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review

The Role of Sugar Uptake and Channelling for Citric Acid Accumulation by *Aspergillus niger*

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Summary

The final yield of citric acid in fermentations by Aspergillus niger is strongly dependent on the type and concentration of the carbon source. Here will be reviewed the current understanding of the mechanism by which the carbon source and its concentration influence citric acid accumulation, which emphasizes a major regulatory point at the level of hexose transport and phosphorylation. Evidence for regulation of the rate of citric acid accumulation and glycolysis by trehalose-6-phosphate will be presented, and a successful recombinant strategy for its relief will be shown.

Keywords: *Aspergillus niger*, citric acid accumulation, hexokinase, glucose transport, trehalose-6-phosphate, regulation

Introduction

The biochemical mechanism by which *Aspergillus niger* accumulates citric acid has attracted the interest of researchers since the late thirties when the conversion of a laboratory observation into a commercial process began. The striking effects of several nutrient parameters on the rate and yield of citric acid production has attracted numerous workers to use these as a target to investigate the mechanism of citric acid accumulation (for review see *1–3*). While some citric acid (up to 200 g L^{-1}, of the carbon concentration applied) can easily be obtained with any *A. niger* under most conditions, yields mimicking those obtained in industry generally require the simultaneous absence of manganese ion, low pH, and an excess of oxygen and sugar. However, serious studies of the biochemical mechanisms which are responsible for this effect are rendered difficult by the interaction of several of these parameters *(4,5)*. We have previously shown that even a strict maintainenace of all the other critical parameters will not lead to high citrate accumulation unless the sugar concentration is maintained at least over 50 g L^{-1}, and thus the sugar concentration is obviously of major importance *(6,7)*. This was proven by establishing a replacement type system, in which *A. niger* was pregrown in a citric acid producing medium but with only 10 g L^{-1} sucrose, and to which then a pulse of sucrose (final concentration 100 g L^{-1}) was added. *A. niger* immediately started to accumulate citric acid under these conditions. As this system is easy to standardize, we have consequently made use of it to investigate the biochemical alterations taking place upon pulsing with sucrose, and consequently to understand the biochemical regulation of citric acid production in more detail.

Triggering of citric acid accumulation by high sucrose concentrations correlates with increased cellular Fru-2,6-P_2 pool levels

In order to obtain a first hint as to the metabolic changes accompanying the triggering of citric acid accumulation, Kubicek-Pranz *et al. (8)* have investigated the changes in the intracellular concentration of selected metabolite levels in mycelia during transfer from 10 to 100 g L^{-1} sucrose during the triggering of citrate accumulation in the system described above. They detected a 2.5- and 7-fold rise in the levels of Fru-2,6-P_2 and citrate, respectively, whereas the concentration of all other metabolites remained unaffected. Only carbon sources which allow high yields of citric acid (*i.e.* glucose, sucro-

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se or others which are taken up rapidly; cf. *1,7,9,10*) produced these effects, whereas pulsing with others (fructose, glycerol) did not. Thus, the concentration of Fru-2,6-P_2 correlates positively with the rate of citrate production. Since Fru-2,6-P_2 antagonizes the inhibition of PFK1 by citrate *(11)*, it is possible that at least one of the triggering effects of a shift to high sucrose concentrations is due to an increased glycolytic flux rate because of decreased feedback inhibition by the accumulated citrate.

Although the effect of the sugar concentration on Fru-2,6-P_2 appears clear, the reason for it is less obvious. In order to understand this rise in Fru-2,6-P_2, the synthesizing enzyme – PFK2 – has been studied with partially purified preparations *(12)*. Its activity – in contrast to the results with the enzyme from *Saccharomyces cerevisiae* – was not modulated by physiological concentrations of various glycolytic metabolites; also phosphorylation of the enzyme did not change its activity. Hence Harmsen *et al. (12)* concluded that the enzyme is mainly modulated by the availability of its substrates, Fru-6-P and ATP. As a consequence, higher pool levels of Fru-2,6-P_2 will accumulate when more Fru-6-P becomes available. This links regulation of PFK2 (and PFK1) to control earlier glycolytic steps. Such interpretation has later on received theoretic support by Biochemical Control Analysis *(13,14)*, where it was concluded that a major part of control of citric acid production *in vivo* must occur at hexose uptake and/or phosphorylation.

Glucose phosphorylation controls the rate of citric acid accumulation

Steinböck *et al. (15)* purified a single hexo/glucokinase from the citric acid producing *A. niger* strain ATCC 11414, and investigated its regulation *in vitro*: the enzyme was inhibited by citrate and by rather high concentrations of trehalose-6-phosphate *(16)*. The inhibition by citric acid was found to be due to chelation of Mg^{2+} which is required to chelate the co-substrate ATP, and was thus considered to be irrelevant under physiological conditions where Mg^{2+} is present in excess. However, the inhibition by trehalose-6-phosphate was considered as potentially important, particularly during growth on high sugar concentrations where the accumulation of trehalose-6-phosphate concentrations as high as needed for inhibition (1.5–2.0 mM) may accumulate. In order to test this hypothesis, we have cloned the *A. niger* trehalose-6-phosphate synthase-encoding *tpsA* gene *(17)*, which encodes a 517-aa polypeptide with 64–70% similarity to trehalose-6-phosphate synthase of *Sacch. cerevisiae*, *K. lactis* and *S. pombe*. Having the *tpsA* gene available we constructed a recombinant strain of *A. niger* carrying a disrupted copy of *tpsA*. The respective strain was then cultivated on citric acid producing media in the presence of varying concentrations of sucrose (10–140 g L^{-1}), and its production of citric acid studied *(16)*: in accordance with our hypothesis, increased rates of citric acid formation were observed at sugar concentrations higher than 75 g L^{-1}, whereas no differences to the control were apparent in media containing lower sugar concentrations. Similarly, a strain bearing multiple copies of *tpsA* and hence overproducing trehalose-6-phosphate synthase exhibited a reduced rate of citrate production only at sugar concentrations higher than 75 g L^{-1}. These findings provide genetic evidence that the cellular level of trehalose-6-phosphate indeed regulates the flux from glucose to citric acid and thus that glucose phosphorylation accounts for the major part of regulation at the early steps of glycolysis, thereby also supporting the conclusions by Torres *et al. (13,14)*.

Intriguingly, Panneman *et al. (18)* more recently reported the isolation and characterization of a specific glucokinase from *A. niger* N400, a strain producing only low levels of citric acid, and provided also indirect evidence – in analogy to *A. nidulans (19)* – for at least one additional »real« hexokinase in this strain. It is currently not clear why Steinböck *et al. (15)* found only a single enzyme with properties both resembling gluco-, as well as, hexokinase in their strain. Hybridization of *A. niger* ATCC 11414 DNA with a *Kluyveromyces lactis* hexokinase-encoding gene as a probe showed hybridization to a single fragment only (F. Narendja and C. P. Kubicek, unpublished data), which would be in accordance with the presence of a single enzyme only, as reported by Steinböck *et al. (15)*. However, using sequence information from the *glkA* gene *(18)*, we were able to amplify the glucokinase-gene from *A. niger* ATCC 11414 as well, thus proving that the respective protein is present in this strain. The roles of the individual hexose phosphorylating enzymes in *A. niger*, therefore, still require clarification. Whatever the results of such an investigation may be, however, the results by Arisan-Atac *et al. (16)* clearly show that the depletion of the mycelia from trehalose-6-phosphate stimulates the glycolytic flux and citric acid accumulation at high sugar concentrations. Since inhibition of hexokinase by trehalose-6-phosphate is the only enzyme step known to be regulated by this metabolite, hexose phosphorylation, therefore, constitutes a major regulatory point in this fermentation.

High sugar concentrations induce a further glucose transporter

Due to the lack of data on intracellular glucose/fructose concentrations in *A. niger*, the biochemical model of Torres *(13,14)* could not differentiate between glucose uptake and its phosphorylation, and glucose transport may therefore also contribute to the overall regulation of glycolytic flux. In fact, from a plot of hexokinase activity *vs.* citric acid production in various 2-deoxiglucose-resistant mutants of *A. niger (15)*, Torres *et al. (20)* calculated that the overall regulation at this step is shared between glucose transport and phosphorylation at a ratio of 1:2. Glucose uptake by *A. niger* was investigated *(20)*. When grown on a low (10 g L^{-1}) glucose concentration, *A. niger* ATCC 11414 contains a single, high-affinity glucose transporter, but an additional low-affinity transporter is formed during growth on a high (150 g L^{-1}) glucose concentration. The activity of both glucose transporters is decreased at low pH and inhibited by citric acid, whereas the activity of the low-affinity transporter is comparatively much less affected. A class of 2-deoxiglucose resistant *(dgr)*-mutants of *A. niger* which produce citric acid at a much lower rate than the parent strain, are impaired in the formation of the low-affinity transporter, but form the high-affinity transpor-

ter in higher activities *(20)*. These data are consistent with the assumption that the low-affinity glucose transporter takes part in the mechanism by which *A. niger* responds to high extracellular glucose concentrations which ultimately lead to citric acid accumulation.

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List of abbreviations

Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; PFK1, 6-phosphofructo-1-kinase; PFK2, 6-phosphofructo-2-kinase; Fru-6-P, fructose-6-phosphate

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Način ugradnje i metabolički put šećera pri nakupljanju limunske kiseline u *Aspergillus niger*

Sažetak

Iskorištenje limunske kiseline dobiveno vrenjem s pomoću Aspergillus niger strogo je ovisno o vrsti i koncentraciji izvora ugljika. U radu je prikazano današnje shvaćanje mehanizma kojim izvor ugljika i njegova koncentracija utječu na nakupljanje limunske kiseline. Prema tom mehanizmu najvažnija je ona regulacija koja se provodi na razini prijenosa heksoze te fosforilacija. Iznesen je i dokaz za regulaciju brzine nakupljanja limunske kiseline te glikolize u prisutnosti trehaloza-6-fosfata, kao i uspješni rekombinantni način kojim se uklanja inhibitorno djelovanje trehaloza-6-fosfata.