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Control of Malate Synthase Formation in *Rhizopus nigricans*

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The control of malate synthase formation in a fumaric acid-producing strain of *Rhizopus nigricans* has been found to be similar in most respects to that of isocitrate lyase, the companion enzyme of the glyoxylate bypass. A basal level is formed in a casein hydrolysate medium, which is repressed by glucose. Utilization of glucose during growth results in relief of glucose repression. Any factor which stimulates growth promotes relief of glucose repression by enhancing the incorporation of repressor metabolites derived from glucose into cell material. Thus, malate synthase formation was enhanced in glucose-containing media by the addition of zinc, or by an increase of the concentration of available nitrogen source in a synthetic medium. Both acetate and glycolate acted as apparent inducers of malate synthase, with glycolate the more effective of the two when added alone. Acetate induction was enhanced by Zn^{++}, however, whereas induction by glycolate was unaffected. This supports the concept that acetate stimulates formation of glyoxylate bypass enzymes by a derepression mechanism, whereas glycolate or a product derived from it acts directly as an inducer. Moreover, it is indicated that the malate synthases induced by acetate and glycolate are separate and distinct, as has been shown in *Escherichia coli*.

The glyoxylate bypass has been shown to occur in a wide variety of microorganisms, and has been assigned an important role in allowing growth on two-carbon compounds. Two enzymes, isocitrate lyase and malate synthase, are operative in the pathway. The concerted action of these enzymes allows a bypass of the decarboxylation steps of the tricarboxylic acid cycle and affords an efficient means of replenishing C_4 acids that are drawn off for biosynthesis. Both enzyme systems are subject to control, and, in general, the enzymes are formed chiefly under conditions requiring the net synthesis of C_4 compounds from acetate. Studies on the metabolic significance and the regulation of the glyoxylate cycle have been reviewed by Kornberg (7, 9), and Kornberg and Elsden (10).

Malate synthase also plays a role in the glycerate pathway (11). It allows the operation of a dicarboxylic acid cycle which allows growth on glyoxylate or precursors of glyoxylate, such as glycolate. It is of interest that the malate synthases operative in the two respective pathways in *Escherichia coli* have been shown by Falmagne, Vanderwinkel, and Wiame (2) to consist of two distinct enzymes.

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While the glyoxylate bypass enzymes have been studied in a wide variety of bacteria, they have been studied much less in fungi. This is in spite of the importance that has been attached to C_2 metabolism in fungi (4). Kornberg (7) has postulated that the glyoxylate bypass may play an important role in the formation of C_4 acids by fungi. A study of the control of the glyoxylate bypass enzymes in fungi has therefore been undertaken. A previous report (15) dealt with the control of isocitrate lyase in *Rhizopus nigricans*. The present report represents an extension of this earlier work to include malate synthase, the companion enzyme.

MATERIALS AND METHODS

Organism. R. nigricans 45, a fumaric acid-producing strain described by Foster and Waksman (5), was used.

Growth conditions and enzyme preparation. Two types of medium were used: one was a casein hydrolysate medium; the other was a synthetic glucose-$(NH_4)_2SO_4$ medium. The basal salts mixture used in both media consisted of: 0.5 g of K_2HPO_4; 0.5 g of $MgSO_4 \cdot 7H_2O$; 0.01 g of $Fe_2(SO_4)_3$; and 1,000 ml of deionized distilled water. To this was added either casein hydrolysate (acid-hydrolyzed, vitamin- and salt-free; Nutritional Biochemicals Corp., Cleveland, Ohio) at a final concentration of 1%, or glucose (final

concentration, 5%) and $(NH_4)_2SO_4$ (final concentration, 0.2%). The media were dispensed in 50-ml amounts to 250-ml Erlenmeyer flasks. Flasks were covered with cotton filter discs (Rapid-Flo, Johnson and Johnson Filter Products Div., Chicago, Ill.) to promote good aeration. Since copious amounts of fumaric acid were produced in the glucose-$(NH_4)_2SO_4$ synthetic medium, 1.5 g of sterile $CaCO_3$ was added to each flask after sterilization to act as a neutralizing agent. Cultures were inoculated with 1.0 ml of a spore suspension standardized to an optical density of 0.3 at 540 mμ. Incubation was carried out at 32 C on a rotary shaker at 250 rev/min.

Growth measurement. Cell weight was determined by filtering the mycelial mass on dried and weighed Whatman no. 1 filter paper and drying to constant weight at 95 C.

Chemical determinations. Residual glucose was determined by the method of Folin and Malmros (3). Protein concentrations of the enzyme preparations were determined by the method of Lowry et al. (13).

Enzyme preparation and assay. Cells were harvested by filtration, washed with distilled water, and suspended in 0.025 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0) to make a thick slurry. This slurry was agitated for 2 min with glass beads (0.2-mm diameter) in an ice-jacketed Waring Blendor according to the method of Lamanna and Mallette (12). The ruptured-cell suspension was then centrifuged at 14,500 × *g* for 30 min at 2 C. Malate synthase was assayed by a modification of the procedure of Dixon and Kornberg (1), whereby the cleavage of acetyl coenzyme A (acetyl-CoA) in the presence of glyoxylate was followed spectrophotometrically by measuring the decrease in optical density at 232 mμ. The reaction mixture contained (in 3.0 ml): 50 μmoles of Tris buffer, *p*H 8.0; 10 μmoles of $MgCl_2$; 0.10 μmoles of acetyl-CoA (Sigma Chemical Co., St. Louis, Mo.), and 0.05 to 0.20 ml of enzyme preparation. Optical density measurements were made with a Beckman DU spectrophotometer equipped with thermospacers at 28 C. Readings at 232 mμ were made every 15 sec for 3 min to determine the initial rate of acetyl-CoA cleavage. Specific activity is expressed as micromoles of acetyl-CoA cleaved per hour per milligram of protein.

RESULTS

Formation of malate synthase in casein hydrolysate medium. Casein hydrolysate medium was selected to determine whether a basal or constitutive level of malate synthase was produced. This medium was selected so there would not be the significant accumulation of repressor substances normally formed from carbohydrates or inducers, such as acetate. The results are shown in Fig. 1, where the rate of malate synthase formation is compared with growth. It is clear that the enzyme is produced without addition of exogenous inducer. Growth preceded enzyme formation, and maximum levels of malate synthase were not reached until the culture approached the stationary phase of growth. This indicates that this enzyme is not essential for growth under these conditions; the situation is similar to that found for isocitrate lyase under the same conditions (15).

The level of enzyme produced in the casein hydrolysate medium could be considered the basal, or constitutive, level, and the effects of various additions to this medium could be conveniently studied.

Glucose repression of malate synthase. Addition of glucose to the casein hydrolysate medium resulted in repression of malate synthase formation. When the concentration of glucose was low enough so that it could be utilized during growth, the repression was relieved. Figure 2 shows the comparison of rates of growth, glucose utilization, and malate synthase formation in a casein hydrolysate medium originally containing 0.03 M glucose. The level of malate synthase remained very low until the glucose concentration dropped below 1 mg/ml. At this point, enzyme synthesis took place at a rate approximately three times that found in the basal casein hydrolysate medium, indicating a derepression. The maximum level of enzyme formed after relief of glucose repression, however, was only slightly higher than that found in the absence of repressor.

Effect of zinc ion. Zinc shows a striking effect on the growth and physiological activities of fungi. In studies on fumaric acid formation from glucose by the organism used here, Foster and Waksman

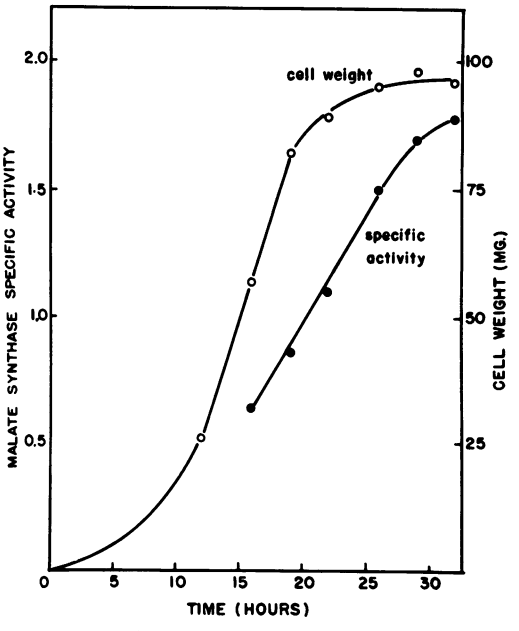

FIG. 1. *Relationship between growth and malate synthase formation. Basal salts mixture containing 1% casein hydrolysate.*

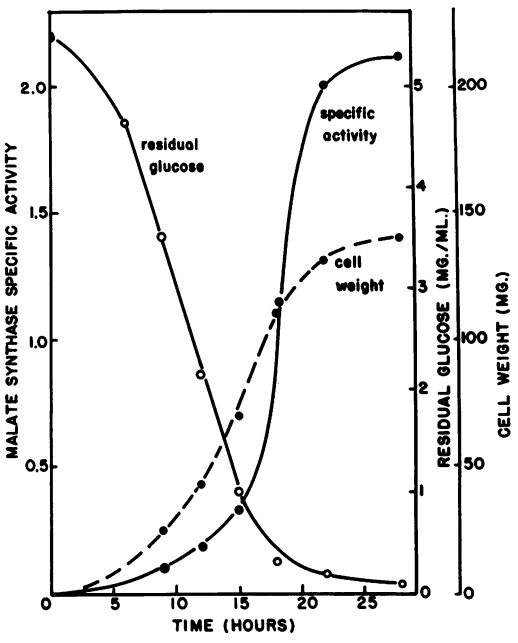

FIG. 2. *Relationship between growth, glucose utilization, and malate synthase formation. Basal salts mixture containing 0.5% casein hydrolysate and 0.03 M glucose.*

(6) showed that addition of Zn^{++} stimulated an increase in cell synthesis, a more rapid and efficient utilization of glucose, and a concomitant decrease in fumaric acid yield. Wegener and Romano (14) presented evidence that Zn^{++} stimulates growth and glucose utilization through a primary effect on ribonucleic acid (RNA) and protein synthesis. With regard to the glyoxylate bypass, increased rates of biosynthesis occasioned by this metal brought about relief of glucose repression of isocitrate lyase, and stimulated induction by acetate (15). Therefore, the effect of Zn^{++} on malate synthase formation was also studied. The data shown in Table 1 confirm that addition of Zn^{++} to a glucose-containing medium results in relief of glucose repression. It is seen that addition of Zn^{++} to the basal casein hydrolysate medium had no significant effect on enzyme formation. However, addition of Zn^{++} to a culture containing 0.075 M glucose resulted in production of malate synthetase at a level equivalent to that found in the nonrepressed culture. Table 1 also shows that the metal brought about complete utilization of added glucose and an increase in cell synthesis. This is consistent with the view that Zn^{++} relieves glucose repression by effecting the removal of catabolite repressor(s) arising from glucose by stimulating the incorporation of such metabolites into cell material.

Formation of malate synthase in synthetic medium. It was of interest to determine the level of malate synthase formed in a synthetic glucose-$(NH_4)_2SO_4$ medium that favors fumaric acid formation, since it was suggested by Kornberg (7) that the glyoxylate bypass may play an important role in the production of this acid by fungi. Therefore, the organism was grown on a medium of high C-N ratio, containing 5% glucose and 0.2% $(NH_4)_2SO_4$. The results obtained in cultures with and without added Zn^{++} are shown in Table 2. It is seen that although malate synthase was produced in the glucose medium without added Zn^{++}, it was produced at a repressed level. A higher level was produced in the culture with added Zn^{++}; again, it is probable that this higher level was occasioned by the increased utilization of glucose and cell synthesis provoked by the addition of this metal.

TABLE 1. *Relief of glucose repression of malate synthase by zinc*

Additions[a]	Malate synthase specific activity[b]	Cell weight	Glucose utilized
		mg	%
None	1.90	101	
$ZnSO_4 \cdot 7H_2O$ (5 ppm)	1.84	98	
Glucose (0.075 M)	0.36	218	61
Glucose (0.075 M) + $ZnSO_4 \cdot 7H_2O$ (5 ppm)	1.98	252	100

[a] Basal salts mixture containing 1% casein hydrolysate; incubation time, 26 hr.

[b] Specific activity is expressed as micromoles of acetyl-coenzyme A cleaved per hour per milligram of protein.

Thus, it appears that factors which stimulate cell synthesis and the removal of glucose or glucose catabolites result in relief of repression of malate synthase. On this basis, it would be expected that an increase in available nitrogen source and the restoration of a C-N ratio more favorable to cell synthesis would also result in enhanced formation of this enzyme. This is realized as shown in Table 3. The level of malate synthase increased as the nitrogen source concentration was increased. Addition of Zn^{++} further stimulated enzyme formation at all nitrogen concentrations employed.

Induction of malate synthase. In most organisms studied, growth in the presence of acetate or glycolate resulted in elevated levels of malate synthase. These two substances have been implicated as inducers, acting directly or indirectly (*see* Discussion). Table 4 shows that acetate and glycolate also stimulate malate synthase in this organism. Acetate added to the casein hydrolysate medium increased the level

TABLE 2. *Effect of zinc on malate synthase formation in glucose-ammonium sulfate synthetic medium*[a]

Determination	$-Zn^{++}$	$+Zn^{++}$
Cell weight (mg)	287	372
Residual glucose (mg/ml)	9.2	0
Malate synthase specific activity[b]	1.04	2.16

[a] Basal salts mixture plus 5% glucose, 0.2% $(NH_4)_2SO_4$; incubation time, 72 hr.

[b] Specific activity is expressed as micromoles of acetyl-coenzyme A cleaved per hour per milligram of protein.

TABLE 3. *Effect of nitrogen source concentration on malate synthase formation*

$(NH_4)_2SO_4$ concn of medium[a] (%)	Malate synthase specific activity[b]	
	$-Zn^{++}$	$+Zn^{++}$
0.2	1.14	2.16
0.6	2.20	3.20
1.0	3.40	5.00
2.0	3.60	8.00

[a] Basal salts mixture containing 5% glucose; incubation time, 72 hr.

[b] Specific activity is expressed as micromoles of acetyl-coenzyme A cleaved per hour per milligram of protein.

of enzyme twofold over the basal level; glycolate addition stimulated enzyme formation nearly fivefold. It is significant that Zn^{++} enhanced the inductive effect of acetate; this parallels what has been reported for isocitrate lyase (15). However, addition of this metal had no effect when glycolate was used as the inducer.

A similar effect of Zn^{++} on acetate and glycolate induction was found in the synthetic medium. In this experiment, cells were grown for 55 hr on the basal salts mixture containing 5% glucose and 0.6% $(NH_4)_2SO_4$. The cells were then washed and replaced with the same mineral salts medium containing various additions. The results are shown in Table 5. Again, glycolate effected a twofold greater induction than did acetate. While addition of Zn^{++} to the acetate medium enhanced induction twofold, Zn^{++} added to the glycolate medium had no significant effect.

This differential effect of Zn^{++} on induction by acetate versus glycolate suggested the possibility that two separate malate synthases, regulated in different ways, exist in *Rhizopus*, just as has been shown in *E. coli* (2). This possibility

TABLE 4. *Malate synthase induction by acetate and glycolate*

Additions to medium[a]	Malate synthase specific activity[b]
None	1.90
$ZnSO_4 \cdot 7H_2O$ (5 ppm)	1.84
Sodium acetate (0.1 M)	3.82
Sodium acetate (0.1 M) + $ZnSO_4 \cdot 7H_2O$ (5 ppm)	5.56
Sodium glycolate (0.1 M)	8.68
Sodium glycolate (0.1 M) + $ZnSO_4 \cdot 7H_2O$ (5 ppm)	8.08

[a] Basal salts mixture containing 1% casein hydrolysate; incubation time, 26 hr.

[b] Specific activity is expressed as micromoles of acetyl-coenzyme A cleaved per hour per milligram of protein.

was investigated by a comparison of the rates of thermal inactivation of acetate- and glycolate-induced enzyme preparations. Accordingly, cells were grown for 19 hr in the basal salts mixture containing 1.0% casein hydrolysate; they were then induced for 9 hr by addition of acetate or glycolate, respectively, at a final concentration of 0.05 M. Samples (1 ml) of enzyme preparations from these cells were incubated in a water bath at 53 C for various period of time, and were then chilled immediately in an ice bath prior to assay. The results of three independent heat-inactivation experiments are shown in Fig. 3. It is clear that the acetate-induced enzyme is more sensitive to heat inactivation than is the glycolate-induced enzyme. Thus, the presence of two distinct malate synthases is indicated.

DISCUSSION

The pattern of control of malate synthase in *R. nigricans* appears, in most respects, to parallel that found for isocitrate lyase (15). A basal level of enzyme is formed in a casein hydrolysate medium, which can be repressed or induced under appropriate conditions. Addition of glucose to the medium results in catabolite repression. Upon exhaustion of glucose, enzyme synthesis is resumed, and a level is reached which is equivalent to that found in a non-repressed system.

In general, factors which stimulate growth bring about relief of glucose repression. Presumably, this comes about by the stimulated incorporation of glucose metabolites into cell material and a resultant decrease in the repressor pool. Thus, addition of Zn^{++}, which has a striking effect on the growth of fungi in glucose-

TABLE 5. *Effect of zinc on acetate and glycolate induction of malate synthase in synthetic medium*

Phase	Additions to medium[a]	Malate synthase specific activity[b]
Primary growth (55 hr)	None	1.92
Replacement (15 hr)	None	2.20
	Sodium acetate (0.1 M)	5.20
	Sodium acetate (0.1 M) + $ZnSO_4 \cdot 7H_2O$ (5 ppm)	8.45
	Sodium glycolate (0.1 M)	8.39
	Sodium glycolate (0.1 M) + $ZnSO_4 \cdot 7H_2O$ (5 ppm)	8.62

[a] Basal salts mixture + 5% glucose and 0.6% $(NH_4)_2SO_4$.
[b] Specific activity is expressed as micromoles of acetyl-coenzyme A cleaved per hour per milligram of protein.

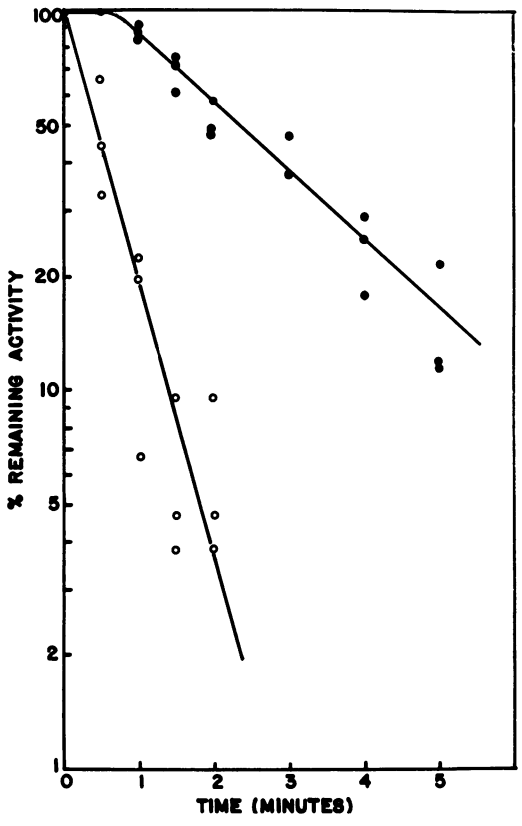

FIG. 3. *Rates of thermal inactivation of malate synthase at 53 C.* (○) *Enzyme preparation from acetate-induced cells.* (●) *Enzyme preparation from glycolate-induced cells.*

containing media, resulted in enhanced enzyme formation. Likewise, an increase in available nitrogen source in a glucose-$(NH_4)_2SO_4$ synthetic medium promoted relief of glucose repression.

Both acetate and glycolate acted as apparent inducers of malate synthase, with glycolate the more effective of the two. There was a salient difference, however, in the effect of Zn^{++} on induction. Acetate induction was significantly enhanced by this metal, whereas induction by glycolate was unaffected. Kornberg (9) has developed the thesis that acetate does not act directly as an inducer of glyoxylate bypass enzymes, but rather indirectly by bringing about a derepression. Part of the evidence derives from the fact that acetate is not effective as an inducer in a mutant of *E. coli* which lacks condensing enzyme (8). Kornberg has further advanced evidence implicating pyruvate or a substance close to it (possibly phosphoenolpyruvate) as the effective repressor (9). The enhanced apparent induction by acetate in the presence of added Zn^{++} can be interpreted to be in favor of this depression mechanism, since this metal further promotes removal of intracellular repressor.

Falmagne, Vanderwinkel, and Wiame (2) have shown that malate synthase induced in *E. coli* by glycolate is different from that induced by acetate; the two enzymes are separable by chromatography, and show different thermoinactivation kinetics, K_m values, and sensitivity to inhibitors. The presence of malate synthase isozymes in *R. nigricans* as well is indicated by differences in rates of heat inactivation between acetate-induced and glycolate-induced enzyme preparations.

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LITERATURE CITED

1. DIXON, G. H., AND H. L. KORNBERG. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. 72:3p.

2. FALMAGNE, P. E., E. VANDERWINKEL, AND J. M. WIAME. 1965. Mise en evidence de deux malate synthases chez *Escherichia coli*. Biochim. Biophys. Acta **99**:246–258.
3. FOLIN, O., AND H. MALMROS. 1929. An improved form of Folin's micromethod for blood sugar determinations. J. Biol. Chem. **83**:115–120.
4. FOSTER, J. W. 1958. An evaluation of the role of molds in the comparative biochemistry of carbohydrate oxidation. Texas Rept. Biol. Med. **16**:79–100.
5. FOSTER, J. W., AND S. A. WAKSMAN. 1939. The production of fumaric acid by molds belonging to the genus *Rhizopus*. J. Am. Chem. Soc. **61**: 127–135.
6. FOSTER, J. W., AND S. A. WAKSMAN. 1939. The specific effect of zinc and other heavy metals on growth and fumaric-acid production by Rhizopus. J. Bacteriol. **37**:599–617.
7. KORNBERG, H. L. 1959. Aspects of terminal respiration in microorganisms. Ann. Rev. Microbiol. **13**:49–78.
8. KORNBERG, H. L. 1963. The role of acetate in isocitrate lyase induction. Biochim. Biophys. Acta **73**:517–519.
9. KORNBERG, H. L. 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. **99**:1–11.
10. KORNBERG, H. L., AND S. R. ELSDEN. 1961. The metabolism of 2-carbon compounds by microorganisms. Advan. Enzymol. **23**:401–470.
11. KORNBERG, H. L., AND A. M. GOTTO. 1961. The metabolism of C_2-compounds in microorganisms. 6. Synthesis of cell constituents from glycolate by *Pseudomonas sp*. Biochem. J. **78**: 69–82.
12. LAMANNA, C., AND M. F. MALLETTE. 1954. Use of glass beads for the mechanical rupture of microorganisms in concentrated suspensions. J. Bacteriol. **67**:503–504.
13. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. WEGENER, W. S., AND A. H. ROMANO. 1963. Zinc stimulation of RNA and protein synthesis in *Rhizopus nigricans*. Science **142**:1669–1670.
15. WEGENER, W. S., AND A. H. ROMANO. 1964. Control of isocitratase in *Rhizopus nigricans*. J. Bacteriol. **87**:156–161.