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Inhibition of Citric Acid Accumulation by Manganese Ions in *Aspergillus niger* Mutants with Reduced Citrate Control of Phosphofructokinase

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Mutant strains of *Aspergillus niger* with reduced citrate control of carbohydrate catabolism (*cic* mutants) grow faster than the parent strain on media containing 5% (wt/vol) citrate. The mutants tolerated a higher intracellular citrate concentration than the parent strain. One mutant (*cic*-7/3) contained phosphofructokinase activity significantly less sensitive towards citrate than the enzyme from the parent strain. When this mutant was grown under citrate-accumulating conditions, acidogenesis was far less sensitive to inhibition by Mn^{2+} than in the parent strain. Some of the *cic* mutants also showed altered citrate inhibition of NADP-specific isocitrate dehydrogenase.

The accumulation of large amounts of citrate by the filamentous fungus *Aspergillus niger* is known to depend on a variety of environmental factors, among which the level of Mn^{2+} ions in the nutrient medium plays a key role. Only when the Mn^{2+} concentration is below 0.02 mM (which does not yet affect growth rate or yield) will citrate accumulate in high amounts (17).

A biochemical explanation of this manganese effect has been presented recently. Mn^{2+} deficiency leads to an increase in protein turnover, which ultimately leads to a high intracellular concentration of NH_4^+ (7, 9); this level of NH_4^+ is able to counteract the inhibition of phosphofructokinase by citrate (4, 5), thereby guaranteeing a high rate of glucose breakdown by the fructose biphosphate pathway, which is necessary for citric acid accumulation (17).

According to this model, Mn^{2+} deficiency is mainly required for eliminating glycolytic feedback control by citrate. If this model is correct, mutants lacking citrate inhibition of phosphofructokinase should accumulate citrate even in the presence of Mn^{2+}.

To test this hypothesis, *A. niger* B60 (7) was propagated on potato dextrose agar for 7 days, and the conidia were harvested, diluted in sterile water containing 0.1% (wt/vol) Tween 80 to give a final density of 2×10^6 conidia per ml, and shaken vigorously to break clumps. Of this suspension, 10 ml was then placed in an open petri dish 14 cm below a germicidal UV lamp and stirred magnetically for 5 min. Subsequently, the suspension was covered with aluminium foil for 1 h and used for the isolation of potential mutants. Their identification was based on the assumption that mutants lacking citrate control of phosphofructokinase would grow faster on a sucrose medium containing citrate, as discussed below. For this reason, the diluted, mutagenized conidium suspensions were plated on solid medium containing 20% (wt/vol) sucrose and 5% (wt/vol) citrate as carbon sources. Other inorganic nutrients and nitrogen source were essentially as used in the citric acid production medium (8), except that the pH was adjusted to 3.5 with H_2SO_4. Oxgall (0.5% [wt/vol]; Sigma Chemical Co., St. Louis, Mo.) was added to the medium to restrict growth to small, defined colonies (15). The fastest-growing colonies were considered to be less affected in carbohydrate breakdown by citrate and thus were picked for further analysis. By this method, the screening of several thousand colonies of *A. niger* B60 allowed the detection of 20 to 30 potential mutants exhibiting an appreciably reduced lag time in growth in the presence of both sucrose and citrate, compared with the parental strain. To eliminate uptake-defective mutants, all colonies were checked for growth on media containing only citrate or glucose as carbon source. None of the mutants exhibited morphological alterations. Twelve mutants were stable after several subcultivations and were used for further investigations.

The parental strain and the putative mutants, which were expected to be characterized by citrate-insensitive carbohydrate catabolism (*cic*), were grown in citric acid-nonaccumulating medium (13) for 40 h. The mycelia were harvested and homogenized by ultrasonication as described previously (13) in 50 mM imidazole buffer (pH 7.8) containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% (wt/vol) glycerol. Phosphofructokinase (EC 2.7.1.11) was purified from the cell extract and assayed as described elsewhere (5). When phosphofructokinase from all 12 stable mutants was investigated with respect to inhibition by citrate, no completely citrate-insensitive enzyme could be found in any *cic* mutant. It is possible that mutants with completely citric-insensitive phosphofructokinase cannot be isolated. In two strains, however, i.e., *cic*-7/3 and *cic*-1, the enzyme was less sensitive than the parental strain enzyme to physiological concentrations of citrate (1 to 3 mM; Fig. 1). When the effect of citrate was studied in relation to the concentration of the substrate fructose 6-phosphate (Fig. 2), the kinetics shown for mutant *cic*-7/3 in the presence of citrate appeared complex; up to a concentration of 1.5 mM fructose 6-phosphate, citrate exerted almost no effect, and it inhibited only at fructose 6-phosphate concentrations higher than 2 mM. Since the intracellular concentration of fructose 6-phosphate in *A. niger* is well below 1.5 mM (4), this mutant appears to contain a phosphofructokinase which is insensitive to citrate under physiological substrate conditions.

The inhibition of glucose catabolism in *A. niger* by citrate is not exerted only at the level of phosphofructokinase.

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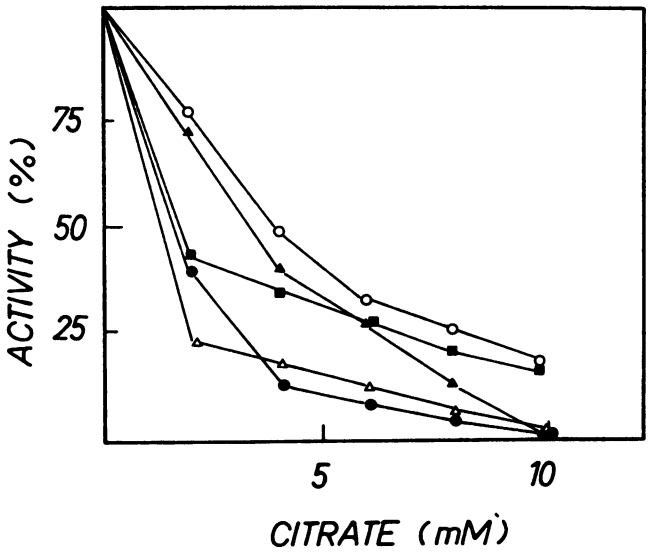

FIG. 1. Inhibition of phosphofructokinase from the B60 parental strain (△) and its *cic* mutants (○, *cic*-7/3; ●, *cic*-6/3; ▲, *cic*-1; ■, *cic*-7/1) by citrate. The enzyme was purified by affinity chromatography as described previously (5). The auxiliary enzymes required for the optical assay were dialyzed before use to remove ammonium sulfate, which otherwise interferes with studies on citrate inhibition of the enzyme. Activities are given as percentages of activity without citrate. V_{max} values of phosphofructokinase of the various strains were as follows (U/mg of protein ± standard deviation): B60, 0.25 ± 0.02; *cic*-7/3, 0.27 ± 0.02; *cic*-6/3, 0.20 ± 0.015; *cic*-1, 0.30 ± 0.02; *cic*-7/1, 0.25 ± 0.02.

Recently, Mischak et al. (14) found the glucose transport system of *A. niger* to be inhibited by citrate. We have further analyzed the *cic* mutants with respect to this inhibition. Two mutants (*cic*-6/3 and *cic*-7/1) actually exhibited a glucose transport system less sensitive towards citrate; that of

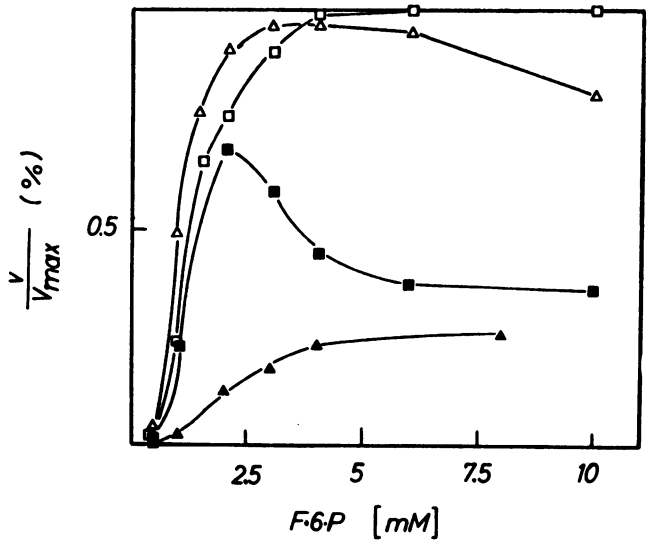

FIG. 2. Substrate kinetics of phosphofructokinase (F-6-P) from *A. niger* B60 parental strain (△, ▲) and the *cic*-7/3 mutant (□, ■) in the absence (△, □) and presence (▲, ■) of 2.5 mM citrate in the test system. All conditions, including V_{max} of both enzyme preparations, are indicated in the legend to Fig. 1.

TABLE 1. Inhibition of glucose transport in *A. niger* B60 and *cic* mutants

Strain	V_{max} of glucose transport[a] with:	
	No citrate	0.25 M citrate
B60	0.840 ± 0.057	0.400 ± 0.040
cic-7/3	0.800 ± 0.060	0.360 ± 0.050
cic-1	0.480 ± 0.035	0.145 ± 0.045
cic-6/3	0.760 ± 0.060	1.020 ± 0.045
cic-7/1	0.860 ± 0.042	0.790 ± 0.037

[a] Millimoles of glucose taken up per gram of mycelium (dry weight) per hour, as extrapolated from the uptake experiment (14). Values are ± standard deviations.

cic-7/3, however, appeared not to have been altered (Table 1).

cic mutants were next examined with respect to inhibition of citric acid accumulation by Mn^{2+} ions. Cultivation of *A. niger* in a citric acid fermentation medium was carried out as described previously (8). Sucrose (14%, wt/vol) was used as a carbon source after removal of manganese and other metal ion impurities from it with Dowex AG 50 W-X8 (8). All the *cic* mutants produced citric acid in high amounts in the absence of Mn^{2+} ions (≥60 g/liter), although some of the mutants did not achieve the titer of the parental strain (Table 2). In the presence of 20 μM Mn^{2+} ions, however, the yield of citric acid was drastically decreased, as previously reported (2, 6, 8). Mutant *cic*-7/3, however, accumulated 50% of the amount produced under deficient conditions, i.e., twice the amount produced by the parent strain B60 under Mn^{2+}-containing conditions (Table 2). During these experiments, citric acid in the culture fluid was quantified by titration with 0.1 N KOH against phenolphthalein, and the validity of this procedure was routinely checked by high-pressure liquid chromatography as described elsewhere (W. Wöhrer, M. Röhr, J. Kominek, and W. Salzbrunn, 2nd European Congress on Biotechnology, abstr., p. 280, 1981). Since citric acid production in the absence of Mn^{2+} has been attributed to the accumulation of intracellular NH_4^+ (7, 9), which antagonizes the inhibition of phosphofructokinase by citrate (4, 5), it is important to note that none of the mutants exhibited elevated intracellular concentrations of NH_4^+ in

TABLE 2. Inhibition of citric acid accumulation by manganese ions and intracellular NH_4^+ pools in *A. niger* B60 and *cic* mutants

Strain	Citric acid accumulation (g/liter)[a] with:		Concn of intracellular NH_4^+ pool[b] (mM)
	No Mn^{2+}	20 μM Mn^{2+}	
B60	85 ± 7	12 ± 3	2.3 ± 0.6
cic-7/3	73 ± 6	39 ± 5	1.9 ± 0.7
cic-6/3	57 ± 9	8 ± 4	2.0 ± 0.6
cic-1	102 ± 12	33 ± 4	2.6 ± 0.5
cic-7/1	114 ± 14	28 ± 6	1.5 ± 0.6

[a] Citrate in the culture fluid was measured under conditions of citric acid production after 150 h in the presence of 20 μM Mn^{2+} and after 240 h of cultivation in the absence of Mn^{2+}. Values are means ± standard deviations of at least three separate runs.

[b] Concentrations of intracellular NH_4^+ pools were determined on cultures grown in citric acid-producing medium for 70 h in the presence of 20 μM Mn^{2+}. Intracellular concentration is indicated as millimolar in the cytosol (average distribution) and was calculated from mycelial dry weight determinations (19) by assuming an intracellular volume of 2.43 ml per g dry weight (20). Values are means ± standard deviations of at least three separate determinations.

the presence of 20 μM Mn^{2+} when extracted and assayed as described in references 4 and 5. Also, none of the mutants appeared to have lost its ability to take up Mn^{2+}, since all *cic* mutants exhibited the characteristic filamentous morphology in Mn^{2+}-containing medium which is dependent on the uptake of Mn^{2+} (6).

The above results showed increased citrate production on Mn^{2+} ion-containing medium by *A. niger* mutants with reduced control of phosphofructokinase by citrate, which is consistent with our previous hypothesis on the mode of action of Mn^{2+} on citric acid accumulation (4, 5, 7, 9). However, the final yield of citrate obtained was still only 50% of that obtained under manganese deficiency. This lack of complete antagonization of the effect of manganese could be explained by the incomplete insensitivity of phosphofructokinase to citrate, which might have led to inhibition at later stages of fermentation, when the intracellular concentration of citrate is very high (9 mM, [5]). On the other hand, it should be noted that manganese deficiency is always accompanied by a number of additional physiological events (i.e., changes in lipid synthesis, membrane and cell wall composition, and respiratory activity [6, 12, 16, 22]) whose role in citric acid accumulation is still not clear but which could also be necessary to obtain high yields.

Another important point in regulation of citric acid accumulation has been postulated to occur at the NADP-specific isocitrate dehydrogenase. Inhibition of this enzyme by citrate has been claimed to be the key event in citric acid accumulation (11). Therefore, the kinetics of citrate inhibition of this enzyme from the *cic* mutants has been investigated (Table 3). The enzymes from two strains (*cic*-6/3 and *cic*-7/1) had a decreased K_i for citrate, which may be interpreted to indicate stronger inhibition by citrate than in parent strain B60. The kinetic properties of the enzyme from mutant strain *cic*-7/3 were not significantly different when compared with those of the parent strain, except for V_{max}, which was clearly increased. This difference might indicate that the enzyme is found in higher amounts in this strain. No correlation was observed between the ability of the various strains to produce citrate in the absence of Mn^{2+} and their sensitivity of NADP-isocitrate dehydrogenase towards citrate. This observation is consistent with our hypothesis that inhibition of this enzyme by citrate has no primary role in acidogenesis in this fungus (13, 17). We have recently been able to support this hypothesis by kinetic studies with purified NADP-specific isocitrate dehydrogenase from *A. niger* (B. Meixner-Monori, C. P. Kubicek, G. Schreferl, W. Harrer, and M. Röhr, Biochem. J., in press), in which it was shown that citrate inhibits this enzyme only at unphysiologically low concentrations of Mg^{2+}. The present results therefore support the hypothesis that relief to citrate inhibition of phosphofructokinase is a major event related to manganese deficiency stimulation of acidogenesis in *A. niger*.

TABLE 3. Kinetics of NADP-specific isocitrate dehydrogenase from *A. niger* B60[a]

Strain	Kinetic values for NADP-specific isocitrate dehydrogenase[b]		
	V_{max} (U · mg^{-1})	K_mIC (mM)	K_iCit (mM)
B60	0.103 ± 0.009	0.05 ± 0.02	2.9 ± 0.6
cic-7/3	0.191 ± 0.015	0.08 ± 0.03	3.6 ± 1.0
cic-6/3	0.121 ± 0.011	0.05 ± 0.01	0.9 ± 0.5
cic-1	0.134 ± 0.016	0.06 ± 0.02	5.7 ± 0.9
cic-7/1	0.194 ± 0.020	0.04 ± 0.02	1.0 ± 0.3

[a] An ammonium-fractionated (46 to 70% saturation) cell extract, which was desalted by being passed over a small column (15 by 80 mm) of Sephadex G 25 (Pharmacia PD 10 columns) was used for the kinetic investigations. Lineweaver-Burk and Dixon plots were used for the determination of V_{max}, K_m, and K_i. Activity was assayed by previously described methods (21), with Mn^{2+} ions as a cofactor (0.5 mM final concentration). Values are means ± standard deviations of at least three separate determinations. Specific activity is expressed as micromoles of substrate converted per minute (=1 U) per milligram of protein, assayed by the method of Bradford (1).

[b] IC, Isocitrate dehydrogenase; Cit, citrate.

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