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Citrate synthase from the thermophilic archaebacteria *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*

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Citrate synthase has been purified to homogeneity from the thermophilic archaebacteria *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*. From the relative molecular masses of the native proteins (85 and 83 kDa, respectively) and of their polypeptide chains (43 and 41 kDa, respectively) it is established that they are dimeric enzymes. The N-terminal sequence of the *Thermoplasma* citrate synthase was determined to be P-E-T-E-E-I-S-K-G-L-E-D-V-N-I-K. These properties are compared with those of citrate synthases from eubacteria and eukaryotes to extend the pattern of structural and functional diversity previously observed for this enzyme in non-archaebacterial species.

Citrate synthase; Archaebacteria; *(Thermoplasma, Sulfolobus)*

1. INTRODUCTION

Citrate synthase catalyses the condensation of acetyl-CoA with oxaloacetate and so effects the entry of carbon into the citric acid cycle. The enzyme possesses a diversity of structure, activity and regulation which correlates strongly with the taxonomic status of the source organism [1–3]. The 'large' hexameric [4] citrate synthases are found only in Gram-negative eubacteria whereas Gram-positive eubacteria and eukaryotes possess 'small', dimeric citrate synthases [5–7]. Only the large citrate synthases are allosterically inhibited by NADH, the small enzymes being isosterically inhibited by ATP [1–3].

With the recognition of the archaebacteria as a phylogenetically distinct lineage, it is important to extend studies of the molecular diversity of citrate synthases to this group of organisms. The enzymes' catalytic and regulatory properties have been studied in halophilic, thermophilic and methanogenic archaebacteria [3,8,9], and in the halophiles [8,10,11] and thermophiles [8,9] the citrate synthases appear to be of the small type. Here, we report the first purification to homogeneity of archaebacterial citrate synthases; the dimeric nature of the enzymes from *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius* is established and the N-terminal sequence of the *T. acidophilum* citrate synthase is reported.

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2. EXPERIMENTAL

T. acidophilum (DSM1728) was grown at 57°C (pH 1.8) as in [12] and *S. acidocaldarius* (DSM639) at 70°C (pH 2.0) according to [13]. Citrate synthase was assayed spectrophotometrically at 412 nm and 55°C [14].

Citrate synthase from *T. acidophilum* was purified by the following procedure. Cells (2 g wet wt) were suspended in 20 ml of 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA and cell extracts

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were prepared at 4°C as in [13]. After centrifugation at 10000 × *g* for 15 min, the resultant supernatant was fractionated with $(NH_4)_2SO_4$ at 4°C, the citrate synthase being precipitated between 50 and 95% (w/v) saturation. All remaining steps were carried out at 25°C. The precipitated protein was resuspended in 25 mM diethanolamine-HCl (pH 9.5) and was then fractionated on a column of Sephacryl S-300 (15 × 800 mm) in the same buffer. Fractions containing citrate synthase activity were pooled and subjected to chromatofocussing on a Pharmacia FPLC Mono P column; after loading and washing with 25 mM diethanolamine-HCl (pH 9.5), protein was eluted with a pH gradient from pH 9.5 to 6.0, generated with 10-fold diluted Pharmacia polybuffer 96. Ammonium sulphate was added to the pool of citrate synthase-containing fractions to 60% (w/v) saturation and this was then applied to a column of phenyl-Sepharose CL-4B (13 × 50 mm) previously equilibrated in 25 mM diethanolamine-HCl (pH 8.0) containing 60% (w/v) saturated ammonium sulphate. Citrate synthase was eluted with a 20 ml gradient of 60 to 0% (w/v) saturated ammonium sulphate in the same buffer. The enzyme was stored at 4°C.

Citrate synthase from *S. acidocaldarius* was purified by the above procedure but with the following modifications. Cell extracts were prepared in 100 mM Tris-HCl (pH 8.0) containing 2 mM EDTA and 0.5 M KCl. Enzyme was precipitated at 50–85% (w/v) ammonium sulphate and, after gel filtration on Sephacryl S-300, was subjected to ion-exchange chromatography on a Pharmacia FPLC Mono Q column previously equilibrated in 25 mM diethanolamine-HCl (pH 9.5). Citrate synthase was eluted with a gradient of 0–0.2 M NaCl in the same buffer and, after chromatofocussing on a Pharmacia FPLC Mono P column and hydrophobic interaction chromatography on phenyl-Sepharose CL-4B as described for the *T. acidophilum* citrate synthase, the enzyme was purified to homogeneity by gel filtration on a Pharmacia FPLC Superose 12 column in 10 mM NH_4HCO_3 (pH 7.8). The purified enzyme was stored at 4°C.

Discontinuous SDS-polyacrylamide gel electrophoresis was carried out as in [15] using a polyacrylamide concentration of 3.75% (w/v) in the spacer gel and 10.0% (w/v) in the resolving gel. Standard proteins used as molecular mass markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (polypeptide chain 36 kDa), carbonic anhydrase (29 kDa) and bovine trypsin inhibitor (20 kDa).

N-terminal protein sequencing was performed by Dr D.J. McKay (University of Calgary Canada) and by Dr L.C. Packman (University of Cambridge, England) using an Applied Biosystems gas-phase sequencer (model 470A) coupled to a 120A PTH-analyser. *T. acidophilum* citrate synthase was dialysed exhaustively against 10 mM ammonium acetate and then taken to dryness. The sample (approx. 60 pmol) was taken up in trifluoroacetic acid and applied to a glass-fibre disc coated with 3 mg Biobrene Plus. Precycling was carried out according to the manufacturer's instructions and sequencing cycles were run on standard 03RPTH software.

3. RESULTS

The purification of citrate synthase from *T. acidophilum* and *S. acidocaldarius* is summarised in table 1. The specific activities of the purified enzymes (41 and 53 μmol CoA produced/min per mg, respectively) are in the same range as those reported for citrate synthases from *Bacillus megaterium, Escherichia coli* and pig heart (22, 80 and 100 μmol/min per mg, respectively). Both archaebacterial enzymes were pure as judged from single bands on SDS-polyacrylamide gel electrophoresis using either a single acrylamide concentration (10%, w/v) or a gradient (8–25%, w/v). By comparison with standard proteins of known sizes, the polypeptide molecular mass value of citrate synthase from *T. acidophilum* is 43 kDa (± 2 kDa) and from *S. acidocaldarius*, 41 kDa (± 2 kDa). The previously determined values of the molecular mass of the native enzymes (85 and 83 kDa, respectively) [8] were confirmed by gel filtration of the pure proteins on the Pharmacia FPLC Superose 12 column; thus it is established that both archaebacterial citrate synthases are dimeric proteins.

The N-terminal sequence of the *T. acidophilum* citrate synthase was determined for two preparations of the enzyme from cells grown independently from two separate freeze-dried cultures of the organism. The sequences obtained were identical:

Table 1

Purification of citrate synthases from *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*

Purification step	Volume (ml)	Total enzymic activity (U)	Total protein (mg)	Spec. act. (U/mg)	Recovery (%)	Overall purification (-fold)
Thermoplasma						
Cell extract	27.5	77.3	371	0.21	100	–
Ammonium sulphate (50–95%)	8.3	39.4	168	0.24	51	1.1
Gel filtration (Sephacryl S-300)	14.6	29.0	30	0.97	38	4.6
Chromatofocussing (Mono P)	3.2	11.5	0.6	19.2	15	91
Phenyl-Sepharose CL-4B	3.0	8.2	0.2	41.0	11	195
Sulfolobus						
Cell extract	85.0	82.0	476	0.17	100	–
Ammonium sulphate (50–85%)	9.2	66.7	287	0.23	81	1.4
Gel filtration (Sephacryl S-300)	48.0	62.5	250	0.25	76	1.5
Ion exchange (Mono Q)	13.5	40.4	51	0.8	49	4.7
Chromatofocussing (Mono P)	2.0	11.2	1.9	5.9	14	35
Phenyl-Sepharose CL-4B	2.9	11.1	0.5	22.2	14	131
Gel filtration (Superose 12)	10.0	10.9	0.2	53.0	13	311

P-E-T-E-E-I-S-K-G-L-E-D-V-N-I-K

This sequence was compared with the complete amino acid sequence of pig heart citrate synthase [16] using the hydrophobicity matrix of Levitt [17] with gap penalties of 8 (in secondary structure):2 (at ends of secondary structural regions):1 (regions not involved in secondary structure). The best alignment obtained was to residues 48–64 of the pig enzyme (fig.1); the possible significance of this result is discussed below.

4. DISCUSSION

The present data represent the first purification to homogeneity and oligomeric characterisation of archaebacterial citrate synthases. The significance of the findings stems from the taxonomically

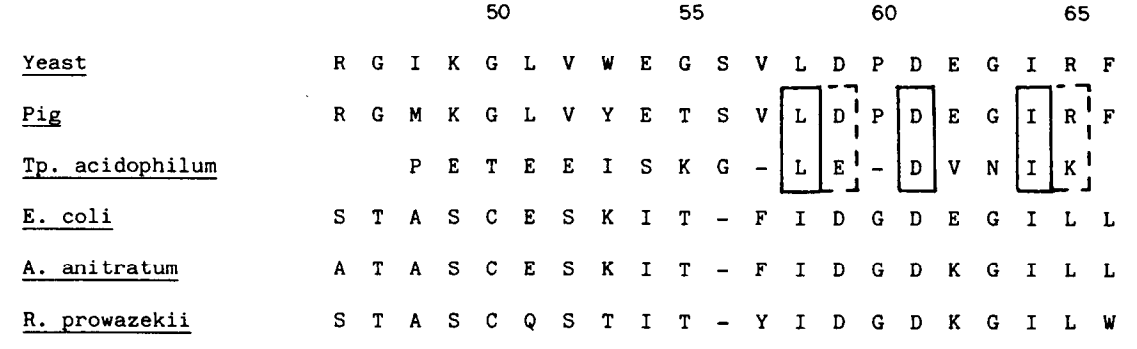

Fig.1. Alignment of the N-terminal sequence of *Thermoplasma acidophilum* citrate synthase with pig heart citrate synthase. A 16-amino-acid N-terminal sequence of *T. acidophilum* citrate synthase was determined and compared with the complete sequence of the pig heart enzyme using the hydrophobicity matrix of Levitt [17] as described in the text. Identical residues between the two sequences are boxed with solid lines and conservative changes with dotted lines. The corresponding sequences of the citrate synthases from yeast, *E. coli, A. anitratum* and *R. prowazekii* are aligned according to [21,22], based on whole sequence homologies. Residue numbers refer to the pig heart sequence.

related diversity of the enzyme from eubacterial and eukaryotic species [1–3]. The two archaebacterial genera used are phylogenetically distinct, *Thermoplasma* being a member of the methanogenic-halophilic branch whereas *Sulfolobus* is a sulphur-dependent archaebacterium. Thus, dimeric citrate synthases are found in both main divisions of archaebacteria, in the Gram-positive eubacteria and in the eukaryotes. Its presence in all three evolutionary kingdoms suggests that the dimer is the ancestral form of the enzyme with the hexamer originating after the emergence of the Gram-negative eubacteria.

Citrate synthase has been sequenced from pig heart [16], yeast [18], *E. coli* [19,20], *Acinetobacter anitratum* [21] and *Rickettsia prowazekii* [22]. For evolutionary comparisons it is imperative to obtain the sequence of an archaebacterial citrate synthase. To this end, we have determined the 16-amino-acid N-terminal sequence of the *Thermoplasma* enzyme from which oligonucleotide probes can be constructed for gene cloning and sequencing. We have been able to compare the limited sequence with that of pig heart citrate synthase on the basis that the three-dimensional structure of the eukaryotic enzyme is known [17] and therefore the search for sequence homology can incorporate penalties for insertions and deletions in regions of secondary structure. The best homology (fig.1) was between residues 10 and 16 of the *Thermoplasma* sequence and 58–65 of the pig enzyme, i.e. three identities and two conservative changes (D/E and R/K).

The alignment is tentative with such a short archaebacterial protein sequence, although it is supported by a number of factors. First, the region of homology suggested between *Thermoplasma* and pig citrate synthases is a sequence also conserved in the yeast, *E. coli* and *A. anitratum* enzymes. Moreover, the region which shows no homology (residues 1–9 of the *Thermoplasma* enzyme) is also non-homologous when the eukaryotic and eubacterial enzymes are aligned [22]. Secondly, the alignment in fig.1 indicates that the archaebacterial enzyme will be approx. 390 residues in length and of ≃44 kDa (cf. 437 amino acids and 49.7 kDa for pig citrate synthase). This smaller size is precisely what is observed by SDS gel electrophoresis and gel filtration.

Clearly, when the complete sequence of an archaebacterial citrate synthase is available, considerable evolutionary and structure-functional information will be generated by comparison with the eukaryotic and eubacterial enzymes.

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