

Aconitase and Citric Acid Fermentation by *Aspergillus niger*

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In view of the often-cited theory that citric acid accumulation is caused by an inhibition of aconitase activity, the equilibrium of the reaction of aconitase was investigated by comparing in vivo steady-state concentrations of citrate and isocitrate in *Aspergillus niger* grown under various citric acid-producing conditions. With the equilibrium catalyzed by the *A. niger* enzyme in vitro, similar values were obtained. The validity of our in vivo measurements was verified by the addition of the aconitase inhibitor fluorocitrate, which appreciably elevated the citrate:isocitrate ratio. The results strongly argue against an inhibition of aconitase during citric acid fermentation.

The biochemical mechanism responsible for citric acid accumulation by *Aspergillus niger* has been the subject of investigation for the last 40 years; in particular, controversial theories have been proposed for the role of tricarboxylic acid cycle in citric acid overflow (12). Aconitase (EC 4.2.1.3), the second enzyme of the citric acid cycle, catalyzes an equilibrium between citrate, *cis*-aconitate, and isocitrate. Because it is the immediate enzyme catalyzing citrate breakdown within the cycle, several workers have claimed that its disappearance (11) or inhibition (3) is the key event causing citric acid to accumulate. This hypothesis seemed to be supported by findings that citric acid accumulation is decreased by the addition of iron (6; L. B. Schweiger, U.S. patent 2,916,420, 1959), a known activator and component of aconitase (8); furthermore, some ions and compounds (e.g., Cu^{2+} and hydrogen peroxide [3]) which inhibit aconitase in vitro stimulate citric acid production (3; Schweiger, U.S. patent). It should be noted in this context that the role of iron in aconitase was recently challenged (15).

We recently offered considerable evidence that an inhibition of α -ketoglutarate dehydrogenase is the true key event for citric acid overflow (4, 5, 7, 12). Inhibition of the tricarboxylic acid cycle at this step would cause a noncyclic operation which then leads to accumulation of its intermediates according to the equilibria of the enzymes involved (5, 7, 12). Because citrate, once it has accumulated to a certain concentration, inhibits its own catabolism, it is the only metabolite which can be further accumulated owing to a continuing supply of its precursors, e.g., acetyl coenzyme A and oxaloacetate (5, 12). Therefore, accumulation of aconitase is not necessary, because the equilibrium of the reaction of the enzyme is strongly in the direction of citrate (2). However, evidence for an active aconitase during citric acid fermentation, apart from a measurement of its total activity (1, 4, 6), has not yet been presented. We thus present here the results from a brief investigation which decisively showed that aconitase is active during citric acid fermentation, regardless of the absence of iron or the presence of copper.

All the investigations reported here were performed with *A. niger* B 60 (13). Its maintenance, conidia production, and conditions for citric acid fermentation in either shake flasks or under pilot plant conditions have been fully described previously (4, 5, 14). A chemically defined medium was used for all experiments, with cation-exchanged sucrose (14%

[wt/vol]) as the carbon source (4, 5). Mycelial dry weight and citric acid were determined as described previously (14).

Because aconitase is an enzyme which catalyzes an equilibrium reaction, its inhibition in vivo can easily be assessed by measuring steady-state in vivo concentrations of its substrates and products and by calculating the mass action ratio and comparing it with the mass action ratio of substrates and products as determined from the reaction of the enzymes in vitro. For this purpose, intracellular citrate and isocitrate were extracted by perchloric acid treatment, neutralized with ethanolic K_2CO_3 , and analyzed by enzymatic analysis (5). Aconitase was extracted from fast-growing non-citric acid-accumulating mycelia by suspension of the mycelia in 50 mM phosphate buffer (pH 7.5), containing 5 mM EDTA to give a ratio of 1:5 (g [wet weight]:ml) and disintegration by ultrasonication (7). The homogenate was centrifuged at $10,000 \times g$ (4°C , 20 min), and the supernatant was saved for the assay of aconitase activity, which was done by the method of Racker (10). For the determination of aconitase equilibrium in vitro, the method of Blair (2) was used. Equilibrium concentrations of citrate and isocitrate were determined by enzymatic analysis (5). Respective aconitase samples were desalted on PD 10 columns (Pharmacia, Uppsala, Sweden), equilibrated with the buffer given above.

Citric acid fermentations were performed on pilot plant scale in the presence of Cu^{2+} and Fe^{3+} and in the absence of both for conditions which should lead to differing activities of aconitase. The corresponding time course of biomass production and citric acid accumulation under the three conditions chosen (Fig. 1) indicated that the addition of Cu^{2+} favored the highest citric acid yield, whereas the presence of Mn^{2+} and, to a lesser extent, Fe^{3+} decreased this yield. Growth was not very different under the three conditions. Thus, the possibility that the differing citric acid yields are due simply to different growth patterns is ruled out.

Aconitase activity could be extracted from the mycelia under all conditions of fermentation, provided the precautions recommended by La Nauze (6) were carefully considered. The activity assayed with isocitrate as the substrate was approximately 2- to 2.3-fold that obtained when citrate was the substrate. This was reasonably close to the value obtained with aconitase from other tissues (8), and we thus assume that citrate dehydratase (EC 4.2.1.4), which has been found in *A. niger* (9), is absent from our strain.

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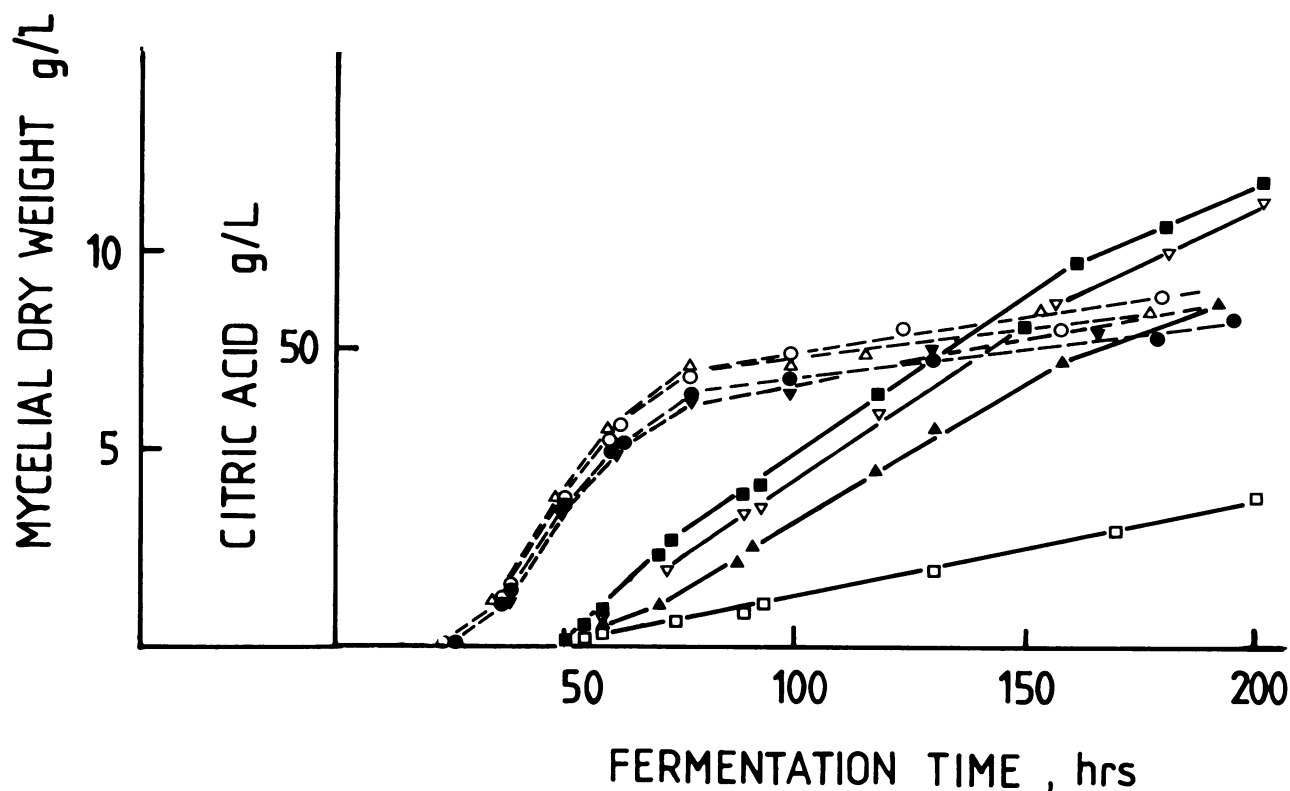


FIG. 1. Mycelial growth and citric acid accumulation by *A. niger* in pilot plant culture in the presence of Cu^{2+} (100 mg/liter) (●, dry weight [g/liter]; ■, citric acid [g/liter]), in the presence of Fe^{3+} (50 mg/liter) (○, dry weight [g/liter]; ▲, citric acid [g/liter]), and in the absence of Cu^{2+} and Fe^{3+} but in the presence of Mn^{2+} (0.1 mg/liter) (△, dry weight [g/liter]; □, citric acid [g/liter]). Control (absence of all added metals): ▼, dry weight (g/liter); ▽, citric acid (g/liter). Conditions for citric acid fermentation were essentially as previously described (14).

Because enzymes in their cellular compartments operate at subsaturating concentrations of their substrates and moreover are subject to interference by various factors or metabolites, we considered it to be of little value to measure the total extractable activity of aconitase. Instead, we measured intracellular concentrations of citrate and isocitrate, the ratio of which should show significant deviations from the theoretical value of 9.3 if aconitase is inhibited (2). The validity of this assumption was initially verified by measuring the effect of the addition of fluorocitrate on the citrate-isocitrate ratio. For this purpose, a final concentration of 1 mM fluorocitrate was added to mycelia (3 g/liter, pregrown for 40 h in citric acid-accumulating medium but then suspended in fresh medium to avoid the competitive action of accumulated citrate on fluorocitrate transport or action), and citrate and isocitrate levels were measured after 1, 2, and 4 h of further cultivation. After 1 h, a ratio of 25.4 was obtained, but at 2 or 4 h, isocitrate decreased below the detection limit (0.03 mM), and thus a ratio of only >56 can be quoted. Nevertheless, the results indicate that our experimental approach is suitable for assessing aconitase inhibition *in vivo*.

Under the conditions for citric acid fermentation shown in Fig. 1, the ratio of citrate:isocitrate was around 10 (Table 1). Although the ratio was somewhat lower in the lowest citric acid-producing culture (0.1 mg of Mn^{2+} per liter added), the differences were far less pronounced (when compared with the effect of fluorocitrate) and thus might be attributable to other factors, such as cellular ionic strength, compartmentation, or others.

To examine whether the equilibrium of *A. niger* aconitase is identical to the theoretical value of 9.3 (2), we reassessed

the equilibrium of the *A. niger* enzyme by the method of Blair (2). The results are documented in Table 2. The equilibrium found by us agreed well with the theoretical value. However, the experimental conditions under which this value was obtained neglected the known chelation of

TABLE 1. Intracellular concentrations of citrate and isocitrate in *A. niger* during citric acid fermentation under different conditions^a

Fermentation condition and time	Concn (mM) ^b		Ratio ^c
	Citrate	Isocitrate	
+ 100 mg of Cu^{2+} per liter			
40 h	2.6	0.22	10
90 h	4.7	0.36	10
130 h	5.2	0.40	13
+ 50 mg of Fe^{3+} per liter			
40 h	2.2	0.27	8
90 h	4.2	0.32	12
130 h	4.5	0.36	12
+ 0.1 mg of Mn^{2+} per liter			
40 h	0.9	0.12	7.5
90 h	2.1	0.15	10
130 h	1.8	0.20	9

^a Conditions were essentially as described in the legend to Fig. 1 by methods reported previously (14).

^b Values are based on average cell concentrations and are the means of three separate determinations.

^c Ratio of mM citrate/mM isocitrate.

TABLE 2. Aconitase equilibrium in vitro^a

Addition	Concn (mM)			Ratio ^b
	Citrate	Isocitrate	Citrate + Isocitrate	
	0.83	0.09	0.92	9.2
2 mM Mg ²⁺	0.915	0.04	0.955	22
5 mM Mg ²⁺	0.92	0.02	0.922	46

^a Experimental conditions were essentially as described by Blair (2), with 1 mM citrate as the initial substrate. Conditions for incubation were 20 mM Tris hydrochloride buffer (pH 7.3) and 28°C.

^b Ratio of mM citrate/mM isocitrate.

intracellular citrate and isocitrate with Mg²⁺. Blair showed that the presence of Mg²⁺ considerably elevates the citrate:isocitrate equilibrium ratio (2). This was also the case with the *A. niger* enzyme (Table 2).

If the results from Tables 1 and 2 are now compared, it becomes evident that neither the addition of Fe³⁺ nor the addition of Cu²⁺ had an effect on the in vivo activity of aconitase, although their effect on citric acid accumulation was well documented (Fig. 1). We have not yet performed investigations on the mechanism of their action, but the concentrations of Fe³⁺ required to decrease citric acid production are at least 1,000-fold higher than those of Mn²⁺, and it is probable that Mn²⁺ impurities in the Fe³⁺ source cause the actual inhibition. Cu²⁺ is able to antagonize the inhibition of citric acid fermentation by Mn²⁺ (12). Further work is needed to validate these assumptions.

From the present work, however, we are able to conclude that inhibition of aconitase is not necessary for citric acid fermentation, thereby disproving previous theories (3, 11).

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