Archaebacterial Citrate Synthases: The Enzymes from the Thermoacidophiles *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* Show pro-S Stereospecificity

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Citrate synthase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* was purified 365-fold to electrophoretic homogeneity. At 40 °C and pH 8.1 the homogeneous enzyme shows a specific activity of 73 units per mg, which corresponds to a turnover number of 44 sec⁻¹. Citrate synthase from *S. acidocaldarius* shows pro-S stereospecificity, as is found with a partially purified preparation of the enzyme from *Thermoplasma acidophilum*, another thermoacidophilic archaebacterium.

Introduction

Citrate synthases catalyse the formation of citrate in a stereospecific manner [1]. Most citrate synthases show pro-S stereospecificity, whereby C-4 and C-5 of citrate is derived from acetyl-CoA [2]. Exceptions are the enzymes found in some strictly anaerobic bacteria [3–5]. These enzymes are pro-R citrate synthases and C-1 and C-2 of citrate are formed from acetyl-CoA. It was speculated that the pro-R citrate synthase might be an ancient form of the enzyme from an early stage of life [5]. In this respect it is of interest to determine the stereospecificity of citrate synthases from archaebacteria, the third urkingdom of life [6].

In an earlier report we described molecular and kinetic properties obtained with partially purified citrate synthases from the thermoacidophilic archaebacteria *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* [7]. In this paper we report on the stereospecificity of the archaebacterial citrate synthases as found with the homogeneous enzyme isolated from *S. acidocaldarius* and a partially purified preparation from *T. acidophilum*. While this work was in progress two procedures for purifying citrate synthase from *Sulfolobus* have been published [8, 9]. The procedure described in this report results in an increased specific activity of the enzyme.

Materials and Methods

Organisms and growth conditions

*Sulfolobus acidocaldarius* (DSM 639) and *Thermoplasma acidophilum* 122-1B3 (ATCC 27658) were grown as described previously [7]. *Streptococcus dia­acetilactis* was kindly provided by Prof. Dr. G. Gottschalk, Göttingen, and was grown in a lactose citrate medium [10].

Chemicals

[1-14C]Acetyl-CoA (5 mCi/mmol, 20 μCi/ml) and [1,5-14C]citric acid (111 mCi/mmol, 50 μCi/ml) were purchased from Amersham Buchler (Frankfurt). All other chemicals were of analytical grade.

Buffers

(A) 1 m potassium phosphate, pH 7.0; (B) 0.1 m Tris/HCl, pH 7.5; (C) 0.05 m Tris/HCl, pH 7.3; (D) 0.01 m Tris/HCl, pH 7.5; (E) 0.1 m Tris/HCl, pH 7.3, containing 0.1 m NaCl; (P) 0.01 m potassium phosphate, pH 7.2.

Determination of enzymatic activities

Citrate synthase was assayed as described previously [7]. One unit of enzyme catalyses the formation of 1 μmol of free CoA. Citrate lyase activity was determined according to Ward and Srere [11].

Protein determination

Protein was estimated by the method of Groves *et al.* [12] with 224 nm and 235 nm as isoabsorbance.

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wavelengths. Bovine serum albumine was used as standard.

**Gel electrophoresis**

Polyacrylamide gel electrophoresis was performed at pH 7.5 according to Maurer [13] and at pH 8 as described by Ornstein and Davis [14]. Both gels contained 7.5% acrylamide. SDS gel electrophoresis was performed in gels containing 7.5 or 10% acrylamide according to Weber and Osborne [15].

**Citrate lyase from Streptococcus diacetilactis**

An extract of *Streptococcus diacetilactis* with citrate lyase activity was prepared according to Harvey & Collins [10].

**Purification of citrate synthase from Sulfolobus acidocaldarius**

**Crude extract**

30 g frozen cells were thawed and suspended in 60 ml of buffer (B). The cells were disrupted in a French press at 22,000 psi. Cell debris was removed by centrifugation at 4 °C and 39,000 × g for 1 h. The supernatant was dialysed against buffer (B).

**Salting-out chromatography**

The crude extract was diluted with buffer (B) to a protein content of 15 mg/ml. Solid (NH₄)₂SO₄ was added to give 50% saturation. After stirring for 30 min the suspension was centrifuged at 4 °C and 39,000 × g for 30 min. The supernatant was applied to a Sepharose Cl-4B column (2.5 × 25 cm) equilibrated with buffer (C) containing 80% (NH₄)₂SO₄. Citrate synthase was eluted with a linear decreasing gradient of (NH₄)₂SO₄ in buffer (C) (80% to 0% (NH₄)₂SO₄, 800 ml). Active fractions were pooled and dialysed against buffer (D).

**Anion exchange chromatography**

The dialysed preparation was applied onto a column of Q-Sepharose fast flow (2.5 × 14 cm) equilibrated with buffer (D). Citrate synthase was eluted with a linear gradient of NaCl (0 to 0.5 M, 400 ml) in buffer (D). Active fractions were combined and dialysed against buffer (C).

Affinity chromatography

The dialysed enzyme was loaded onto a Red Sepharose Cl-6B column (2.5 × 14 cm) equilibrated with buffer (C). The column was washed with about 250 ml of buffer (C) until no protein was eluted. Citrate synthase was eluted with a linear gradient of ATP (0 to 5 mM, 800 ml) in buffer (C). Active fractions were pooled and concentrated to 6.5 ml by ultrafiltration.

**Gel chromatography**

The concentrated solution was applied to a Sephacryl S-200 column (2.6 × 90 cm) equilibrated with buffer (E). A flow rate of 20 ml/h was maintained and the enzyme was passed twice over the column. The active fractions were pooled and concentrated to 2 ml by ultrafiltration.

**FPLC gel filtration**

Citrate synthase was further purified by gel filtration using a Pharmacia FPLC Superose 12 column equilibrated with buffer (P). Portions of 300 μl (= 3 mg of protein) were chromatographed with a flow rate of 1.2 ml/min. Active fractions were combined and concentrated by ultrafiltration. The enzyme was stored at −20 °C.

**Partially purification of citrate synthase from Thermoplasma acidophilum**

The crude extract and protamine sulfate precipitation were done as described by Großebüter and Görisch [7]. The preparation was purified by a salting-out chromatography as described above but with the following modifications. (NH₄)₂SO₄ was added to the extract (0.57 U/mg) to give 65% saturation. Precipitated protein was removed by centrifugation. The column was equilibrated with buffer (C) containing 95% (NH₄)₂SO₄ and the gradient started with the same concentration. This procedure resulted in a 4.1-fold purification with a recovery of 67%.

**Synthesis of [14C]citrate from [1-14C]acetyl-CoA by citrate synthase from Sulfolobus, Thermoplasma and pig heart**

[14C]Citrate was synthesized as described by Gottschalk and Barker [4]. The reaction mixtures contained in 2 ml of buffer (C) 50 μl of [1-14C]acetyl-CoA, 100 μl of 25 mM oxaloacetate, 100 μl of 0.1 M
MgCl₂, 5 μg of avidin, and about 1 unit of the respective citrate synthase.

The archaebacterial enzymes were incubated at 40 °C while pig heart citrate synthase was incubated at room temperature. After 30 min 100 μl of 0.1 M citrate was added. Protein was denatured in a boiling water bath and removed by centrifugation. The supernatant was applied to a column of Dowex 1-X8 (0.8 × 4 cm) in the formiate form. After washing the column with 5 volumes of 1 M formic acid, citrate was eluted with 4 N formic acid. Samples of each fraction (50 μl) were removed and the radioactivity determined. The radioactive fractions were combined and evaporated to dryness. The labelled citrate was dissolved in 2 ml of buffer (C).

Cleavage of [¹⁴C]citrate by citrate lyase

The reaction was performed according to Gottschalk and Barker [4]. The mixture contained in a total volume of 2 ml of buffer (C) 100 μl of 0.1 M MgCl₂, 100 μl of the citrate lyase extract, 200 μl of NAD⁺ (20 mg/ml), 300 μl of [¹⁴C]citrate solution, and about 5 units of malate dehydrogenase. After incubation for 30 min at room temperature enzyme was inactivated in a boiling water bath and removed by centrifugation. The supernatant was applied to a Dowex 1-X8 column. The column was washed with 10 ml of water.

Acetate, malate and citrate were eluted by a stepwise gradient with 0.2 M, 1 M and 4 M formic acid. The radioactive fractions of each step of the gradient were pooled and the total radioactivity was determined.

Results and Discussion

Purification of citrate synthase from Sulfolobus acidocaldarius

Citrate synthase was purified 365-fold with a recovery of 14% (Table I). The purified enzyme has a specific activity of 73 units/mg, corresponding to a turnover number of 44 sec⁻¹. The homogeneity of the preparation was demonstrated by polyacrylamide gel electrophoresis in different systems. In the presence as well as in the absence of SDS a single protein band was detected.

The most efficient step in the purification procedure of citrate synthase from S. acidocaldarius was the affinity chromatography with Red Sepharose Cl-6B. Usually proteins are eluted from dye-linked agarose by their substrates or by salt. In the case of citrate synthase, elution by the substrate acetyl-CoA is too expensive for practical purposes. As citrate synthase from S. acidocaldarius is inhibited by ATP competitively with respect to acetyl-CoA [7], acetyl-CoA can be replaced by ATP to elute the enzyme from Red Sepharose Cl-6B. Recently a purification protocol for citrate synthase of S. acidocaldarius DSM 639 was published by Smith et al. [8]. The enzyme was purified 310-fold and showed a somewhat lower specific activity of 58 units/mg. The results cannot be compared directly as the method of the protein determination was not given. The specific activity of our preparation from S. acidocaldarius however is significantly higher than that reported by Löhlein-Werhan et al. [9] for S. solfataricus.

Citrate synthase from S. acidocaldarius shows a relative molecular mass of 72,000 as estimated by gel filtration, whereas SDS gel electrophoresis showed a protein band corresponding to a relative molecular mass of 35,000 for the subunit. The enzyme therefore belongs to the group of the small, dimeric citrate synthases. These data are in agreement with earlier observations of us [7] and the results of Smith et al. [8].

Stereospecificity of the archaebacterial citrate synthases

[¹⁴C]Citrate was synthesized from oxaloacetate and [1,1⁴C]acetyl-CoA by the Citrate synthases of

Table I. Purification of citrate synthase from S. acidocaldarius.

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<tbody>
<tr>
<td>Crude extract</td>
<td>75</td>
<td>2715</td>
<td>543</td>
<td>0.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Salting-out chromatography</td>
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<td>380</td>
<td>380</td>
<td>1</td>
<td>70</td>
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<tr>
<td>O-Sepharose</td>
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<td>106</td>
<td>329</td>
<td>3.1</td>
<td>61</td>
<td>16</td>
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<tr>
<td>Red-Sepharose</td>
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<td>13.9</td>
<td>237</td>
<td>17</td>
<td>44</td>
<td>85</td>
</tr>
<tr>
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<td>109</td>
<td>38</td>
<td>20</td>
<td>190</td>
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<tr>
<td>FPLC Superose 12</td>
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<td>1</td>
<td>76</td>
<td>73</td>
<td>14</td>
<td>365</td>
</tr>
</tbody>
</table>
Table II. Cleavage of $^{14}$C-citrate by citrate lyase. The $^{14}$C-citrate samples, except $[1,5-^{14}\text{C}]$citrate, were enzymically prepared from $[1-^{14}\text{C}]$acetyl-CoA and oxaloacetate. Citrate was isolated and cleaved by citrate lyase as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Labelled citrate</th>
<th>Radioactivity (cpm $\times 10^{-5}$) in Cleavage products</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1,5-^{14}\text{C}]$citrate</td>
<td>7.9</td>
</tr>
<tr>
<td>$[^{14}\text{C}]$citrate formed by the enzyme from $S$. acidocaldarius</td>
<td>2.4</td>
</tr>
<tr>
<td>$T$. acidophilum</td>
<td>2.2</td>
</tr>
<tr>
<td>pig heart</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Radioactivity (about 90 cpm) was in the range of background radioactivity.

$S$. acidocaldarius, $T$. acidophilum and from pig heart. Samples of these $[^{14}\text{C}]$ preparations and $[1,5-^{14}\text{C}]$citrate were cleaved by citrate lyase from $S$. diacetilactis in the presence of malic dehydrogenase and NADH. The products were separated on an anion exchange column. The cleavage of all three enzymically synthesized $[^{14}\text{C}]$citrate samples gave yield to $[^{14}\text{C}]$acetate as the only labelled product. When $[1,5-^{14}\text{C}]$citrate was cleaved, equal amounts of labelled acetate and malate were obtained, Table II.

Thus the citrate synthases of the two thermoacidophilic archaebacteria $S$. acidocaldarius and $T$. acidophilum show the same stereospecificity as the pig heart enzyme, belonging to the pro-S citrate synthases. It will be of interest to learn if pro-R citrate synthases can be found in other archaeabacteria or if this type of enzyme is restricted to some anaerobic eubacteria [16].

Acknowledgements

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