

Contribution of arginase to manganese metabolism of *Aspergillus niger*

Sarita Keni · Narayan S. Punekar

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Abstract Aspects of manganese metabolism during normal and acidogenic growth of *Aspergillus niger* were explored. Arginase from this fungus was a Mn[II]-enzyme. The contribution of the arginase protein towards *A. niger* manganese metabolism was investigated using arginase knockout (D-42) and arginase over-expressing (Δ XCA-29) strains of *A. niger* NCIM 565. The Mn[II] contents of various mycelial fractions were found in the order: D-42 strain < parent strain < Δ XCA-29 strain. While the soluble fraction forms 60 % of the total mycelial Mn[II] content, arginase accounted for a significant fraction of this soluble Mn[II] pool. Changes in the arginase levels affected the absolute mycelial Mn[II] content but not its distribution in the various mycelial fractions. The *A. niger* mycelia harvested from acidogenic growth media contain substantially less Mn[II] as compared to those from normal growth media. Nevertheless, acidogenic mycelia harbor considerable Mn[II] levels and a functional arginase. Altered levels of mycelial arginase protein did not significantly influence citric acid production. The relevance of arginase to cellular Mn[II] pool and homeostasis was evaluated and the results suggest that

arginase regulation could occur via manganese availability.

Keywords *Aspergillus niger* · Arginase · Manganese metabolism · Acidogenic growth

Introduction

Manganese is an essential micronutrient found in almost all biological systems where it functions as a co-factor for many enzymes (Crowley et al. 2000). Cellular Mn[II] ions also interact with various metabolites to form low molecular weight complexes; some of these function in protection against oxidative stress (McNaughton et al. 2010). On the other hand, excessive Mn[II] can be toxic and inhibits growth (Kosman 1994). Aspects of manganese metabolism with respect to growth, development and secondary metabolite production have been studied in a few fungi (Auling 1994). A lipid phosphatase (Cdc1p) located in the yeast endoplasmic reticulum is implicated in Mn[II] metabolism but does not affect cellular Mn[II] levels (Paidhungat and Garrett 1998). The *cdc1* homolog of *Neurospora crassa* (*frost*) is involved in manganese sensitivity and hyphal branching (Sone and Griffiths 1999). Various mushrooms have been analyzed for their Mn[II] contents because of their role in bioaccumulation of metal ions (Tuzen 2003). Our current understanding of manganese metabolism is

S. Keni · N. S. Punekar (✉)
Department of Biosciences and Bioengineering, Indian
Institute of Technology Bombay, Powai, Mumbai 400076,
India
e-mail: nsp@iitb.ac.in

largely confined to bacteria (Foster et al. 2014) and among fungi *Saccharomyces cerevisiae* is well represented (Reddi et al. 2009). Mn[II] transporters from the yeast and homologs from *N. crassa* and *Aspergillus fumigatus* have also been studied (Bowman et al. 2012; Pinchai et al. 2010).

A large number of enzymes require divalent metal ions for their activity, but very few of them are absolutely specific for Mn[II] (Crowley et al. 2000). Table 1 summarizes the available literature on enzymes from Aspergilli that can use Mn[II] ions. With very few exceptions, arginase is a strict Mn[II] enzyme across organisms (Ash 2004; Mendz et al. 1998). The *Aspergillus niger* arginase may therefore find a place in Table 1 as a prominent member with tightly bound Mn[II] ions. Arginase hydrolyzes arginine to ornithine and urea. Despite its catabolic role, significant basal levels of arginase are found in fungal mycelia grown on minimal media (Dave et al. 2012; Davis 1986). For these reasons, *Aspergillus niger* arginase could represent a significant cellular pool of Mn[II] and its levels could potentially influence fungal manganese metabolism. While the global effects of manipulating Mn[II] transport are

obvious, the impact of interfering with cellular Mn[II] binding proteins on manganese homeostasis is not.

In the present study, the contribution of *A. niger* arginase towards manganese metabolism was investigated using arginase knockout (D-42) and arginase over-expressing (Δ XCA-29) strains of *A. niger*. The cellular arginase protein content influenced the mycelial Mn[II] levels. This report for the first time demonstrates the involvement of arginase in the manganese homeostasis of any organism.

Materials and methods

Strains and growth conditions

The three different fungal strains used in this study were *A. niger* NCIM 565 (the parent strain), D-42 (an arginase knockout) and Δ XCA-29 (a strain that constitutively expresses arginase) (Dave et al. 2012). Preparation of spore inoculum and media (normal minimal medium—MM and acidogenic medium—AM) was as earlier (Punekar et al. 1985); all the media were prepared in de-ionized water. The Mn[II]

Table 1 Enzymes from Aspergilli that require Mn[II] for activity

Enzyme ^a	Metal ions that can substitute Mn[II]	Reference
Alkaline manganese peroxidase	None	Kanayama et al. (2002)
Exo-polygalacturonase	None	Maller et al. (2013)
Manganese superoxide dismutase	None	MacKenzie et al. (2005)
Prolidase	None	Jalving et al. (2002)
Adenylate cyclase	Not studied	Xu et al. (1989)
Carboxymethylcellulase	Not studied	Siddiqui et al. (1997)
Galactofuranosyltransferase	Not studied	Komachi et al. (2013)
NADH:Ubiquinone oxidoreductase	Not studied	Promper et al. (1993)
Oxaloacetase	Not studied	Ruijter et al. (1999)
Glutamine synthetase	Mg[II]	Punekar et al. (1985)
Malic enzyme	Mg[II]	Jernejc and Legisa (2002)
NADP ⁺ isocitrate dehydrogenase	Mg[II]	Kubicek and Rohr (1986)
Oxalate decarboxylase	Mg[II]	Emiliani and Bekes (1964)
PEP carboxykinase	Mg[II]	Kawasaki et al. (1995)
β -Glucosidase	Fe[II]	Gong et al. (2014)
Chitin synthase	Mg[III], Ca[II], Co[III]	Tatsuno et al. (1997)
Citrate transporter	Mg[III], Zn[II], Fe[III]	Netik et al. (1997)
Glucoamylase	Ca[II]	Benassi et al. (2014)

^a The enzymes have been grouped according to their Mn[II] requirement

concentration measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; $9.2 \pm 0.7 \mu\text{M}$, $n = 4$) in typical MM preparations was close to the value calculated from the MM composition ($10.8 \mu\text{M}$). While no explicit Mn[II] additions were made, the traces contributed by other media components to AM amounted to $0.1 \pm 0.1 \mu\text{M}$, $n = 5$ (inductively coupled plasma mass spectrometry, ICP-MS). Mycelial growth was carried out in shake flasks (220 rpm) at 30°C , either for 4 days (on MM) or for 8 days (on AM). For experiments involving pure arginase protein, *Escherichia coli* BL21 (DE3) (expressing *A. niger* arginase cDNA from pETNat) was grown in Luria–Bertani broth (with ampicillin at 0.1 mg/ml) at 37°C (Jayashri et al. 2009).

A. niger arginase purification

The *E. coli* BL21 (DE3) harboring pETNat was induced with 0.4 mM IPTG for 3 h at 37°C (Jayashri et al. 2009). Purification of expressed arginase was performed in three steps: ammonium sulfate precipitation, ion-exchange chromatography using DEAE Sephacel and gel filtration chromatography. All steps except gel filtration were performed at 4°C . The cell lysates were prepared in Buffer A (200 mM imidazole-HCl, 12 mM MnSO_4 , 2 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.5). The crude extract was subjected to 0–30 % and further 30–60 % ammonium sulfate fractionation. The pellet obtained was dissolved in Buffer B (25 mM imidazole-HCl, 1.2 mM MnSO_4 , 2 mM 2-mercaptoethanol, pH 7.5) and loaded onto DEAE Sephacel (Pharmacia LKB, Uppsala, Sweden) column. Elution was performed with a linear gradient of zero to 0.8 M KCl and arginase activity eluted around 0.4 M KCl. The peak activity fractions were pooled, ammonium sulfate precipitated and loaded onto Superdex 200 (HiLoad 16/60 prep grade, GE Healthcare, United Kingdom) gel filtration column, using 25 mM HEPES-NaOH at pH 7.5. The Mn[II] ions were not included in this final step of purification. This ensured that purified arginase protein containing only tightly bound metal ions was isolated. Suitably chosen active fractions from this column when pooled, provided the purified arginase protein. The purified arginase protein was a hexamer with a subunit molecular weight of about 40 kDa .

Preparation of arginase apo-enzyme

De-ionized water was used throughout for metal ion stoichiometry and Mn[II] interaction studies. Whenever the purified protein was needed for Mn[II] interaction/specificity studies MnSO_4 was omitted from the gel filtration buffer (as above). The apoenzyme form was prepared using a reported protocol (Lopez et al. 2005) with some modifications. Pure arginase protein was incubated with 25 mM EDTA and 3.0 M guanidinium hydrochloride in 10 mM Tris HCl (pH 7.5) for 1.0 h at 25°C . The sample was immediately desalted using HiPrep 26/10 desalting column (GE Healthcare) into 25 mM HEPES NaOH buffer (pH 7.5) at 4°C . The protein thus obtained had negligible arginase activity in absence of MnSO_4 and was used as the apoenzyme.

Mn[II] estimation and calculations

All glassware required during mycelial growth and other steps of Mn[II] estimation were washed with 20 % HNO_3 and rinsed with de-ionized water. The mycelia after filtration on muslin cloth were washed (five times with de-ionized water), blot dried and stored at -20°C . Corresponding samples were also dried in a hot air oven to constant weight and stored at -20°C . Crude extracts were prepared in Buffer A (but without MnSO_4), from wet weight samples by crushing frozen mycelia in a pre-chilled mortar and pestle, using acid washed sea sand. For obtaining the soluble fraction devoid of macromolecule ligands, crude extracts were ultra-filtered using a 3 kDa cut off filtration device (Pall Corporation, USA). The strategy for fractionation and the actual fractions analyzed are shown Fig. 1.

For Mn[II] analysis, the samples were digested in a microwave digester. The samples (about 1.0 g of mycelia or 1.0 ml of protein/extract + 6 ml HNO_3 + 0.4 ml H_2O_2 + 6 ml H_2O) were digested at 800 W (20 min), 1600 W (20 min), 0 W (20 min). The digested sample was analyzed by ICP-AES or ICP-MS.

The following conversion factors were used to compute physiological Mn[II] concentrations. (i) For mycelia grown on MM, 1.0 g dry weight = 2.54 ml intracellular volume (Slayman and Tatum 1964), (ii) for mycelia grown on AM, 1.0 g wet weight = 0.175 ml intracellular volume (Legisa and Kidric

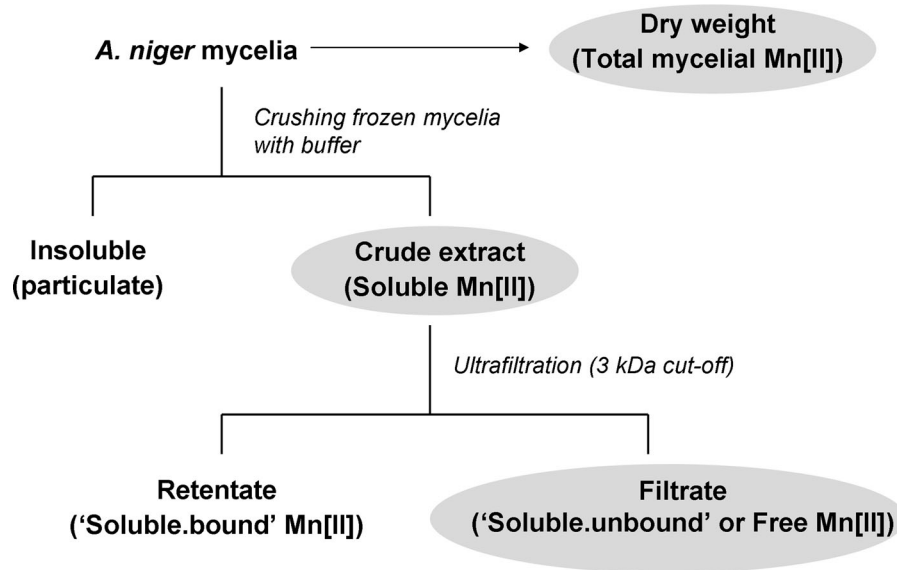


Fig. 1 Strategy to assess the distribution of Mn[II] ions in mycelial fractions. The Mn[II] concentration in the fractions highlighted in gray were experimentally measured

1989). The following average wet and dry weight inter-conversions were arrived at experimentally. (i) For mycelia grown on MM, 1.0 g dry weight = 5.0 g wet weight, (ii) for mycelia grown on AM, 1.0 g dry weight = 2.9 g wet weight. The proportion of Mn[II] in various fractions was calculated using the following formulae:

- (1) Soluble fraction = $\frac{\text{Total soluble Mn[II] in } \mu\text{M}}{\text{Total mycelial Mn[II] in } \mu\text{M}}$
- (2) ‘Soluble.unbound’ fraction
= $\frac{\text{ng Unbound Mn[II]/mg protein}}{\text{ng Total soluble Mn[II]/mg protein}} \times \text{Soluble fraction}$
- (3) ‘Soluble.bound’ fraction = Soluble fraction – ‘Soluble.unbound’ fraction

Arginase assay

Arginase activity was monitored by measuring urea according to Archibald’s method (Dave et al. 2012). The standard assay conducted at 37 °C involved a pre-incubation step with 0.8 mM MnSO₄ for 10 min, followed by addition of buffered arginine (150 mM) and incubation for 15 min. Unless stated otherwise, arginase assays were performed at pH 7.5 and in presence of 1 mg/ml BSA. One unit (U) of arginase is

the amount of enzyme that liberates 1.0 μmol of urea per min under the respective assay conditions.

Estimations

Citric acid in the spent medium was estimated by an enzymatic (citrate lyase) method as earlier (Dave et al. 2012). Protein estimation was done with the Bradford method (Bradford 1976) using bovine serum albumin as a standard. For metal stoichiometry analysis, arginase protein concentration was additionally measured using the extinction co-efficient (280 nm) of 25,440 M⁻¹ (obtained from ExPASy ProtParam software).

Western blot

Arginase protein from *A. niger* crude extracts was detected after the samples were run using native polyacrylamide gel electrophoresis. An enriched anti-arginase from rabbit serum served as the primary antibody. The anti-rabbit IgG tagged with alkaline phosphatase was used and detected using Nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate (Roche Diagnostics, Mannheim, Germany). The band intensities were quantified using ImageJ software.

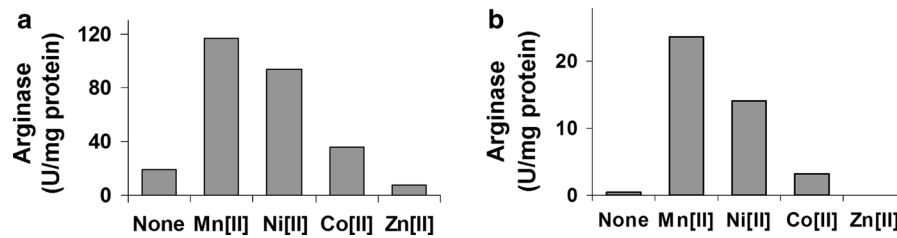


Fig. 2 Metal ion specificity of *A. niger* arginase. The arginase activity was measured in the presence of different metal ions (0.8 mM, as chlorides) for **a** purified arginase and **b** apoenzyme

Results and discussion

A. niger arginase is a Mn[II] activated enzyme

The *A. niger* arginase was expressed in *E. coli* BL21 (DE3) and purified in a buffer without Mn[II] ions. This enzyme showed significant activity even without Mn[II] added in the standard assays (see Materials and methods). The activity was enhanced further (about 5-fold) by including 0.8 mM of Mn[II] ions in the assay (Fig. 2a). Analysis of such an enzyme preparation by ICP-MS indicated that Mn[II] was the major metal ion tightly bound to the enzyme (Table 2) and a Mn[II] stoichiometry of about 3 Mn[II] ions per hexamer could be calculated. This stoichiometry is lower than that observed for mammalian arginases (one Mn[II] per subunit) (Orellana et al. 2002), whereas a much lower value is reported for the *N. crassa* arginase (Davis 1986).

The *A. niger* enzyme preparation (containing only the tightly bound Mn[II]) was also assayed by addition of different metal ions; the activity with Mn[II] was the highest followed by Ni[II] and Co[II], while Zn[II] showed a slight inhibitory effect (Fig. 2a). However, the apo-enzyme form generated by stripping off the tightly bound Mn[II] ions, was virtually inactive in the absence of added metal ions. The apo-enzyme was maximally activated by Mn[II] followed by Ni[II] and

form of purified arginase. Data are representative of two independent experiments (each performed in duplicates)

Co[II] (Fig. 2b). Clearly, the *A. niger* arginase prefers Mn[II] for optimal activity. The protein does retain tightly bound Mn[II] and is best activated by pre-incubation with Mn[II] ions; the pre-incubation may allow the weaker Mn[II]-binding sites to be occupied. Removal of the tightly bound metal ion appears to affect arginase irreversibly since the activity of the apoenzyme could not be fully reconstituted by pre-incubation with Mn[II] ions (Fig. 2). The metal ion preference is similar to that for well-studied arginases (Ash 2004), whereas *Helicobacter pylori* and *Bacillus anthracis* arginases are exceptions and prefer Co[II] and Ni[II], respectively (Mendez et al. 1998; Viator et al. 2008).

Arginase and mycelial Mn[II] levels in *A. niger*

The *A. niger* arginase is a Mn[II] enzyme and displays Mn[II] specificity in vitro. It was of interest to see if this translates into an effect on Mn[II] metabolism of the fungus. The contribution of the arginase protein towards *A. niger* manganese metabolism was investigated using arginase knockout (D-42), arginase over-expressing (Δ XCA-29) strains and the parent *A. niger* NCIM 565. Both the arginase activity (Dave et al. 2012) as well as the arginase protein (Fig. 3) in the three strains grown on MM, followed the pattern, D-42 (none) < NCIM 565 < Δ XCA-29. Since these strains differ only in their arginase protein contents, any change in Mn[II] levels could be attributed either directly or indirectly to arginase itself. The total Mn[II] content of D-42 strain was 10 % lower while that of Δ XCA-29 strain was 15 % higher than in the parent strain (Fig. 4a).

A systematic analysis of the Mn[II] content of various *A. niger* mycelial fractions (Fig. 1) was

Table 2 Estimation of metal ions bound to purified *A. niger* arginase

Metal ion ^a	Me[II] subunit ⁻¹	Me[II] hexamer ⁻¹
Mn[II]	0.45	2.72
Co[II]	0.01	0.06

^a The divalent metal ions (Me[II]) were estimated by ICP-MS

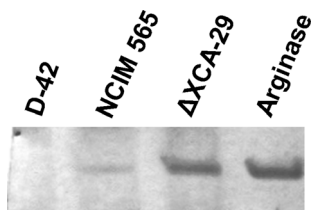


Fig. 3 Western blot of crude extracts of the three strains of *A. niger*. Crude extracts of strain D-42, strain NCIM 565, strain Δ XCA-29 were loaded as equal amount of protein (45 μ g). Pure arginase protein (0.19 μ g) served as positive control

undertaken. The total mycelial Mn[II] was broadly divided into particulate and soluble components. The soluble component consists of Mn[II] ions: (a) bound to various macromolecules ('soluble.bound') and (b) free in solution consisting of all Mn[II] species either unbound or complexed with small cellular metabolite chelators ('soluble.unbound'). This ill-defined Mn[II] buffer consists of small molecules such as amino acids, glutathione, organic acids and inorganic ligands (Foster et al. 2014). The arginase content of *A. niger* mycelia significantly affected the total soluble as well as the 'soluble.unbound' Mn[II] levels (Fig. 4). This was anticipated since arginase is a cytosolic protein. Accordingly, the total soluble Mn[II] in D-42 strain was 30 % lower than in the parent strain; and it was 55 % higher in the Δ XCA-29 strain (Fig. 4b). The 'soluble.unbound' Mn[II] levels in these three strains also followed a similar pattern (Fig. 4c). The results indicate that arginase protein levels influence mycelial Mn[II] levels. It is therefore likely that arginase may well function as a Mn[II] reservoir in *A. niger*. Fungal cytosolic arginine concentration during growth on MM (as was done here) is too low for significant catabolism by arginase (Davis 2010). Therefore, the effect of arginase on manganese metabolism appears to be independent of its catalytic function. This is the first study of arginase involvement in manganese metabolism and the effects are significant. The only other example which considered the effect of a Mn[II]-enzyme on Mn[II] metabolism is manganese superoxide dismutase (in yeast, *E. coli* and *Deinococcus radiodurans*); but there appears to be no agreement on the extent of superoxide dismutase contribution to cellular Mn[II] pools (Sharma et al. 2013; Tabares and Un 2013).

Interestingly, changes in the arginase content affected the absolute Mn[II] levels but not its distribution in the three fractions (Fig. 5). About 60 % of

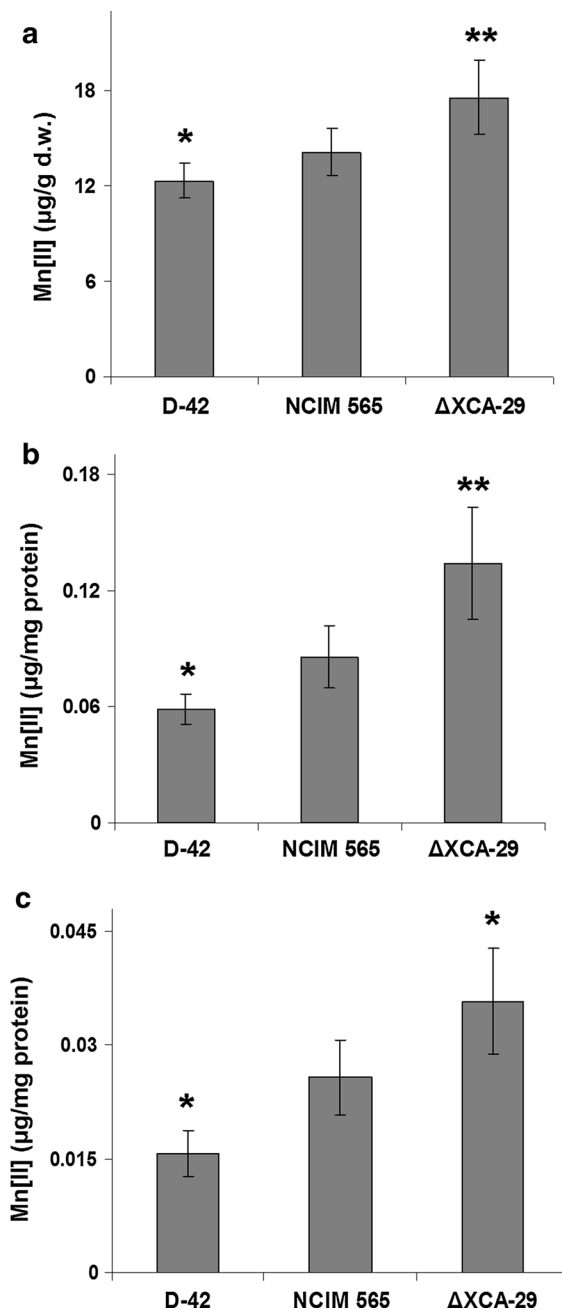


Fig. 4 Mn[II] contents in various fractions of *A. niger* mycelia. **a** Total mycelial Mn[II] normalized with mycelial dry weight (d.w.), * $P < 0.01$, $n = 13$, ** $P < 0.05$, $n = 8$, **b** total soluble Mn[II] normalized with protein content in the crude extract, * $P < 0.1$, $n = 6$; ** $P = 0.05$, $n = 6$, **c** 'soluble.unbound' Mn[II] normalized with protein content in the corresponding crude extract, * $P < 0.1$, $n = 4$. Results are presented as average \pm SEM. P values are from paired *t* tests performed with corresponding Mn[II] values of NCIM 565 within the same experiment. Samples for **a** and **b** were analyzed by ICP-AES and **c** by ICP-MS

the total Mn[II] was in the soluble fraction while the remaining 40 % as the insoluble (particulate) form. About 20 % of the total Mn[II] was unbound to any macromolecular ligand ('soluble.unbound'). This distribution of Mn[II] ions in the soluble versus the insoluble fractions is similar to that of *Saccharomyces carlsbergensis* (Okorokov et al. 1977). A higher level of control(s) seems to operate in sub-cellular manganese distribution in fungi.

Arginase kinetics under physiological Mn[II] concentrations

From the Mn[II] measurements reported above, the physiological concentrations of Mn[II] in *A. niger*

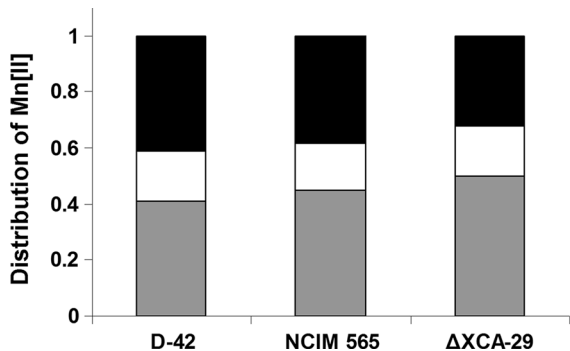


Fig. 5 The distribution of mycelial Mn[II] in *A. niger*: The proportions of Mn[II] in the 'soluble.bound' (gray), 'soluble.unbound' (white) and insoluble (black) fractions have been shown. The total soluble component includes the 'soluble.bound' and the 'soluble.unbound' fractions. The proportions were derived from data in Fig. 4, see "Materials and methods" section for details of calculations

NCIM 565 could be computed, albeit with certain assumptions about intracellular volume (see Materials and methods). The total mycelial Mn[II], soluble Mn[II] and 'soluble.unbound' Mn[II] concentrations of 100, 60 and 20 μM, respectively were estimated. The total *A. niger* mycelial Mn[II] concentration is comparable to that in *S. cerevisiae* (Luk and Culotta 2001). Much higher concentrations were found in *D. radiodurans* (radiation resistance; Sun et al. 2010) and mushrooms (metal bioaccumulation; Tuzen 2003).

Near physiological concentrations of arginine and Mn[II] when used, are known to significantly influence the observed kinetic parameters in some arginases (Carvajal et al. 1982; Davis et al. 1978; Maggini et al. 1992). A knowledge of mycelial Mn[II] distribution permitted the kinetic evaluation of *A. niger* arginase under near-physiological conditions. The enzyme would experience around 20 μM of Mn[II] ions in the cytosol. Therefore, arginine saturation was performed at this near in vivo Mn[II] concentration; the saturation was hyperbolic with a K_M for arginine of 20 mM and a V_{max} of around 200 U/mg protein (Fig. 6a). The near-physiological Mn[II] concentrations appear adequate for efficient functioning of arginase over a wide range of arginine concentrations. At 150 mM arginine, pure arginase displayed a hyperbolic activity pattern over an initial range of Mn[II] concentrations; a Mn[II] dissociation constant ($K_{Mn[II]}$) of 15 μM was obtained (Fig. 6b). Higher concentrations of Mn[II] were inhibitory with a K_I of 250 μM. Since the pure protein contains tightly bound Mn[II] as well (see earlier section), the $K_{Mn[II]}$ determined by Mn[II] saturation represents the affinity

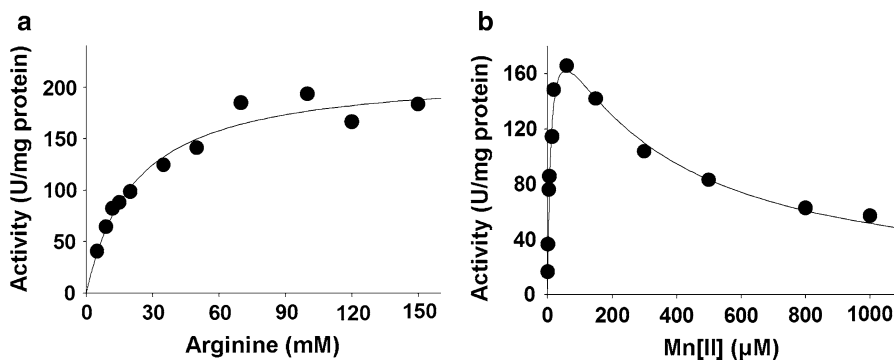


Fig. 6 Arginase kinetic features under simulated physiological conditions. **a** Arginine saturation of purified arginase with 20 μM Mn[II] in the assay. **b** Mn[II] saturation kinetics using 150 mM arginine. Circles represent actual data points while the

line represents the best fit using SigmaPlot 11.2 Enzyme Kinetics software. Data are representative of three independent experiments, each performed in duplicates

of arginase to loosely bound Mn[II]. Interestingly, the $K_{Mn[II]}$ closely mirrors the ‘soluble.unbound’ Mn[II] concentration in *A. niger*. Yet another key enzyme from this fungus namely, glutamine synthetase also responds to Mn[II] concentration in the lower μM range (Punekar et al. 1985). That arginase activity in vivo can be modulated by changes in Mn[II] availability appears feasible. This may hold particular significance when the fungus experiences diverse Mn[II] environments.

The *A. niger* arginase is a cytosolic enzyme. Based on the observed values for $K_{Mn[II]}$ and [Mn[II]] (15 μM and 20 μM , respectively), the Mn[II] binding equilibrium for arginase (at the loosely Mn[II]-binding site) could be estimated. About one half of the total arginase protein in *A. niger* (grown on MM) would be in fully Mn[II]-bound state. The rest would contain only the tightly bound Mn[II]. The actual proportion of fully Mn[II]-saturated arginase will depend on the concentration of free Mn[II] in the fungal cytoplasm. The cytosolic manganese availability can vary because of factors such as sequestration by the fungal vacuole (Okorokov et al. 1977; Reddi et al. 2009), buffering by polydisperse metal ion buffers and other environmental cues (Foster et al. 2014). For instance, the Mn[II] levels in a bacterial cytosol can vary; the Mn[II] is elevated in response to oxidants, to correctly metallate manganese superoxide dismutase (Imlyay 2014).

Acidogenesis—an altered Mn[II] metabolism?

Acidogenesis (citric acid production) is a well known example of Mn[II]-deficient growth of *A. niger*. This process is highly sensitive to the presence of Mn[II] ions in the growth medium; presence of ≥ 50 ppb of Mn[II] reduce citric acid yields significantly in various strains of *A. niger* (Karaffa and Kubicek 2003). However, the primary roles of Mn[II] deficiency in citric acid accumulation remain unclear even today. We therefore analyzed *A. niger* NCIM 565 mycelia, harvested during maximal growth phase on AM (48 h) and MM (24 h), for their Mn[II] content. The mycelia committed to acidogenic growth contained about 8-fold lower Mn[II] content (in both the total and the soluble fractions) when compared to normal mycelia (Table 3). The proportion of Mn[II] ions in the mycelial soluble fraction was similar when the fungus was grown either on AM or on MM. Nevertheless, this

Table 3 Mn[II] levels in acidogenic mycelia of *A. niger* NCIM 565

Fraction	Content ($\mu\text{g Mn[II]}/\text{g d.w.}$)		Concentration ^a (μM)	
	On MM	On AM	On MM	On AM
Total mycelial	14.2	1.8	100	65
Soluble	8.7	1.3	60	48

^a See “Materials and methods” section for conversion factors used for calculating mycelial concentrations. Mycelia grown on MM and AM were analyzed by ICP-AES and ICP-MS, respectively

Mn[II] content of acidogenic mycelia is notable as it is found despite the fact that AM is a manganese limited growth medium (see Materials and methods). The growth of *A. niger* is very sensitive to Mn[II] availability and its ability to cadge this micronutrient was exploited for manganese bioassay (Sulochana and Lakshmanan 1968). Efficient and specific manganese transporter(s) is reported to function under citric acid production conditions (Hockertz et al. 1987). The degradation of Mn[II] transporters may be inhibited in yeast starved of manganese (Bleackley and Macgillivray 2011). Such factors may be at play and thereby resulting in significant mycelial Mn[II] levels during acidogenic growth of *A. niger*.

Arginase during acidogenic growth

In terms of mechanistic details, citric acid fermentation by *A. niger* continues to be an enigma. Many morphological, physiological and biochemical studies are reported over the years to explain acidogenesis. These include bulbous, branched mycelia with pellet morphology, metabolic and enzyme regulation at the level of glycolysis, Krebs cycle and sub-cellular compartments, altered membrane phospholipids and β -glucan content of the cell wall etc., (Karaffa and Kubicek 2003). These effects are a cumulative outcome of the fermentative growth of *A. niger* on AM and not addressed by manganese limitation alone. Despite extensive physiological/biochemical literature the metabolic circuits leading to acidogenesis are ill defined; the contribution from nitrogen metabolism is poorly understood (Kubicek and Rohr 1986; Paggianni et al. 2005; Punekar et al. 1985). A possible involvement of *A. niger* arginase in the acidogenic metabolism is suggested by the following:

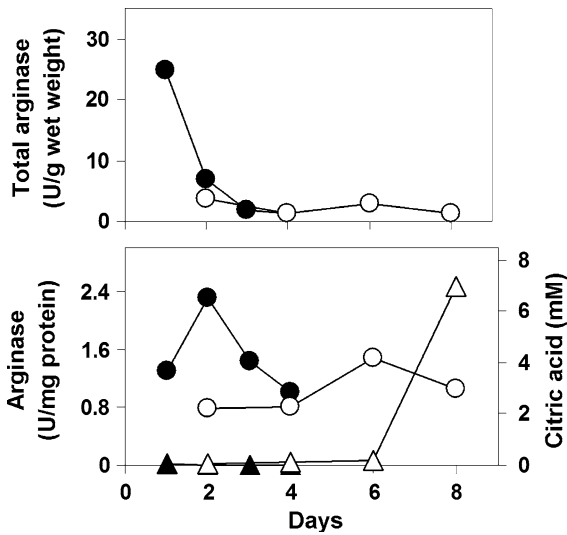


Fig. 7 Arginase activity during acidogenic and normal growth of *A. niger*. Arginase total activity (top panel) and arginase specific activity (bottom panel) in mycelia grown on AM (open circles) and MM (filled circles) were measured. The arginase assays were performed at pH 10.6 using 1 mM MnSO₄. Citric acid concentration in the spent medium at the corresponding time points for AM (open triangles) and MM (filled triangles) is shown in the bottom panel. Data are representative of three independent experiments, each performed in duplicates

(a) acidogenesis is a manganese sensitive process and manganese deficiency leads to elevated amino acid pools (and arginine in particular) during acidogenic growth (Kubicek and Rohr 1986), (b) arginase is a Mn[II] enzyme and its levels influence the fungal Mn[II] pool (this study) and (c) arginase is the sole route for arginine degradation in this fungus (Dave et al. 2012).

Arginase activity could be detected in *A. niger* NCM 565 mycelia throughout the course of growth on

AM. The arginase content (expressed as total U/g wet weight) was lower in acidogenic mycelia when compared to mycelia grown on MM (Fig. 7, top panel). However, the arginase specific activity patterns were comparable on the two growth media (Fig. 7, bottom panel). Growth on AM results in very different mycelia morphology and significantly lowered protein content and intracellular volume per unit biomass (see Materials and methods). Clearly, active arginase protein does exist during acidogenic growth and sufficient Mn[II] is available in these mycelia (see Table 3; Figs. 6a, 7) for arginase to function in vivo. Therefore, it is unlikely that elevated arginine pools observed during acidogenic growth is due a compromised arginase activity because of Mn[II] deficiency.

Comparable citrate yields (between 6 and 10 mM) were obtained for NCIM 565, D-42 and ΔXCA-29 strains of *A. niger*, suggesting that lack or over-expression of arginase did not affect the ability of the fungus to produce citric acid. The *A. niger* NCIM 565 studied here produces citric acid but is not an industrial strain. While it may be worthwhile extending this study to other high yielding *A. niger* strains, that there is a strain-to-strain variation in the manganese effect (for example, see Gupta and Sharma 1995) makes the analysis difficult.

Arginase and Mn[II] toxicity

Variations in mycelial Mn[II] pools consequent to altered arginase levels can potentially influence aspects of fungal physiology including Mn[II] toxicity. The effect of a range of Mn[II] concentrations on the growth of *A. niger* on MM was assessed. Despite their differences in mycelial Mn[II] levels, the three

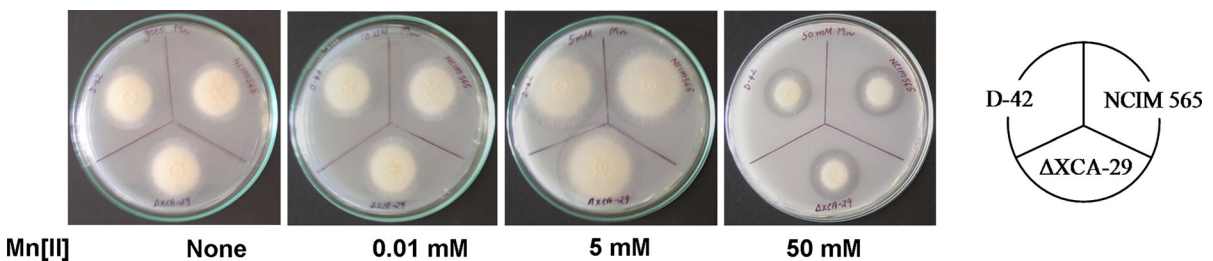


Fig. 8 Effect of Mn[II] on growth of *A. niger* strains. Radial growth (after point inoculation with equal number of conidia) was recorded at 60 h as a function of Mn[II] concentration supplemented to MM. High Mn[II] concentrations made the

MM turbid and subsequent radial growth was surrounded by zones of clearance possibly due to chelation of Mn[II] ions by secreted organic acids. (0.01 mM of Mn[II] is normally present in MM)

A. niger strains (D-42, NCIM 565 and ΔXCA-29) showed similar growth and Mn[II] toxicity profiles (Fig. 8). Concentrations higher than 5 mM were toxic to *A. niger*. The intracellular Mn[II] contents are affected in the Mn[II] transport mutants of *S. cerevisiae* and *D. radiodurans* and they display significantly altered growth responses to Mn[II] toxicity (Lapinskas et al. 1995; Sun et al. 2010). The mycelial Mn[II] pool changes due to differing arginase levels may not be this drastic and the effects may become apparent only under specific physiological states. Fungal vacuoles play a significant role in sequestering Mn[II] ions (Okorokov et al. 1977) and may account for masking any growth effects in these *A. niger* strains. Nevertheless, a change in cellular Mn[II] levels may have implications for manganese homeostasis (Reddi et al. 2009) and/or mis-metallation of enzymes (Foster et al. 2014).

Conclusions

Access to three *A. niger* strains (D-42, NCIM 565 and ΔXCA-29) which differ in their arginase content served to evaluate the contribution of arginase to manganese metabolism of this fungus. Changes in the cellular arginase protein and the total Mn[II] content were directly correlated. The *A. niger* arginase is a Mn[II]-enzyme and forms an important fraction of soluble Mn[II] pool of the mycelia. Despite the manganese deficient nature of AM, acidogenic mycelia harbor significant Mn[II] levels and a functional arginase. This study brings out the relevance of arginase in cellular Mn[II] homeostasis and suggests that arginase regulation could occur via manganese availability.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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