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Correlation between manganese-deficiency, loss of respiratory chain complex I activity and citric acid production in *Aspergillus niger*

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Abstract. The correlation between manganese deficiency, loss of mitochondrial respiratory chain NADH : ubiquinone oxidoreductase (complex I) activity and citric acid overproduction in the *Aspergillus niger* strain B 60 was analysed. With increasing manganese-supplementation of the production medium the loss of complex I activity and the production of citric acid was reduced. Addition of manganese during growth stopped further loss of complex I activity and further increase of citric acid production. A possible causality between complex I deficiency and citric acid overproduction is discussed.

Key words: *Aspergillus niger* – Citric acid production – NADH : ubiquinone reductase – Manganese

Citric acid is commercially produced in large amounts by incomplete catabolism of glucose in the fungus *Aspergillus niger*. Many aspects favouring this metabolite overproduction have been studied, e.g. medium composition, fermentation parameters and defective regulations in the glycolytic and citric acid metabolic pathways. However, the reason why glucose is incompletely oxidized has not been found (Kubicek and Röhr 1986). Our studies focuss on the alteration of the mitochondrial respiratory chain occurring simultaneously with the onset of the citric acid overproduction (Wallrath et al. 1991; Schmidt et al. 1992).

Mitochondrial respiration in filamentous fungi, like *A. niger*, is not only mediated by the three proton-pumping respiratory complexes, NADH : ubiquinone oxidoreductase (complex I), ubiquinol : cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV) (Hatefi 1985), but also by non proton-pumping, alternative respiratory enzymes. Two alternative NADH : ubiquinone oxidoreductases, one facing the matrix and the other gacing the intermembrane space of mitochondria, oxidize internal and cytosolic NADH, respectively. An alternative ubiquinol oxidase catalyzes the direct oxidation of ubiquinol by oxygen. Compared to the proton-pumping respiratory complexes the alternative respiratory enzymes have lower affinities to their reduced substrates. Therefore these enzymes are believed to function as outlet valves for electrons in case of an overflow of reducing equivalents in the cells (Lambers 1982).

Previously we reported the concomitant loss of respiratory complex I activity and the onset of citric acid overproduction in *A. niger* strain B 60 (Wallrath et al. 1991; Schmidt et al. 1992). We suggested that the alternative, matrix facing NADH : ubiquinone oxidoreductase takes charge of the reoxidation of all mitochondrial NADH. The low affinity to NADH of this enzyme, however, leads to an imbalanced NAD/NADH ratio. The excretion of the reduced metabolite citric acid may be a remedy to avoid further NADH accumulation.

According to Kubicek and Röhr (1977) manganese-deficiency is a prerequisite for the onset of citric acid overproduction in *A. niger*. The purpose of this work was therefore to elucidate a possible correlation between manganese deficiency, loss of complex I and citric acid overproduction.

Materials and methods

Aspergillus niger strain B 60, a kind gift of Dr. M. Röhr, Institute of Biochemical technology and Microbiology, University of Technology, Vienna, was used throughout all expeiments. Culture conditions and analytical procedures were as described previously (Wallrath et al. 1991). Manganese was added to the production medium as $MnCl_2 \times 4\,H_2O$.

Results

Figure 1 shows the time courses of growth and citric acid production during fermentations on production medium without manganese or supplemented with 10 μM or 30 μM Mn^{2+}, respectively, prior to inoculation. During trophophase citric acid production was similar under all three fermentation conditions, but the switch from tro-

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phophasic to idiophasic growth occured later with rising concentrations of manganese. During idiophase, inverse correlation between citric acid production and manganese supplementation was observed. The time courses of enzymatic activities of complex I and the alternative NADH : ubiquinone oxidoreductases during these fermentations are shown in Fig. 2. The less manganese was available, the faster and the more complete was the loss of complex I activity. The activity of the alternative NADH : ubiquinone oxidoreductases showed an opposite behaviour. During fermentation without manganese a constantly high activity was observed, whereas addition of manganese led to decreased activity correlating fairly well with the amount of manganese available. The activities of the respiratory complexes III and IV and the alternative ubiquinol oxidase did not alternate in dependence on the amount of manganese added to the medium (data not shown, see also Wallrath et al. 1991).

We also analysed the effect of 30 µM Mn^{2+} added to cultures growing already for 66 or 90 h in the absence of manganese. With the manganese becoming available citric acid overproduction ceased in favour of increased growth. It appears that the earlier the manganese was added, the stronger was its effect (Fig. 3). The complex I activity was stabilized after addition of manganese, while further manganese-deficiency led to complete loss of the enzyme. The alternative NADH : ubiquinone oxidoreductases again changed in an opposite manner. Their activities decreased after the addition of manganese. Again manganese-addition had no effect on the activities of the complexes III and IV and the alternative ubiquinol oxidase (data not shown).

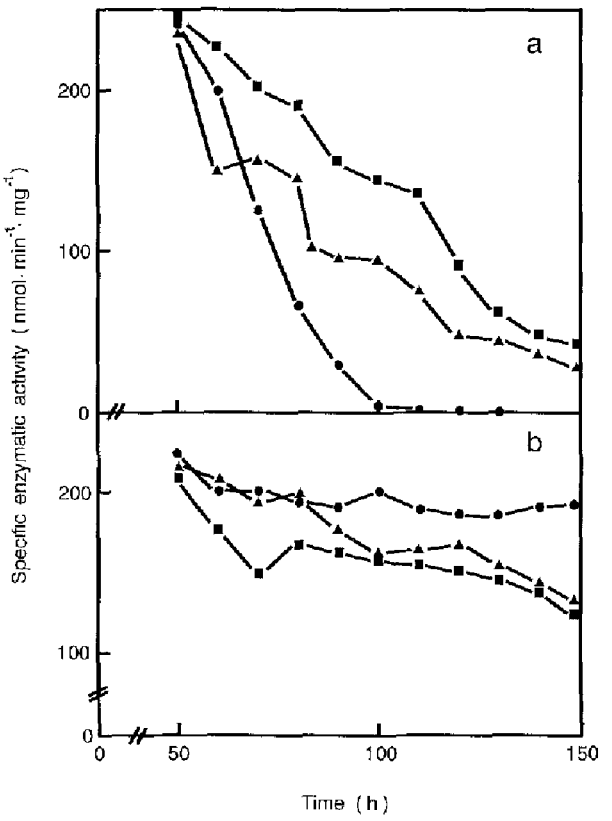

Fig. 2. Activities of complex I (**a**) and the alternative NADH : ubiquinone oxidoreductases (**b**) of *Aspergillus niger* B 60 grown in the production medium without mangenese (●), with 10 µM (▲) or 30 µM (■) manganese

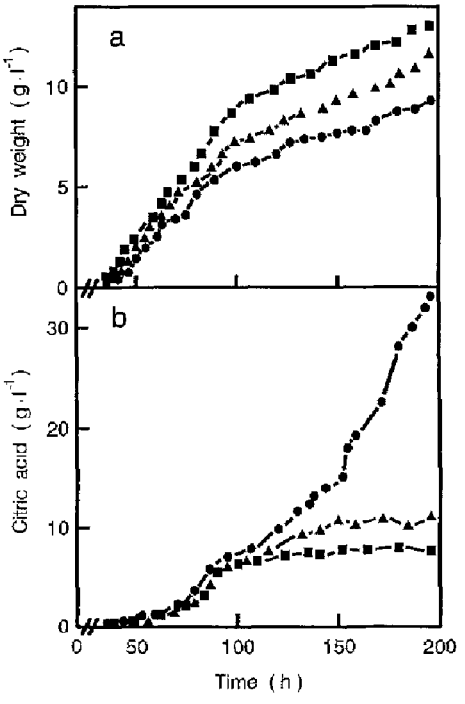

Fig. 1. Growth (**a**) and citric acid production (**b**) of *Aspergillus niger* B 60 in the production medium without mangenese (●), with 10 µM (▲) or 30 µM (■) manganese

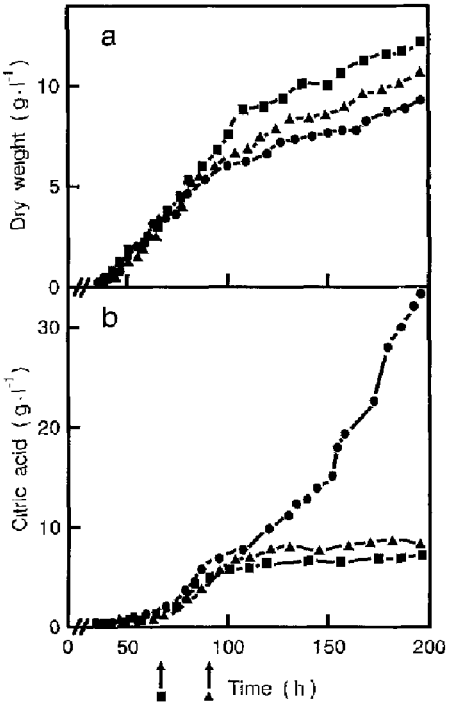

Fig. 3. Growth (**a**) and citric acid production (**b**) of *Aspergillus niger* B 60 in the production medium without manganese (●), or with addition of 30 µM manganese after 66 h (■) or 90 h (▲) of growth. The *arrows* indicate the time of manganese-addition

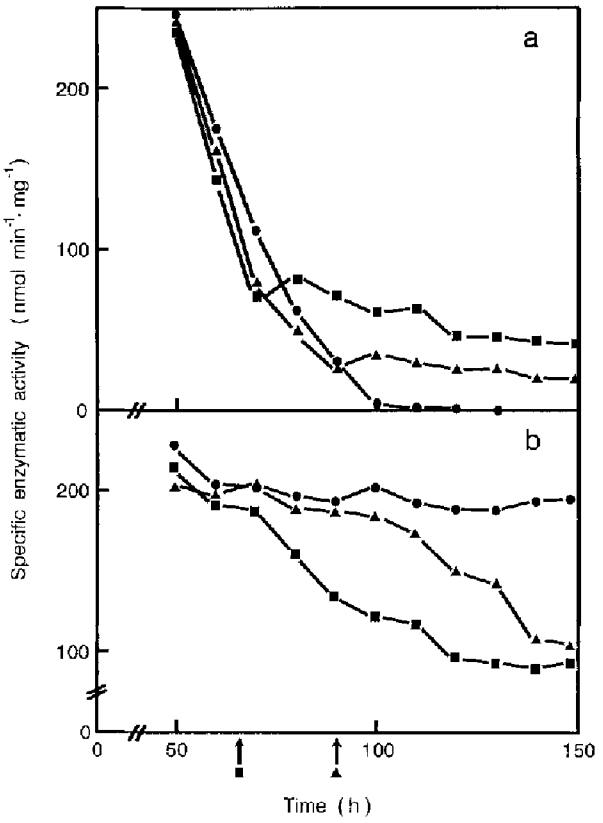

Fig. 4. Activities of complex I (*a*) and the alternative NADH : ubiquinone oxidoreductases (*b*) of *Aspergillus niger* B 60 grown in the production medium without mangenese (●) or with addition of 30 µM manganese after 66 h (■) or 90 h (▲) of growth. The *arrows* indicate the time of manganese-addition

Discussion

The experiments demonstrate a close correlation between (i) manganese-deficiency, (ii) loss of respiratory chain complex I, (iii) maintenance of high levels of the alternative NADH : ubiquinone oxidoreductases, probably as counteraction, and (iv) increased production of citric acid. This correlation further supports our suggestions to explain citric acid overproduction in *Aspergillus niger* (Wallrath et al. 1991). A loss of complex I activity leads to a disregulation of the intramitochondrial NAD/NADH ratio with the consequence of a block in the dehydrogenation reactions of the citric acid cycle and accumulation and secretion of citric acid to keep the NAD/NADH ratio in balance.

Manganese-deficiency has certainly multiple effects. Most manganese-dependent enzymatic processes are located inside the mitochondrion and the intracellular manganese concentration is highest in the mitochondrial matrix (Williams 1982; Archibald 1986). Therefore, manganese-deficiency as the main prerequisite for citric acid overproduction in *A. niger* should affect mitochondrial biogenesis and metabolism above all. RNA polymerases and protein kinases are known as manganese-dependent enzymes (Garraway and Evans 1984). The latter are discussed to be involved in translational control. A reduced rate of DNA synthesis (Hockertz et al. 1987) and a disfunction of ribosomal activity (Ma et al. 1985) are other manganese-dependent effects observed for citric acid accumulation *A. niger*. A reduced activity of the manganese-dependent mitochondrial processing protease (Hartl and Neupert 1990) represents another possible affected area, which might foil a correct formation of complex I. Among the enzymes of the mitochondrial respiratory chain manganese-deficiency selectively affects complex I of *A. niger*. We consider the pronounced complexity of this respiratory complex (Weiss et al. 1991) to be responsible for this selectivity.

Recently the mitochondrial acyl-carrier protein (ACP) was found to be a peripherally located subunit of complex I in *Neurospora crassa* (Sackmann et al. 1991) as well as in bovine heart (Runswick et al. 1991). Pulse-labelling of cellular protein of *A. niger* strain B 60 grown under citric acid producing conditions revealed the accumulation of only membrane bound subunits of complex I (Schmidt et al. 1992), implying the absence of the peripheral subunits and, hence, also the ACP. Assuming the ACP to be a complex of a synthetic pathway that exists to satisfy special needs of lipids for the mitchondrion (Mikolajczyk and Brody 1990), the loss of complex I should have consequences for the mitochondrial inner membrane. For respiratory deficient *A. niger* mutants alterations of the content of cardiolipin, a mitochondria-specific phospholipid, has been reported (Mandal et al. 1978). Cardiolipin synthesis itself requires manganese (Daum 1985). In conclusion, an interrelation between manganese-deficiency and mitochondrial lipid biosynthesis, on one hand, and loss of complex I and citric acide accumulation, on the other hand, seems to be likely.

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