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EXPERIMENTAL ARTICLES

Biochemical Characterization of the Yeast *Yarrowia lipolytica* Overproducing Carboxylic Acids from Ethanol: Nitrogen Metabolism Enzymes

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Abstract—A comparative assay of nitrogen metabolism enzymes in the *Yarrowia lipolytica* mutant N1 grown under conditions promoting the overproduction of either α-ketoglutaric acid (KGA) or citric acid showed that the overproduction of KGA correlates with an increase in the activities of the NAD- and NADP-linked glutamate dehydrogenase, glutamic–pyruvic transaminase, and glutamic–oxaloacetic transaminase reactions. These reactions are likely to be responsible for the overproduction of KGA by this mutant. In contrast, the overproduction of citric acid correlated with a decline in the activities of the NAD- and NADP-linked glutamate dehydrogenases and with an increase in the activities of glutamine synthetase and glutamate synthase.

Key words: yeasts, ethanol, overproduction of carboxylic acids, nitrogen metabolism.

Under certain cultivation conditions, the *Yarrowia lipolytica* mutant N1 grown on ethanol overproduces some intermediates of the tricarboxylic acid (TCA) cycle, α-ketoglutaric acid (KGA) and citric acid in particular [1]. The major necessary condition for the overproduction of citric acid is a shortage of ammonium ions in the medium. In contrast, KGA is overproduced when ammonium ions are in excess, whereas thiamine is scarce. These data suggest that the activities of nitrogen metabolism enzymes in yeast cells overproducing different carboxylic acids must differ.

The aim of this work to verify this suggestion.

MATERIALS AND METHODS

The mutant strain N1 of *Yarrowia lipolytica* used in this study and cultivation conditions were described in detail earlier [1, 2].

The initial concentration of thiamine in the cultivation medium favorable to KGA oversynthesis was 3 μg/l, while in the complete medium and in the medium favorable to citric acid oversynthesis it was 200 μg/l. The concentration of ammonium ions for KGA oversynthesis was maintained at a level of 1–1.2 g/l, while for citric acid oversynthesis it was at a level close to zero. The concentration of ammonium ions in the medium was controlled using an Orion-720 ion-meter. The pO_2 of the medium was maintained at a level of 50% saturation by an automated oxystat system. Ethanol was added to the medium in batches so that its concentration in the medium did not exceed 2.5 g/l.

The content of carboxylic acids in the culture liquid was determined as described in the aforementioned work [1]. The cell-free homogenate of yeast cells was prepared as described elsewhere [3].

The NAD- and NADP-linked glutamate dehydrogenases (EC 1.4.1.2 and EC 1.4.1.4, respectively) were assayed spectrophotometrically by the oxidation of NAD(P)H or reduction of $NAD(P)^+$ [4]. The activities of glutamine synthetase (EC 6.3.1.2) and NAD(P)-linked glutamate synthase (EC 1.4.7.1) were also determined as described by Holmes *et al.* [4]. L-Aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1), L-alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2), and L-alanine:glyoxylate aminotransferase (EC 2.6.1.44) were assayed by the method of Baars *et al.* [5].

The respiration rate of intact and permeabilized yeast cells was determined at 28°C using a Clark-type oxygen electrode. Cells (1–2 mg dry wt per 1 ml) were permeabilized by incubating them at 30°C in a 50 mM K-phosphate buffer (pH 7.5) containing 20% glycerol. Then the suspension was supplemented with 0.2–0.3 mg/ml digitonin. The time of incubation with digitonin depended on the growth phase of cells.

Protein was quantified by the method of Lowry *et al*.

RESULTS AND DISCUSSION

Glutamate dehydrogenases. It is generally accepted that the major role in the assimilation of ammonium ions by yeasts is played by NADP-linked glutamate dehydrogenases [6–10], whereas NAD-linked glutamate dehydrogenases play a role in the dis-

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Activities (U/mg protein) of nitrogen metabolism enzymes in the *Y. lipolytica* N1 cells grown on ethanol under different cultivation conditions

Enzyme	Complete medium			KGA overproduction			Citric acid overproduction			
	1	2	3	1	2	3	1	2	3	4
Desaminating glutamate dehydrogenase (NAD^+)	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Desaminating glutamate dehydrogenase ($NADP^+$)	0.000	0.000	0.000	0.000	0.012	0.022	0.000	0.000	0.000	0.000
Aminating glutamate dehydrogenase (NADH)	0.056	0.034	0.040	0.236	0.153	0.147	0.000	0.000	0.000	0.000
Aminating glutamate dehydrogenase (NADPH)	0.182	0.263	0.169	0.165	0.019	0.011	0.096	0.011	0.000	0.000
Glutamine synthetase	0.004	0.006	0.005	0.021	0.011	0.021	0.036	0.053	0.084	0.110
Glutamate synthase (NADH)	0.030	0.035	0.026	0.000	0.000	0.000	0.000	0.056	0.052	0.050
Glutamate synthase (NADPH)	0.057	0.044	0.033	0.029	0.031	0.022	0.062	0.040	0.072	0.081
Aspartate aminotransferase	0.370	0.520	0.330	0.433	0.487	0.495	0.270	0.272	0.171	0.141
Alanine aminotransferase	0.230	0.295	0.230	0.060	0.135	0.286	0.050	0.000	0.000	0.000
Alanine–glyoxylate transaminase	0.100	0.092	0.084	0.086	0.051	0.032	0.048	0.000	0.000	0.000

Note: Complete medium: 1, 2, and 3 correspond, respectively, to cultivation times of 12, 20, and 42 h (stationary growth phase). KGA overproduction: 1, 2, and 3 correspond, respectively, to cultivation times and KGA concentration in the medium equal to 24 h (0 g/l KGA), 72 h (10 g/l KGA), and 120 h (30 g/l KGA). Citric acid overproduction: 1, 2, 3, and 4 correspond, respectively, to cultivation times and concentrations of citric acid (CA) in the medium equal to 19 h (0 g/l CA), 30 h (5 g/l CA), 50 h (49 g/l CA), and 75 h (75 g/l).

similation of ammonium ions, irrespective of their concentration in the medium [10, 11]. The role of glutamine synthetase and glutamate synthase in ammonium metabolism is believed to be insignificant.

Our measurements of enzymatic activities in the ethanol-grown cells of *Y. lipolytica* N1 cultivated under different conditions (table) showed that the activities of both NAD- and NADP-linked desaminating glutamate dehydrogenases were close to zero under all of the cultivation conditions studied. In cells grown in the complete medium, the activity of NADPH-linked aminating glutamate dehydrogenase was three times that of the NADH-linked enzyme. In cells overproducing KGA, the activity of NADPH-linked aminating glutamate dehydrogenase decreased by an order of magnitude, whereas the activity of the NADH-linked enzyme remained high. In cells overproducing citric acid, the activities of both NADH- and NADPH-linked aminating glutamate dehydrogenases were close to zero.

These data show that yeast cells overproducing KGA contain functionally active NADH-linked aminating glutamate dehydrogenase. During the oversynthesis of citric acid, all glutamate dehydrogenases are suppressed.

These differences can be explained by the different concentrations of nitrogen in the cultivation medium. Indeed, the concentration of ammonium ions favorable to KGA oversynthesis is 20–30 mM [1]. In this case, the calculated concentration of NH_4^+ ions in cells must be 4 mM, which is close to the K_m of glutamate dehydrogenase. The decrease in the concentration of ammonium ions in the medium to 15 mM slowed down the overproduction of KGA. Under the conditions of citric acid overproduction, the concentration of NH_4^+ ions in the medium is close to zero, which completely suppresses glutamate dehydrogenases.

Of interest is the fact that the activity of NADPH-linked glutamate dehydrogenase is high during the unlimited growth of cells, whereas the activity of NADH-linked glutamate dehydrogenase is high during the limited growth of cells, such as in the case of KGA overproduction.

Glutamine synthetase and glutamate synthase. In an alternative pathway of glutamate metabolism, it is aminated by glutamine synthetase to glutamine, whose amino group is then transferred to KGA by glutamate synthase [12]. This system is believed to have a higher affinity for ammonium ions than has the NADP-dependent glutamate dehydrogenase and thereby must be more efficient at low concentrations of ammonium ions. Some researchers believe that this system is the major pathway of ammonium assimilation by yeasts of the genera *Candida* and *Schizosaccharomyces* [4, 13] and the subsidiary ammonium assimilation pathway in the yeast *Saccharomyces cerevisiae* [8, 14].

According to our measurements (table), the activity of glutamine synthetase was high in citric acid–overproducing cells (i.e., under ammonium deficiency) and low in yeast cells grown in the complete medium and under conditions of KGA overproduction.

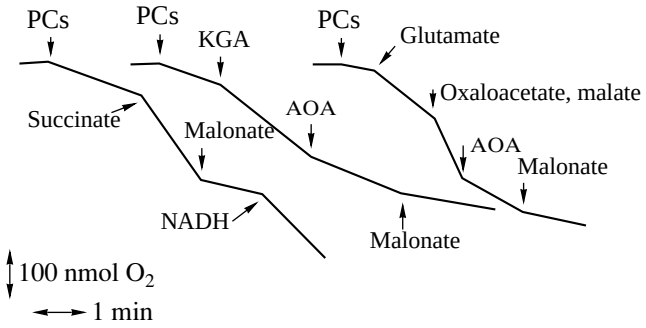

Fig. 1. The oxidation of some substrates by the *Y. lipolytica* N1 cells grown under the conditions promoting KGA overproduction. The arrows indicate the addition of permeabilized cells (PCs) at a concentration of 1.5 mg dry wt/ml, KGA at a final concentration of 3 mM, succinate (3 mM), glutamate (3 mM), oxaloacetate (3 mM), malate (3 mM), NADH (0.5 mM), malonate (3 mM), and aminooxyacetate (AOA) (2 mM). The respiration of cells was measured in a medium containing 200 mM NH_4HCO_3 and 20 mM KH_2PO_4 (pH 7.5), as described in the Materials and Methods section.

The activities of NADH- and NADPH-linked glutamate synthases were high in yeast cells grown in the complete medium and under conditions of citric acid overproduction. In KGA-overproducing cells, the activity of NADPH-dependent glutamate synthase was low and the activity of the NADH-dependent enzyme was zero.

Thus, the activity of the glutamine synthetase–glutamate synthase system increases at low concentrations of ammonium ions in the medium. In the absence of NADH- and NADPH-dependent glutamate dehydrogenases (as in cells overproducing citric acid), this system may play the major role in nitrogen metabolism. It should be noted that the source of glutamate for the synthesis of glutamine still remains unknown.

Transamination. The role of transaminases (aminotransferases) in the nitrogen metabolism of yeasts is far from understood. The relevant data available in the literature are mainly concerned with the cell localization of these enzymes [10, 11, 15].

The *Y. lipolytica* N1 cells grown on ethanol in the complete medium or in the medium favorable to KGA overproduction exhibited high activities of glutamic–oxaloacetic transaminase (aspartate aminotransferase) and glutamic–pyruvic transaminase (alanine aminotransferase) and low activity of alanine–glyoxylate transaminase. The activities of the first two enzymes increased in the course of cultivation. At the same time, yeast cells overproducing citric acid exhibited only high activity of aspartate aminotransferase, which decreased in the course of cultivation (table).

The high activity of aminotransferases in yeast cells grown in the complete medium (table) suggests that aminotransferase reactions, malate–aspartate shuttle in particular, play an important part in the ethanol metabolism of yeasts and animals [16].

The high activity of aspartate and alanine aminotransferases in KGA-overproducing cells (table) suggests an important role of these enzymes (together with malate dehydrogenase [2]) in the process of KGA oversynthesis. Indeed, the highly active aspartate and alanine aminotransferases in cells grown under nitrogen excess in the medium must be provided with oxaloacetate and pyruvate generated at high rates in the left part of the TCA cycle and with glutamate generated in the right part of this cycle. Both aspartate and alanine aminotransferases produce KGA at high rates and thereby may be responsible for KGA overproduction.

The analysis of carboxylating and transphosphorylating enzymes in the KGA-overproducing *Y. lipolytica* cells showed high activities of decarboxylating malate dehydrogenase (EC 1.1.1.38), phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31), pyruvate carboxylase (EC 6.4.1.1), PEP carboxykinase (EC 4.1.1.49), PEP carboxytransphosphorylase (EC 4.1.1.38), and acetyl-CoA carboxylase (EC 6.4.1.2) [17]. In contrast, *Y. lipolytica* cells overproducing citric acid exhibited zero activities of transphosphorylating enzymes and PEP carboxylase and high activity of acetyl-CoA carboxylase.

The high activities of the aforementioned carboxylating and transphosphorylating enzymes in KGA-overproducing cells are likely to be necessary to provide for the synthesis of sufficient amounts of oxaloacetate and pyruvate, which, in turn, are necessary to provide for the high activities of aspartate and alanine aminotransferases. The suppression of these aminotransferases, as in yeast cells overproducing citric acid, reduces the activity of the enzymes generating oxaloacetate (or malate) and pyruvate.

Alternatively, the high aminotransferase activities in KGA-overproducing cells may be necessary to reduce the high level of ammonium ions in these cells through the intense synthesis of amino acids, including aspartate and alanine, in transamination reactions. Such a situation was observed in ammonia-stressed hybridoma cells [18].

The possibility also cannot be excluded that part of the glutamate formed by aminotransferases is converted to arginine, which is a precursor of polyamines and urea [19].

The low constitutive level of aspartate aminotransferase activity in citric acid–overproducing cells likely maintains nitrogen metabolism in these cells at a minimal necessary level.

The involvement of aminotransferases in the oxidative metabolism of KGA-overproducing cells. Aminotransferases are presently considered to play a central part in the oxidative and energy metabolism of eukaryotes [20]. In view of this, it was of interest to study the oxidation of some substrates directly related to transamination reactions.

Intact KGA-overproducing cells were able to oxidize only ethanol and acetate. The permeabilized cells

were able to oxidize not only these substrates but also the substrates that are typically oxidized by yeast mitochondria (succinate, exogenous NADH, pyruvate + malate) and some others (Fig. 1). The oxidation of succinate was inhibited by malonate, a specific inhibitor of succinate dehydrogenase localized in mitochondria. The inhibition of the endogenous respiration of permeabilized cells by malonate indicated that succinate is the major endogenous substrate in the cells, which is oxidized through the mitochondrial respiratory chain.

The question here arises as to the source of succinate, since the activity of the possible succinate-producing enzymes (α-ketoglutarate dehydrogenase, isocitrate lyase, and malate synthase) in KGA-overproducing cells is low [2]. As can be seen from Fig. 1, the permeabilized KGA-overproducing cells were able to oxidize KGA, the oxidation being sensitive to aminooxyacetate (an inhibitor of transamination reactions) and malonate. The malonate-induced inhibition of the oxidation of KGA, as well as of glutamate in the presence of oxaloacetate or malate (Fig. 1), was indicative of the possible formation of succinate during the oxidation of these substrates. The sensitivity of cell respiration on these substrates to aminooxyacetate suggested the involvement of the amination and transamination reactions in the synthesis of succinate. At low activity of α-ketoglutarate dehydrogenase, the glutamate formed by glutamate dehydrogenase may give rise to γ-aminobutyrate in the reaction catalyzed by glutamate decarboxylase (EC 4.1.1.15). The next enzyme of this pathway, 4-aminobutyrate aminotransferase (EC 2.6.1.19), must also be functional under conditions of nitrogen excess.

It should be noted that the overproduction of KGA by *Y. lipolytica* is always accompanied by the excretion of a small amount (1–1.5 g/l) of pyruvate (Fig. 2). The addition of malonate to the KGA-overproducing yeast culture grown on ethanol did not influence the excretion of KGA but stimulated the excretion of pyruvate. Since *Y. lipolytica* grown on ethanol produces acetyl-CoA from acetate in reactions catalyzed by alcohol and aldehyde oxidoreductases, the enhanced excretion of pyruvate in the presence of malonate can be accounted for by the operation of the left part of the TCA cycle in the reverse (reductive) direction. This is confirmed by the high activity of fumarate hydratase with malate and the presence of fumarate reductase in KGA-overproducing cells [2].

The operation of the left part of the TCA cycle in the reductive direction must lead to the formation of an additional amount of succinate. When the oxidation of succinate is inhibited by malonate or the concentration of ammonium ions in the medium is low, one or both reactions catalyzed by aspartate and alanine aminotransferases would reverse and begin to produce either oxaloacetate or pyruvate or both. As a result, the excretion of pyruvate into the medium increases.

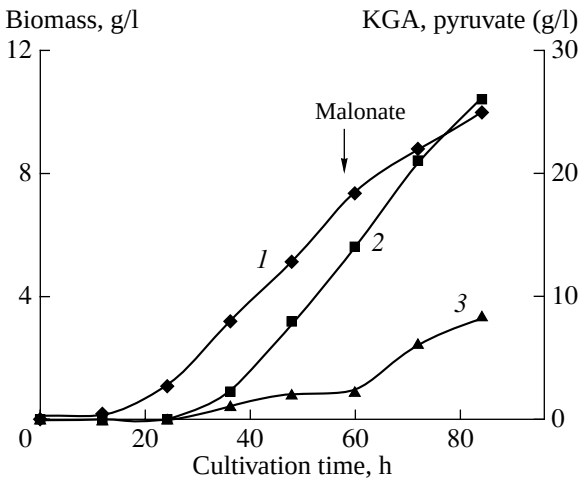

Fig. 2. The effect of the addition of malonate (3 mM) on (*1*) the growth of *Y. lipolytica* N1 on ethanol and the excretion of (*2*) KGA and (*3*) pyruvate.

In the *Y. lipolytica* cells overproducing citric acid, succinate is likely produced in a different way. Namely, active acetyl-CoA carboxylase in such cells [17] may successively give rise to propionyl-CoA, methylmalonyl-CoA, succinyl-CoA, and succinate.

Thus, the data obtained indicate that transamination reactions may play a central part not only in the nitrogen metabolism but also in the oxidative and energy metabolisms of *Y. lipolytica* cells grown at high concentrations of ammonium ions in the medium.

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