ELSEVIER

Biochimica et Biophysica Acta 1326 (1997) 287–294

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Uptake and export of citric acid by *Aspergillus niger* is reciprocally regulated by manganese ions

Angela Netik [a], Nestor V. Torres [b], Jose-Maria Riol [b], Christian P. Kubicek [a,*]

[a] *Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU Wien, Getreidemarkt 9 / 172.5, A-1060 Wien, Austria*

[b] *Grupo de Tecnologia Bioquimica y Control Metabolico, Departamento Bioquimica Biologia Molecular, Universidad de la Laguna, La Laguna 38206, Santa Cruz de Tenerife, Canary Islands, Spain*

Received 19 November 1996; revised 11 February 1997; accepted 11 February 1997

Abstract

The uptake as well as the export of citric acid by *Aspergillus niger* occur by active, ΔpH-driven, H^+-symport dependent systems. They are inhibited by nonmetabolizable tricarboxylic acid analogues and phthalic acid, and by several other mono-, di- and tribasic organic acids. However, citrate export could only be demonstrated in a mycelium cultivated under manganese-deficient growth conditions, whereas the uptake of citrate from the medium was only detectable upon precultivation of *A. niger* in a medium supplemented with Mn^{2+} ions. In addition, the uptake of citrate was dependent on the presence of Mn^{2+} ions in the assay, and inhibited by EDTA. This requirement for Mn^{2+} could also be partially fulfilled by Mg^{2+}, Fe^{2+} or Zn^{2+}, whereas Cu^{2+} ions inhibited citrate transport. The observed divergent effects of manganese ions on citrate uptake and citrate export may be a major reason for the well documented requirement for manganese deficiency of citric acid accumulation.

Keywords: Citric acid; Manganese; (*Aspergillus niger*)

1. Introduction

Aspergillus niger has been widely used during the past 50 years as a commercial producer of citric acid. The complexity of this process and its dependency on several unusual nutritional conditions has attracted numerous biochemical investigations [1,2].

Despite this interest in the regulation of citric acid biosynthesis and breakdown, however, the mechanism of secretion of citric acid has not been studied to any extent. Although the concentrations for intracellular citric acid quoted by different authors differ considerably (2–30 mM; cf. [3–5]), they are orders of magnitude lower than the extracellular concentration ($>$ 100 g/l which is equivalent to $>$ 0.5 M) and thus suggest the occurrence of an active export. The application of sensitivity analysis to a comprehensive mathematical model of the metabolism of *A. niger* in conditions of citric acid production identified citrate transport as one of the control points in citric acid accumulation [6]. The observation that a reduction of the extracellular accumulation of citric acid is not always reflected in a similar reduction of its intracellular citric acid pool concentration [3,5] illustrates this point.

* Corresponding author. Fax: +43 1 5816266.
E-mail: ckubicek@fbch.tuwien.ac.at

PII S0005-2736(97)00032-1

Export of organic acids (i.e. succinate and malate) by *Saccharomyces cerevisiae* has been shown to occur by an active process depending on membrane energization [7,8]. In bacteria, some metabolite efflux processes appear to be mediated by an inversion of the uptake system [9,10], although this has been shown to be less likely for glutamate secretion by *Corynebacteria* [11–13]. In this paper, we report the properties of the citrate export as well as the citrate uptake system from a citric acid producing strain of *A. niger*.

2. Materials and methods

2.1. Materials

All chemicals used were standard analytical grade and provided by Merck (Darmstadt, FRG) or Sigma (St. Louis, MO). For citric acid fermentations, food quality crystalline beet sugar (Leopoldsdorfer Zuckerfabrik, NÖ, Austria) was used, which was decationized by treating 2 l of a 20% (w/v) sucrose thrice (for 10 min each) in batch with 100 g of Dowex AG 50W-X8 prior to its use for nutrient medium preparation.

2.2. Organism and media

A. niger ATCC 11414, a strain producing high yields of citric acid, was used as the parent strain in the course of this study. Conditions for its maintenance, inoculum preparation, and growth on different carbon compounds have been described previously [14,15]. Citric acid fermentations were carried out in 1 l wide-mouthed shake flasks containing 100 ml of medium, agitated (250 rpm) on a rotary shaker at 30°C for up to 240 h.

2.3. Extraction and quantification of citric acid

Mycelia were harvested by suction filtration on a precooled linen cloth, immediately immersed in ice-cold 0.1 M HCl containing 0.1 M NaCl, stirred for 5 min at 4°C, filtered as described above, and washed with the same solution thrice. This procedure removed more than 98% of the extracellular citrate, whereas it did not lead to leakage of intracellular citrate into the medium (data not shown). An intracellular volume of 1.3 ml/g dry weight was used for the calculations [16]. Extra- and intracellular citrate were quantified by enzymatic analysis [17], using a commercial test kit (Boehringer-Mannheim, Mannheim, FRG).

2.4. Assay of citrate export

Mycelia were harvested by suction filtration and washed with 500 ml of tap water to remove $>95\%$ of the citrate adsorbed to the mycelia. They were then suspended to a final biomass density of 3–4 g dry weight/ml in 10 mM sodium phosphate, pH 5.0, and aliquots of 10 ml of this suspension was pipetted into 100 ml Erlenmeyer flasks. Samples of 1.0 ml were withdrawn after appropriate intervals of incubation, centrifuged in an Eppendorf centrifuge ($5000 \times g$, 3 min), and citrate assayed in the supernatant by enzymatic analysis [17], using a commercial test kit (Boehringer-Mannheim, Mannheim, FRG).

2.5. Assay of citrate uptake

Uptake of citric acid by mycelia of *A. niger* was studied using [1,5-^{14}C]citric acid (4.1 GBq/mmol, Amersham Life Science, Cardiff, UK) at a final concentration of 100 μM, unless otherwise stated. To this end, they were pregrown on 1 or 14% (w/v) sucrose for 28 h or 40 h, respectively (at which time they were approximately in the middle of their growth phase). The mycelium from one flask was harvested by suction filtration, and washed with 500 ml of tap water. It was then suspended in 10 mM sodium phosphate, pH 3.3, to give a mycelial suspension of approximately 2 g dry weight per liter. Portions of 10 ml of this suspension were distributed into several 100 ml Erlenmeyer flasks, and incubated in a shaking water bath for 15 min. This time was necessary for the fungus to adapt to the new conditions and guaranteed linear uptake rates. The suspension was then pulsed with 0.37 MBq [1,5-^{14}C]citric acid, and the uptake – and $^{14}CO_2$ evolution, if required – measured as described previously [18]. All data were related to the amount of mycelial biomass dry weight, which was determined from separate samples of the initially prepared mycelial suspension.

3. Results

3.1. Manganese deficient cultivation is required for citrate export

Our initial strategy to demonstrate citrate efflux from *A. niger* was based on loading mycelia with different total concentrations of [1,5-^{14}C]citrate, and measuring the kinetics of subsequent release of radioactive citrate into the medium. However, whereas mycelia pregrown on glucose or citrate (as sole carbon source under otherwise complete nutrient conditions) took up radioactive citrate, we could not detect its release into the medium within subsequent 90 min (Fig. 1a). Since less than 15% of the label taken up was released as CO_2 (data not shown), this suggests a high metabolic stability of citrate in these cells. Incubation of heat-inactivated mycelia with [1,5-^{14}C]citrate did not show any 'uptake' hence providing evidence that the measured uptake rate was not only due to adsorption to the fungal cell walls.

In contrast, mycelia cultivated in a medium that stimulated citric acid production, take up no [1,5-^{14}C]citrate (Fig. 1a). Several control experiments were carried out to ensure that this was not due to a carry-over of and thus competition by unlabelled citrate from the medium.

As an alternative strategy to measure citrate export, we replaced thoroughly washed, citric acid producing mycelia in buffer and followed the export of citrate by enzymatic analysis. The release of citrate into the medium under these conditions was not due to leakage from the cells or release of adsorbed material, since it could be instantly inhibited by the uncoupling agent 2,4-dinitrophenol and the cytochrome-C oxidase inhibitor NaN_3. Citrate export thus appears to be energy dependent. Furthermore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, a protonophore) drastically inhibited citrate transport suggesting that it occurs by proton symport. On the other hand nigericin, a ionophore which dissipates the ΔpH, had only a very slight inhibitory effect which indicates that citrate import is independent of ΔpH (Table 1). Citrate export showed highest activity at an extracellular pH of 2.0, and only a fourth of that rate was observed at pH 7.0.

Table 1
Effect of selected inhibitors on the export of citric acid [a]

Compound	Concn. (M)	Export activity (nmol/min per mg cell dry weight)
Control	–	0.521 (±0.032)
H^+	10^{-7}	0.264 (±0.019)
H^+	10^{-2}	1.031 (±0.021)
NaN_3	10^{-3}	< 0.030
CCCP	10^{-5}	< 0.030
2,4-DNP	10^{-4}	0.117 (±0.010)
Nigericin	10^{-4}	0.323 (±0.016)

[a] All experiments were carried out at pH 5, unless otherwise indicated. Values are means of at least three separate experiments, and standard deviations are given in parentheses.

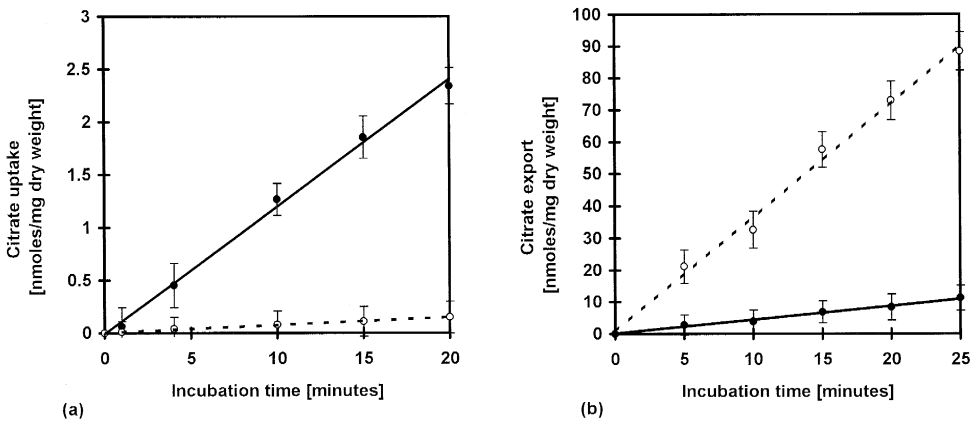

Fig. 1. Citric acid uptake (a) and export (b) by mycelia cultivated in a citric acid producing medium in the presence ($5 \cdot 10^{-5}$ M; full symbols and solid lines) or absence (empty symbols and dotted lines) of Mn^{2+} ions, respectively. Vertical bars show the standard deviation ($n = 4$).

Citrate export could be observed with mycelia which had been precultivated under normal growth conditions on glucose or citrate as a carbon source. We found that the manganese ion concentration of the nutrient medium was exclusively responsible for this effect: decreasing the Mn^{2+} ion concentration in the growth medium resulted in clearly measurable citrate export, e.g. 0.74 and 1.37 nmol/min per mg dry weight for 10^{-7} and 10^{-8} M, respectively. This effect was dependent on the manganese ion concentration in the growth medium and could not be counteracted by appropriate alterations of assay conditions, as the omission of manganese ions from the export assay, or their chelation by EDTA. Consistent with this, the addition of manganese ions to Mn^{2+}-deficiently grown mycelia during the export assay did not inhibit their citrate export activity. It should be noted that the intracellular citrate concentration in mycelia pregrown in the presence of $5 \cdot 10^{-5}$ M Mn^{2+} and practically lacking citrate export activity was 42% of that measured in Mn^{2+}-deficient grown (and citrate exporting) mycelia (1.7 vs. 4.1 mM).It is unlikely that this concentration difference could explain the absence of export activity in the presence of Mn^{2+} by a shortage of substrate availability.

1,2,3-Benzenetricarboxylic acid and tricarballylic acid, nonmetabolizable inhibitors of mitochondrial citrate translocation [19], inhibited citrate export when added at final concentrations of 50 mM (63 and 17% inhibition, respectively).

3.2. Manganese ions are required for citrate uptake

Since the export of citrate was detectable only under manganese deficient growth conditions, we investigated whether manganese ions also influenced citrate uptake. This was indeed the case: at concentrations below 10^{-8} M Mn^{2+}, virtually no citrate uptake was measurable (Fig. 2). Citrate uptake recovered in a concentration-dependent manner with increasing Mn^{2+} concentrations, and was comparable to the control (i.e. the uptake rate under normal growth conditions) at about $5 \cdot 10^{-5}$ M. Hence manganese ions influenced the formation of the uptake and the export system for citrate in a reciprocal manner.

Interestingly, the effect of Mn^{2+} on citrate uptake depended both on its presence during growth as well as in the uptake assay: whereas the uptake of citrate

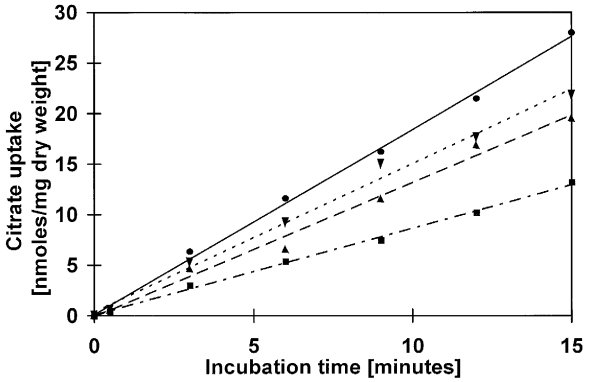

Fig. 2. Reversal of EDTA inhibition of citric acid uptake by Mn^{2+} ions. ●, control (no EDTA, no extra Mn^{2+}; ■, 2 mM EDTA; ▲, 2 mM EDTA plus 10^{-6} M Mn^{2+}; ▼, 2 mM EDTA plus $5 \cdot 10^{-5}$ Mn^{2+}.

by mycelia pregrown in the presence of manganese ions could be inhibited by the addition of EDTA, and this inhibition could be counteracted by addition of increased Mn^{2+} concentrations (Fig. 3), addition of manganese ions to manganese deficiently grown mycelia did not restore citrate uptake (data not shown). This counteraction in the assay system was rather specific for manganese ions, since other cations such as Mg^{2+}, Zn^{2+}, or Fe^{3+} could only partially restore citrate transport, and high concentrations were even inhibitory (data not shown). Cu^{2+} strongly inhibited citrate uptake at all concentrations tested (73 and 88% inhibition at $1.5 \cdot 10^{-5}$ and $7.5 \cdot 10^{-5}$ M, respectively). In summary, we conclude from these

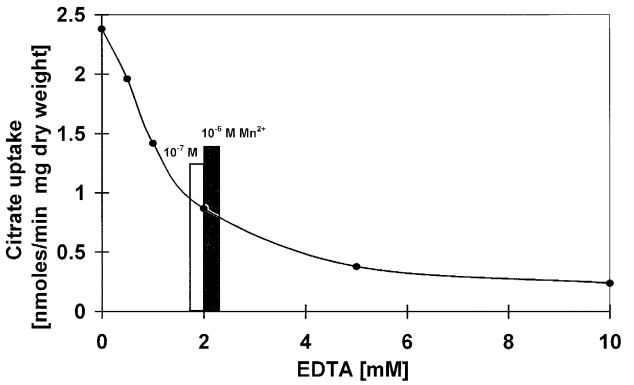

Fig. 3. Inhibition of citric acid uptake by EDTA and its reversal by Mn^{2+}. The effect of addition of 10^{-7} M and 10^{-6} M Mn^{2+} in the presence of 2 mM EDTA is shown by an empty and a solid bar, respectively.

data that the import of citrate by *A. niger* requires the presence of manganese ions, both for formation as well as for activity.

3.3. Citric acid import by A. niger is catalyzed by a constitutive inducible system

Having found conditions where citrate uptake by *A. niger* was clearly demonstrable, we examined some properties of this uptake system. A variation of the concentration of citrate in this system and statistic evaluation of the data according to a nonlinear least square estimation procedure within the mathematical package (Wolfram Research Trade Center Drive, Champain, IL) yielded data in favour of a single uptake system characterized by a K_m of 220 ($\pm$15) μM and V_{max} of 3.6 nmol/min per mg dry weight (Fig. 4). The permease was present during growth on glucose, but its activity increased about 3-fold upon growth on citrate. The effect of 2,4-dinitrophenol, NaN_3, CCCP, nigericin and pH on citrate import is shown in Fig. 5. The inhibition by these compounds and the effect of pH suggests that it occurs by proton symport and independent of ΔpH, similarly as for citrate export.

In order to investigate the specificity of citrate transport by *A. niger*, the ability of several tri-, di- and monocarboxylic acid metabolites to compete with uptake of [1,5-^{14}C]citrate was investigated (Table 2). Most of them were found to be competitors, succinate, malate and pyruvate being strongest. Among nonmetabolizable analogs of citric acid, tricarballylic acid, 1,2,3-benzenetricarboxylic acid and phthalic acid all were inhibitors of citrate uptake.

As the addition of 50 mM tricarballylic acid inhibited citrate import more strongly than citrate export

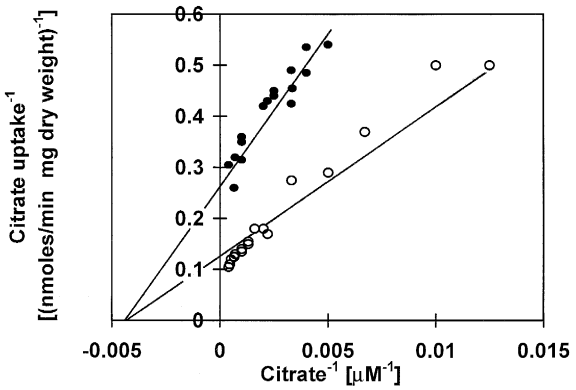

Fig. 4. Lineweaver–Burk plot of the effect of the concentration of citrate on the activity of the citrate uptake system. Full symbols: mycelia pregrown on glucose; empty symbols: mycelia pregrown on citrate. K_m values calculated were 220 ($\pm$40) and 280 ($\pm$45) μM, and V_{max} 3.6 and 8.9 nmol/min per mg dry weight for glucose- and citrate-grown mycelia, respectively. Nonlinear least-squares estimation was carried out on both data sets yielding $r = 0.972$ and $r = 0.99$, respectively.

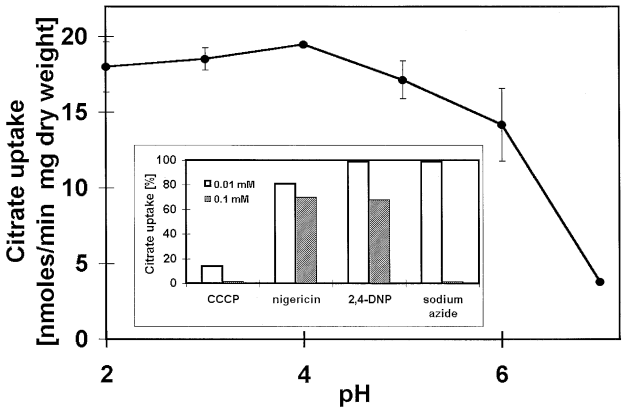

Fig. 5. Effect of pH and some agents interfering with H^+ formation and compartmentation on the uptake of citrate. The inset shows a bar chart of the relative effect of CCCP, nigericin, 2,4-DNP and NaN_3. The activity of the citrate uptake system in the absence of any inhibitor is taken as 100%.

Table 2
Effect of organic acid metabolites and nonmetabolizable analogues on citrate uptake [a]

Component	Uptake activity (nmol/min per mg cell dry weight)
Control	1.57 ($\pm$0.05)
Isocitrate	0.41 ($\pm$0.12)
Malate	0.11 ($\pm$0.09)
Succinate	0.15 ($\pm$0.09)
α-Ketoglutarate	0.48 ($\pm$0.13)
Lactate	0.60 ($\pm$0.08)
Pyruvate	0.25 ($\pm$0.11)
1,2,3-Benzenetricarboxylate	0.39 ($\pm$0.07)
Tricarballylate	0.12 ($\pm$0.05)
Phthalate	0.08 ($\pm$0.03)

[a] Control experiments were carried out with mycelia cultivated in the presence of $5 \cdot 10^{-5}$ M Mn^{2+} ions. Citrate concentration was 50 μM in all cases. Organic acids were tested at final concentrations of 50 mM. Values are means of at least three separate experiments, and standard deviations are given in parentheses.

(see above), we investigated whether its addition to mycelia pregrown at a suboptimal manganese ion concentration (i.e. 10^{-7} M; at which both the import as well as the export system are present) has an effect on the citrate export rate. In fact, the rate of citrate export was increased from 0.19 to 0.25 nmol/min per mg dry weight, thereby indicating that the relative uptake rates of the two systems influence each other.

4. Discussion

Excretion of organic acid metabolites by yeast has been reported to occur as a result of passive diffusion of the undissociated acid through the plasma membrane [7,8]. According to its three pK_a values citric acid is almost completely dissociated at the intracellular pH (6.5–7.0), whereas it is almost undissociated at the pH of the medium during citric acid fermentation (1.7–2.0). Mattey [20] hypothesized that citrate^{2-} is excreted by passive diffusion along a gradient of dissociated citric acid. In view of the present findings that (a) CCCP, 2,4-DNP and sodium azide immediately blocked citrate excretion, and (b) 30% of the excretion observed at an external pH of 2 was still observed at pH 7, which is even higher than the intracellular pH of *A. niger* under these conditions [4], we assume that citrate export occurs by an active transport system. These findings add to those for glutamate efflux by *C. glutamicum* and to those for other amino acids for which also active transport systems have been reported [11,12,21–23], hence expanding the list of metabolite overproductions which occur by active excretion.

Also in accordance with the situation in amino acid overproducing bacteria, citric acid import by active transport could be demonstrated in *A. niger*. The situation in *A. niger* therefore seems to differ from that of *Candida utilis* [24], which contains two systems, a high-affinity system depending on proton symport, and a low-affinity facilitated diffusion system acting on undissociated and charged citric acid. An intriguing property of the *A. niger* uptake system was its dependency on Mn^{2+} which could only partially be fulfilled by other divalent metal ions. The citrate uptake system of *Bacillus subtilis* [25] – but not that of other bacteria [26–29] and neither of *C. utilis* [24] – has also been described to be dependent on divalent metal ions. One possibility for this observation could be either a requirement of the permease for metal ion-chelated citrate, as many other enzymes reacting with tricarboxylic acids [29], or Mn^{2+} symport. Cotransport of citrate and Mg^{2+} or Fe^{2+} has been reported in other *Bacillus subtilis* and *Escherichia coli*, respectively [30,31]. An active transport system for manganese ions has been demonstrated in *A. niger* [28], and it is intriguing that its activation by citrate has been reported (Auling, G. and Seehaus, D., unpublished data; cited in [32]). While further experiments would be necessary to support the existence of a symport with Mn^{2+} ions, a brief calculation of the free and chelated forms of Mn^{2+} in the presence of citrate and EDTA shows (Torres, N., data not given) that EDTA competes with citrate for this metal ion. A Lineweaver–Burk plot of the affinity of the uptake system for citrate in the presence of 2 mM EDTA and $5 \cdot 10^{-5}$ M Mn^{2+} shows a similar K_m yet a lower V_{max}, and this V_{max} correlates with the one which is obtained if only the Mn^{2+}-citrate chelate is plotted as a substrate. These data support the assumption that the citrate uptake system requires preferably the metal ion chelated form of citrate as a substrate, and that the nonchelated form is neither transported nor inhibits transport.

The most intriguing aspect which emerged from this study is the reciprocal regulation of citrate efflux and uptake by manganese ions, i.e. the stimulation of formation of the latter and the inhibition of formation of the former in the presence of manganese, and vice versa. These findings are in accordance with the theoretical prediction [6] that passage of citrate through the plasma membrane is one of the more critical events in citric acid accumulation, and link these findings to the well-known negative effect of Mn^{2+} ions on citric acid accumulation [1,2]. One may argue that this observation may be an artefact, because the uptake system will simultaneously pump the exported citrate back into the cell in the presence of Mn^{2+} ions, hence pretending inactivity of the export system, and vice versa. However, as complexing the available Mn^{2+} by EDTA does not lead to citrate export in mycelia pregrown in the presence of manganese this explanation is unlikely. One other possibility would be differential transcriptional regulation of the uptake and the excretion system by manganese, in analogy to the Fe-citrate carrier system

of *E. coli* [33]. Yet a further explanation may be deduced from earlier findings that Mn^{2+} deficiency leads to a pronounced alteration in *A. niger* membrane lipid composition [34]. It has already been shown that such an alteration affects the properties of membrane bound enzymes such as permeases significantly, and it may therefore be speculated that the absence of manganese ions leads to carrier inversion [9,10], in which an inversed uptake system now pumps citrate out of the cells. The observed requirement of the uptake system for free manganese ions would not contradict this possibility: assuming that all the extracellular Mn^{2+} would be taken up by e.g. 2 g (dry weight)/l of fungal mycelia (which have an internal volume of 1–2 ml/g), the intracellular concentration should be between 10^{-6} and 10^{-5} M even at an external manganese ion concentration as low as 10^{-8} M and thus high enough for Mn^{2+} symport [2]. The carrier-inversion hypothesis would also form a basis to explain the well-known ability of various membrane affecting agents such as lower alcohols, fatty acids and quaternary ammonium compounds to antagonize the presence of manganese ion impurities in citric acid fermentation substrates (for review see [1]). In order to safely distinguish between the possibility of two differently regulated transport systems explanations (e.g. one of import and one for export) and the carrier inversion model, however, a molecular characterization of the *A. niger* citrate carrier(s) will be necessary. A reciprocal regulation of citrate uptake and export by Mn^{2+} may be beneficial to the fungus because it enables the recruition of Mn ions by secreted citrate when their external availability is low, whereas it allows their uptake once they are accumulated at certain concentrations.

Acknowledgements

This work has been supported by a research grant from the Ministerio de Educacion y Ciencia de Espana, CICYT, Ref. 96-1458, and by the ÖAD (Acciones Integradas Project No. 11). The authors greatly appreciate the practical help of Markus F. Wolschek during the early stages of this work, the helpful comments and discussion of the manuscript by Carlos Glez-Alcon, Dr. Maria de la Luz Perez Pont, and Dr. W. Burgstaller.

References

[1] M. Roehr, C.P. Kubicek and J. Kominek (1992) in *Aspergillus*: Biology and Industrial Application (J.W. Bennett and M.A. Klich, eds.), pp. 91–131, Butterworth-Heinemann, Stoneham, UK.
[2] C.P. Kubicek (1996) in Commercial Fermentations and their Nutritional Needs (T. Nagodawithana and G. Reed, eds.), Esteekan Assoc. Inc., Milwaukee, USA, in press.
[3] C.P. Kubicek, M. Röhr, Appl. Environ. Microbiol. 50 (1985) 1336–1339.
[4] M. Legisa, J. Kidric, Appl. Microbiol. Biotechnol. 31 (1989) 453–457.
[5] C. Prömper, R. Schneider, H. Weiss, Eur. J. Biochem. 216 (1993) 223–230.
[6] N.V. Torres, C. Regalado, A. Sorribas and M. Cascante (1993) in Modern Trends in Biothermokinetics (E. Shuster, ed.), pp. 115–124, Plenum Press, New York.
[7] A.F. Duro, R. Serrano, Curr. Microbiol. 6 (1981) 111–114.
[8] J.M. Salmon, Biochim. Biophys. Acta 901 (1987) 30–34.
[9] W.N. Konings, Trends Biochem. Sci. 8 (1985) 317–319.
[10] M.H. Saier, Jr. (1979) in The bacteria (I.C. Gunsalus, J.R. Sokatch and L.N. Ornston, eds.), Vol. 7, pp. 167–227, Academic Press, New York.
[11] M. Gutmann, C. Hoischen, R. Krämer, Biochim. Biophys. Acta 1112 (1992) 115–123.
[12] C. Hoischen, R. Krämer, Arch. Microbiol. 151 (1989) 342–347.
[13] C. Hoischen, R. Krämer, J. Bacteriol. 172 (1990) 3409–3416.
[14] C.P. Kubicek, M. Röhr, Eur. J. Appl. Microbiol. 4 (1977) 167–173.
[15] E.M. Kubicek-Pranz, M. Mozelt, M. Röhr, C.P. Kubicek, Biochim. Biophys. Acta 1033 (1990) 250–255.
[16] G.J.G. Ruyter, J. Visser, J. Microbiol. Methods 25 (1996) 295–302.
[17] S. Dagley (1974) in Methods in Enzymatic Analysis, (H.U. Bergmeyer, ed.), 2nd Edn., Vol. 2, pp. 1607–1612, Verlag Chemie, Weinheim, FRG.
[18] I. Arisan-Atac and C.P. Kubicek (1996) Microbiol. UK, in press.
[19] F. Bisaccia, A. De Palma, F. Palmieri, Biochim. Biophys. Acta 977 (1989) 171–176.
[20] M. Mattey, FEMS Microbiol. Lett. 2 (1977) 71–74.
[21] H. Ebbinghausen, B. Weil, R. Krämer, Appl. Microbiol. Biotechnol. 31 (1989) 184–190.
[22] S. Bröer, R. Krämer, Eur. J. Biochem. 202 (1991) 131–135.
[23] S. Bröer, L. Eggeling, R. Krämer, Appl. Environ. Microbiol. 59 (1993) 316–321.
[24] F. Cassio, C. Leao, Appl. Environ. Microbiol. 57 (1991) 3623–3628.
[25] J. Bergsma, W.N. Konings, Eur. J. Biochem. 134 (1983) 151–156.
[26] M.E. Van der Rest, T. Abee, D. Molenaar, W.N. Konings, Eur. J. Biochem. 195 (1991) 71–77.
[27] M.E. Van der Rest, D. Molenaar, W.N. Konings, J. Bacteriol. 174 (1992) 4892–4898.

[28] S. Hockertz, J. Schmid, G. Auling, J. Gen. Microbiol. 133 (1987) 3513–3519.
[29] J.P. Glusker, Curr. Top. Cell. Regul. 33 (1992) 169–184.
[30] S. Silver, M. Walderhaug, Microbiol. Rev. 56 (1992) 195–208.
[31] K. Willecke, E.-M. Gries, P. Oehr, J. Biol. Chem. 248 (1973) 807–814.
[32] G. Auling (1994) in Metal Ions in Fungi (G. Winkelmann and D.R. Winge, eds.), pp. 215–236, Marcel Dekker, New York.
[33] M. Ochs, A. Angerer, S. Enz, V. Braun, Mol. Gen. Genet. 250 (1996) 455–465.
[34] O. Meixner, H. Mischak, C.P. Kubicek, M. Röhr, FEMS Microbiol. Lett. 26 (1985) 271–274.