SIDEROPHORE IRON TRANSPORT FOLLOWED BY MÖSSBAUER SPECTROSCOPY

Berthold MATZANKE and Günther WINKELMANN

Institut für Biologie I, Mikrobiologie I, Universität Tübingen Auf der Morgenstelle 1, 7400 Tübingen, FRG

Received 7 May 1981

1. Introduction

Siderophore iron transport into fungal cells has been largely investigated by the use of radioactively-labelled siderophores. Thus, it was observed that siderophore iron transport into iron-starved fungal cells is dependent on aerobic conditions and inhibited by respiratory poisons and uncouplers [1]. Furthermore, kinetic studies revealed saturation kinetics and a competitive behaviour during uptake [2], indicating that siderophore iron transport is mediated by specific membrane components.

The specificity of siderophore uptake was confirmed by comparing the transport of ferrichrome and enantio-ferrichrome into cells of *Neurospora crassa, Aspergillus quadricinctus* [3] and *Penicillium parvum* [4]. These results showed that fungi are able to distinguish between the naturally occurring Δ-*cis*-ferrichrome and the synthetic Δ-*cis*-enantio-ferrichrome, indicating a stereoselective recognition of siderophores.

Studies on isolated cytoplasmic membranes and membrane vesicles, prepared from the cell wall-less mutant *Neurospora crassa slime,* revealed that the first step during siderophore iron transport is a specific binding to the cytoplasmic membrane [5].

To analyse further events during siderophore iron transport into the fungal cell, we decided to follow the fate of siderophores by Mössbauer spectroscopy. Mössbauer spectroscopic data exist from a variety of purified microbial iron-transport compounds, e.g., ferrichrome A [6], ferrioxamine [7] and enterobactin [8]. In some cases the existence of iron-storage material has been detected by Mössbauer spectroscopy in whole cells [9,10], but the use of Mössbauer spectroscopy to follow the transport of iron-transport molecules into the cell had not been reported.

This work includes spectra of purified, ^{57}Fe-enriched coprogen at room temperature and at 78 K, as well as spectra from lyophilized cells of *Neurospora crassa (arg-5 ota aga)* which had been incubated for 10 min and 120 min in a medium containing [^{55}Fe]coprogen. These results indicate that the chelate molecule is accumulated as a whole and that reduction of the chelated iron (III) is not transport rate limiting. Therefore, we suggest that besides their property to solubilize and transport iron into fungal cells, siderophores function also as iron storage molecules, from which iron is subsequently released for growth and cell metabolism.

2. Materials and methods

2.1. *Isolation and preparation of coprogen*

For production of deferri-coprogen, *Neurospora crassa* 74A was grown under iron limitation for 5 days in a 30 l fermenter (New Brunswick NJ) with aeration, using a mineral medium as in [11]. Coprogen, obtained from the culture filtrate after adding $FeCl_3$, was purified on CM-32 Sephadex (Deutsche Pharmacia, Freiburg), Servachrome XAD-2 (Serva, Heidelberg) and LH-20 (Deutsche Pharmacia). Deferricoprogen was prepared from coprogen according to [11]. [^{57}Fe]Coprogen was obtained by adding $^{57}Fe_2(SO_4)_3$ (95.1%) (Rohstoff-Einfuhr, Düsseldorf) to an equivalent amount of deferricoprogen.

2.2. *Uptake measurements*

Neurospora crassa arg-5 ota aga, a mutant which can be grown deferri-coprogen free [3], was incubated with [^{57}Fe]coprogen (50 μmol, 1 l) and filtered after 10 min, 30 min and 120 min. The mycelia were washed with cold NaCl solution (0.9%), frozen at 78 K, lyophilized and stored under liquid nitrogen. The filtered medium was collected and the residual

 Elsevier/North-Holland Biomedical Press

[^{57}Fe]coprogen and deferri-coprogen was isolated and determined colorimetrically as in [11].

Uptake of coprogen was additionally determined using radioactive [^{55}Fe]coprogen, prepared from deferri-coprogen and $^{55}FeCl_3$ (carrier-free) (Amersham/Buchler, Braunschweig).

2.3. *Mössbauer measurements*

Mössbauer measurements were made at room temperature and at 78 K with a conventional constant acceleration Mössbauer spectrometer (Elscint) containing a 2.5 mCi ^{57}Co on Rh source which was held at room temperature. The lyophilized material was filled into a plexiglassholder (10 mm thick, 22 mm diam.).

2.4. *Susceptibility measurements*

The susceptibility was determined with a Gouy balance, using 3 different field strength and a temperature interval from 80–320 K. The diamagnetic part of the sample was determined by extrapolation of the total susceptibility to $1/T = 0$ using the χ, $1/T$ diagram [12].

3. Results

The Mössbauer spectrum of pure coprogen (fig.1) shows a broad doublet which could not be fitted by Lorentzians, with a quadrupole splitting of 0.49 mm/s and an isomer shift of 0.40 mm/s. This shift is characteristic for Fe^{3+} high spin. By lowering the temperture to 78 K (fig.2A), shift and quadrupole splitting changes slightly ($\delta = 0.44, \Delta = 0.57$ mm/s). Additional 4 lines with less intensity occure, indicating a magnetic hyperfine field (HF) structure.

The Mössbauer spectra of freeze dried cells of *Neurospora crassa* at 78 K (fig.2B,C) show different

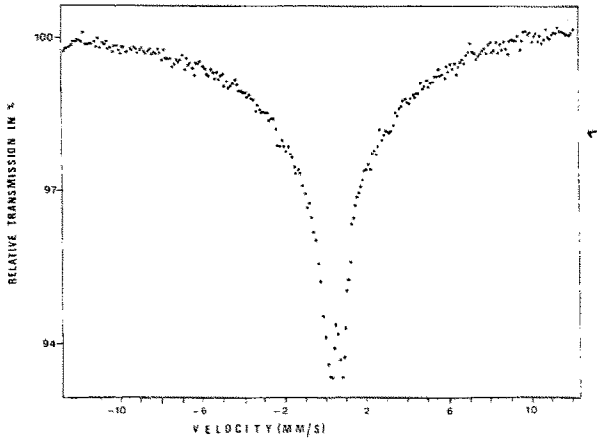

Fig.1. Mössbauer spectrum of pure coprogen at room temperature, calibrated with α-iron.

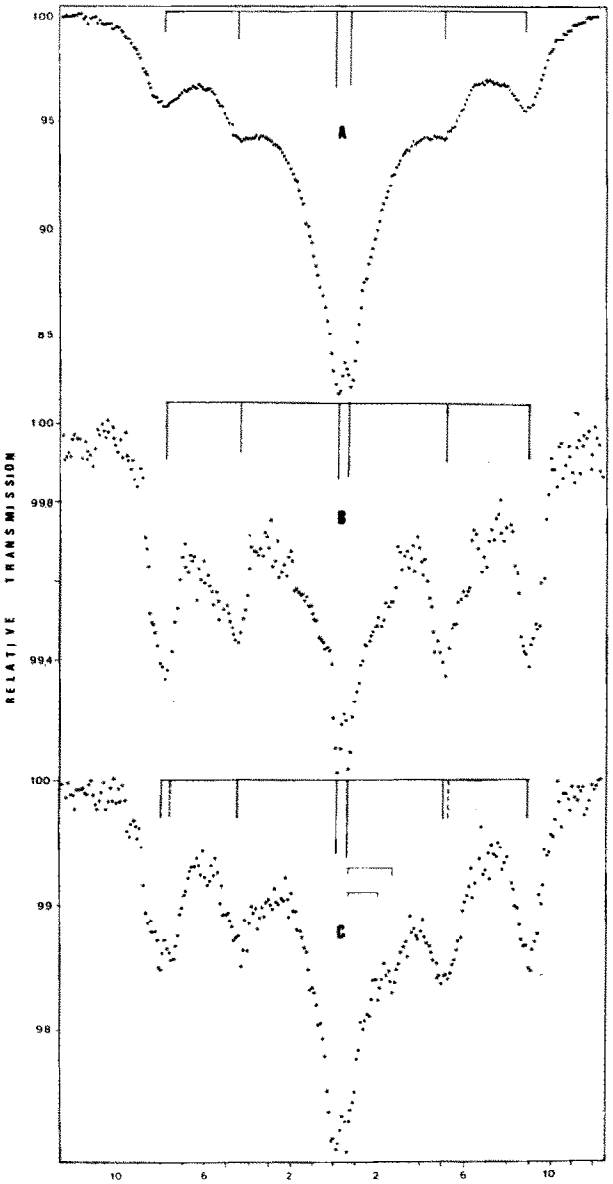

Fig.2. Mössbauer spectra at 78 K of pure coprogen (A) and of freeze dried cells from *Neurospora crassa arg-5 ota aga* after [^{57}Fe]coprogen uptake during 10 min (B) or 120 min (C). The uptake was stopped by washing and quick freezing to 78 K. Sample (B) contained 1.3 g, sample (C) 1.0 g freeze dried cells/cm^2 with ~0.117 and 2.85 mg ^{57}Fe/g. The position and intensities of the lines are shown on the top of the figures.

absorptions depending on the time of incubation with [^{57}Fe]coprogen. The 78 K spectrum after 10 min uptake (fig.2B) differs significantly from the spectrum of pure coprogen. There are differences in the relative intensities of the 4 outer absorptions and the central doublet is more compact by ~0.095 mm/s.

The spectrum from cells incubated for 30 min (not shown) shows additional absorptions which may be assigned to different Fe^{2+} and Fe^{3+} compounds, but valid answers require quantitative spectra analysis of pure coprogen and an examination of separated cell components, especially cytoplasma and membranes.

The spectrum of cells incubated for 120 min (fig.2C) contains 2 additional absorptions which are typical for Fe^{2+} high spin. Furthermore, the right part of the central coprogen doublet (δ = 0.40 mm/s, Δ = 0.49 mm/s) is more intense than the left, which may be explained by the superposition of two Fe^{2+} doublets (δ = 1.01 mm/s, Δ = 1.37 mm/s; δ = 1.35 mm/s, Δ = 2.05 mm/s, shown by horizontal bars in fig.2C). The 4 outer absorptions, typical for coprogen at 78 K, contain additional maxima. Absorption peaks and absorption areas of the 120 min spectrum (fig.2C) indicate that coprogen is still present at high concentrations (~90%).

Uptake measurements with radioactive [^{55}Fe]coprogen (fig.3) showed a rapid stepwise accumulation of the chelate into the cells of *N. crassa arg-5 ota aga.* After an 120 min incubation the labelled coprogen was quantitatively removed from the incubation medium. However, a certain amount of deferri-coprogen (~9%) was detected in the culture filtrate. As this mutant of *Neurospora* is unable to synthesize deferri-coprogen unless supplied with ornithine, the deferri-coprogen in the medium resulted from iron release of [^{55}Fe]-coprogen within the cells.

On the basis of the susceptibility measurements an effective magnetical moment of 5.48 Bohr magnetons was obtained, which is typical for Fe^{3+} high spin compounds. The diagram in fig.4 shows that there was no deviation from the Curie's law within 80–320 K, indicating that neither a phase transition nor a magnetic ordering exists in the measuring range.

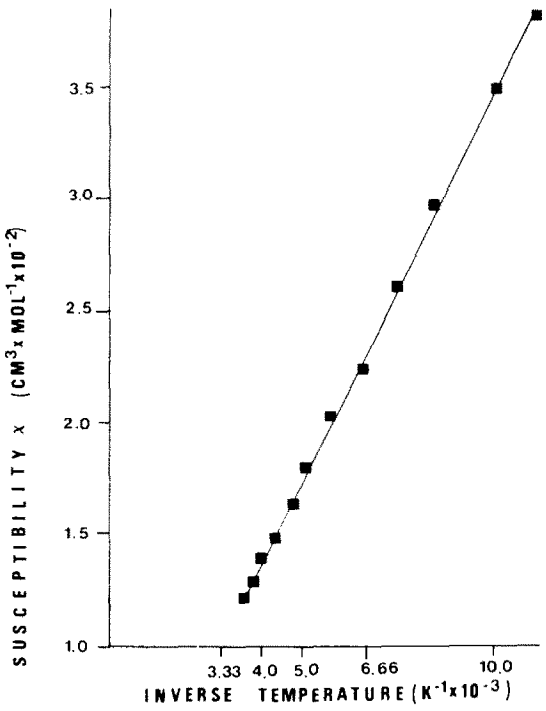

Fig.3. Experimental values of magnetic susceptibility temperature dependence of coprogen, obtained by the Gouy-method.

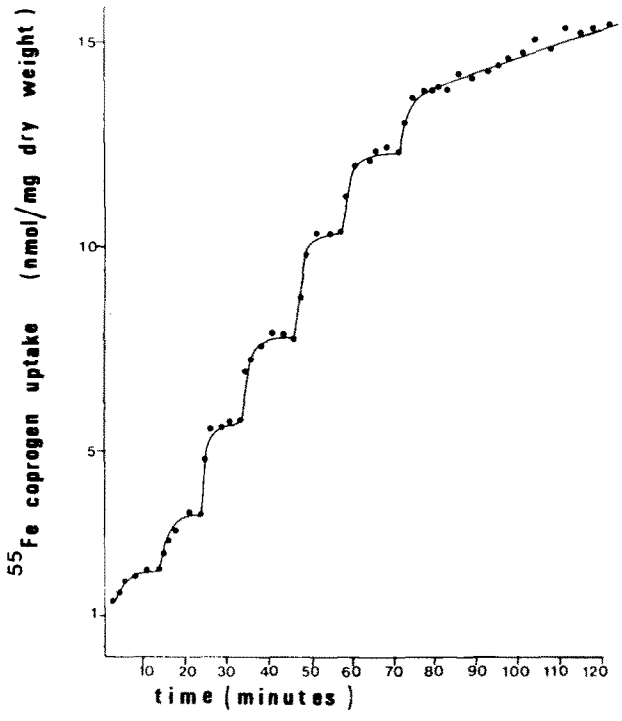

Fig.4. Uptake of iron from [^{55}Fe]coprogen by *Neurospora crassa arg-5 ota aga* added to iron-deficient culture (24 h old) and incubated for 120 min at 27°C. Samples were taken at intervals, filtered, washed and counted in a liquid scintillation counter.

4. Discussion

The magnetic hyperfine structure in the 78 K spectrum of pure coprogen may result from a magnetic ordering or a magnetic relaxation slower than the ^{57}Fe Larmor precession time, so that the nuclear moment may couple with the electronic moment. As the first possibility can be excluded by the susceptibility measurements, the latter argument will be correct. A similar situation was reported [6] for ferrichrome A. There, the polycrystalline paramagnetic sample exhibited temperature-, field- and concentration-dependent hyperfine structure. Changes in the spectra with temperature and field were attributed to electronic relaxation. The spectra of coprogen differ significantly from those of ferrichrome A. A complete quantitative analysis of the coprogen spectra is in progress.

Differences between the spectrum of pure coprogen and the spectrum of mycelia after 10 min uptake may be explained by changed relaxation behavior due to different iron distances [6], as coprogen was highly diluted and in a different environment within the cells.

As the positions of the 4 outer absorption lines are identical in both spectra, the 10 min spectrum is dominated by coprogen. The lower intensities of the 4 outer lines of the 120 min spectrum indicate an increase in coprogen within the cells. The 2 additional absorption lines, observed in fig.2C (—) may possibly be interpreted as a superposition of different HF spectra of coprogen, caused by coupling of all 3 Kramer's states of the electronic shell with the nucleus. A similar effect has been observed on Fe^{3+} samples, highly diluted by Al_2O_3 [13].

These results are evidence that siderophores, like coprogen, were taken up as intact chelate molecules by *Neurospora crassa*, without being decomplexed in appreciable amounts. Therefore, reduction to Fe^{2+} [14] is not rate limiting for coprogen iron uptake, as suggested in [15]. Furthermore, the accumulation of high amounts of siderophores into fungal cells suggest that siderophores are not only synthesized to solubilize iron and transport iron through the cytoplasmic membrane, but may also be regarded as iron-storage molecules. Even after 120 min uptake the amount of reduced iron and the amount of excreted deferri-coprogen remained small (<10%) compared to the total amount of coprogen taken up. As uptake measurements were carried out with iron-starved cells, siderophore uptake, iron storage and iron release may be excessive. But it is assumed that these functions are also operative during normal growth [16]. Although ferritin-like iron storage proteins have been observed in *Phycomyces* [17] and *E. coli* [9], the occurrence of siderophore iron-storage proteins among microorganisms remains an open question. These results support the view that siderophores function to solubilize, transport and store iron in order to enable continuous growth during varying growth conditions.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76). We thank R. B. Johnson for reading the manuscript.

References

[1] Emery, T. (1971) Biochemistry 10, 1483–1487.
[2] Winkelmann, G. (1974) Arch. Microbiol. 98, 39–50.
[3] Winkelmann, G. (1979) FEBS Lett. 97, 43–46.
[4] Winkelmann, G. and Braun, V. (1981) FEMS Microbiol. Lett. in press.
[5] Müller, G. and Winkelmann, G. (1981) FEMS Microbiol. Lett. in press.
[6] Wickmann, H. H., Klein, M. P. and Shirley, D. A. (1966) Phys. Rev. 152, 345–357.
[7] Bock, J. L. and Lang, G. (1972) Biochim. Biophys. Acta 264, 245–251.
[8] Spartalian, K., Oosterhuis, W. T. and Neilands, J. B. (1975) J. Chem. Phys. 62, 3538–3543.
[9] Bauminger, E. R., Cohen, S. G., Dickson, D. P. E., Levy, A., Ofer, S. and Yariv, J. (1980) Biochim. Biophys. Acta 623, 237–242.
[10] Bauminger, E. R., Cohen, S. G., Labenski de Kanter, F., Levy, A., Ofer, S., Kessel, M. and Rottem, S. (1980) J. Bacteriol. 141, 378–381.
[11] Wiebe, C. and Winkelmann, G. (1975) J. Bacteriol. 123, 837–842.
[12] Tasaki, A., Otsuka, J. and Kotani, M. (1967) Biochim. Biophys. Acta 140, 284–290.
[13] Gütlich, P., Lenk, R. and Trautwein, A. (1978) in: Mössbauer Spectroscopy and Transition Metal Chemistry (1978) Inorganic Chemistry Concepts 3, p. 96.
[14] Ernst, J. F. and Winkelmann, G. (1977) Biochim. Biophys. Acta 500, 27–41.
[15] Emery, T. and Emery, L. (1973) Biochem. Biophys. Res. Commun. 50, 670–675.
[16] Charlang, G., Ng, B., Horowitz, N. H. and Horowitz, R. (1981) Mol. Cell. Biol. in press.
[17] Spartalian, K., Smarra, N. and Oosterhuis, W. T. (1974) AIP Conf. Proc. 18, 1326–1329.