar cultures grown on yeast and malt extract, with ^{57}Fe added as ferric citrate. Fortunately, accumulation of ^{57}Fe was high enough to yield Mössbauer spectra of acceptable intensity (see Fig. 1 and 2).

Analysis of the Mössbauer spectra. The magnetic spectra of siderophores in frozen aqueous solutions at 4.2 K were not well resolved, indicating residual spin-spin relaxation because of interaction of neighboring siderophores (15). Such spectra were not suitable for comparison with spectra of cells and spores. Since the cell fluids were rich in protein, the siderophore molecules were magnetically diluted, which slowed down spin-spin relaxation. To simulate this situation, we mixed bovine serum albumin with aqueous solutions of ferricrocin (Fig. 1A), ferrichrysin (Fig. 1B), and ferrirubin (data not shown). The Mössbauer spectra of these three siderophores were very similar. The envelope of the ferricrocin pattern (solid line in Fig. 1A) was used to subtract the siderophore subspectrum from the complex spore spectra. Ferrichrysin and ferrirubin are the main siderophores of *A. ochraceus*. These two ferrichrome-type siderophores differ from ferricrocin in the cyclic hexapeptide backbone or the C-terminal residues of the hydroxamate ligands. However, line positions and overall splittings were the same for all three ferrichrome-type siderophores. Thus, Mössbauer spectroscopy can be used to determine the content of ferrichrome-type siderophores, but differentiation of different ferrichromes cannot be achieved. Mössbauer spectroscopy enables a facile discrimination between siderophores and ferritin and other iron metabolites, since temperature and concentration dependencies, as well as overall splittings, are different.

For the spectrum shown in Fig. 1C, spores of *N. crassa* 74A were used. Three components can be discerned directly by inspection of the spectrum: a magnetically split species which spans from −8 to +9.5 mm s^{-1} and two components which are between 0 and +1 mm s^{-1}. The magnetically split subspectrum does not fit ferritins or bacterial iron storage proteins. In particular, the internal magnetic fields differ considerably: they are on the order of 48 to 49 T for ferritins and 43 T for bacterial iron storage proteins, whereas the value for the magnetic species observed in the spores of *N. crassa* is 56 T. Moreover, bacterial iron storage proteins isolated from *Escherichia coli* and *Mycoplasma capricolum* are not magnetically split at 4.2 K. Horowitz et al. (11) have identified ferricrocin in spores of *N. crassa*. In fact, it turned out that the magnetic species was easily stripped from the measured spore spectrum in Fig. 1C by using the theoretical envelope of the ferricrocin spectrum (Fig. 1A). Therefore, this species exhibits the same spectroscopic features as does

ferricrocin. The contribution of this component to the total absorption area is 47%. To confirm the spectroscopic result, we isolated the siderophore from spore extracts. In solvent systems 1 and 2, the R_f values of the siderophore isolated from spores were identical to those of ferricrocin. In addition, a minor component could be detected by thin-layer chromatography of *N. crassa* extracts which probably corresponds to ferrichrome C (which was already reported by Horowitz et al. [11]).

After subtraction of the ferricrocin spectrum (Fig. 1A) from the spore spectrum (Fig. 1C), the residual spectrum (Fig. 2A) was fitted (Fig. 2A) by Lorentzians: $\delta_1 = 0.13$ mm s^{-1}, $\Delta E_{Q1} = 0.65$ mm s^{-1}, $\Gamma_{1/2} = 0.46$ mm s^{-1}, 20.9% of total absorption area; $\delta_2 = 0.50$ mm s^{-1}, $\Delta E_{Q2} = 1.09$ mm s^{-1}, $\Gamma_{1/2} = 0.45$ mm s^{-1}, 32.1% of total absorption area. The Mössbauer parameters of these two spectral components were not consistent with those reported for bacterial iron storage proteins ($\delta \approx 0.51$ mm s^{-1}, $\Delta E_Q \approx 0.65$ mm s^{-1}) (3, 9, 18). Thus, from the spectral analyses, we have to conclude that spores of *N. crassa* do not contain ferritins or bacterial iron storage proteins.

To investigate the role of ferricrocin, we followed the course of germination of ^{57}Fe-enriched conidiospores. A spectrum is shown (Fig. 2B) of young mycelia harvested after 12 h of growth from a low-iron mineral salt medium which was inoculated with ^{57}Fe-enriched spores. The spectrum exhibits two components of which the Mössbauer parameters ($\delta_1 = 0.17$ mm s^{-1}, $\Delta E_{Q1} = 0.64$ mm s^{-1}, $\Gamma_{1/2} = 0.33$ mm s^{-1}, 32.7% of total absorption area; $\delta_2 = 0.53$ mm s^{-1}, $\Delta E_{Q2} = 1.01$ mm s^{-1}, $\Gamma_{1/2} = 0.40$ mm s^{-1}, 67.3% of total absorption area) are very similar to those found in Fig. 2A. However, no magnetically split component could be detected. We assume that the lack of ferricrocin in young mycelia germinated under low-iron-stress growth conditions is caused by the consumption of the iron pool. This is an indication that the siderophore ferricrocin may serve as an iron storage peptide in spores of *N. crassa*. In iron-deficient cultures of *N. crassa*, we could demonstrate that ferricrocin uptake yields very poor metabolization of iron, whereas coprogen-bound iron is shunted quickly into intracellular metabolism (B. F. Matzanke, E. Bill, A. X. Trautwein, and G. Winkelman, submitted for publication). This finding again provides evidence for a role of ferricrocin in the iron storage of *N. crassa*.

In Fig. 2C, a spectrum is depicted of spores from *A. ochraceus* gold. Two components are discernible: a poorly defined absorption between 0 and +1 mm s^{-1} and a magnetically split absorption pattern. Again, this magnetic species can be subtracted easily from the total absorption area by using the ferricrocin spectrum, or, likewise, the spectrum of ferrichrysin or ferrirubin. This ferrichrome-type siderophore represents 74% of the total iron content of the spores of *A. ochraceus*. A principal component was isolated from a methanol-chloroform extraction of the spores that exhibited features similar to asperochromes D_1 to D_3 on thin-layer chromatography (13) as follows: (numbers in parentheses correspond to asperochromes): with solvent system 1, $R_f = 0.40$ (0.39), and with solvent system 2, $R_f = 0.14$ (0.13, 0.16, and 0.15). No ferritin could be detected. Since the absorption pattern of the other species in the central part of Fig. 2C is poorly resolved, we cannot positively exclude the existence of a compound in spores of *A. ochraceus* that exhibits spectral features similar to bacterial iron storage proteins. An iron storage function of the ferrichrome-type siderophore in *A. ochraceus* is likely. This result indicates that a ferritin-substituting function of siderophores is not restricted to *N. crassa* but may be a general feature of siderophore-producing ascomycetes.

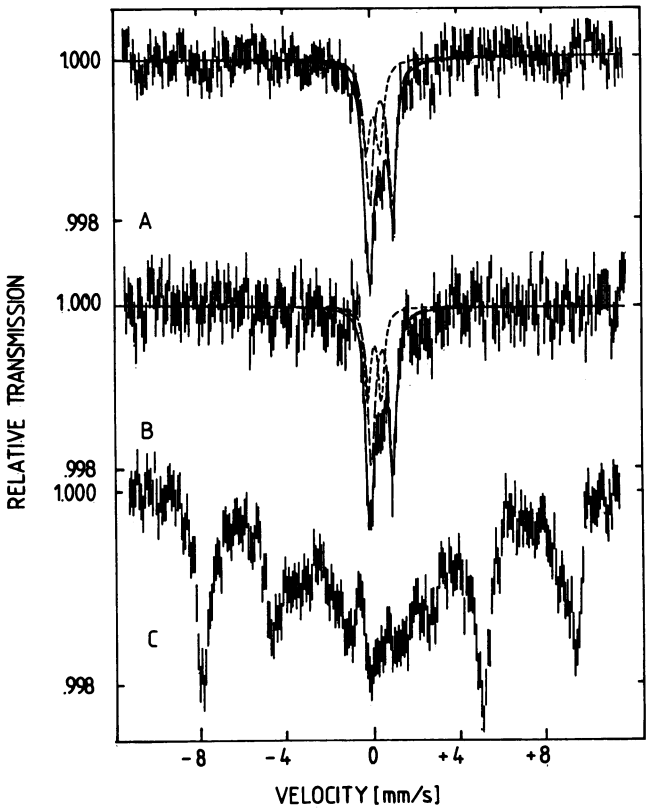

FIG. 2. Mössbauer spectra of spores and germ tubes recorded at 4.2 K in a magnetic field in which $H_{app} = 20$ mT perpendicular to the γ ray. (A) Spectrum of *N. crassa* spores after stripping. Experimental conditions were as described in the legend to Fig. 1; the contribution of ferricrocin was subtracted from the spore spectrum, and the resulting metabolite spectrum was fitted with two quadruple doublets of Lorentzian curves. (B) Spectrum obtained from germ tubes. Spores were enriched with ^{57}Fe as described in the legend to Fig. 1 and were allowed to germinate for 12 h. (C) Spectrum of spores of *A. ochraceus* prepared by the same procedure as that described for *N. crassa*.

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