Purification and Some Properties of Citrate Synthase from Ammonia-Oxidizing Chemoautotrophic *Nitrosomonas europaea* ATCC 25978

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Abstract: Ammonia-oxidizing chemoautotrophic *Nitrosomonas europaea* ATCC 25978 exhibited remarkable oxaloacetate-oxidative activity. Citrate(sii)-synthase [EC 4.1.3.7.] was purified as an electrophoretic homogeneous protein. The molecular weight of the enzyme was estimated to be about 295,000 (α,β) by gel filtration, suggesting that the enzyme consisted of two different subunits (α; 65,000, β; 75,000), as demonstrated by SDS-PAGE. The N-terminal sequences of α and β type enzyme proteins were Ala-Leu-Val-Ser-Leu-Arg-Gln-Leu-Leu. The isoelectric point of the enzyme was pH 5.2. The enzyme was stable up to 35°C and in a pH range between 6.0-8.0. The optimum pH for the reaction was 6.5-8.5 and the optimum temperature was 30-40°C. The apparent *Km* values for oxaloacetate and acetyl-CoA were about 0.025 and 0.080 mM, respectively. The enzyme was not inhibited by ATP (1 mM), NADH (1 mM) or 2-oxoglutaric acid (10 mM). Activation by metal ions (0.1 mM) was not observed.

Key words: *Nitrosomonas europaea*, ammonia-oxidizing bacteria, citrate synthase, nitrifying bacteria.

Introduction

Citrate(sii)-synthase [EC 4.1.3.7. citrate oxaloacetate-lyase] catalyzes the first reaction of the tricarboxylic acid (TCA) cycle and thus plays an important role in cellular metabolism. The enzyme catalyses the reaction: Acetyl-CoA + Oxaloacetate + H₂O → Citrate + CoA-SH. Bacterial citrate synthases display a marked but still partly unexplained diversity in their molecular and regulatory properties. The citrate synthases of gram-negative bacteria are allosteric enzymes, whose activity is inhibited strongly and specifically by the end-product of the TCA-cycle, NADH, whereas those of gram-positive bacteria are inhibited by ATP.

We have previously shown that ammonia-oxidizing chemoautotrophic *Nitrosomonas europaea* ATCC 25978 was found capable of growing more rapidly in a medium containing oxaloacetate or pyruvate than one consisting entirely of mineral salts. In *Nitrosomonas*, carbon assimilation proceeds chiefly via the ribulose biphosphate (RuBP) carboxylase pathway and only in part via the phosphoenolpyruvate carboxylase pathway.

Although citrate synthase from chemosynthetic thiobacilli has been studied, no data are available for this enzyme and its role in the metabolism of *Nitrosomonas*. Several obligately autotrophic bacteria, such as *Nitrosomonas*, lack 2-oxoglutarate dehydrogenase. Until now, no one has succeeded in obtaining a pure sample...
of a *Nitrosomonas* citrate synthase, largely owing to the poor growth of the cells in conventional media.

The present study examines the purification and various properties of isolated citrate synthase from *N. europaea* ATCC 25978.

**Materials and Methods**

**Nitrocoorganisms and culture conditions**

*Nitrosomonas europaea* ATCC 25978\(^{19}\) was used throughout this study. The culture medium (P-medium) was composed of: Na\(_2\)HPO\(_4\), 13.5 g; KH\(_2\)PO\(_4\), 0.7 g; (NH\(_4\))\(_2\)SO\(_4\), 2.5 g; MgSO\(_4\)·7H\(_2\)O, 0.1 g; NaHCO\(_3\), 0.5 g; CaCl\(_2\)·2H\(_2\)O, 5 mg; chelated iron (Fe-EDTA, Dojindo Laboratories Co., Ltd., Japan), 1.0 mg; and deionized water, 1 liter\(^{11}\). The cultivation system used in this experiment was the same as described previously\(^{16}\). Cells from liquid culture were sedimented by conditions at 12,000 × g for 30 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 140,000 × g for 30 min and the resultant supernatant was used as a crude enzyme solution. Solid ammonium sulfate was added to the cell-free extract. The 40–60% ammonium sulfate precipitate was collected by centrifugation (9,500 × g, 10 min) and dissolved in a small volume of 10 mM Tris–HCl buffer (pH 7.5). The enzyme solution was dialyzed overnight against the same buffer, then applied to a DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Sweden) column (1.6 × 30 cm) equilibrated with the 10 mM Tris–HCl buffer (pH 7.5). The elution was performed with a linear gradient of NaCl (0 to 0.7 M) in the same buffer at a flow rate of 12 ml/h, and the elute was collected in 4-ml fractions. The active fractions were collected and dialyzed overnight with the same buffer. The enzyme solution (15 ml) was concentrated to 2 ml by osmotic ultracentrifugation using Ficoll 400 (Pharmacia), then applied to a Cellulofine GCL-2000-m (Seikagaku Kogyo Co., Ltd., Japan) column (0.9 × 60 cm) equilibrated with P-buffer. The elution was done with the same buffer at a flow rate of 6 ml/h and the enzyme fractions were pooled. The enzyme solution was concentrated to one milliliter by Ficoll 400, then applied on to FPLC (Fast protein Liquid Chromatograph, Pharmacia) with Mono Q column (0.5 × 5 cm) bufferized with P-buffer. The elution of the enzyme was performed by a linear gradient of NaCl (0 to 0.1 M) in the same buffer. The purified enzyme fractions were collected, dialyzed against 10 mM Tris–HCl buffer (pH 7.5).
Polyacrylamide gel disc electrophoresis

The homogeneity of the enzyme preparation was examined by polyacrylamide disc gel electrophoresis (PAGE). Electrophoresis was done in 7.5% polyacrylamide gel in Tris-glycine buffer (pH 8.6) with a current of 2.0 mA per tube for 1.2 h at 0°C by the method of Hames. Gels were stained for protein with Coomassie brilliant blue G-250.

Determination of molecular weight

The molecular weight of the purified enzyme was estimated by gel filtration using a Superose 12 (Pharmacia) according to the method by Andrews. The column (1.0 x 30 cm) was equilibrated with P-buffer containing 0.1 M sodium chloride. The purified enzyme was eluted in 0.25-ml fractions at a flow rate of 15 ml/h. Apoferritin; horse spleen (MW, 443,000), β-amylase (MW, 200,000), alcohol dehydrogenase (MW, 150,000), albumin; bovine serum (MW, 66,000), carbonic anhydrase (MW, 29,000) and cytochrome c (MW, 12,400) were used as marker proteins (Pharmacia).

The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn using SDS-PAG PLATE 4/20 (Daiich Pure Chemical Co., Ltd., Japan) with Tris-glycine buffer (pH 8.4) containing 0.1% SDS. The electrophoresis was performed at 60 mA for 60 min. The marker proteins used were myosin (MW, 212,000), α2-macroglobulin (MW, 170,000), β-galactosidase (MW, 116,000), transferrin (MW, 76,000), and glutamic dehydrogenase (MW, 53,000) (Pharmacia). The proteins were detected by the silver stain method.

Isoelectric focusing

Isoelectric focusing was performed using AMPHOLINE PAGE PLATE (pH 3.9-9.5, Pharmacia) gels on an ATTO AE-3230 apparatus. The gels were prefocused at 300 V for 60 min and focused at 300 V for 120 min at 4°C. Anode and Cathode solutions were 1.0 M H₃PO₄ and 1.0 M NaOH, respectively. After electrofocusing, the protein in the gels was stained with silver stain. pH marker proteins with values of 3.5-9.5 were used as references.

Determination of the amino acid sequence

The N-terminal amino acid sequence of the α and β subunits were determined by a protein sequencer (ABI model 477 A, Applied Biosystems Inc., New York, USA).

Results

Purification of citrate synthase

The results of a purification done as described in Materials and Methods are summarized in Table 1. The fractions with enzyme activity were eluted at around 0.2 M NaCl from a DEAE-Sepharose CL-6B column as described previously. The purification fold at this step was 28.1. The active fraction was applied on a Cellulofine GCL-2000-m column. The purification fold at the step was

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>310.4</td>
<td>28.23</td>
<td>0.09</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate ppt (40-60% saturation)</td>
<td>44.5</td>
<td>16.29</td>
<td>0.37</td>
<td>4.1</td>
<td>57.7</td>
</tr>
<tr>
<td>DEAE Sepharose CL-6B</td>
<td>3.25</td>
<td>8.22</td>
<td>2.53</td>
<td>28.1</td>
<td>29.1</td>
</tr>
<tr>
<td>Cellulofine GCL-2000-m</td>
<td>1.56</td>
<td>5.28</td>
<td>3.39</td>
<td>37.7</td>
<td>18.7</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.152</td>
<td>3.39</td>
<td>22.30</td>
<td>247.8</td>
<td>12.1</td>
</tr>
</tbody>
</table>
The specific activity of the purified enzyme obtained by the next Mono Q column was 22.3 unit/mg, which was 247.8-fold higher than that of the cell-free extract, with 12% recovery. The isoelectric point of the enzyme was pH 5.2 (data not shown).

Fig. 1. Polyacrylamide gel electrophoresis of citrate synthase.
(A): Electrophoresis of native enzyme on polyacrylamide gel. (B): Electrophoresis of denatured enzyme and molecular markers on SDS polyacrylamide gel. Electrophoresis was carried out as described in Materials and Methods. The arrow indicates the position of the enzyme protein.

Fig. 2. Estimation of molecular weight of citrate synthase.
(A): Gel filtration on a column of Superose 12. (B): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Symbols: ○, citrate synthase; (α) MW 65,000, (β) MW 75,000. Conditions of gel filtration and electrophoresis were as described in the Materials and Methods.
**Enzyme purity and molecular weight**

The purified enzyme migrated as a single protein band on disk electrophoresis at pH 8.6 (Fig. 1A). The molecular weight of the native enzyme was estimated at 295,000 by gel filtration (Fig. 2A). The molecular weight of the denatured enzyme was estimated by SDS-PAGE. Two protein bands

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**Fig. 3.** Effect of pH on activity and stability of citrate synthase.

(A): The enzyme reaction was carried out as described under Materials and Methods except that the pH was changed as indicated. (B): Enzyme solution was incubated at various pHs at 4°C for 24 h and then the residual activity was assayed at pH 8.0. Buffers used were 50 mM Britton-Robinson buffer (●), 50 mM Tris-HCl buffer (○), and 50 mM Na2HPO4-NaOH buffer (▲).

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**Fig. 4.** Effect of temperature on activity and stability on citrate synthase.

(A): The enzyme reaction was carried out as described Materials and Methods except that the temperature was changed as indicated. (B): Enzyme solution was incubated at various temperature in 20 mM potassium phosphate buffer, pH 8.0, for 10 min and then the residual activity was assayed at 35°C.
were detected on the gel (Fig. 1B), and results showed that the enzyme consisted of two different subunits with a molecular weight 65,000 (α) and 75,000 (β) (Fig. 2B). Thus citrate synthase of strain ATCC 25978 are "large" enzymes (αβ).

**Effect of pH on the activity and stability**

The enzyme was most active at pH values ranging between 6.5-8.5, when the enzyme activity was measured under standard assay conditions (Fig. 3A).

The enzyme was stable in the pH range of 6-8 when kept at 4°C for 24 h (Fig. 3B).

**Effect of temperature on the activity and stability**

The enzyme showed the highest activity at 35°C under the standard assay condition (Fig. 4A). The enzyme was incubated at various temperature (20-70°C) for 10 min in 0.1 M Tris–HCl buffer (pH 8.0), and the remaining activity was assayed by the standard assay method. The enzyme was stable at temperatures below 40°C (Fig. 4B).

**Effect of various compounds**

The effects of metal ions and inhibitors on the enzyme activity were assayed. Each compound examined was added to the enzyme, followed by standing for 10 min at 35°C, and then the activity was measured (data not shown). The activity of the enzyme was not significantly inhibited by the following compounds at 1 mM: FeCl₂, CoCl₂, EDTA, NaN₃, CH₂COOH, NH₂OH, KCN, o-phenanthroline, ATP, NADH, and 10 mM: 2-oxoglutaric acid. Some compounds such as CuSO₄, HgCl₂ and CdCl₂ significantly inhibited the activity at concentration of 0.1 mM.

**Effect of substrate concentration on enzyme activity**

The Michaelis constants for oxaloacetate and acetyl-CoA in citrate synthesis were calculated from the Lineweaver-Burk plots, respectively, using purified enzyme. The apparent Kₘ values of the enzyme for oxaloacetate and acetyl CoA were 0.025 mM and 0.080 mM, respectively (Fig. 5A and 5B).
N-terminal amino acid sequence

The N-terminal sequence of the enzyme proteins determined was Ala-Leu-Val-Ser-Leu-Arg-Gln-Leu-Leu. The sequences from those of both α and β type enzyme proteins are similar.

Discussion

Citrate synthases occur naturally in two sizes: "large" (α₂β₂) and "small" (αβ). The large enzyme (MW, 240,000–280,000) is found in gram-negative bacteria and is usually sensitive to feedback inhibition by the end-product of the TCA cycle, NADH. The much smaller NADH-insensitive enzyme (MW, 60,000–100,000) is observed in gram-positive bacteria21–24.

The molecular weight of citrate synthase of N. europaea ATCC 25978 was estimated to be about 295,000 by gel filtration (Fig. 2A). Similar results on molecular size have been reported for citrate synthases of Azotobacter vinelandii8 and Acinetobacter anitratus8. The Kₘ value for oxaloacetate of the strain ATCC 25978 enzyme (0.025 mM) is similar to that of the A. vinelandii enzyme (0.020 mM)9. The value for acetyl-CoA of strain ATCC 25978 (0.080 mM) is also similar to that of A. vinelandii (0.080 mM).

The citrate synthases of gram-negative bacteria are inhibited by NADH21, whereas those of gram-positive bacteria are inhibited by ATP77. The citrate synthases from facultatively anaerobic gram-negative bacteria are also inhibited by 2-oxoglutarate22. The citrate synthases of obligatorily autotrophic thiobacilli, such as T. denitrificans and T. neapolitanus, were inhibited by 2-oxoglutarate17. The citrate synthase of strain ATCC 25978, unlike those of obligatorily autotrophic thiobacilli, was not inhibited by 2-oxoglutarate (10 mM). The citrate synthase from facultatively autotrophic thiobacilli, such as T. novellus, was not inhibited by NADH, ATP or 2-oxoglutarate17.

In these respects, the citrate synthase of strain ATCC 25978 resembled to that of T. novellus. T. novellus, and contrasted with obligatorily autotrophic thiobacilli, by possessing a complete TCA cycle even when grown autotrophically17. We have examined the citrate synthase from N. europaea and found that it was unusual in not being inhibited by any of the three inhibitors, ATP, NADH and 2-oxoglutarate. No homologies could be found between the N-terminal amino acid sequences of citrate synthases from the gram-negative bacteria E. coli23 and P. aeruginosa4 and that of citrate synthase (two subunits; α and β) purified from strain ATCC 25978 in this study. The sequences from those of both α and β subunits are similar. In order to elucidate the physiological and biochemical functions of the both subunits, molecular cloning experiments of the gene coding for the enzyme are now in progress and will be reported elsewhere.

From our observations, we conclude that new patterns of structure and regulation are to be found in obligatorily autotrophic N. europaea ATCC 25978 citrate synthase.

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References

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