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EXPERIMENTAL ARTICLES

Metabolism of *Yarrowia lipolytica* Grown on Ethanol under Conditions Promoting the Production of α-Ketoglutaric and Citric Acids: A Comparative Study of the Central Metabolism Enzymes

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Abstract—A comparative study of the enzymes of tricarboxylic acid (TCA) and glyoxylate cycles in the mutant *Yarrowia lipolytica* strain N1 capable of producing α-ketoglutaric acid (KGA) and citric acid showed that almost all enzymes of the TCA cycle are more active under conditions promoting the production of KGA. The only exception was citrate synthase, whose activity was higher in yeast cells producing citric acid. The production of both acids was accompanied by suppression of the glyoxylate cycle enzymes. The activities of malate dehydrogenase, aconitase, NADP-dependent isocitrate dehydrogenase, and fumarase were higher in cells producing KGA than in cells producing citric acid.

Key words: oversynthesis, yeasts, central metabolism.

Our previous study showed that the degree of aeration and the concentration of NH_4^+ ions in the medium differently influence the growth, respiration, and the cytochrome system of the mutant *Yarrowia lipolytica* strain grown on ethanol under conditions promoting the production of α-ketoglutaric acid (KGA) and citric acid [1]. These data suggest that yeast cells producing KGA may differ from cells producing citric acid in the activity of the central metabolism enzymes, in particular, those which are involved in the tricarboxylic acid (TCA) and glyoxylate cycles.

The aim of the present work was to study the activity of the enzymes of these two cycles in yeast cells producing KGA and citric acid.

MATERIALS AND METHODS

The mutant *Yarrowia lipolytica* strain N1 was grown at 28°C in a 6-l ANKUM-2M fermentor in mineral Reader medium containing ethanol as the carbon and energy source [1]. Ethanol was added in portions, as it was consumed from the medium. To promote the production of KGA, the concentration of thiamine in the growth medium was reduced to 3 μg/l. For comparison, the concentration of this vitamin necessary for the normal growth of *Y. lipolytica* is 200 μg/l. The concentration of ammonium ions in the medium under conditions of citric acid synthesis was close to zero, while it was to be maintained at a level of 0.8–1.0 g/l to enhance the production of KGA.

The concentration of KGA and citric acid in the growth medium was determined as described earlier [1].

To assay enzymes, cells were collected by centrifugation at 3000 *g* for 5 min (4°C), washed with 0.9% NaCl, and disrupted using a rotary mill with glass beads. Cells were disrupted for 5 min in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.01 mM dithiothreitol. The homogenate was centrifuged at 3000 *g* for 5 min and then at 7000 *g* for 15 min. The supernatant, hereafter referred to as cell-free extract, was used to assay the enzymes of the TCA and glyoxylate cycles.

The activities of succinate oxidase and NADH oxidase were determined at 28°C in 50 mM Tris–HCl buffer (pH 7.8) with 5 mM $MgSO_4$ using a Clark-type oxygen electrode. Citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3), NAD- and NADP-dependent isocitrate dehydrogenases (EC 1.1.1.41 and EC 1.1.1.42, respectively), α-ketoglutarate dehydrogenase (EC 1.2.4.2), succinate dehydrogenase (EC 1.3.99.1), fumarase (EC 4.2.1.2), malate dehydrogenase (EC 1.1.1.37), isocitrate lyase (EC 4.1.3.1), and malate synthase (EC 4.1.3.2) were assayed as described in the publications [2–9]. The activity of fumarate reductase (EC 1.3.99.1) was determined by measuring the oxidation rate of reduced benzyl viologen [10]. NADH dehydrogenase was assayed by measuring the oxidation rate of NADH in 50 mM potassium phosphate buffer, pH 7.5, at 340 nm.

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Enzymatic activities (U/mg protein) in the *Y. lipolytica* N1 cells grown on ethanol under different cultivation conditions

Enzyme	Cultivation conditions									
	Complete medium			KGA production			Citric acid production			
	1	2	3	1	2	3	1	2	3	4
Citrate synthase	0.57	0.72	1.35	0.92	0.75	0.67	1.26	1.91	3.25	2.95
Aconitase	0.38	0.93	0.33	0.36	0.71	0.34	0.39	0.18	0.09	0.05
NAD-dependent ICD	0.03	0.05	0.05	0.03	0.02	0.02	0.02	0.02	0.01	0.01
NADP-dependent ICD	0.31	0.22	0.17	0.30	0.17	0.16	0.14	0.11	0.07	0.06
Isocitrate lyase	0.25	0.19	0.06	0.18	0.09	0.05	0.25	0.14	0.12	0.09
KGA dehydrogenase	0.02	0.02	0.01	0.01	0.01	0.00	0.02	0.01	0.01	0.00
Succinate oxidase	0.11	0.10	0.13	0.10	0.12	0.09	0.03	0.03	0.01	0.00
Succinate dehydrogenase	0.22	0.18	0.05	0.20	0.26	0.14	0.12	0.04	0.00	0.00
Fumarase with malate	0.66	0.84	0.63	0.32	0.47	0.68	0.32	0.22	0.21	0.20
Fumarase with fumarate	0.39	0.47	0.33	0.36	0.45	0.62	0.44	0.30	0.26	0.21
Fumarate reductase	0.10	0.09	0.04	0.10	0.06	0.09	0.03	0.02	0.01	0.00
Malate dehydrogenase	35.4	25.1	14.1	40.5	44.9	50.4	32.5	14.9	6.3	2.9
Malate synthase	0.13	0.05	0.02	0.05	0.02	0.02	0.05	0.03	0.02	0.02
NADH oxidase	0.25	0.25	0.18	0.15	0.09	0.05	0.12	0.08	0.02	0.01
NADH dehydrogenase	0.25	0.41	0.27	0.29	0.38	0.23	0.23	0.18	0.15	0.13

Note: 1, 2, 3, and 4 stand for the linear, retardation, stationary, and late stationary growth phases, respectively. The production of KGA and citric acid began in the retardation growth phase, was intense in the stationary phase, and continued (in the case of citric acid) in the late stationary phase. In the complete medium, KGA and citric acids were not produced.

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

RESULTS AND DISCUSSION

Citrate Synthase and Aconitase

Citrate synthase is a key enzyme of central metabolism responsible for the assimilation of ethanol. The mutant *Y. lipolytica* strain N1 possesses highly active citrate synthase, especially under conditions promoting the production of citric acid (see table). Under nitrogen deficiency in the medium, the activity of citrate synthase began increasing after 40–48 h of cultivation, together with the production of citric acid.

These data suggest that citrate synthase is regulated by the concentration of ammonium ion in the medium. This suggestion is confirmed by the measurements of citrate synthase activity in cells grown in the complete medium (see table). After the yeast transition to the stationary growth phase induced by the exhaustion of nitrogen sources in the thiamine-sufficient medium, the activity of citrate synthase increased in the same way as in the case of cells producing citric acid. As opposed to the other enzymes presented in the table, the activity of citrate synthase increased with decreasing concentrations of nitrogen in the medium. This suggests that the ammonium ion may serve as a negative modulator of citrate synthase.

At oxygen concentrations in the medium below 20% saturation, the activity of citrate synthase falls and the production of citric acid ceases [11]. Therefore, the production of citric acid is controlled not only by the concentration of nitrogen in the medium but also by the concentration of dissolved oxygen. Unlike the production of citric acid, the production of KGA is not inhibited by low oxygen concentrations in the medium [1], although the activity of citrate synthase in this case decreases (see table).

Active citrate synthase is necessary for the production of citric acid from ethanol, since this enzyme is the main source of carbon for the synthesis of citric acid under nitrogen limitation conditions. As was shown earlier [1], the yield of citric acid with respect to the ethanol consumed may reach 80–90%. We believe that the extensive synthesis of citric acid is dictated by the necessity of regenerating the cellular pool of CoA through the saturation of citrate synthase with its substrate, acetyl-CoA.

Nitrogen deficiency not only induces citrate synthase and, hence, citric acid synthesis but also restricts the consumption of ATP in biosynthetic reactions. Under nitrogen deficiency and the attendant suppression of metabolism, the synthesis of acetyl-CoA becomes the main ATP-consuming reaction. Citrate synthase in yeast cells producing citric acid may be induced just by a deficiency of ATP and NADH, which are inhibitors of this enzyme.

The activity of aconitase may also depend on the concentration of nitrogen in the medium, albeit not so strongly as the activity of citrate synthase. Indeed, in nitrogen-limited cells from the retardation growth phase, the activity of aconitase was two times lower than in actively growing cells (see table). Furthermore, the aconitase activity of cells producing citric acid was 3 to 4 times lower than that of KGA-producing cells and cells grown in the complete medium.

The activity of aconitase in KGA-synthesizing cells remained at a high level throughout the period of synthesis (recall that KGA is produced at a relatively high concentration of nitrogen in the medium). In the yeast grown in the complete medium, the activity of aconitase decreased during transition to the stationary growth phase induced by exhaustion of nitrogen in the medium.

The relative activity of citrate synthase was considerably higher in yeast cells producing citric acid (the ratio of citrate synthase to aconitase is 10 : 1 to 50 : 1) than in yeast cells producing KGA (the ratio of citrate synthase to aconitase is 1 : 1 to 2 : 1). This may explain the extensive synthesis and excretion of citric acid by yeast cells grown under nitrogen deficiency in the medium.

Thus, as opposed to the effect of ammonium concentration on citrate synthase, its effect on the activity of aconitase is indirect, being most likely determined by a general decrease in the metabolic activity of yeast cells growing under nitrogen deficiency.

Isocitrate-metabolizing Enzymes

Yeast cells can metabolize isocitrate produced by aconitase with the involvement of at least four enzymes, mitochondrial NAD- and NADP-dependent isocitrate dehydrogenases (ICDs), cytoplasmic NADP-dependent ICD, and peroxysomal isocitrate lyase. Alkane-grown yeast cells may also contain peroxysomal NADP-dependent ICD [12].

The activity of mitochondrial NAD-dependent ICD was relatively low in both KGA- and citric acid–producing yeast cells, as well as in cells grown in the complete medium (see table). The activity of NADP-dependent ICD was considerably higher than that of NAD-dependent ICD, especially in the KGA-producing cells. The activity of isocitrate lyase tended to decrease in the course of yeast cultivation in the complete medium and was low in the KGA- and citric acid–producing cells.

Thus, yeast cells synthesizing KGA and citric acid contain active NAD- and NADP-dependent isocitrate dehydrogenases and isocitrate lyase. It is believed that the allosteric NAD-dependent ICD regulates the flow of metabolites through the TCA cycle and the respiratory chain, AMP and the ammonium ion being positive modulators, and citric acid and ATP being negative modulators. The low activity of NAD-dependent ICD under all growth conditions (see table) suggests that this enzyme does not play any important part in the metabolism of ethanol in *Y. lipolytica*. The low activity of NAD-dependent ICD was also observed in the *Candida tropicalis* and *Saccharomycopsis lipolytica* cells grown on *n*-alkanes [12, 13].

Presumably, isocitrate is mainly metabolized with the involvement of NADP-dependent ICD, whose activity is considerably higher than that of NAD-dependent ICD (see table). The cytoplasmic NADP-dependent ICD may be the most important enzyme of isocitrate metabolism, since the KGA produced in the cytoplasm does not need energy for its exchange transport from mitochondria. Cytoplasmic KGA may be the main source of glutamate in yeast cells, since their glutamate dehydrogenase is localized in the cytoplasm. For instance, the double *Saccharomyces cerevisiae* mutant lacking mitochondrial isocitrate dehydrogenases was found to be able to grow on nonfermentable carbon sources without adding glutamate to the growth medium [14]. This indicates that the cytosolic NADP-dependent ICD of yeasts may provide both for the function of the TCA cycle and for the synthesis of glutamate.

To be functional, the isocitrate lyase of yeasts grown under thiamine or nitrogen deficiency requires isocitrate in sufficient amounts, since the K_m of this enzyme with respect to isocitrate is considerably higher than that of NADP-dependent ICD [15]. Being involved in the glyoxylate cycle, isocitrate lyase, together with malate synthase, plays a significant part in biosynthetic processes during the active growth of yeasts on C_2-compounds, providing mitochondria with the easily metabolizable succinate. On the other hand, the relatively low activity of isocitrate lyase in yeast cells producing KGA and citric acid (see table) indicates that this enzyme is not crucial to the syntheses of KGA and citric acid.

α-Ketoglutarate Oxidation

As can be seen from the table, the activity of α-ketoglutarate dehydrogenase (KGA dehydrogenase) in yeast cells grown under thiamine deficiency was relatively low. This is not surprising, if we take into account the fact that thiamine deficiency leads to the overproduction of KGA in the mutant strain. It should, however, be noted that the low activity of KGA dehydrogenase was also observed in the mutant N1 cells grown in the complete medium and under nitrogen deficiency, as well as in the parent strain grown on glucose and *n*-alkanes in the complete medium and under conditions promoting KGA synthesis [16, 17] and in the *S. cerevisiae* cells grown on acetate and glycerol [18].

The low activity of the preceding enzyme of the TCA cycle, NAD-dependent ICD, suggests that the activity of the subsequent enzyme, KGA dehydrogenase, may also be low, provided there is no other source of α-ketoglutarate in the cell. The high activity of

NADP-dependent ICD in KGA-producing cells shows that it is this enzyme that serves as the source of KGA in these cells. The KGA produced in the cytoplasm is either excreted into the medium or is metabolized in a way independent of KGA dehydrogenase. For instance, KGA can be reductively aminated by glutamate dehydrogenase. Under the conditions of KGA overproduction, yeast cells can both excrete and aminate KGA, while they only aminate it under nitrogen limitation conditions (i.e., under the conditions of citric acid overproduction).

Succinate Oxidation

The succinate oxidase and succinate dehydrogenase activities of yeast cells were three times higher under conditions promoting KGA production than under conditions promoting citric acid production (see table). In the late terms of citric acid production, these activities decreased to zero. The low activities of succinate oxidase and succinate dehydrogenase in the early terms of citric acid production suggest that it is nitrogen deficiency that suppresses these two enzymes.

When the activity of KGA dehydrogenase is low, isocitrate lyase is the main source of succinate in yeast cells. However, as mentioned above, the activity of isocitrate lyase in the retardation and stationary growth phases is low, and this enzyme has a low K_m value with respect to isocitrate [15]. This suggests that the high rate of succinate oxidation is provided either by the hypersynthesis of isocitrate lyase, as was shown for yeast cells grown on *n*-alkanes [15], or through the functioning of other anaplerotic enzymes, such as fumarate reductase (table).

Fumarate and Malate Metabolism

The activity of fumarase was high in all types of cells (see table), particularly in those grown in the complete medium and under the conditions of KGA overproduction. Taking into account the low activity of the preceding enzymes of the TCA cycle, these data suggest that the fumarate hydratase reaction proceeds towards the formation of fumarate. This suggestion is in line with the above inference that the functioning of fumarate reductase leads to the additional production of succinate.

Like isocitrate, malate can be metabolized with the involvement of several enzymes, such as NAD- and NADP-dependent malate dehydrogenases (MDH) and peroxysomal malate synthase. The activity of NAD-dependent MDH was high in all types of cells (see table). Even in the late terms of citric acid production, when the activity of this enzyme considerably decreased, it still remained at a sufficiently high level, exceeding the activity of all other NAD-dependent enzymes of the TCA cycle. This can be explained by the following reasons.

First, intermediates of the TCA cycle must be translocated into and from mitochondria. The translocases of dicarboxylic acids are activated only by inorganic phosphate, while those of tricarboxylic acids, are activated by inorganic phosphate and malate (as well as by malonic acid in the case of α-ketoglutarate). Such a transport system, which is based on the exchange translocation of malate and the tricarboxylic acid anion at a ratio of 1 : 1, operates in many yeasts, including citrate-producing ones [19]. This implies that yeast cells, especially those which overproduce KGA and citric acid, must maintain the cellular pool of malate at a sufficiently high level dependent on the concentration of translocated organic acids.

Second, the high activity of citrate synthase in yeast cells producing KGA and citric acid (see table) indicates that such cells must possess a high pool of oxalacetate necessary for the normal functioning of this enzyme. Some fraction of mitochondrial malate is produced by fumarase, whose activity in cells producing KGA and citric acid is sufficiently high. On the other hand, some amount of malate present in the cytoplasm and peroxysomes is likely produced by malate synthase, which is also involved in the regeneration of CoA necessary in great amounts for yeast cell growth on ethanol. It should, however, be noted that the activity of malate synthase under the conditions of KGA and citric acid production is relatively low and does not depend on the concentration of nitrogen in the medium (see table). Therefore, it remains unclear which of the aforementioned enzymes provide malate dehydrogenase with its substrate (malate or oxalacetate).

NADH Oxidation

Yeast mitochondria are able to rapidly oxidize exogenous NADH via NADH dehydrogenase localized on the outer surface of the internal mitochondrial membrane. The main goal of this oxidation is to regenerate the cytoplasmic pool of NAD^+.

As can be seen from the table, the activity of NADH oxidase was maximum in yeast cells grown in the complete medium. The activity of NADH dehydrogenase in yeast cells producing citric acid was about two times as low as in the two other types of cells. As was shown in the previous work [1], the respiration rate of citric acid-producing cells was 2 to 3 times higher than in KGA-producing cells. The intense respiration of the citric acid-producing cells was not due to the active oxidation of succinate or a NAD-dependent substrate, since the succinate oxidase and the NADH oxidase activities of the citric acid–producing cells were lower than those of the KGA-producing cells.

The high activities of the enzymes of the reductive branch of the TCA cycle (fumarase, succinate dehydrogenase, and malate dehydrogenase reducing oxalacetate to malate) suggest that a considerable fraction of cellular NADH is spent for the reduction of oxalacetate

to malate rather than is oxidized through the respiratory chain. This suggestion is confirmed by the fact that the respiration rate of KGA-producing cells is lower than that of citric acid–producing cells [1].

CONCLUSION

The data presented indicate that the production of citric acid and α-ketoglutarate considerably affects the enzymes of central metabolism. The low activity of these enzymes in citric acid–producing cells may be due to suppression of the total metabolic activity of these cells because of the exhaustion of nitrogen from the growth medium. At the same time, the direct effect of low ammonium concentrations on the activity of some enzymes cannot be excluded. The increase in the activity of citrate synthase and the attendant oversynthesis of citric acid can be considered to be a defense response of yeast cells to the stress factor (a high concentration of ethanol in the growth medium).

Similarly, the production of KGA may also represent a defense response of yeast cells to the excess concentration of ammonium ions under the conditions of thiamine-limited growth. In this case, ammonium ions are spent in great amounts for the amination of α-ketoglutarate into glutamate, which is further converted in a series of transamination reactions.

As was shown for *S. cerevisiae*, the blockade of one of the TCA cycle enzymes may significantly affect the activity of mitochondrial enzymes [14]. For instance, the mutants of this yeast lacking NAD-dependent isocitrate dehydrogenase were found to have a twofold more active citrate synthase than the parent strain, whereas the activities of the other enzymes of the TCA cycle remained unchanged. Our experiments also show that the mutant *Y. lipolytica* strain N1 possesses low NAD-dependent isocitrate dehydrogenase activity and high citrate synthase activity under the conditions of KGA and citric acid oversynthesis and during growth in the complete medium (see table).

Along with the low activity of NAD-dependent isocitrate dehydrogenase, the mutant strain N1 grown on ethanol has the low activity of KGA dehydrogenase. The activities of these two enzymes in the parent strain grown on *n*-alkanes are also low [16, 17, 20]. These data allow the low activities of NAD-dependent isocitrate dehydrogenase and KGA dehydrogenase and the high activity of citrate synthase to be considered as a distinguishing characteristic of the group of alkane-oxidizing yeasts. The low activities of the first two enzymes are compensated for by the high activities of citrate synthase and NADP-dependent isocitrate dehydrogenase. It should, however, be noted that strain N1 may also implement another compensatory mechanism. The high activities of malate dehydrogenase and fumarase, as well as the presence of fumarate reductase activity, allow the suggestion to be made that some amount of succinate is produced in the TCA cycle operating in the reductive direction.

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