Biol Metals (1989) 2:108–113

BIOLOGY OF METALS

Iron limitation and its effect on membrane proteins and siderophore transport in *Neurospora crassa*

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Summary. Cells of the fungus *Neurospora crassa* were grown under iron-deficient and iron-sufficient conditions and their plasma membrane proteins were compared. Three strains were studied: *N. crassa* 74A (wild type), a siderophore-free mutant *N. crassa (arg-5 ota aga)* as well as a 'slime' variant of *N. crassa* which lacks a cell wall. Plasma membranes were purified, solubilized and analyzed by one-dimensional SDS/polyacrylamide gel electrophoresis yielding approximately 50 distinct protein bands with molecular masses in the range 14–160 kDa. Iron-sufficient and iron-deficient growth resulted in nearly identical plasma membrane protein profiles in all strains. Although minor alterations in the proportion of certain proteins could be detected, significant overproduction of certain membrane proteins during iron limitation could not be observed. Transport of ^{55}Fe-labeled siderophores seems to be correlated to the degree of iron limitation. For example, transport rates were enhanced fivefold after 16 h of growth in iron-deficient medium compared to growth in iron-sufficient medium. Extraction and HPLC measurement of siderophores from conidiospores yielded approximately 10^{-15} mol/spore, indicating that germination tubes and young cells used for transport measurements are not iron-deficient. It is suggested that the putative transport systems for siderophores in fungal plasma membranes are constitutively expressed and enhanced uptake of siderophores during iron limitation is rather the result of cellular transport regulation mechanisms.

Key words: Membrane proteins — Iron deficiency — Iron storage — *Neurospora crassa*

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Introduction

In a variety of fungi, iron limitation results in the biosynthesis of siderophores. Thus ferrichromes, coprogens, fusarinines and rhodotorulic acids have been isolated from filamentous and yeast-like fungi of different genera. Structures and functions of fungal siderophores have been compiled recently (Winkelmann et al. 1987). However, iron limitation in fungi has so far not been reported to induce overproduction of iron-regulated membrane proteins. In contrast, enterobacteria are known to biosynthesize both siderophores and their corresponding outer membrane receptors concomitantly in low-iron media. Moreover, in enterobacteria the transcription units for siderophore biosynthesis and outer membrane receptors are often located on the same gene cluster or are even transcribed as an operon by a single promotor as found in the ColV plasmids (Williams 1979; Gross et al. 1984; DeLorenzo and Neilands 1986). Attempts to identify plasma membrane proteins involved in siderophore transport in fungi have so far been unsuccessful. One reason for this may be that fungal cytoplasmic membranes are not equivalent structures to the bacterial outer membrane and that there is no functional similarity between outer membrane receptors and fungal siderophore transport systems. Another reason, however, may be that fungal membrane proteins involved in siderophore transport are constitutively expressed after germination and growth from conidia. Despite the fact that conidia always contain considerable amounts of iron, e.g. as siderophores (Matzanke et al. 1987), iron transport systems in fungi are immediately active after germination from spores. In an attempt to identify iron-regulated membrane proteins in fungi, we compared the plasma membrane proteins originating from three

different strains of *N. crassa*: *N. crassa* 74A (wild type), *N. crassa (arg-5 ota aga)* and the cell-wall-less 'slime' variant of *N. crassa (fz sg os-1)* grown in iron-sufficient and iron-deficient media.

Materials and methods

Strains and growth conditions. The 'slime' variant *Neurospora crassa (fz sg os-1)* was from the Fungal Genetic Stock Center (Department of Microbiology, University of Kansas Medical Center, USA). The cultures were maintained at 27° C on agar slants containing yeast extract (0.4%), malt extract (1.0%), glucose (0.4%), agar (1.5%) and mannitol (2%) (Müller and Winkelmann 1981). Cultures were transferred every two weeks to a fresh agar medium. The filamentous strains *Neurospora crassa* 74A (wild type) and *N. crassa (arg-5 ota aga)* were gifts from R. H. Davis (Irvine, CA, USA). The strains were maintained on YMG agar containing 0.4% yeast extract, 1% malt extract, 0.4% glucose and 1.5% agar. Conidiospores were harvested after 1 week of growth at 27° C.

Iron-deficient and iron-sufficient media. The media for all *N. crassa* strains consisted of an asparagine/salt/glucose medium containing per liter 2.5 g L-asparagine, 1 g $K_2SO_4 \cdot 3H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 10 µg biotin and 2% glucose (autoclaved separately). The pH was adjusted to 5.5 with 1 M HCl. In order to remove residual iron completely, the medium and, separately, glucose or manitol were passed through a Chelex-100 column. Iron-sufficient media were prepared by adding $FeCl_3$ (50 µM) for the wild type and slime mutant and 20 µM iron citrate (1:10) for the siderophore-free *ota* mutant (Winkelmann and Zähner 1973; Winkelmann 1979b). The slime variant of *N. crassa* was first grown in a complex medium consisting of the same asparagine/salt/glucose medium as used for iron-deficient cultures but containing additionally 0.75% yeast extract and 0.1% peptone. Mannitol (2%) was added to obtain the correct osmolarity. After 2 days of growth in complex medium at 30° C and gentle rotation, 10 ml was transferred to 100 ml iron-deficient and iron-sufficient media. From these precultures 50 ml was used for inoculation of 500 ml iron-deficient and iron-sufficient medium. The cultures were incubated for a further 5 days at 30° C on a rotary shaker. The media for *N. crassa (arg-5 ota aga)* were as described for the wild type except that 50 mg arginine and 100 mg putrescine were added per liter. Each medium was inoculated with 10^9 conidiospores, freshly harvested from YMG-agar and washed twice with 0.9% NaCl. After growth for 10 h at 27° C on a rotary shaker in the final medium, the young mycelia were used for preparation of membranes or transport measurements.

Isolation of plasma membranes from filamentous strains. Isolation of plasma membranes from *N. crassa* 74A (wild type) and *N. crassa (arg-5 ota aga)* was carried out according to the procedure of Bowman et al. (1981). Low-iron medium was inoculated with conidia and incubated for approximately 10–16 h. The cells were sedimented by centrifugation ($4000 \times g$, 10 min) and washed twice by repeated suspension in 0.9% NACl and centrifugation ($4000 \times g$, 10 min). To digest the cell wall, the washed cells were resuspended in 40 ml medium A (0.59 M sucrose, 5 mM EDTA, 50 mM NaH_2PO_4, pH 6.5) containing 200000 units β-glucuronidase, type H1 (Sigma, St. Louis, MO, USA) and 70 µl mercaptoethanol, and incubated for a further 3 h at 30° C and 120 rpm. After the treatment with glucuronidase, the sphaeroplasts were sedimented by centrifugation ($4000 \times g$ 10 min), washed with 100 ml 0.68 M sucrose and recentrifuged at $4000 \times g$. The pelleted sphaeroplasts were then resuspended in 20 ml medium B (0.33 M sucrose, 1 mM EGTA, 0.3% bovine serum albumin, pH 7.1) and homogenized in a glass/teflon homogenizer. To remove cell debris, the cell lysate was centrifuged at $1000 \times g$ for 10 min. Mitochondria were removed from the supernatant by sedimentation at $15000 \times g$ for 30 min and then at $12000 \times g$ for 30 min. Finally the plasma membranes were sedimented at $40000 \times g$ for 45 min, and subsequently used for SDS-gel electrophoretic analysis.

Isolation of plasma membranes from slime mutant. Membranes of the slime mutant were isolated after 5 days of growth in iron-sufficient or iron-deficient medium ($A_{650} = 0.7$). The cells were sedimented at $650 \times g$ (10 min) and washed twice by repeated suspension in ice-cold medium A and sedimented ($200 \times g$, 10 min). The cells were then resuspended in medium B and treated further as described for the spaeroplasts of the filamentous strains.

SDS polyacrylamide gel electrophoresis. Electrophoresis was performed on 10% acrylamide/0.17% bis(acrylamide)/SDS slab gels. The samples were mixed with 50 µl sample buffer, containing (50 mM Tris/HCl, pH 6.8, 10% glycerol, 0.1 M dithiothreitol, 2.5% sodium dodecylsulfate, and 0.1 mg/ml bromphenol blue), then heated for 10 min at 100° C. A voltage of 100 V (start) with a constant current of 30 mA was applied for 3.5 h. For molecular mass determination, an electrophoresis kit (low-molecular-mass proteins, Pharmacia, Freiburg, FRG) was used. Gels were stained with Comassie blue G250 (Serva).

Transport assays. Transport measurements in *N. crassa arg-5 ota aga* were carried out as described earlier (Konetschny-Rapp et al. 1988). The filtration method, however, is not applicable to the fragile cells of the slime mutant so we developed a sedimentation procedure to measure transport of siderophores in this mutant. To a suspension of 5 ml cells, grown in iron-deficient or iron-sufficient medium [^{55}Fe]siderophores (50 nmol) were added. Samples (1 ml) were taken at intervals, mixed with 100 µl NaN_3 (1 mM) and centrifuged at $650 \times g$ for 10 min. After careful aspiration of the supernatant, the cells were resuspended in 0.05 M Tris/HCl pH 7.5 containing 0.25 M mannitol and sedimented at $650 \times g$ for 10 min. After removing the supernatant the pellet was then dissolved in a liquid scintillation cocktail and the radioactivity of the cells was counted in a liquid scintillation counter.

Measurement of siderophores in conidiospores. Three different extraction procedures were used. In the first method the spores were heated in 2 ml distilled water in a closed vial at 100° C for 10 h and the siderophore content was determined by HPLC from the supernatant after concentration. In the second method water was substituted by 75% methanol. The third method consisted of a chloroform/methanol extraction procedure at room temperature as described earlier (Matzanke et al. 1987). HPLC separation of siderophores was performed according to Konetschny-Rapp et al. (1988), but in the present investigation an isocratic separation with methanol/water (20:80) as a solvent was used. Integration of HPLC peaks and counting of spores allowed the quantification of ferricrocin/conidiospore.

Results

Sodium dodecyl sulfate/polyacrylamide gel electrohporesis of plasma membrane proteins isolated from *N. crassa* 74A and from the mutant *N. crassa (arg-5 ota aga),* designated as "ota", are shown in Fig. 1. A variety of protein bands are seen possessing approximate molecular masses of 14–160 kDa as calculated by a low-molecular-mass calibration kit. A comparison of membrane proteins isolated from both iron-sufficient as well as from iron-deficient cells revealed no significant differences in the protein pattern, suggesting that there is no overproduction of membrane proteins under iron limitation. As shown in Fig. 2, cells of *N. crassa* slime revealed a very similar protein pattern of membrane proteins compared to the filamentous strains. Again there was no overproduction of membrane proteins when the cells were grown in iron-deficient medium. In membranes from older cultures of the slime mutant some proteolytic degradation of proteins was observed. Therefore in some cases bovine serum albumin was added during membrane preparation.

Siderophores of the ferrichrome family, such as ferrichrome, ferricrocin or ferrichrysin are equally well taken up by *N. crassa*. Therefore, any of these can be used to measure transport activity.

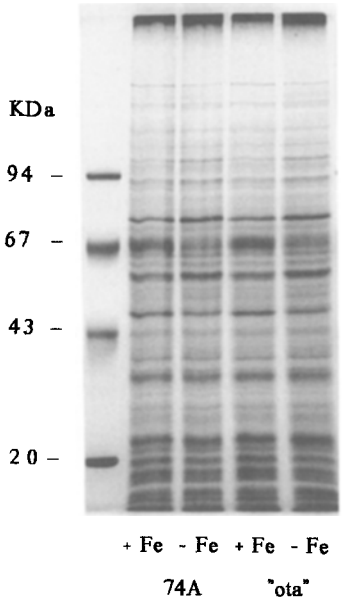

Fig. 1. SDS/polyacrylamide gel electrophoresis of plasma membrane proteins of *N. crassa* 74A (wild type) and *N. crassa (arg-5 ota aga),* designated "ota", isolated from cells grown in the presence (+Fe) or in the absence (−Fe) of iron. Assignment of the molecular masses was done by using a low-molecular-mass calibration kit: phosphorylase *b* (94.0 kDa), bovine serum albumin (68 kDa), ovalbumin (43.0 kDa), soy bean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Membranes were isolated as described in Materials and methods. 10% polyacrylamide gels were used

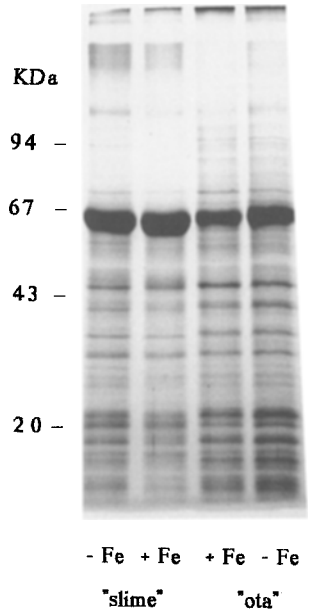

Fig. 2. SDS/polyacrylamide gel electrophoresis of SDS-solubilized plasma membranes of *N. crassa* slime and *ota* mutants isolated from cells grown in the presence (+Fe) and in the absence (−Fe) of iron. Conditions are as described in Fig. 1

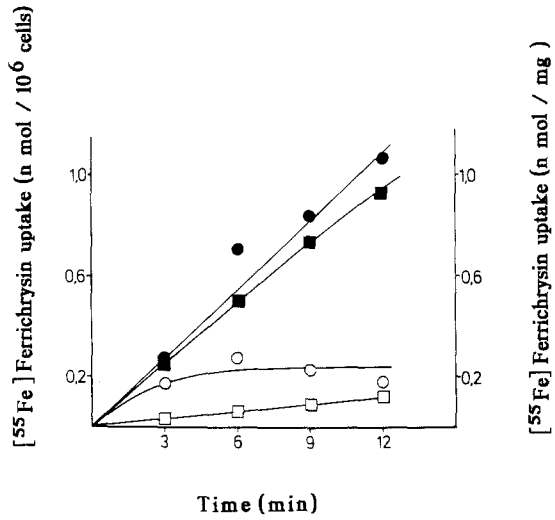

Fig. 3. Transport of [^{55}Fe]ferrichrysin in the two strains of *N. crassa*: *N. crassa (arg-5 ota aga)* grown in iron-deficient (■) and iron-sufficient (□) medium; *N. crassa* (slime mutant) grown in iron-deficient (●) and iron-sufficient (○) medium. Transport of siderophores was measured by the filter method in the case of the filamentous strain and by the sedimentation method in the case of the more fragile slime variant, as described in Materials and methods. Uptake is expressed as nmol ferrichrysin taken up/mg dry mass for the filamentous strain and nmol/10^6 cells for the slime variant

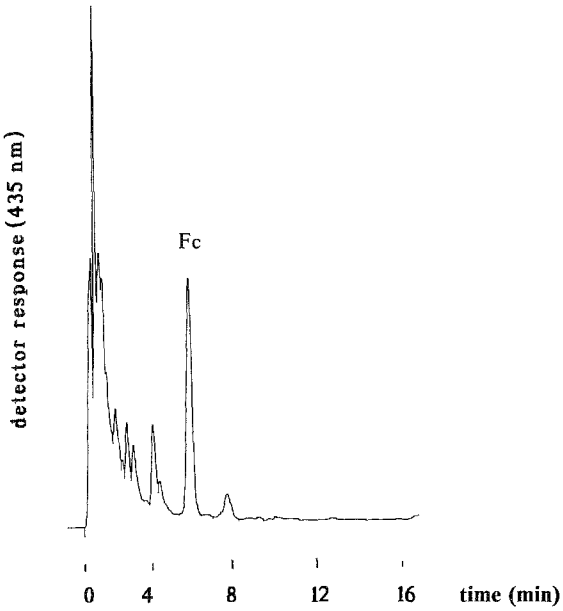

Fig. 4. HPLC separation of siderophores isolated from conidiospores of *N. crassa (arg-5 ota aga)* grown in YMG-agar. Conidia were harvested by shaking with 0.9% NaCl solution containing 0.1% Tween 80. The suspension was passed through a glass-wool filter and washed repeatedly by centrifugation in distilled water. Extraction of siderophores as well as separation and quantification by HPLC was as described in Materials and methods. Fc = ferricrocin

Transport of [^{55}Fe]ferrichrysin in the filamentous strain *N. crassa (arg-5 ota aga)* and in the yeast-like slime variant *N. crassa (fz sg os-1)* revealed significant differences depending on whether iron was present in the cultivation medium or not (Fig. 3). From the transport kinetics obtained, it is clear that uptake of labeled siderophores is increased approximately fivefold in iron-depleted cells which corresponds exactly to earlier observations. Thus in both strains, the transport of siderophores is significantly reduced when the cells have previously been grown in iron-supplemented medium.

All extraction procedures revealed ferricrocin as the predominant siderophore in conidiospores. Although some minor peaks were additionally detected, coprogen could not be found in any of the three extracts. The amount of coloured lipophilic compounds increased when chloroform/methanol extraction was used (data not shown). Water and methanol extraction gave nearly identical results and the highest amount of ferricrocin (Fig. 4). Therefore, the calculation of ferricrocin was based on the water-extracted samples. The yield of ferricrocin was 0.65 μmol/0.5 ml spore suspension (0.6×10^9 spores) which corresponded to approximately 10^{-15} mol ferricrocin/conidiospore.

Discussion

Previous reports from this laboratory have shown that *N. crassa* is able to take up iron from two structurally distinct siderophore families: the ferrichromes and the coprogens (Winkelmann 1974, 1979a; Huschka et al. 1985, 1986). Representatives of both families (ferricrocin and coprogen) are synthesized and excreted as desferri-siderophores by *N. crassa* during growth in low-iron media. Transport studies in fungi have revealed that these siderophores are taken up by membrane-located and energy-consuming transport systems (Huschka et al. 1983). However, proteins of the fungal siderophore transport system have not been characterized or isolated so far. In contrast, bacteria of the family Enterobacteriaceae such as *Escherichia coli* are known to overproduce iron-regulated membrane proteins under iron limitation. These outer membrane proteins function as siderophore receptors (FhuA for ferrichrome, FhuE for coprogen, FepA for enterobactin, FecA for ferric dicitrate) or colicin and phage receptors (Braun and Winkelmann 1987). The FhuA receptor is known to recognize the fungal ferrichromes. The FhuE receptor is specifically designed for uptake of coprogen (Hantke 1983), which is the principal siderophore of *N. crassa* and related fungi (Hossain et al. 1987). However, unlike the enterobacterial siderophore receptors which reside in the outer membrane, the siderophore transport systems of fungi are components of the plasma membrane. It is therefore presumed that there is little structural and functional correspondence between bacterial outer membrane receptors and fungal plasma membrane proteins involved in siderophore transport.

As shown in the present study, overproduction of membrane proteins in response to a low content of iron in the cultivation medium seems not to occur in plasma membranes of fungi. The filamentous strains of *N. crassa* are generally grown from conidiospores which have recently been shown spectroscopically to contain a siderophore (ferricrocin) as the main iron-storage compound (Matzanke et al. 1987). Thus, if cells are grown from conidia, a considerable amount of iron should be available to the growing cells and it seems doubtful whether young mycelia of *N. crassa* are really iron-deficient at an early stage of growth. Transport of siderophores can be measured after only a few hours of germination. Although at this stage of growth, iron stores may be depleted and iron may already be incorporated into various enzymes, this does not mean that the

cells suffer from iron deficiency. This, however, would suggest that siderophore transport systems in fungal plasma membranes are expressed constitutively in the presence of intracellular iron. It is also conceivable that the disappearance of intracellular ferricrocin by intracellular metabolic events is the signal for activating siderophore transport systems. Thus, intracellular siderophores, such as ferricrocin, may exert some kind of regulatory function on fungal transport systems during the process of siderophore uptake.

As was shown by Stotish and Somberg (1981), the actual number of different plasma membrane proteins in a slime mutant of *N. crassa* (FGSC No. 326) is about 180, as determined by two-dimensional polyacrylamide gel electrophoresis. About 40% of these are exposed to the exatracellular surface. The present investigation provides evidence that iron-dependent alterations in plasma proteins could not be observed. We have recently shown that coprogen linked to a photoaffinity label reduces coprogen uptake during illumination by approximately 50%, suggesting that siderophore transport may be specifically inactivated by covalent linkage of coprogen (Bailey et al. 1986). Although an assignment of plasma membrane proteins to the process of siderophore transport could not be achieved in the present investigation, evidence has been presented that cells of *N. crassa* respond to environmental iron deficiency by changes of transport rates but not by changes in the plasma membrane protein pattern. The occurrence of ferricrocin in spores of *N. crassa* has been reported earlier (Horowitz et al. 1976; Matzanke et al. 1987). Ferricrocin has also been found as the main intracellular iron-storage compound in mycelia of *N. crassa* (Matzanke et al. 1988), suggesting that ferricrocin is biosynthesized mainly for iron-storage purposes in mycelia and then transferred to the conidiospores during sporulation. The present investigation also revealed that the siderophore-free mutant *N. crassa (arg-5 ota aga)* is unable to sporulate when grown on ornithine-free agar media, suggesting an intimate connection between siderophore biosynthesis and spore formation. As coprogen could not be found among the siderophores of conidiospores, it appears that the main function of coprogen is iron accumulation during mycelial growth, whereas ferricrocin is used as an iron-storage compound. The amount of ferricrocin determined in spores was approximately 10^{-15} mol/spore. Mössbauer spectroscopic measurements have shown that ferricrocin represents 47% of the total iron content of spores and that it is completely metabolized during germination. As germ tubes and young mycelia use the siderophores of spores for growth and still contain a certain amount of non-siderophore iron, iron deficiency is not prevalent at this stage of growth. The enhanced uptake of siderophores found in young mycelia might originate from internal signals, e.g. a decreasing ferricrocin content, and seems not to be the result of overproduction of membrane transport proteins. The fact that siderophore transport in fungi proceeds in the presence of sufficient iron deposited in conidiospores suggests that the corresponding transport systems seem to be constitutive rather than induced by iron deficiency.

Acknowledgements. This work was supported by the *Deutsche Forschungsgemeinschaft* (Wi-628/3-2). The assistance of A. Chapeaurouge in the HPLC separation is gratefully acknowledged.

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Received June 19, 1989