Stereospecificity of Citrate Synthetase in Relation to Glutamate Biosynthesis by Extracts of Chloropseudomonas ethylicum

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The synthesis of citric and glutamic acids by extracts of Chloropseudomonas ethylicum was studied with labeled precursors. When acetyl-coenzyme A-\(1^{14}\)C was used as substrate, only 0.1% of the total radioactivity was found in the C-5 position of citric acid; whereas, with oxalacetate-4-\(1^{14}\)C as substrate, 100% of the total radioactivity was found in C-5. These results demonstrated that the Chloropseudomonas citrate synthetase had an absolute stereospecificity, identical to that of the pig heart synthetase. The distribution of radioactivity in the glutamic acid synthesized from acetyl-coenzyme A-\(1^{14}\)C was 0% in C-1 and 94.0% in C-5; whereas the glutamic acid formed from oxalacetate-4-\(1^{14}\)C contained 89.6% in C-1 and 0.5% in C-5. This distribution is entirely consistent with the biosynthesis of glutamic acid from citric acid via aconitase, \(\Delta_5\)-isocitrate, and L-glutamate dehydrogenases. The presence of L-glutamate dehydrogenase in extracts was demonstrated. The stereospecificity of the citrate synthetase and the pattern of glutamate labeling further establish that the aconitase of Chloropseudomonas is completely stereospecific.

Recent studies have shown that the citrate synthetase of some species of anaerobic bacteria possesses an unusual stereospecificity. This was first revealed by Gottschalk and Barker (7), who found that the citrate synthetase of Clostridium kluveri had essentially the reverse, or unusual, stereospecificity of that found for the pig heart enzyme (9). On the other hand, Stern, Hegre, and Bambers (11), using a different strain of C. kluveri, found that the citrate synthetase displayed the usual stereospecificity. However, both groups found that about 5 to 8% of the radioactivity of the labeled precursors, oxalacetate-4-\(1^{14}\)C and acetate-\(1^{14}\)C, did translocate to the opposite carboxyl of glutamate to that predicted from an absolutely stereospecific course of citrate synthesis. Gottschalk and Barker (8) have shown that three other anaerobic bacteria (C. acidi-urici, C. cylindrosporum, and Desulfovibrio vulgaris) possess a citrate synthetase with the unusual or reverse stereospecificity. Five other anaerobes examined, including two other species of Clostridium, possessed the usual type of citrate synthetase.

Callyle and Fuller (2) have reported the presence of citrate synthetase, aconitase, and \(\Delta_5\)-isocitrate dehydrogenase in cell extracts of Chloropseudomonas ethylicum, a green obligately photosynthetic anaerobic microorganism. Like C. kluveri, it requires both \(CO_2\) and acetate (or a precursor of acetate, e.g., ethyl alcohol) as carbon sources for growth; whereas C. kluveri derives its reducing power by alcohol oxidation, C. ethylicum utilizes the photosynthetic process. It was therefore of interest to examine the stereospecificity of the citrate synthetase of C. ethylicum.

The results reported in this paper demonstrate that Chloropseudomonas citrate synthetase is absolutely stereospecific and has the usual stereospecificity, identical to the pig heart enzyme. In addition, the presence of L-glutamate dehydrogenase has been demonstrated, and it is shown that glutamate biosynthesis from labeled precursors does occur via the citrate pathway.

MATERIALS AND METHODS

Organisms and growth conditions. A lyophilized preparation of C. ethylicum, which had been grown as described (2), was kindly provided by R. C. Fuller, Aerobacter aerogenes NCTC 418 was grown on the citrate medium of Dagley and Dawes (4) and C tetanomorphum H1 was grown as described by Barker et al. (1). Preparation of cell extracts. Cell extracts were prepared by hand grinding 0.5 g of lyophilized Chloropseudomonas cells with 3 g of glass beads (size 12) for 5 min at 5 C in 1 ml of 0.05 M tris(hydrox-
zymethyl)aminomethane (Tris)-chloride buffer (pH 7.4) containing 0.02 M 2-mercaptoethanol. An additional 4.0 ml of buffer was added, and the suspension was centrifuged for 20 min at 27,000 × g at 0 C. A. aerogenes extract was prepared by treating 3 g of wet cells in 10 ml of 0.2 M Tris-chloride buffer (pH 8.0) for 3 min at 5 C with a Branson Sonifier. Cell debris was settled by centrifugation as described above.

Enzyme assays. Citrate synthetase was assayed according to the procedure of Stern et al. (12). Glutamate dehydrogenase was measured spectrophotometrically at 340 nm by (i) the reduction of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of α-ketoglutarate and NH₄Cl and (ii) the reduction of nicotinamide adenine dinucleotide phosphate (NADP) by L-glutamate.

Materials. E. coli L-glutamate decarboxylase was obtained from Worthington Biochemical Corp. (Freehold, N.J.) and glutamate-oxalacetate transaminase from Boehringer & Co. Acetyl-coenzyme A (CoA)-¹⁴C was a product of New England Nuclear Corp. (Boston, Mass.) and aspartic acid-⁴-¹⁴C was a product of Calbiochem (Los Angeles, Calif.).

Synthesis of radioactive citric and glutamic acids from acetyl-CoA-¹⁴C. The reaction mixture contained: Tris-chloride buffer (pH 7.4), 100 μmoles; potassium oxalacetate, 20 μmoles; acetyl-CoA-¹⁴C 5 μmoles (2.5 μc); and Chloropseudomonas extract, approximately 20 mg of protein in a total volume of 1.8 ml.

Synthesis of radioactive citric and glutamic acids from aspartic acid-⁴-¹⁴C. The reaction mixture contained: Tris-chloride buffer (pH 7.4), 100 μmoles; potassium α-ketoglutarate, 25 μmoles; acetyl-CoA, 5 μmoles; aspartic acid-⁴-¹⁴C, 5 μmoles (2.5 μc); glutamate-oxalacetate transaminase, 0.2 mg; and Chloropseudomonas extract, approximately 20 mg of protein in a total volume of 1.8 ml.

Ammonia, NADP, and Mg²⁺ were deliberately omitted from both of the reaction mixtures to minimize further metabolism of citrate via aconitate, isocitrate, and glutamate dehydrogenases. However, trace amounts of these compounds in the crude cell extract permitted some glutamate formation from accumulated citrate. Both reactions were run in air at 30 C for 10 min and were terminated by heating at 100 C for 3 min. The clear supernatant solutions were placed on Whatman 3 MM paper and were chromatographed in sec-butyl alcohol-formic acid-water (67:11:22). The radioactive citric and glutamic acids were located in a strip scanner and were eluted with 10 ml of water. The glutamic acid was further purified by electrophoresis on Whatman 3 MM paper in pyridine-acetic acid (pH 3.5) for 3 hr at 2,700 V. The citric acid, which gave only a single radioactive spot after chromatography in two different solvent systems, was used without further purification.

Degradation of radioactive citric and glutamic acids. Citric acid-⁴-¹⁴C was incubated with an extract of A. aerogenes containing citrateg and oxalacetate decarboxylase (3) and was thereby almost quantitatively converted to CO₂ (C-5), pyruvate (C-4, C-3, C-6), and acetate (C-1 and C-2). The incubation was carried out in Warburg flasks containing: Tris-chloride buffer (pH 8.0), 100 μmoles; potassium citrate, 5 μmoles; and citric acid-⁴-¹⁴C in a volume of 1.5 ml in the main compartment. One side arm contained 0.3 ml (approximately 10 mg of protein) of A. aerogenes extract and the other contained 0.2 ml of 30% trichloroacetic acid. The center well contained 0.15 ml of Hymamine. The reaction was initiated by tipping the enzyme and was allowed to proceed for 15 min at 30 C before being terminated by tipping the acid. Shaking was continued for an additional 30 min to absorb the evolved CO₂. The method gave a recovery of 80% with authentic citric acid-⁵-¹⁴C, and all values were corrected for this recovery. The C-1 carboxyl of glutamic acid was converted to CO₂ by glutamate decarboxylase and chromatography of the decarboxylated residue was carried out as described by Stern et al. (11). Degradation of the C-5 carboxyl of glutamic acid was carried out as described by Gottschalk and Barker (7) by using a suspension of C. tetanomorphum. Results were corrected for the 65% recovery obtained with authentic glutamic acid-⁵-¹⁴C.

Analytical methods. Protein was determined by the biuret method (6) and radioactivity was measured as described by Stern et al. (11).

RESULTS

Glutamate dehydrogenase. As shown in Table 1, cell-free extracts of Chloropseudomonas were capable of oxidizing NADPH in the presence of α-ketoglutarate and ammonium chloride. There was essentially no oxidation of reduced nicotinamide adenine dinucleotide (NADH) under the same conditions. When α-ketoglutarate or ammonium chloride were omitted, no oxidation of NADPH occurred. Cell extracts were also capable of reducing NADP, but not nicotinamide adenine dinucleotide (NAD), in the presence of L-glutamate. Thus, L-glutamate dehydrogenase, which catalyzes the following reaction, was present in the extracts:

\[ \text{L-glutamate} + \text{NADP} + \alpha \text{-ketoglutarate} + \text{NH}_4^+ + \text{H}^+ \]

The rate of the reverse reaction was approximately 15 times faster than the rate of the forward reaction. The observed activity of L-glutamate dehydrogenase represents a minimal value, since the specific activity of the citrate synthetase of the lyophilized cell extract was only 25% of the value given by Calley and Fuller (2) for frozen cell extracts. It was also noted that sonic treatment of lyophilized cells, even in the presence of mercaptoethanol, resulted in almost complete loss of citrate synthetase activity.

Labeling of citric acid. The C-5 of citrate was converted to CO₂ as described. When acetyl-CoA-¹⁴C was the precursor, only 0.1% of the
Table 1. Presence of L-glutamate dehydrogenase in cell extracts

<table>
<thead>
<tr>
<th>Expt</th>
<th>Systema</th>
<th>Rateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>5.13</td>
</tr>
<tr>
<td></td>
<td>Complete, NADH2 for NADPH2</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Complete less NH4Cl</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Complete less α-ketoglutarate</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>Complete</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Complete, NAD for NADP</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Complete (NADP) less glutamate</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Complete (NAD) less gluta- mate</td>
<td>0.00</td>
</tr>
</tbody>
</table>

a The complete system for experiment 1 contained: Tris-chloride buffer (pH 7.0), 100 μmoles; NADPH2 or NADH2, 0.25 μmole; NH4Cl, 25 μmoles; potassium α-ketoglutarate, 25 μmoles; and cell extract, 0.62 mg of protein. The complete system for experiment 2 contained: Tris-chloride buffer (pH 8.0), 100 μmoles; NADP or NAD, 0.5 μmole; potassium L-glutamate, 25 μmoles; and cell extract, 2.1 mg of protein. Volume 1.0 ml. Temperature, 22 C.

b Rate = μmoles of pyridine nucleotide oxidized or reduced per hour per milligram of protein.

total radioactivity was found in C-5; whereas, when oxalacetate-4-14C was the precursor, 100% of the radioactivity was present in C-5 (Table 2).

Table 2. Amount of radioactivity in C-5 of citric acid-14C synthesized by cell extracts

<table>
<thead>
<tr>
<th>Carbon atoms</th>
<th>Acetyl-CoA-1-14C</th>
<th>Oxalacetate-4-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpmb</td>
<td>%</td>
<td>dpmb</td>
</tr>
<tr>
<td>Total</td>
<td>115,347 100</td>
<td>51,272 100</td>
</tr>
<tr>
<td>C-5</td>
<td>131 0.1</td>
<td>52,595 102.5</td>
</tr>
</tbody>
</table>

* Disintegration per minute = dpm.

Table 3. Distribution of radioactivity in glutamic acid-14C synthesized by cell extracts

<table>
<thead>
<tr>
<th>Carbon atoms</th>
<th>Acetyl-CoA-1-14C</th>
<th>Oxalacetate-4-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpmb</td>
<td>%</td>
<td>dpmb</td>
</tr>
<tr>
<td>Total</td>
<td>6,920 100</td>
<td>18,880 100</td>
</tr>
<tr>
<td>C-1</td>
<td>0 0</td>
<td>16,830 89.6</td>
</tr>
<tr>
<td>C-2 to C-5</td>
<td>6,168 89.4</td>
<td>0 0</td>
</tr>
<tr>
<td>C-5</td>
<td>6,500 94.0</td>
<td>99 0.5</td>
</tr>
</tbody>
</table>

* Disintegration per minute = dpm.

FIG. 1. Pathway of conversion of acetyl-CoA-1-14C (C) and oxalacetate-4-14C (C) to glutamate via citrate. The numbers refer to the C atoms of citrate which is written in the Fischer projection with the – COOH group on top and the oxalacetate-derived aconitase-active – CH2-COOH group on the bottom, and with both projecting behind the plane of the paper.

pounds probably arose by conversion of oxalacetate-14C via the stepwise activities of L-malate dehydrogenase, fumarase, and succinate dehydrogenase.

Discussion

Calley and Fuller (2) have demonstrated that extracts of Chloropseudomonas possess all the enzymes of the citric acid cycle, except α-ketoglutarate dehydrogenase, in Chloropseudomonas; these com-
attempt was made by these workers to determine whether glutamate dehydrogenase was present, but the enzymes of the glyoxylate cycle could not be detected. We have demonstrated the presence of glutamate dehydrogenase in cell-free extracts, thus indicating that synthesis of glutamic acid is possible via the citric acid cycle mechanism. This result has been further confirmed by the finding that Chloropseudomonas extracts synthesized radioactive citric and glutamic acids from acetyl-CoA-1-14C and oxalacetate-4-14C. The fact that the organism does not have a complete citric acid cycle indicates that citrate synthetase, aconitase, isocitrate, and glutamate dehydrogenases constitute a biosynthetic route for the formation of glutamic acid.

The usual stereospecific course of citrate synthesis (Fig. 1) from acetyl-CoA-1-14C and oxalacetate leads to the formation of citric acid-1-14C which is converted via aconitase, isocitrate, and glutamate dehydrogenases to glutamic acid-5-14C; when acetyl-CoA and oxalacetate-4-14C are the precursors, then the products are citric acid-5-14C and glutamic acid-1-14C. The results of experiments with Chloropseudomonas extract (Tables 2 and 3) are essentially in accord with this formulation. Thus, one can conclude that the citrate synthetase of Chloropseudomonas has the usual stereospecificity (identical to pig heart synthetase) and is 100% stereospecific. It is noteworthy that no translocation of carbon radioactivity occurs on conversion of labeled acetate or oxalacetate to glutamate, as was seen in C. kluyveri (7, 10, 11). In this respect, Chloropseudomonas duplicates the behavior of C. thermoaceticum (J. R. Stern, Bacteriol. Proc., p. 75, 1966).

It can also be concluded from the data in Table 3 that the aconitase of Chloropseudomonas is 100% stereospecific and is identical to pig heart aconitase (5, 9).

Acknowledgments

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Literature Cited