AN ATTEMPT TO LOCALIZE IRON-CHELATE BINDING SITES ON CYTOPLASMIC MEMBRANES OF FUNGI

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1. Introduction

Sideramine producing fungi like *Aspergillus, Ustilago* or *Neurospora* show specific iron-chelate uptake when grown under iron-deficient conditions [1–3].

Analysis of the transport behavior revealed saturation kinetics, which could be abolished by uncoupling agents, respiratory poisons and SH-alkylating compounds [4], indicating active accumulation. Incubation experiments with double labeled [^{55}Fe/^{14}C]coprogen on *Neurospora* gave evidence that the whole iron-chelate is taken up, although the chelator is subsequently excreted into the medium [5]. There is, however, no report showing the participation of the cytoplasmic membrane during the actual iron-chelate uptake nor is there any report on binding sites of fungal cytoplasmic membranes. We find, that membranes of *Neurospora crassa sg fz os-1*, called 'slime' possessing no cell-wall when grown in high osmotic media, exhibit significant binding of sideramines. Membranes extracted with 2-chlorethanol revealed specific binding of different iron-chelates irrespective of whether these chelates were added in vivo or in vitro. The binding capacity is lost in aqueous media. A protective effect of sideramines against SH-alkylating agents was observed which could be used in an attempt to label the proteins possibly involved in the iron-chelate uptake system of fungal cytoplasmic membranes.

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2. Materials and methods

2.1. *Culture and growth conditions*

Neurospora crassa sg fz os-1 (slime) was obtained from the Fungal Genetics Stock Center, Arcata, USA. The cell-wall-free mutant was cultivated in complex medium containing yeast extract (0.1%), peptone (0.15%) and sorbitol (4%). Iron-deficient cultures were obtained in minimal medium as described earlier [3] containing additionally sorbitol (4%).

2.2. *Iron-chelates*

Coprogen was isolated from culture filtrates of *Neurospora crassa* 74A, purified on DOWEX XAD-2, CM-cellulose and on silica-gel plates. Ferrirubin was a gift from Professor Keller-Schierlein, Zürich. Ferrichrome was from Professor J. B. Neilands, Berkeley. Ferrichrysin and ferrioxamine B was from the stock of the Institut für Biologie, Lehrstuhl Mikrobiologie I.

2.3. *Chemicals and radiochemicals*

If not otherwise stated all chemicals were from Merck, Darmstadt. $^{55}FeCl_3$ in 1 M HCl (carrier-free), [^{14}C]NEM (*N*-[ethyl-1-^{14}C]-maleimide) was from NEN Chemicals, Dreieichenhain and [1-^{14}C] acetic acid was purchased from Amerham Buchler, Braunschweig. EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) was from Bio. Rad. Lab., Munich. *p*HMB (*p*-hydroxymercuribenzoic acid), Servachrom A-Hg as well as CPG-170, DOWEX-XAD-2 (125–150 μM), CM-cellulose (CM 32, Servacel) and DEAE-cellulose (DEAE-32, Servacel) were obtained from Serva, Heidelberg, CM–Sephadex and LH-20 Sephadex was from Pharmacia, Frankfurt.

2.4. *Preparation of cytoplasmic membranes*

Highly purified cytoplasmic membranes were obtained according to the method of Brunette and Till [6] in a two-phase system consisting of 10 ml 30% polyethyleneglycol and 10 ml 20% Dextran T-500.

2.5. *Solubilization of cytoplasmic membranes*

Membrane solubilization was performed according to Zahler and Hoelzl Wallach [7]. Freshly purified cytoplasmic membranes were mixed with redistilled 2-chlorethanol by adding the solvent dropwise whilst stirring with a glass rod until a nearly clear, slightly reddish, suspension was obtained. This suspension revealed a protein content of approximately 9 mg/ml and a protein/phosphate ratio of about 7. The suspension was centrifuged further at 10 000 × *g* for 10 min until a clear solution was obtained. The solubilized membranes were used for iron-chelate binding studies. Characteristic values of solubilized membrane solution were 1.2 mg protein/ml and protein/phosphate = 1.3.

2.6. *Binding studies*

Binding studies were performed with an equilibrium-dialysis apparatus (Dianorm) equipped with 200 μl Teflon cells possessing a dialysis area of 1.54 cm^2. The dialysis membranes (Union Carbide Corp.) having an exclusion value of 10 000–20 000 dalton were stable in 2-chlorethanol/water mixtures.

2.7. *Determination of iron-chelate binding sites by in vivo-labeling with SH-alkylating agents*

Two methods were performed:

2.7.1. [^{14}C]NEM-labeling

The [^{14}C]NEM-labeling was performed according to Fox and Kennedy [8]. An amount of 200 ml iron-deficient cells of *Neurospora crassa* slime was split into two 100 ml portions (A and B). Both cultures were sedimented by centrifugation (300 × *g*, 5 min) and resuspended in 5 ml medium. To A 1 ml coprogen (1 μmol) and to B 1 ml medium was added and both cultures were incubated at 27°C for 5 min. Subsequently both assays were treated with *p*HMBS (10^{-3} M) for 3 min and then cooled in an ice-bath. After centrifuging at 300 × *g* for 5 min, the sedimented cells were washed twice with 5 ml ice cold medium, resuspended in 5 ml medium, and incubated further 5 min at 27°C. Then [^{14}C]NEM (10^{-3} M, 10 nCi/μmol) was added to each incubation assay and the reaction was stopped by adding 10 μl mercaptoethanol. The cells were sedimented, washed twice with medium and disrupted in a glass-Teflon Potter homogeniser.

2.7.2. *N*-(*p*HMBS)-[^{55}Fe]coprogen B-labeling

Two portions of 100 ml cell-suspensions were sedimented, washed and resuspended as described above.

To A 1 ml medium and to B 1 ml coprogen (500 nmol) were added and incubated for 10 min at 27°C. Both assays were then incubated with 20 nmol *N*-(*p*HMBS)-[^{55}Fe]coprogen B (200 Ci/μmol) for 10 min at 27°C. The reaction was stopped in an ice-bath. The cells were washed, disrupted and the membranes were purified and solubilized as described.

2.8. *Preparation of [^{14}C]coprogen*

Introduction of a [^{14}C]label into coprogen was achieved by coupling [1-^{14}C]acetic acid (57 μCi/μmol) with the free amino group of coprogen B (desacetyl coprogen) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as carboxyl activator [9]. The preparation assay contained 15 mg coprogen B, 4.4 mol [1-^{14}C]acetic acid and 50 mg EDAC, at pH 4.7. Solubilized EDAC was added dropwise with gentle magnetic stirring. After standing overnight [^{14}C]coprogen was purified on DEAE–Sephadex to remove unreacted acetic acid, on CM-cellulose to remove excess coprogen B and finally on DOWEX XAD-2 to separate labeled coprogen from other byproducts. The specific activity of [^{14}C]coprogen was 57 μCi/μmol.

2.9. *Synthesis of* N-*(pHMB)-[^{55}Fe]coprogen*

The carboxyl group of *p*HMB was coupled to the amino group of coprogen B using EDAC as described for [^{14}C]coprogen. The reaction product was purified and treated with 8-hydroxy-quinoline to remove iron and then incubated with $^{55}FeCl_3$ carrier free to obtain *N*-(*p*HMB)-[^{55}Fe]coprogen.

2.10. *Non-permeable pHMB–agarose*

*p*HMB coupled to 3,3′-diaminopropylamine-agarose was obtained form Serva (Heidelberg) as Servachrom A-Hg, 50–150 μm.

2.11. *Quantitative determinations*

Sideramines were determined in an Eppendorf-photometer using the absorption coefficients given by Neilands [10]. Phosphate was determined according to the method of Bartlett [11]. Proteins were measured after Lowry et al. [12].

3. Results

If cytoplasmic membranes of *Neurospora crassa* slime were solubilized in 2-chlorethanol/water 9:1 (v/v) and analyzed on LH-20 using the same solvent for elution, we generally obtained good protein/lipid separation (fig.1). Most, if not all, phospholipids were eluted after the fifteenth fraction. Some phosphate remained in the protein fraction. As lipids are completely solubilized in chlorethanol, no micelle formation, which might lead to incorrect distribution of labeled iron-chelates, is possible.

Figure 2 shows the result of the label distribution

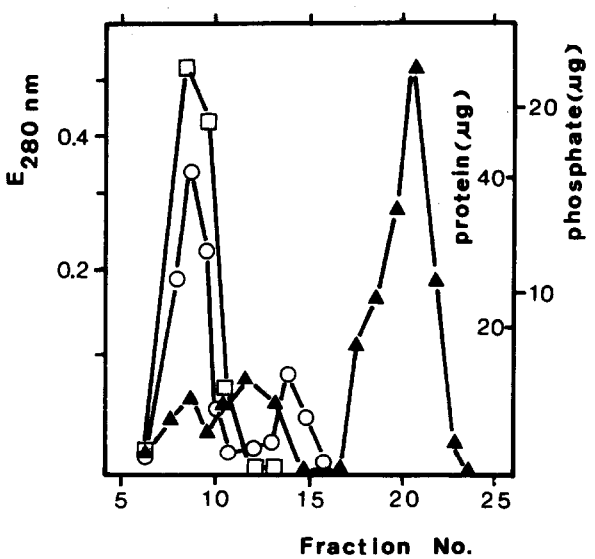

Fig.1. Separation of lipids and proteins from cytoplasmic membranes on LH-20 Sephadex column. Plasma membranes from *Neurospora cr.* slime were isolated and purified using the two-phase system as described in Materials and methods. After solubilization in 2-chlorethanol/water 9:1 (v/v) 1 ml of this solution was separated on a LH-20 Sephadex column (500 × 15 mm) using the same solvent as the eluting solvent. From each fraction (2.5 ml) protein (0.5 ml) and phosphate (0.2 ml) was determined. (○) A_{280}, (□) protein, (▲) phosphate.

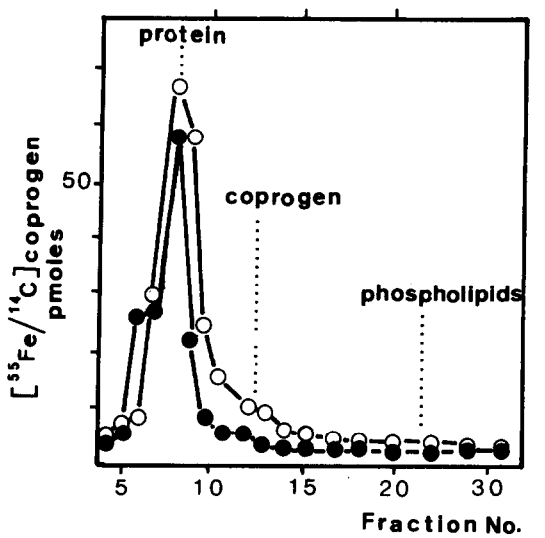

Fig2. Separation of membrane proteins after in vivo binding of double labeled [^{55}Fe/^{14}C]coprogen. A cell suspension of *Neurospora cr.* slime grown in 10 ml iron-deficient minimal medium (1.5 × 10^8 cells) was incubated with [^{55}Fe/^{14}C] coprogen (500 nmol) at 27°C with gentle rotation for 10 min. After washing the cells with cold minimal medium (200 ml), the cells were disrupted and the membranes isolated and purified as described. The membranes were then solubilized in 2-chlorethanol/water 9:1 (v/v), fractionated on LH-20 Sephadex with the same solvent, and the radioactivity was determined. (○) ^{14}C, (●) ^{55}Fe.

when cells of *Neurospora cr.* slime were previously incubated with [^{55}Fe/^{14}C]coprogen. The results indicate that the whole iron-chelate is associated with membrane proteins. From fig.2 we infer that binding and decomplexation of iron-chelates are not necessarily linked events in iron-chelate uptake.

The binding properties of the free chelator and of the iron-containing chelate differ greatly, as shown in fig.3. With the use of [^{14}C]coprogen and desferri-[^{14}C]coprogen, distinct separation was obtained on CPG 170. These results are evidence that only the iron-containing chelates show high protein-binding properties, whereas the iron-free chelators are eluted after the protein fraction. Desferri-coprogen is eluted together with non-bound coprogen, which was also present in this preparation. Solubilized membrane fractions with bound [^{14}C]coprogen, obtained after separation on LH-20, were filled in an equilibrium apparatus and dialyzed against different solvents.

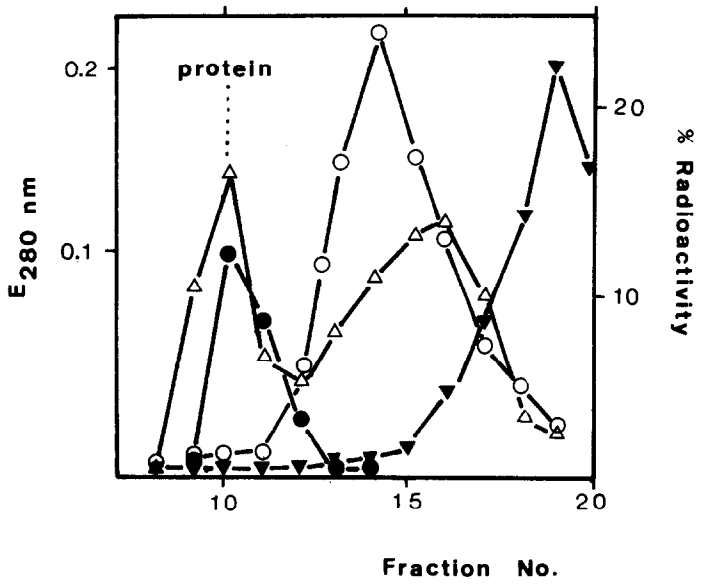

Fig.3. Separation of membrane proteins after in vitro binding of labeled iron-chelates. Membranes of iron-deficient cells of *Neurospora cr.* slime were solubilized in 2-chlorethanol/water 9:1 (v/v) and incubated with various labeled iron-chelates (5 nmol/0.5 mg protein) at 20°C for 10 min. The incubation assay was then separated on a CPG-170 column (450 × 6 mm) using 2-chlorethanol as eluting solvent. The radioactivity is given as a percentage of the total. (△) [^{14}C]coprogen, (○) desferri-[^{14}C]coprogen, (▼) [^{55}Fe]ferrioxamine, (●) A_{280}.

Table 1
The influence of the solvent on binding of [^{14}C]coprogen[a] during equilibrium dialysis (protein-free compartment)

Solvent	Radioactivity (cpm)
2-Chlorethanol	44
H_2O	615
HCl (0.01 M)	47
NaOH (0.01 M)	485

[a] The protein compartment contained solubilized membranes in 2-chlorethanol/water 9:1 with bound labeled coprogen (LH-20 Sephadex fraction)

Table 1 shows that the protein/coprogen associate is stable in 2-chlorethanol and also when dialyzed against acidified water, but dissociates in the presence of neutral or alkaline water.

Table 2 shows the results of equilibrium dialysis

Table 2
Specificity of chelate binding by membrane proteins in vitro

[^{55}Fe]Chelate	% Binding
Ferrirubin	51
Ferrichrome	28
Coprogen	22
Ferrichrysin	10

Table 3
Effect of in vivo pre-treatment on the in vitro binding of [^{55}Fe]coprogen

Pre-treatment	Binding (pmol/μg protein)	(%)
	16	100
NEM (2×10^{-2} M)	0.9	5.7
Servachrom A-Hg (1 mg/ml)	10.7	67
Ferrirubin (100 nmol)	0	0
100°C, 30 min	0	0

Table 4
Labeling of membrane binding sites in vivo by SH-alkylating agents

Labeling method	Coprogen-protected (cpm)	Non-protected (cpm)
[^{14}C]NEM	10 230	5400
N-(*p*HMB)-[^{55}Fe]Coprogen	10 100	32 000

experiments performed with solubilized membranes in the presence of various sideramines. The observed binding specificity of labeled sideramines agrees with the observed order of sideramine uptake on intact cells [3]. However, ferrichrome binding is unexpectedly high and cannnot be correlated to the in vivo uptake behavior.

The binding properties of solubilized membranes are shown in table 3. Binding of [^{55}Fe]coprogen in vitro is completely abolished after boiling for 30 min. SH-Alkylating agents differ in their inhibitory action. NEM (2×10^{-2} M) inhibits more strongly than does the bulky agarose-coupled *p*HMB (Servachrom A-Hg). Furthermore, ferrirubin is an effective inhibitor of coprogen-binding [3] as inferred earlier from kinetic measurements in vivo.

The inhibitory action of SH-alkylating agents during binding of coprogen in vitro led us to an attempt to localize the binding site for coprogen on intact cells of *Neurospora cr.* slime. The cells were incubated with coprogen and then treated with the relatively impermeable, non-radioactive *p*HMB. After washing and further incubating in fresh medium the coprogen protected sites were metabolically released by transport to the inside of the cells. The unprotected binding sites were then labeled with a [^{14}C]NEM pulse in order to achieve specific labeling of the binding protein. A typical result of these experiments is shown in table 4.

The results indicate that when coprogen binding sites are previously protected by coprogen, high [^{14}C] NEM label can be obtained. The membranes of these experiments were isolated as described in Materials and methods, then solubilized by SDS (1%) and counted for radioactivity.

In another experiment we tried to label the coprogen binding site directly by incubating the cells with [^{55}Fe]coprogen coupled to *p*HMB (*N*-(*p*HMB)-[^{55}Fe]coprogen B). In this case, high protein labeling

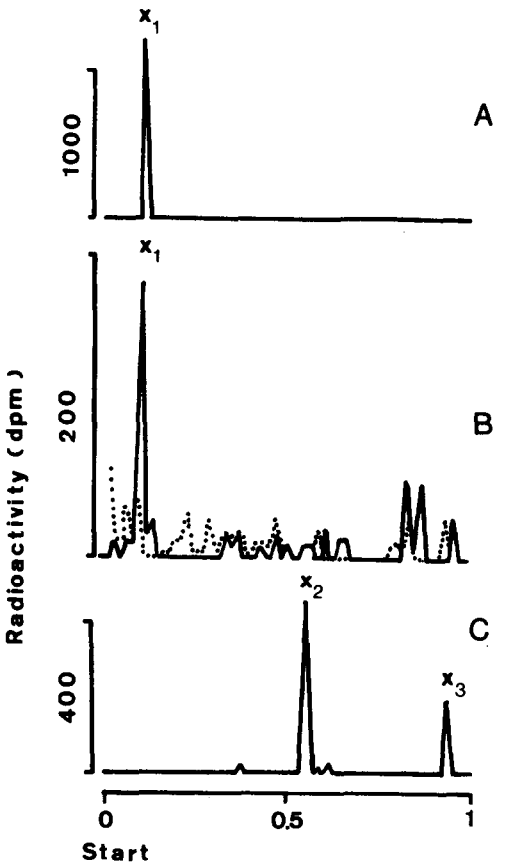

Fig.4. Polyacrylamide gel electrophoretic separation of selectively labeled membrane proteins with and without protection by previous incubation with coprogen. Part A: *N*-(*p*HMB)-[^{55}Fe]coprogen labeled membrane protein (X_1) obtained after in vivo incubation without coprogen. When coprogen was added prior to *N*-(*p*HMB)-[^{55}Fe]coprogen, no prominent radioactive protein was observed. Part B: [^{14}C]NEM labeled membrane proteins obtained after protection with coprogen, treatment with *p*HMB and further incubation to liberate the binding sites for [^{14}C]NEM-labeling. Without coprogen protection (dotted line). Part C: Labeled membrane protein fragments (X_2,X_3) obtained by the [^{14}C]NEM method and subsequent treatment with mercaptoethanol.

was achieved (table 4) when the cells were not previously protected by coprogen. Gel-electrophoretic separation of [^{14}C]NEM-labeled and *N*-(*p*HMB)-[^{55}Fe] coprogen-labeled proteins (fig.4) gave identical molecular weights (X_1 = 380 000). Treatment of labeled membranes with mercaptoethanol led to disappearence of this high molecular species (X_1) and to the appearance of two peaks with lower molecular weights (X_2,X_3). Contrary to X_1 and X_2, the X_3-peak could not be stained with Coomassie Blue G-250 and therefore is probably not a protein.

4. Discussion

This is the first report describing the interrelationship between iron-chelates and cytoplasmic membranes of fungi which we have deduced earlier from kinetic measurements [3,4]. Binding of sideramines is observed in vivo and in vitro, indicating that the responsible binding sites are identical. The use of an organic solvent like 2-chlorethanol seems to promote binding, as dialysis against water leads to loss of binding affinity. In addition to low polarity, acidic conditions also seem to be necessary for binding. Zahler and Hoelzl Wallach [7] have pointed out that 2-chlorethanol promotes α-helix and β-conformation and does not impair the activity of ribonuclease. Thus, it seems quite reasonable that binding of iron-chelates requires special environmental conditions which cannot occur in aqueous media. The corresponding results of the in vivo labeling methods with SH-alkylating agents are further evidence that binding to the cytoplasmic membrane is the first step during iron chelate uptake in fungi. However further work is necessary to demonstrate binding properties of membranes reconstituted with isolated binding-proteins.

Acknowledgements

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References

[1] Wiebe, C. and Winkelmann, G. (1975) J. Bacteriol 123, 837–842.
[2] Emery, T. (1971) Biochemistry 10, 1483–1487.
[3] Winkelmann, G. (1974) Arch. Microbiol. 98, 39–50.
[4] Ernst, J. and Winkelmann, G. (1974) Arch. Microbiol. 100, 271–282.
[5] Winkelmann, G. and Zähner, H. (1973) Arch. Mikrobiol. 88, 49–60.
[6] Brunette, D. M. and Till, J. E. (1971) J. Membr. Biol. 5, 215–224.
[7] Zahler, P. H. and Hoelzl Wallach, D. F. (1967) Biochim. Biophys. Acta 135, 371–374.
[8] Fox, C. F. and Kennedy, E. P. (1965) Proc. Natl. Acad. Sci USA 54, 891–899.
[9] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[10] Neilands, J. B. (1966) Struct. Bond. 1, 59–108.
[11] Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.