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Evidence for a Cytoplasmic Pathway of Oxalate Biosynthesis in *Aspergillus niger*

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Oxalate accumulation of up to 8 g/liter was induced in *Aspergillus niger* by shifting the pH from 6 to 8. This required the presence of P_i and a nitrogen source and was inhibited by the protein synthesis inhibitor cycloheximide. Exogenously added $^{14}CO_2$ was not incorporated into oxalate, but was incorporated into acetate and malate, thus indicating the biosynthesis of oxalate by hydrolytic cleavage of oxaloacetate. Inhibition of mitochondrial citrate metabolism by fluorocitrate did not significantly decrease the oxalate yield. The putative enzyme that was responsible for this was oxaloacetate hydrolase (EC 3.7.1.1), which was induced de novo during the pH shift. Subcellular fractionation of oxalic acid-forming mycelia of *A. niger* showed that this enzyme is located in the cytoplasm of *A. niger*. The results are consistent with a cytoplasmic pathway of oxalate formation which does not involve the tricarboxylic acid cycle.

Oxalic acid is a common metabolic product found in the culture fluid of several fungi; in some of them it has been implicated in the mechanism of plant pathogenesis (1, 11). In *Aspergillus niger*, it may occur as an unwanted by-product of citric acid fermentation, which, because of its toxicity, must be completely removed (18). Oxalic acid biosynthesis is favored by a high, almost alkaline medium pH (3, 8, 9), and therefore can present a problem when molasses is used as a carbon source because molasses has a high buffering capacity between pH 5 and 6. Thus, knowledge concerning regulation of oxalate biosynthesis in *A. niger* should make a beneficial contribution to improvements in citric acid fermentation.

The pathway of oxalate biosynthesis, however, has remained controversial; and different mechanisms may even occur in one organism, depending on the nutritional conditions (3, 4, 14). In *A. niger* current evidence favors a role of oxaloacetate hydrolase (EC 3.7.1.1) in the splitting of oxaloacetate to form oxalate, whereas in other fungi, oxidation of glyoxalate seems to be involved (1, 9, 11). Both mechanisms are based on the assumption that the tricarboxylic acid cycle or the glyoxalate cycle is involved. However, *A. niger* contains a cytoplasmic, constitutive pyruvate carboxylase (EC 6.4.1.1), and is therefore capable of forming oxaloacetate without the reactions of the tricarboxylic acid cycle (8, 18).

In the present report we provide evidence for the biosynthesis of oxalate in *A. niger* by a pathway which involves hydrolysis of oxaloacetate and which is entirely located in the cytoplasm.

MATERIALS AND METHODS

Strain. The citric acid-producing strain *A. niger* B60 was used throughout this study. The origin and method of maintenance of this organism has been documented (19).

Methods of cultivation. Conditions for cultivation of *A. niger* in a synthetic medium supporting citric acid accumulation have been described previously (6). During the course of these studies, all experiments were carried out in 1-liter shake flasks with 200 ml of medium. A 2-liter bench top fermentor equipped with an oxygen electrode was used to vary the dissolved oxygen tension, and the oxygen tension was modified by altering the impeller speed.

pH shift experiments. pH shift experiments were carried out as described recently (13). For these experiments, the whole culture broth of a citric acid-producing culture (48 h; pH 1.8) was titrated with 1 N NaOH to the pH indicated below. The pH was kept at this level throughout the following experiment (maximum of 24 h) by the continuous addition of 1 N NaOH. Cycloheximide was added immediately before NaOH was added to give a final concentration of 50 μg/ml. With the exception of the experiments concerned with the optimization of oxalate formation, all preparations also contained 2.5 g of $(NH_4)_2SO_4$ and 2.5 g of KH_2PO_4 per liter. These were added as solids immediately before the start of the pH shift experiment.

Preparation of cell extracts. Cell extracts used for the determination of enzyme activities were prepared by grinding the mycelia in liquid nitrogen and subsequent ultrasonication as reported previously (12). Preparation of cell extracts for subcellular fractionation was carried out by nitrogen cavitation (16), as modified recently (12, 17).

Enzyme assays. Oxaloacetate hydrolase was assayed by determining the decrease in the A_{255} due to oxaloacetate cleavage (9). Other enzymes were assayed as described previously (6). One unit was always defined as the formation of 1 μmol of product (or equivalent) per min under the assay conditions. For oxaloacetate hydrolase, a molar absorption coefficient for oxaloacetate of 1.1 mM/cm was used (9). Specific activities are given as units per milligram of protein. Protein was determined by the method described by Bradford (2), and bovine serum albumin was used as a standard.

Labeling of oxalate. Labeling of oxalate during biosynthesis was carried out with $NaH^{14}CO_3$ (53 mCi/mmol). Cultures were pregrown for 48 h and subjected to pH shifting as described above. Samples (5 ml) of culture broth were then quickly transferred into 25-ml Erlenmeyer flasks and agitated at 200 rpm (28°C) in a water bath for an additional 60 min. Then, a pulse of 0.4 μCi of $NaH^{14}CO_3$ per ml, which was diluted with cold $NaHCO_3$ to give a final concentration

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TABLE 1. Formation of organic acids by *A. niger* under various conditions following a pH shift to 6.0

Condition[a] (% saturation)	Formation (g/liter) of the following organic acid:		
	Citrate	Gluconate	Oxalate
Aeration (100%)	<0.1	22.8	<0.1
Aeration (20%)	0.3	3.0	0.13
Aeration (100%) plus $(NH_4)_2SO_4$	2.4	10.7	0.3
Aeration (100%) plus $(NH_4)_2SO_4$ and KH_2PO_4	0.86	0.35	2.2

[a] pH shift experiments were carried out in a 2-liter fermentor as described in the text, and the acids formed were described in the text. The acids formed after 20 h of incubation were measured by high-pressure liquid chromatography. In the case of the salt (phosphate nitrogen) additions, a concentration of 2.5 g of each was used per liter. Prolonged cultivation of the starting culture without a pH shift produced only citric acid but less than 0.1 g of gluconic acid or oxalic acid per liter. The values presented are from single experiments only, but repetition yielded results consistent with the results given here.

of 50 mM, was added; the flasks were covered with a U-tube containing KOH (to avoid the evaporation of $^{14}CO_2$ into the laboratory air); and shaken for 30 min. Thereafter, the whole contents of one flask were filtered; the acids that formed were separated by thin-layer chromatography as described by Lenz et al. (9); and samples from the previously calibrated positions of oxalate, acetate, and malate were scraped from the plate and counted in a liquid scintillation counter. Several tracks (corresponding to 500 μl of fermentation fluid) had to be scraped from the plate to obtain sufficient material for one determination. In the case of mycelia, they were washed twice with cold nutrient medium and then counted. In some experiments, they were extracted with 5 ml of ethyl acetate per g (wet weight) of mycelia for 2 h, and the ethyl acetate extract was measured separately for radioactivity.

Determination of oxalate and other fermentation acids. Organic acids were analyzed by high-pressure liquid chromatography on a chromatographic column (HPX-87-H; Bio-Rad Laboratories, Richmond, Calif.) by using 0.01 N H_2SO_4 as the mobile phase. In selected cases, oxalate was alternatively determined by enzymatic analysis (5) by using a test kit (Boehringer GmbH, Mannheim, Federal Republic of Germany). Consistent results were obtained by both methods. Enzymatic analyses of other organic acids were carried out as described previously (7).

RESULTS

Oxalate accumulation requires phosphate and nitrogen. Oxalate accumulation by *A. niger* has been reported to occur only at a pH close to or above neutrality (3, 9). We thus attempted to induce de novo oxalic acid accumulation in a culture of *A. niger* that had produced citric acid for 48 h by the pH shift method, which has recently been applied successfully for gluconic acid accumulation (13). By so doing, however, gluconate was the major organic acid accumulated, and only traces of oxalic acid were found (Table 1). A decrease in the dissolved oxygen tension (to decrease the affinity of the extracellular glucose oxidase for glucose) decreased gluconate production, but had little effect on oxalate accumulation. However, the addition of P_i and nitrogen into the medium used for the pH shift experiment resulted in a considerable accumulation of oxalic acid. No oxalate was ever detected (<0.1 g/liter) in control flasks which were not subjected to the pH shift. The pH optimum for oxalate accumulation was 6, and as much as 5 g/liter was found after 20 h (Fig. 1). Further incubation (to 50 h) led to maximum concentrations of 8 g/liter. The accumulation of oxalate was induced de novo by the pH shift, since the addition of cycloheximide completely inhibited the accumulation of oxalate.

Exogenous $^{14}CO_2$ is not incorporated into oxalate. Since we had available an experimental system by which oxalate accumulation could be induced, we investigated the pathway of oxalate formation. Three different possibilities were considered: (i) formation of oxalate by splitting of the oxaloacetate which does not enter the tricarboxylic acid cycle; (ii) formation of oxalate by splitting of the oxaloacetate which arises from the tricarboxylic acid cycle; and (iii) formation of oxalate from glyoxalate via the glyoxalate cycle. In all these schemes it is presumed that $^{14}CO_2$ is used as a substrate by pyruvate carboxylase to form specifically labeled oxaloacetate. The distribution of the ^{14}C label under the three conditions given above is given in Fig. 2. It is evident that formation of oxalate via the glyoxalate cycle can easily be discriminated from the route of formation of oxalate via oxaloacetate hydrolysis. The two possibilities involving oxaloacetate hydrolysis, i.e., involvement or noninvolvement of the tricarboxylic acid cycle, did not yield labeled oxalate. These two possibilities can be distinguished from each other, however, by looking at the effect of fluorocitrate, which inhibits aconitase and therefore should decrease the yield of oxaloacetate, if its formation requires the operation of the tricarboxylic acid cycle.

Results of an experiment with $NaH^{14}CO_3$ (Table 2) showed that oxalate was labeled only slightly, whereas a clear label was observed in malate and acetate and in the mycelium. In particular, we noted that the specific activity of acetate found in the culture fluid was very similar to that of the added $^{14}CO_2$, which supports its origin by the first mechanism given above. Fluorocitrate, when added at a concentration (1 mM) which reduced the incorporation of [^{14}C] leucine into cellular protein by 35 to 40% (unpublished data), had little effect on the labeling pattern of the acids secreted by *A. niger*. This is consistent with the fact that oxalate is formed from oxaloacetate before it enters the tricarboxylic acid cycle.

Although the acetate in the medium always contained a specific activity consistent with its formation from oxaloacetate, its amount was never more than a fourth to a third of that of oxalate. This indicates that metabolism of the acetate arises during the splitting of oxaloacetate. We found a significant ^{14}C label in an ethyl acetate extract of *A. niger*,

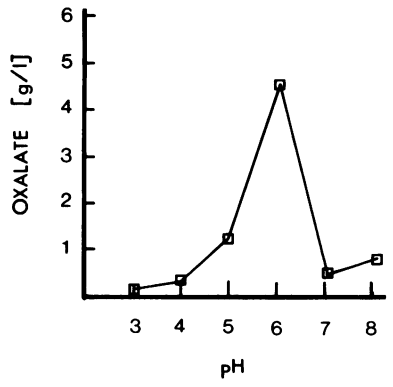

FIG. 1. Influence of the pH of the final incubation medium on the accumulation of oxalate by *A. niger*.

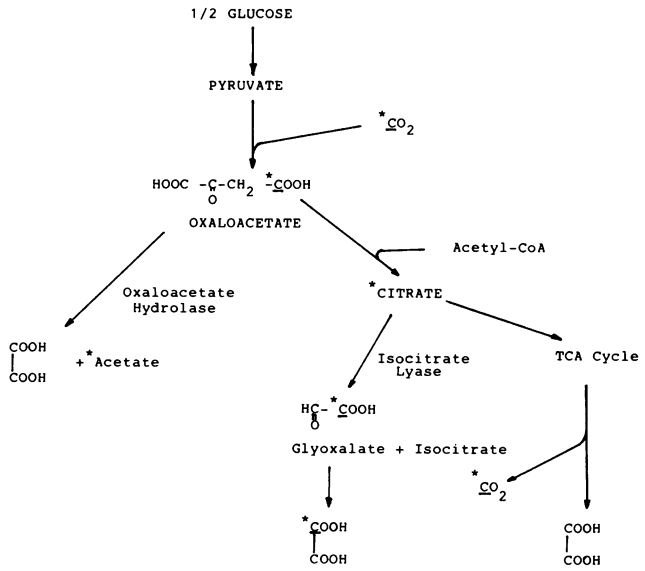

FIG. 2. Discrimination between three modes of oxalate biosynthesis by *A. niger* by using $^{14}CO_2$ incorporation in oxalate and inhibition of the tricarboxylic acid cycle by fluorocitrate. The distribution of labeled ^{14}C from $^{14}CO_2$ is indicated by asterisks and by underlining of the respective carbon. Abbreviations: CoA, coenzyme A; TCA, tricarboxylic acid.

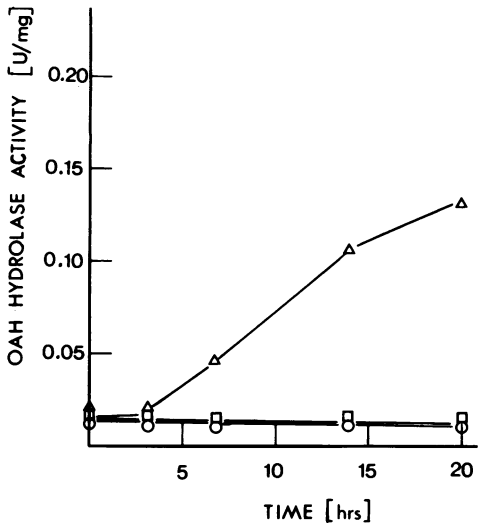

FIG. 3. Induction of oxaloacetate hydrolase (OAH) in *A. niger* by a shift in pH to 6. Activity is given in units per milligram of protein. Conditions were as follows: (i) shift up in pH to 6 in the presence of 2.5 g of $(NH_4)_2SO_4$ and KH_2PO_4 per liter (△); the same experiment as described for (i), but including 50 μg of cycloheximide per ml (□); the same experiment as described for (i), but without $(NH_4)_2SO_4$ and KH_2PO_4 (○).

which presumably contained polyketids and lipids, indicating that acetate is converted to acetyl coenzyme A under these conditions. We have previously shown (17) that *A. niger* contains an inducible acetyl coenzyme A kinase (EC 6.2.1.1) in its cytoplasm.

Oxaloacetate hydrolase is an inducible, cytoplasmic enzyme in *A. niger*. The results reported so far have shown that (i) oxalate accumulation is specifically induced by shifting of the pH to 6.5 and (ii) oxalate arises from oxaloacetate, which is formed by pyruvate carboxylase, which is located in the cytoplasm of *A. niger*. The corresponding scheme for oxalate accumulation would be strongly supported if oxaloacetate hydrolase would exhibit the same properties. This could be demonstrated by the fact that the enzyme was not detectable during the growth of *A. niger* at or below pH 4 and was induced on a shift of the pH to 6 (Fig. 3). Induction was blocked by the addition of cycloheximide, indicating the de novo synthesis of oxaloacetate hydrolase. When the mycelium was homogenized under conditions that preserved subcellular compartmentalization, oxaloacetate hydrolase was always detected in the lightest fraction on subcellular fractionation (Table 3). This behavior correlated with that of other enzymes of known cytoplasmic location (i.e., pyruvate kinase, glucose-6-phosphate dehydrogenase), whereas citrate synthase, which occurs in the mitochondria, was found at a high percentage in the heavier fractions. Hence, oxaloacetate hydrolase appears to be a cytoplasmic enzyme in *A. niger*.

DISCUSSION

Results of this study support a pathway of oxalate accumulation by *A. niger* which is summarized in Fig. 4. This pathway occurs completely in the cytoplasm and does not require the entry of carbon into the mitochondria. In this respect, the origin of the CO_2 required for the pyruvate carboxylase reaction is unclear and cannot be deduced from the results presented here. During citric acid accumulation, the CO_2 is provided by the pyruvate dehydrogenase reaction in the mitochondrium. However, although pyruvate dehydrogenase is active during oxalate formation in *A. niger* (unpublished data) it is not absolutely required, since oxalate formation accounts for only a small portion of the total

TABLE 2. Distribution of ^{14}C incorporated from $NaH^{14}CO_3$ into acids and total mycelial material formed in a short-term, resting cell experiment with *A. niger* involving a pH shift to 6[a]

Condition[b]	^{14}C distribution (cpm/μmol) in[c]:			
	Acetate	Malate	Oxalate	Mycelium
$NaH^{14}CO_3$	14,066 (0.06)	15,466 (0.15)	31.4 (1.37)	10,316
$NaH^{14}CO_3$ plus fluorocitrate (1 mM)	10,300 (0.09)	15,583 (0.12)	39.2 (1.30)	8,964

[a] *A. niger* was exposed to $NaH^{14}CO_3$ as described in the text. The radioactive acids were counted as described in the text. The organic acids that were formed were also measured by enzymatic analysis. Counts per minute were for 1 ml of culture fluid, and corresponding acid concentrations were used to calculate the counts per minute per micromole. The concentration of individual acids at time zero of the labeling experiment was always considered. The counts per minute incorporated into the mycelium were for 1 ml of cultural suspension. The amount of original label of $NaH^{14}CO_3$ in the experiment for which the results are shown here was 16,044 cpm/μmol. The results are taken from one of three experiments, which all gave consistent results.

[b] Fluorocitrate was added 1 h before $NaH^{14}CO_3$ was added.

[c] Values in parentheses are concentration (in micromolar). Values for mycelium are in counts per minute per milliliter of broth.

TABLE 3. Distribution of several enzymes between cytosolic and mitochondrial fractions in *A. niger*[a]

Enzyme	Sp act (μmol min^{-1} mg of protein^{-1}) of enzymes from the following fraction:	
	Mitochondrial	Cytosolic
Oxaloacetate hydrolase	<0.005	0.180
Glucose-6-phosphate dehydrogenase	<0.010	1.380
Citrate synthase	0.850	0.130
Pyruvate kinase	<0.010	0.634

[a] Cell extracts were prepared under conditions that preserved intracellular compartmentalization, as described in the text. Mycelia were harvested 16 h after the beginning of the pH shift.

carbon metabolized, and other reactions might be involved as well. During our short-term labeling studies, we observed that the label of [1-^{14}C]glucose was incorporated into oxalate in approximately the same low proportion as $^{14}CO_2$, which indicates an origin within the pentose phosphate pathway. More investigations with differentially labeled carbon sources are required to elucidate further the origin of the carbon dioxide needed for oxalate formation. Although such an investigation has already been carried out by Cleland and Johnson (3), the results of this study are different from those presented by Cleland and Johnson (3), since they found that oxalate is mainly formed (at neutral pH) by the oxidation of glycolaldehyde, which is generated via pentose catabolism. However, although Cleland and Johnson (3) also carried out their studies in a replacement system, they used rather long labeling times (several hours). In their studies, glucose was already converted to gluconate before significant oxalate was observed in the medium; thus, they might have been dealing with oxalate formation from gluconate, which could explain the differences in the results of their study and this one.

The operation of the cytoplasmic pathway of oxalate biosynthesis raises the question as to the physiological role and regulation of oxalate formation. Mass and coenzyme balances show that:

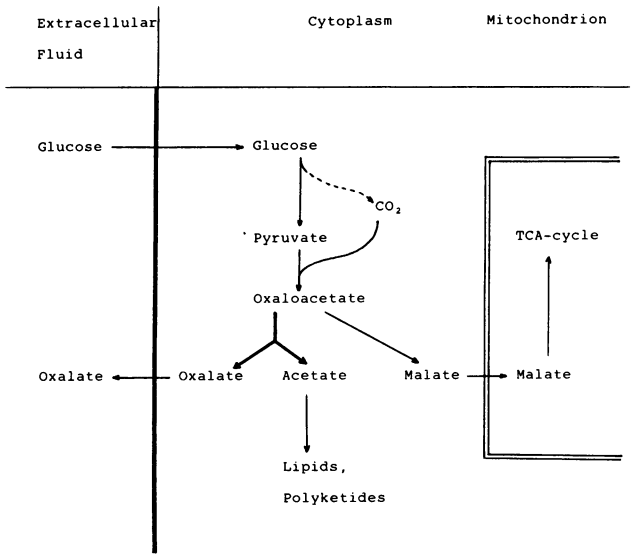

FIG. 4. Simplified metabolic scheme of oxalate biosynthesis in *A. niger*. TCA, Tricarboxylic acid.

$$1 \text{ glucose} + 2CO_2 + 2NAD \rightarrow 2 \text{ oxalate} + 2 \text{ acetate} + 2NADH.$$

Hence, the pathway does not produce ATP (pyruvate carboxylase requires 1 ATP per oxaloacetate formation) but generates a surplus of NADH. Since the cytoplasmic malate dehydrogenase is fairly active under conditions of oxalate formation (unpublished data) it is not clear why oxaloacetate is not reduced to malate (the K_m for oxaloacetate is far lower than that of malate dehydrogenase of oxaloacetate hydrolase) (9, 10). Although malate was found in the medium, it did not account for more than 0.9 g/liter. Apparently, the pH shift to neutrality causes some alteration in the regulation of oxaloacetate metabolism. Lenz et al. (9) have suggested that the rise in pH may increase the intracellular concentration of oxaloacetate, which in turn induces oxaloacetate hydrolase. Although the induction of this enzyme has been shown here, we are unaware whether oxaloacetate was responsible for it. A significant rise in its concentration (up to 10-fold) (7) might decrease malate dehydrogenase activity, which is subject to substrate inhibition (10). Such an increase, however, remains to be demonstrated.

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