A Specific Transport System for Manganese in the Filamentous Fungus *Aspergillus niger*

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The existence of energy-dependent Mn^{2+} influx in *Aspergillus niger* ATCC 11414 was demonstrated both with mycelia grown for production of citric acid and in resting mycelia suspended in 'Good'-buffers. A specific high-affinity transport system was detected at submicromolar concentrations of $^{54}Mn^{2+}$ which functioned independently of the transport of Mg^{2+} and Ca^{2+} , but was preferentially inhibited by Zn^{2+} , Cu^{2+} , Cd^{2+} . The rate of Mn^{2+} transport was suboptimal in the citric acid production medium (pH 3.8 or lower) as the pH optimum was found to be 5.6 in the buffer experiments.

INTRODUCTION

Evidence for Mn^{2+} as an important regulator of metabolism has been obtained both in fungi (Williams, 1982; Garraway & Evans, 1984) and in bacteria such as *Bacillaceae* (Silver & Jasper, 1977), Coryneform (Auling, 1983; Plönzig & Auling, 1987) and certain lactic acid bacteria (Archibald, 1986). The question arises as to how the Mn^{2+} essential for these organisms can be acquired by them, as there is usually a great excess of Mg^{2+} in normal growth media. Several energy-dependent Mn^{2+} -specific transport systems have been demonstrated in bacteria (Silver, 1978) and have also been reported for eukaryotes like *Euglena gracilis* and human KB cells (Silver & Jasper, 1977). So far no specific Mn^{2+} transporter and very few energy-dependent influx systems for other divalent cations of the transition metals have been reported for filamentous fungi such as *Neocosmospora vasinfecta* (Paton & Budd, 1972); *Neurospora crassa* (Mohan *et al.*, 1984) and *Aspergillus parasiticus* (Failla & Niehaus, 1986), obviously due to difficulties encountered with transport assays with mycelial growth.

More attention has been paid to yeasts and yeast-like fungi which possess energy-dependent influx systems for various metal ions. The relatively non-specific divalent cation transport system in *Saccharomyces cerevisiae* (Borst-Pauwels, 1981) contrasts with the more specific uptake systems observed in *Candida utilis* (Failla *et al.*, 1976; Parkin & Ross, 1985). A specific Mn^{2+} transporter has recently been demonstrated in this yeast (Parkin & Ross, 1986).

The rationale behind the present study was that energy-linked transporters which exhibit both high affinity and high specificity are generally detected only in cells grown at very low metal concentrations, which presumably induce such pathways. Recently we have observed that growing *Aspergillus niger* in the mineral medium of Shu & Johnson (1948) for production of citric acid induces typical symptoms of Mn^{2+} deficiency such as altered morphology and inhibition of DNA formation within 24 h of inoculation with spores (Hockertz *et al.*, 1987). Therefore such Mn^{2+} -depleted mycelia, from the early growth phase, should be used for examination of the uptake of Mn^{2+} by *A. niger* both in the citric acid production medium and after resuspension in 'Good'-buffers.

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Abbreviation: CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

METHODS

Organism and growth. The wild-type *A. niger* ATCC 11414, better known as *A. niger* Wisconsin 72-4 (Clark *et al.*, 1966), was obtained from Dr W. Ahrens (Joh. A. Benckiser GmbH, Ludwigshafen, FRG) and maintained on yeast agar (l^{-1} : 4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar). Cultures (50 ml) were grown in baffled Erlenmeyer flasks in the mineral medium of Shu & Johnson (1948) which contained (l^{-1}): 2.5 g KH_2PO_4 ; 0.25 g $MgSO_4 \cdot 7H_2O$; 2.5 g NH_4NO_3 ; 0.24 mg $CuSO_4 \cdot 5H_2O$; 1.1 mg $ZnSO_4 \cdot 7H_2O$; 1.24 mg $FeCl_3 \cdot 6H_2O$ (pH = 3.8), supplemented with 14% (w/v) sucrose.

The cultures were inoculated with 10^5 conidia ml^{-1} and incubated at 27 °C with shaking (250 r.p.m.). Commercial-grade sucrose was purified by passage through a cation-exchange resin (Chelex 100, Bio-Rad no. 142-2852). All glassware, including that used for uptake experiments, was treated with hot nitric acid to remove contaminating trace elements. Determination of biomass as well as of sucrose or citrate in the culture filtrate was done as described by Hockertz *et al.* (1987).

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Calbiochem-Behring. Antimycin A, HEPES, MES and PIPES were supplied by Sigma. Myxothiazol was a kind gift of H. Reichenbach (Gesellschaft für Biotechnologische Forschung mbH, Braunschweig-Stöckheim, FRG). Standard chemicals were obtained from Merck.

Uptake of Mn^{2+} . Uptake by growing mycelia was examined in the citric acid production medium of Shu & Johnson (1948). Cultures from the early-exponential phase were used for the uptake experiments when a cell mass of 1.2 g dry wt l^{-1} was reached after 24 h growth. For assays with growing mycelia 10 ml portions were transferred to 100 ml baffled Erlenmeyer flasks for further incubation at 27 °C and 200 r.p.m. on a model G 76 gyratory water bath shaker. No significant delay of growth was induced by this transfer from the main culture to the smaller volume of the uptake assay. The biomass increased from 1.2 to 1.37 g l^{-1} within the 2 h duration of the uptake experiments, indicating that the Mn^{2+} -depleted culture of *A. niger* had the same generation time (14.3 h) in the uptake vessel as in the main culture.

For uptake experiments with resting mycelia, cultures were grown under Mn^{2+} depletion as above for 24 h. Portions (10 ml) were washed by two low-speed centrifugations (10 min, 1100 *g* at room temperature). The residual pellets were resuspended to the original volume in the appropriate buffer (Good *et al.*, 1966) each time, as indicated in the text. For energization of the cells the buffer contained 14% (w/v) sucrose (purified to remove trace-elements as above) unless otherwise stated. De-energization was achieved either by omitting sucrose or flushing the uptake vessels with nitrogen. The experimental format above was used for the uptake assays.

Both growing and resting mycelia were allowed to equilibrate for 20 min before adding Mn^{2+} . The inhibitor CCCP and the uncoupler 2,4-dinitrophenol (DNP) were added to growing mycelia simultaneously with Mn^{2+} . The respiration inhibitors antimycin A and myxothiazol were added to resting mycelia simultaneously with Mn^{2+} . For the competition experiments solutions of the chloride salts of Mg^{2+} , Ca^{2+} , Co^{2+} , Ni^{2+} , Fe^{2+} , Zn^{2+} , Cu^{2+} , Cd^{2+} and Mn^{2+} were added to the uptake vessels 1 min before the addition of Mn^{2+} to give a final concentration as indicated in Table 1.

$^{54}MnCl_2$ (Amersham, specific activity 100 $\mu Ci \mu g^{-1}$, 3.7 MBq μg^{-1}) was added to give a final $^{54}Mn^{2+}$ concentration of 1.85 pmol ml^{-1} unless otherwise stated. All sampling was done with Gilson pipettes using disposable tips which were cut to widen the opening sufficiently for sucking of the mycelial portions. In our system the use of young Mn^{2+} -depleted mycelia, harvested 1 d after germination, yielded suspensions homogeneous enough for uptake experiments (see Results).

At the beginning and the end of each experiment 0.1 ml samples were pipetted into vials for determination of total radioactivity within the culture. $^{54}Mn^{2+}$ was counted using a 12SW/3aW3 sodium iodide crystal with a RCA multiplier (Harshaw Chemie) and a LP 4840 Nokia Multichannel Pulse Analyzer. Counting efficiency was 21% for ^{54}Mn (c.p.m./d.p.m.). Binding of $^{54}Mn^{2+}$ to filters was about 0.05% of the total radioactivity added to the uptake vessels. The amount of unspecific binding to the cell surface was calculated by determination of the $^{54}Mn^{2+}$ content of cell samples at the beginning of the experiment. It was subtracted from the radioactivity contained in each cell sample to obtain uptake by cells unless otherwise stated.

RESULTS

 Mn^{2+} uptake by growing mycelia

Uptake of Mn^{2+} by exponentially growing *A. niger* cultures undisturbed by any manipulations or washing procedures was examined initially. As it is difficult to determine with precision the very low manganese content in the citric acid production medium, we took the observation of inhibited DNA synthesis (Hockertz *et al.*, 1987) as evidence that manganese deficiency was fully achieved. An abnormal morphology with short, thickened hyphae which aggregated to small spherical pellets (about 0.2 mm diameter) was induced in *A. niger* by Mn^{2+} deficiency. Such

Fig. 1. Effects of inhibitors on Mn²⁺ uptake in growing mycelia of *A. niger*. Values are corrected for growth during the uptake experiment, but not for specific binding to the cell surface. ■, Mn²⁺ uptake in the absence of DNP; ▲, uptake in the presence of 1 mM-DNP; △, uptake in the presence of 10 mM-DNP; □, uptake at 4 °C. The values shown are the mean $\pm$ SE of three experiments.

Table 1. Effect of divalent cation competition on ⁵⁴Mn²⁺ uptake by (a) growing or (b) resting mycelia of *Aspergillus niger*

Values shown (mean $\pm$ range of two experiments) are for uptake after 30 min in the presence of competing cation as indicated. The control value for uptake in the absence of any competing cation was 10.75 ± 0.95 nmol Mn²⁺ (g dry wt)⁻¹. ND, not determined.

Competing cation	Final cation concn	⁵⁴ Mn ²⁺ uptake (percentage of control)		
		(a) in Mineral medium (pH 3.8) + 14% sucrose		(b) in MES buffer (pH 5.6) + 14% sucrose
		1 mM	10 μ M	10 μ M
Mg ²⁺		100 $\pm$ 5	ND	100 $\pm$ 5
Ca ²⁺		94 $\pm$ 5	ND	92 $\pm$ 4
Co ²⁺		60 $\pm$ 4	ND	63 $\pm$ 2
Ni ²⁺		51 $\pm$ 2	ND	47 $\pm$ 2
Fe ²⁺		35 $\pm$ 3	80 $\pm$ 4	36 $\pm$ 2
Zn ²⁺		15 $\pm$ 4	70 $\pm$ 3	6 $\pm$ 2
Cu ²⁺		5 $\pm$ 1	50 $\pm$ 3	6 $\pm$ 1
Cd ²⁺		1 $\pm$ 0	30 $\pm$ 1	5 $\pm$ 1
Mn ²⁺		4 $\pm$ 1	ND	3 $\pm$ 0

Mn²⁺ depleted mycelia were metabolically active and could be handled reproducibly. These cells displayed a biphasic uptake of Mn²⁺ (Fig. 1). First, Mn²⁺ was rapidly bound during the initial 10 min after the addition of the cation. This initial binding of the metal to the anionic sites of the fungal cell wall still occurred at 4 °C and reached about 0.8 $\pm$ 0.2% of the total radioactivity in the experiments. However, although DPN was markedly inhibitory (Fig. 1), neither KCN nor sodium arsenate (both 10 mM) inhibited the second phase of Mn²⁺ uptake in growing mycelia (data not shown). The failure to observe inhibition by KCN or sodium arsenate under these experimental conditions might be explained by the rather acid pH of the production medium (3.8 at the beginning and falling to even lower values). However, KCN (10 mM) was able to inhibit Mn²⁺ uptake at pH 5.6. When the specificity of Mn²⁺ transport was studied in the presence of a 10⁵ molar excess of competing cations ⁵⁴Mn²⁺ uptake was unaffected by Mg²⁺ and Ca²⁺, but more or less suppressed by Cd²⁺, Cu²⁺, unlabelled Mn²⁺ and Zn²⁺ (Table 1). Oxygen uptake measurements revealed no toxicity of the competing cations, although Cd²⁺ and Co²⁺ produced a slight inhibition of respiration (less than 20% compared with the control) when the single chloride salts were added at a concentration of 1 mM.

Fig. 2

Fig. 3

Fig. 2. Effects of inhibitors on Mn^{2+} uptake in resting mycelia of *A. niger*. The data in the graph represent values corrected for unspecific binding. ■, Mn^{2+} uptake without inhibitory factors; ●, uptake in the absence of sucrose; ○, uptake in the presence of sucrose under nitrogen gassing; □, uptake in the absence of sucrose and with the addition of $200\ \mu M$ -antimycin A; ▲, uptake in the presence of 0.1% w/v myxothiazol. The values in the curve for uptake without inhibitory factors represent the mean $\pm$ SE for 16 experiments (for clarity error bars have been omitted from the curves which show inhibition of uptake).

Fig. 3. Effect of substances that alter membrane integrity on Mn^{2+} uptake. Uptake of Mn^{2+} at the pH optimum (pH 5.6) as control (■). Either $0.5\ M$ -toluene (□) or $5\ M$ -ether (○) was added after 1 h and the efflux of Mn^{2+} after this addition was measured. Values shown are the mean range of two experiments.

The uptake kinetics were determined from the linear rates of uptake over 10 min from Mn^{2+} concentrations in the range of 0.5 to $5.0\ \mu M$. The mean data of three separate experiments were plotted by the Lineweaver-Burk method, and the resultant straight line was fitted by the least-squares method. The intercepts on the y - and x -axes gave estimates of the maximal rate of transport, V_{max} , and apparent half-saturation constant, K_t , respectively. The degree of uncertainty in these estimates was quantified by calculating the standard error of the estimates, giving a mean value for K_t of $3.04\ \mu M\ Mn^{2+}$, range 2.93 to $3.15\ \mu M$, and for V_{max} of $6.12\ pmol\ (g\ dry\ wt)^{-1}\ min^{-1}$, range 5.63 to $6.61\ pmol\ (g\ dry\ wt)^{-1}\ min^{-1}$.

Mn²⁺ uptake by resting mycelia

Mn^{2+} uptake by *A. niger* was highly dependent upon the pH of the suspending medium, as was shown with washed mycelia resuspended in the buffers of Good *et al.* (1966), which have negligible metal-complexing capacity. $^{54}Mn^{2+}$ uptake was optimal between pH 5.6 and 5.9 and reduced to about 40% and 15% at pH 7.5 and 3.8 respectively. All further experiments with resting mycelia were done at pH 5.6, which resulted in $^{54}Mn^{2+}$ uptake after 2 h amounting to about 90% of the total radioactivity added, compared with 15% uptake observed with growing mycelia at pH 3.8. With resting mycelia, binding of $^{54}Mn^{2+}$ to the cell surface was low ($1.3 \pm 0.5\%$ of total radioactivity in the experiment) in the presence of sucrose and equalled that in assays with growing mycelia, but increased nearly fivefold when sucrose was omitted.

The energy-dependence of $^{54}Mn^{2+}$ uptake in *A. niger* observed previously with growing mycelia was further substantiated by results from experiments with metabolic inhibition of resting mycelia (Fig. 2). Compared to the control, anaerobic conditions established by gassing with nitrogen reduced uptake to 7% and omission of sucrose reduced it to 14%. Addition of myxothiazol ($2\ mg\ ml^{-1}$) suppressed $^{54}Mn^{2+}$ uptake to 14%, and complete inhibition was observed by the omission of sucrose and the addition of $200\ \mu M$ -antimycin A.

Since carrier-mediated transport systems require intact membranes as well as a functional 'permease' for substrate translocation we examined the effect of substances which alter membrane integrity. Treatment with toluene or diethyl ether released most of the recently translocated $^{54}Mn^{2+}$ from preloaded mycelia of *A. niger* (Fig. 3).

The effect of bivalent cations on ⁵⁴Mn²⁺ uptake was also studied with resting mycelia (Table 1). Uptake was unaffected by a 5000-fold molar excess of Mg²⁺ and Ca²⁺. Strong inhibition was observed by Zn²⁺, Cu²⁺, Cd²⁺ and unlabelled Mn²⁺, and moderate inhibition by Co²⁺, Ni²⁺ and Fe²⁺.

DISCUSSION

The paucity of information relating to metal ion uptake of filamentous fungi partly arises from problems associated with mycelial growth. The energy-dependent Mn²⁺-influx system demonstrated for *A. niger* is the first Mn²⁺-transport system known so far for filamentous fungi (Gadd, 1986), but can be compared with that recently described by Parkin & Ross (1986) for the yeast *C. utilis*.

As has been shown in other papers on metal ion uptake a fast, but limited adsorption preceded the energy-dependent Mn²⁺ uptake in *A. niger*. The very low adsorption values in our experiments with growing mycelia (about 1% only) were probably due to the presence of Mg²⁺ in the growth medium which had preferably saturated the anionic sites on the fungal cell surface able to bind Mn²⁺. As the resting mycelium of *A. niger* adsorbed only limited amounts of Mn²⁺ on its surface during the uptake assays in MES buffer too, either Mn²⁺ appears to behave differently from other cations such as copper or the application of Mn²⁺-depleted cells from the early exponential phase of growth and the presence of very high amounts of sucrose (14%) favoured uptake. Obviously due to masking of a low influx by adsorption, copper uptake could not be demonstrated with resting mycelium of *A. niger* (Townsend & Ross, 1986) although MES buffer was used as in this study. Such difficulties, which arise from high binding of the cation to be tested, might be circumvented by protoplasting as was shown in copper uptake studies on *Penicillium ochro-chloron* (Gadd & White, 1985).

The energy dependence of Mn²⁺ uptake by *A. niger* was shown by its sensitivity to low temperature and to the absence of oxygen or sucrose, and also well as by its sensitivity to both the uncoupling agent DNP and to the respiration inhibitor KCN. A branched respiration chain seems to be present in *A. niger* as Kubicek *et al.* (1980) observed a salicyl hydroxamic acid-sensitive bypass of respiration (Edwards & Kwiecinski, 1973) during the late trophophase in strain B 60. In this paper myxothiazol, which has been identified as an inhibitor of fungal respiration (Gerth *et al.*, 1980), caused a strong inhibition of Mn²⁺ influx in *A. niger* ATCC 11414. Myxothiazol, like other β -methoxyacrylates, has been classified as a group-I inhibitor of the mitochondrial bc₁ complex (Jagow & Link, 1986) which blocks reduction of the iron-sulphur centre (cytochrome c₁) and of cytochrome *b* via the Q_o site. The residual uptake of Mn²⁺ in the presence of a comparatively high concentration of myxothiazol (2 mg ml⁻¹) may be explained by the observation that group-I inhibitors of the mitochondrial bc₁ complex permit reduction of cytochrome *b* in a group-III inhibitor sensitive pathway by reversed electron flow through the b_h centre (Jagow & Link, 1986). However, Mn²⁺ influx in *A. niger* was fully suppressed by combining the action of a group-III inhibitor (antimycin A) with deprivation of energy supply through omission of sucrose.

We have not yet studied the possibility of an energy-linked system for Mn²⁺ efflux in *A. niger*, which has been described in bacteria (Schmid & Auling, 1987) and yeasts (Nieuwenhuis *et al.*, 1981; Parkin & Ross, 1986). However, as treatment of preloaded cells of *A. niger* for disintegration of membranes by toluene or diethyl ether (Jackson & De Moss, 1965; Vosberg & Hoffmann-Berling, 1971; Failla *et al.*, 1976) gave rise to substantial efflux, only a small portion of the recently translocated Mn²⁺ seems to be bound in *A. niger*. This finding contrasts the situation in *S. cerevisiae* where only one-fourth of the accumulated Mn²⁺ is left osmotically free due to sequestering in polyphosphates within the vacuoles (Okorokov *et al.*, 1977).

Mn²⁺ uptake, at the concentrations studied here, appeared to be via a highly specific mechanism, unlike the low-affinity divalent cation transporter previously observed in *S. cerevisiae* (Borst-Pauwels, 1981). As Mn²⁺ uptake was largely unaffected by a 10⁵ molar excess of both Mg²⁺ and Ca²⁺, the degree of cation specificity is in the range of that reported for Mn²⁺ transport in *Escherichia coli* (Silver *et al.*, 1970) and greater than that observed for Mn²⁺

transport in *C. utilis* (Parkin & Ross, 1986). The cation competition experiments with resting mycelia indicated that the high-affinity Mn^{2+} transport in *A. niger* was inhibited by Cd^{2+} , Zn^{2+} and Cu^{2+} to the same extent. Cd^{2+} inhibition of high-affinity Mn^{2+} uptake seems to be a common feature of bacterial systems (Tynecka *et al.*, 1981; Archibald & Duong, 1984; Laddaga *et al.*, 1985; Schmid & Auling, 1987). Mn^{2+} transport in *C. utilis* is competitively inhibited by Zn^{2+} with an inhibitory constant greater than K_i for Mn^{2+} (Parkin & Ross, 1986) and Cu^{2+} inhibits Mn^{2+} uptake in the diatom *Thalassiosira* (Sunda & Huntsman, 1983). Addition of Cu^{2+} can be used to antagonize contaminations of Mn^{2+} , which are deleterious in citric acid fermentations with *A. niger* (Kubicek & Röhr, 1986). Our data indicate that this antagonism is due to competition for uptake by a 'micronutrient' transport system.

The kinetic data obtained for Mn^{2+} transport with growing mycelia of *A. niger* characterize this system as a 'micronutrient' transport system which is still rapid under conditions of citric acid fermentation, although not working at optimum pH. Comparison of these data with those reported by Parkin & Ross (1986) for the Mn^{2+} transport of *C. utilis* reveal similar velocity, but lower affinity. However, the affinity constant is comparable with those reported for the Zn^{2+} micronutrient systems of *C. utilis* and *Aspergillus parasiticus* (Failla *et al.*, 1976; Failla & Niehaus, 1986).

Thus in conclusion, there exists a Mn^{2+} transport in *A. niger* which is energy-dependent, substrate-specific and of high affinity. This system is easily detected at submicromolar concentrations of the metal using manganese-depleted cells which are able to produce citric acid.

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