Biosynthesis of Glutamic Acid in \textit{Saccharomyces}: Accumulation of Tricarboxylic Acid Cycle Intermediates in a Glutamate Auxotroph

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Aconitaseless glutamic acid auxotroph MO-1-9B of \textit{Saccharomyces} grew in glutamic acid-supplemented minimal medium, but failed to grow when glutamic acid was substituted by proline, arginine, ornithine, or glutamine. This mutant was also unable to utilize lactate or glycerol as a carbon source. Under a glutamic acid-limiting condition, by using acetate-1-$^{14}$C as tracer, the mutant accumulated rather large amounts of $^{14}$C-citric acid and $^{14}$C-succinic acid when compared with the wild-type strain. Under excess glutamic acid supplementation, accumulation of citric acid and succinic acid was considerably reduced. When $^{14}$C-glutamic acid-(U) was used as tracer, $^{14}$C-$\alpha$-ketoglutaric acid, $^{14}$C-citric acid, and $^{14}$C-succinic acid were accumulated in the mutant. The citric acid peak was the largest, followed by $\alpha$-ketoglutaric acid and succinic acid. In the wild-type strain under similar conditions, only small amounts of $^{14}$C-citric acid and $^{14}$C-succinic acid and no $^{14}$C-$\alpha$-ketoglutaric acid were accumulated.

Mutants blocked in the tricarboxylic acid cycle were studied first in \textit{Escherichia coli} (5). Subsequently, the tricarboxylic acid cycle mutants and the sporulation deficiency in \textit{Bacillus} have been studied at length by different investigators (3, 4, 8). The first report of an aconitaseless glutamic acid auxotroph (\textit{glu}1) in \textit{Saccharomyces}, a fermentative yeast, appeared several years ago (7). We report here the growth characteristics and the accumulation properties of a \textit{glu}1 mutant.

\textbf{MATERIALS AND METHODS}

\textbf{Cultures.} A glutamate auxotroph of \textit{Saccharomyces}, designated MO-1-9B (\textit{glu}1, \textit{try}, \textit{his}) was obtained from M. Ogur, and a wild-type strain of the same organism designated WL-1 were used.

\textbf{Media and growth conditions.} Stock cultures were maintained on a nutrient medium (peptone, 6 g; yeast extract, 4 g; MgSO$_4$·7H$_2$O, 1 g; KH$_2$PO$_4$, 2 g; glucose, 40 g; agar, 25 g; distilled water, 1,000 ml). Minimal medium was composed of Difco yeast nitrogen base without amino acid, 6.7 g; glucose, 10 g; water, 1 liter. Tryptophan (25 mg%) and histidine (25 mg%) were added routinely in the minimal medium for the growth of mutant MO-1-9B. High levels of tryptophan and histidine were added to minimize the biosynthetic reactions of their respective pathways in addition to satisfying the nutritional requirements. Glutamic acid and other amino acids, as called for in a given experiment, were added to the minimal medium to satisfy the growth requirement of strain MO-1-9B. Cultures were grown at 30 C in a New Brunswick Scientific Co., water bath shaker.

\textbf{Nutritional requirement.} Growth response of strain MO-1-9B to glutamic acid and other amino acids was measured in 25-ml test tubes, each of which contained 10 ml of minimal medium supplemented with specific amino acid. Growth studies were performed in duplicate tubes and repeated more than once. Each tube was inoculated with 0.1 ml of inoculum from a suspension of approximately 1 $\times$ 10$^7$ cells per ml. Tubes were incubated at 30 C, and the optical density was determined at 8-h intervals by using a Coleman spectrophotometer at 540 nm.

\textbf{Isolation and identification of radioactive products.} Wild-type and mutant strains were grown in Erlenmeyer flasks, each containing 50 ml of minimal medium and minimal medium supplemented with 2 mg (or as described in a given experiment) of glutamic acid per 100 ml in the case of the mutant. To the growth medium, 25 $\mu$Ci of sodium acetate-1-$^{14}$C or 10 $\mu$Ci of $^{14}$C-glutamic acid-(U) was added, and the incubation continued for 72 h. Each of the tracer experiments was performed in duplicate flasks and repeated more than once. The cells were removed by centrifugation in a Sorvall RC-2 refrigerated centrifuge for 10 min at 13,800 $\times$ g. Dry weight of cell mass...
was determined, and the spent medium was lyophilized to dryness with 50 mg of the appropriate synthetic carrier. The residue was redissolved in a small volume of water, neutralized, and chromatographed on a column (1 x 30 cm) of Dowex 1-formate by using a continuous gradient to 6 N formic acid (2, 6). Fractions (5-ml volume) were collected by using a Gilson Medical Electronics fraction collector. A sample from each fraction was evaporated to dryness, and its radioactivity was determined in a Beckman liquid scintillation spectrometer by using 10 ml of a toluene-ethanol solvent system (1). A second sample from each fraction was dried and titrated with 0.01 N NaOH (Dowex 1-formate column) or with alcoholic KOH (silicic acid column) in the presence of phenothalein indicator to determine the carrier acid. Chromatography of the radioactive product and the carrier material on silicic acid column was carried out by the procedure of Ramsey (9). The $R_f$ values of the radioactive products, after thin-layer chromatography (Eastman Kodak silica gel), were determined by using a Packard radiochromatogram scanner, and the $R_f$ values of the carrier acid were determined by spraying the thin layer with a 0.4% bromophenol blue solution.

**Chemicals.** Radioactive materials were purchased from the New England Nuclear Corp., and other chemicals were obtained from the Sigma Chemical Co.

**RESULTS**

**Nutritional requirement of strain MO-1-9B.** Mutants were inoculated in minimal medium and minimal medium containing varying amounts (1 and 2 mg/100 ml, respectively) of glutamic acid. Proline, arginine, ornithine, and glutamine (up to 5 mg/100 ml each) were substituted for glutamic acid in different experiments. No growth was observed in the minimal medium over a 48-h period, nor when proline, ornithine, arginine, or glutamine were substituted for glutamic acid. The mutant grew only in the presence of glutamic acid, and the growth in the glutamic acid was concentration dependent. The mutant failed to grow in the nutrient medium when glucose was substituted by sodium lactate or glycerol (Table 1). However, the wild-type strain grew well under similar conditions.

**Accumulation of tricarboxylic acid cycle intermediates in the culture supernatant fluid in experiments with $^{14}$C-acetate.** Mutant MO-1-9B was grown in a 250-ml Erlenmeyer flask containing 50 ml of the minimal medium with 2 mg of glutamic acid per 100 ml and 25 $\mu$Ci of sodium acetate-$^14$C as described under Materials and Methods. The wild-type strain was grown under similar conditions without glutamic acid. Culture supernatant fluids were chromatographed on a column of Dowex 1-formate with 50 mg of carrier citric acid, and the fractions were analyzed for the radioactivity and the carrier acid. Accumulations of different products are given in the radioactive profiles (Fig. 1). Particularly significant is the large peak between fractions 30 to 55 and a concomitant reduction in the peaks between fractions 1 and 9, as well as between fractions 21 and 26, of the mutant profile as compared with the wild type. The radioactive product in fractions 30 to 50 of the wild-type profile and fractions 30 to 55 of the mutant profile was identified as citric acid by the coincidence of the titration peak of the carrier citric acid and the radioactivity. Fractions containing the radioactivity and the carrier citric acid from the mutant profile were combined, lyophilized, and rechromatographed on a column of silicic acid. Both the radioactivity and the carrier citric acid were eluted in the same fractions (Fig. 2). The radioactive product and the carrier citric acid also exhibited similar mobility upon thin-layer chromatography in six different solvent systems (Table 2). By using similar procedures, the radioactive material in fractions 30 to 50 of the wild-type profile was identified as citric acid.

The radioactive peak contained in fractions 18 to 22 of the mutant profile was characterized as succinic acid based on chromatography with carrier succinic acid on a column of silicic acid (Fig. 3). The similarity was also demonstrated by the chromatographic property on a column of Dowex 1-formate. By using similar procedures, the radioactive material in fractions 18 to 22 of the wild-type profile (Fig. 1) was also characterized as succinic acid. No other tricarboxylic acid cycle intermediate was present in the remaining unidentified peaks of the mutant.

In an effort to examine the effect of glutamic acid in vivo on the accumulation products, the mutant was grown in 2 and in 25 mg of glutamic acid per 100 ml, respectively. A significant re-

### Table 1. Growth response of MO-1-9B

| Supplement in minimal medium or carbon source | Absorbance at 540 nm$^\circ$ |
|----------------------------------------------|-----------------------------|
| None                                         | 0.04                        |
| Glutamic acid (1 mg/100 ml)                   | 0.15                        |
| Glutamic acid (2 mg/100 ml)                   | 0.30                        |
| Arginine (5 mg/100 ml)                        | 0.04                        |
| Glutamine (5 mg/100 ml)                       | 0.04                        |
| Prolene (5 mg/100 ml)                         | 0.04                        |
| Ornithine (5 mg/100 ml)                       | 0.04                        |
| Glycerol-nutrient                            | 0.03                        |
| Lactate-nutrient                              | 0.03                        |

$^\circ$ Readings were taken every 8 h for 48 h. These results are representative of three or more observations.
duction was observed for the accumulation of citric acid and succinic acid in the experiments where the mutant was grown in the higher concentration of glutamic acid (Table 3). Accumulation of citric acid and succinic acid was inversely related to the concentrations (1 to 25 mg/100 ml) of glutamic acid used in various experiments. No such reduction was observed in the case of the wild-type strain grown in the presence of increasing concentrations of glutamic acid.

**Experiments with \(^{14}\)C-glutamic acid.** Wild-type and MO-1-9B strain were grown, respectively, in minimal medium- and glutamic acid (2 mg/100 ml)-supplemented media each containing a tracer quantity (10 \(\mu\)Ci) of \(^{14}\)C-glu-
Radioactivity from fractions 30 to 55 of the mutant profile (Fig. 1) and the carrier citric acid were rechromatographed on a column of silicic acid. Fractions were analyzed for radioactivity (counts/min) and the carrier (KOH).

Table 2. Rₜ values of the radioactive materials isolated from the mutant MO-1-9B and carrier citric acid and α-ketoglutaric acid

| Solvent systems                        | Mutant material (fraction 30–55) | Carrier citric acid | Mutant material (fraction 70–90) | Carrier α-ketoglutaric acid |
|----------------------------------------|-----------------------------------|---------------------|-----------------------------------|-----------------------------|
| 3-Butanol-methyl ketone-formic acid-water (40:30:15:15) | 0.54                             | 0.53                | 0.57                              | 0.57                        |
| Ether-benzene-formic acid-water (21:9:7:2) | 0.39                             | 0.39                |                                    |                             |
| 1-Butanol-formic acid-water (5:1:1)    | 0.44                             | 0.42                | 0.55                              | 0.54                        |
| 1-Butanol-acetic acid-water (4:1:1)   | 0.38                             | 0.39                | 0.46                              | 0.44                        |
| 3-Butanol-formic acid-water (67:11:22) | 0.50                             | 0.51                | 0.52                              | 0.49                        |
| Ethylacetate-formic acid-water (10:2:2) | 0.70                             | 0.70                | 0.75                              | 0.74                        |

Radioactive peak in fractions 70 to 90 of the mutant profile coincided with the titration peak of the carrier α-ketoglutaric acid. This peak was absent from the wild-type profile. Radioactive material in fractions 70 to 90 and carrier α-ketoglutaric acid exhibited identical profiles after chromatography on a column of silicic acid. The radioactive material was further characterized as α-ketoglutaric acid in five different solvent systems by use of thin-layer chromatography.

Culture supernatant fluids were processed as described in Materials and Methods and chromatographed on columns of Dowex 1-formate. Each fraction was analyzed for radioactivity and carrier acid (Fig. 4). The radioactive peak in fractions 70 to 90 of the mutant profile coincided with the titration peak of the carrier α-ketoglutaric acid. This peak was absent from the wild-type profile. Radioactive material in fractions 70 to 90 and carrier α-ketoglutaric acid exhibited identical profiles after chromatography on a column of silicic acid. The radioactive material was further characterized as α-ketoglutaric acid in five different solvent systems by use of thin-layer chromatography.

Table 3. Effect of glutamic acid on the accumulation of ¹⁴C-citric acid and ¹⁴C-succinic acid in vivo

| Glutamic acid (Concn in medium) | ¹⁴C-citric acid (counts/min)* | % Reduction | ¹⁴C-succinic acid (counts/min)* | % Reduction |
|--------------------------------|------------------------------|-------------|-------------------------------|-------------|
| 2 mg/100 ml                    | 524,570                      | 0           | 70,700                        | 0           |
| 25 mg/100 ml                   | 8,855                        | 98          | 10,320                        | 85          |

* Based on per milligram (dry weight) of cells. These results are representative of three or more observations.
GLUTAMIC ACID BIOSYNTHESIS

Radioactive citric acid and succinic acid were identified in both the wild-type profile and in the mutant profile in chromatographic systems. As in the case of the $^{14}$C-acetate-labeled experiment, the $^{14}$C-citric acid peak was the largest and the $^{14}$C-succinic acid peak was the smallest for the mutant strain.

Accumulation of $^{14}$C-citric acid and $^{14}$C-succinic acid in the wild-type organism was significantly less compared with the mutant strain. No other tricarboxylic acid cycle intermediate was present in the culture supernatant fluid of the mutant strain when grown in the presence of $^{14}$C-acetate or $^{14}$C-glutamic acid.

Fig. 4. Column (Dowex 1-formate) chromatographic profile of the carrier $\alpha$-ketoglutaric acid ($\alpha$-KG) and the radioactivity from the culture supernatant fluids of the wild-type (upper frame) and mutant strain MO-1-9B (lower frame) grown in the presence of $^{14}$C-glutamic acid-(U). Individual fractions were analyzed for radioactivity (counts/min) and the carrier (NaOH).
DISCUSSION

Mutant MO-1-9B responds satisfactorily to glutamic acid supplementation; however, the inability to substitute proline, ornithine, arginine, or glutamine for glutamate is not understood, except that these may be related to the respiratory deficiency (inability to grow in lactate or glycerol media). The mutant accumulates significantly large amount of citric acid in the culture supernatant fluid as compared with the wild-type strain. It is believed that the mutant is able to synthesize 14C-citric acid in vivo by utilizing 14C-acetate and oxaloacetate (generated by the degradation of glutamic acid supplement via succinic acid), but it fails to utilize any citric acid because of the lack of aconitase activity (7). Consequently, excess citric acid is excreted into the medium. As expected in the wild-type organism, the tricarboxylic acid cycle being unimpaired, there is very little accumulation of citric acid. Formation and accumulation of 14C-succinic acid in the mutant experiments where 14C-acetate was used as tracer was not apparent. Suppression of 14C-citric acid and 14C-succinic acid accumulation under excess glutamic acid supplementation may be due to the inhibition or repression, or both, of the enzymes responsible for the formation of these intermediates. Accumulation of ketoglutaric acid 14C-α, 14C-succinic acid and 14C-citric acid, but not 14C-aconitic acid, in experiments where 14C-glutamic acid was used as tracer is consistent with degradation of glutamic acid to citric acid via succinic acid. The glu1 mutant is particularly interesting because of its metabolic block in the tricarboxylic acid cycle, causing the glutamic acid requirement and the ability of the mutant to accumulate a significant amount of key tricarboxylic acid cycle intermediates.

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