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Rhodothermus marinus: a thermophilic bacterium producing dimeric and hexameric citrate synthase isoenzymes

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Abstract Two separate citrate synthases from the extremely thermophilic bacterium *Rhodothermus marinus* have been identified and purified. One of the enzymes is a hexameric protein and is the first thermostable, hexameric citrate synthase to be isolated. The other is a dimeric enzyme, which is also thermostable but possesses both citrate synthase and 2-methyl citrate synthase activities. 2-Methyl citrate synthase uses propionyl-coenzyme A as one of its substrates and in *Escherichia coli*, for example, it has been implicated in the metabolism of propionate. However, no growth of *R. marinus* was observed using minimal medium with propionate as the sole carbon source, and both hexameric and dimeric enzymes were produced irrespective of whether propionate was included in the growth medium. The data are discussed with respect to the evolutionary relationships between the known hexameric and dimeric citrate synthases and 2-methyl citrate synthase.

Key words Metabolism · 2-Methyl citrate synthase · Protein thermostability · *Rhodothermus* · Thermophile

Introduction

Rhodothermus marinus is an extremely thermophilic bacterium with a phylogenetic position close to the root of the *Flexibacter-Cytophaga-Bacteroides* group (Andrésson and Fridjonsson 1994), but distant from most thermophilic bacteria (e.g., *Aquifex*, *Thermus*, and *Thermotoga*) and Archaea, and also distant from the root of life as proposed by Woese et al. (1990). This organism was selected as an interesting candidate for the isolation of a bacterial (as opposed to archaeal) thermophilic citrate synthase; this choice was based on the organism's phylogenetic position, and also based on the fact that it is a Gram-negative bacterium, allowing the identification of a thermostable, hexameric enzyme.

Citrate synthase (EC 4.1.3.7) catalyzes the reaction: Acetyl-CoA + oxaloacetate + H_2O → citrate + CoA. Due to its central role in metabolism, being the first enzyme in the citric acid cycle, it has been studied from a variety of organisms. Citrate synthases have been shown to be either homodimeric or homohexameric proteins consisting of polypeptide subunits of M_r equaling 42–50 kDa. Eukarya, Archaea, and Gram-positive bacteria possess the homodimeric form of the enzyme, whereas Gram-negative bacteria generally have a hexameric citrate synthase. However, in the latter group a few exceptions are reported. For instance, the thermophilic bacterium *Thermus aquaticus* has been found to posses only the dimeric form (Weitzman 1978), as have the intracellular parasites *Coxiella burnetti* and *Rickettsia prowazekii* (Phibbs and Winkler 1982; Heinzen et al. 1987).

Recently, the Gram-negative bacterium *Escherichia coli* was shown to possess both the dimeric and hexameric form of citrate synthase (Patton et al. 1993; Gerike et al. 1998). In this case, the dimeric form was capable of accepting both acetyl-CoA and *n*-propionyl-CoA as substrates, showing that the enzyme possessed both citrate synthase (EC 4.1.3.7) and 2-methylcitrate synthase (EC 4.1.3.31) activity. Further investigations showed that some Archaea (*Pyrococcus furiosus* and *Thermoplasma acidophilum*) and a Gram-positive bacterium (*Arthrobacter* strain DS2-3R) possess dimeric citrate synthases that share the same double-substrate specificity (Gerike et al. 1998). These findings, together with sequence and structural comparisons, led to the proposal that citrate synthase and 2-methylcitrate synthase have arisen from one protein, with the retention of a common catalytic mechanism.

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In the present paper we give further evidence for this proposal, showing that *R. marinus* produces both dimeric and hexameric citrate synthases, with the dimeric enzyme accepting both substrates acetyl-CoA and *n*-propionyl-CoA. Interestingly, both enzymes are highly thermostable, providing a novel system in which to investigate, in the one organism, the nature and significance of inter-subunit interactions in protein thermostability.

Materials and methods

Microorganism and cultivation conditions

R. marinus strain R-10 (DSM 4252) was cultivated at 65°C in complex M162-medium (Degryse et al. 1978) supplemented with 2% (w/v) NaCl and 5 g/l xylan. Cultivation was performed in 1-l shake-flasks (300 ml of medium), on a shaking glycerol bath, or in a stirred-tank reactor (2-l volume, pH 7.2). Cultivations were also performed in the same medium supplemented with 10 mM sodium-propionate. A minimal medium (RSNO 1599) (Blücher et al. 2000) was used for a cultivation trial with 10 mM sodium-propionate as the only carbon source.

Purification of native citrate synthases

R. marinus cells were lysed using a single-shot cell disrupter (Constant Systems, Warwick, UK) in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 25 mM NaCl, followed by centrifugation (20,000 *g*, 20 min). The supernatant was applied to a Matrex Gel Red A (Amicon, UK) column (60 ml, d = 3 cm, h = 8.5 cm) equilibrated with the above Tris/EDTA/NaCl buffer, and unbound protein was removed by washing in three column volumes of the same buffer. Citrate synthase was biospecifically eluted by 1 mM CoA and 5 mM oxaloacetic acid in the Tris/EDTA/NaCl buffer. Citrate synthase-containing fractions were concentrated using Vivaspin concentrators (20 ml, MWCO 30 kDa; Vivascience, Lincoln, UK), and the buffer changed to 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 25 mM NaCl, 0.1 M KCl. The concentrate was loaded onto a Superdex-200 gel filtration column (Amersham-Pharmacia-Biotech., Amersham, UK), pre-equilibrated with the above Tris/EDTA/NaCl/KCl buffer, and the proteins were eluted at a flow rate of 1 ml/min. The column was calibrated with the following M_r markers: thyroglobulin (663 kDa, V_e = 45.8 ml), apoferritin (443 kDa, V_e = 51.4 ml), β-amylase (200 kDa, V_e = 58.0 ml), alcohol dehydrogenase (150 kDa, V_e = 69.8 ml), and serum albumin (66 kDa, V_e = 77.6 ml), and with blue dextran (V_0 = 39.4 ml).

N-terminal sequence analysis

Purified citrate synthase was electrophoresed through a 10% (w/v) polyacrylamide gel according to Laemmli (1970), and subsequently transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA., USA) by semidry electroblotting (Novablot, Pharmacia, Uppsala, Sweden). The membrane was stained with Coomassie Brilliant Blue, and the protein bands were sequenced using a 470A Applied Biosystems gas phase sequencer coupled to a phenylhydantoin analyzer.

Assay and kinetic analysis of citrate synthase and 2-methylcitrate synthase

Citrate synthase activity was assayed according to Srere et al. (1963), detecting the reaction product CoA, using the chromogenic thiol-specific reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The enzyme activity is described i n units (U), defined as the rate of produced reaction product in µmol/min. The assay mixture (1 ml) contained 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propane sulfonic acid) (EPPS), pH 8.0, 2 mM EDTA, 100 mM KCl, 0.1 mM DTNB, 0.2 mM oxaloacetic acid, and 0.2 mM acetyl-CoA. The assay temperature was 55°C unless otherwise stated. In 2-methylcitrate synthase assays, the acetyl-CoA was exchanged for *n*-propionyl-CoA, but otherwise performed as described above.

For the determination of kinetic parameters, the concentration of one substrate was varied while the second was kept at a saturating concentration (approximately $20 \times K_m$). K_m and V_{max} values were calculated from these data by the direct linear plot of Eisenthal and Cornish-Bowden (1974) using Enzpack3 computer software (Biosoft, Cambridge, UK).

Optimum temperature of enzymic activity

The dependence of catalytic activity on temperature was studied by assaying the initial citrate synthase activity (as described above) in the temperature range 40°–90°C, but in this case the EPPS buffer was changed to 50 mM sodium phosphate, pH 7.0, 2 mM EDTA, 0.1 M KCl.

Thermal inactivation

Thermal inactivation studies were carried out by incubating citrate synthase (2 µg/ml) in 50 mM sodium phosphate buffer, pH 7.0, 2 mM EDTA, 0.1 M KCl, at a defined temperature. Aliquots were removed at specified time intervals and rapidly cooled in ice. The remaining enzyme activity (incubated on ice for at least 1 h) was measured under the assay conditions described above.

The transition temperature (T_m) of the citrate synthase (0.1 mg/ml in 20 mM Tris-HCl buffer, pH 8.0, 2 mM EDTA, 0.25 mM NaCl, 0.1 M KCl) from the folded to unfolded state was measured by differential scanning calorimetry (DSC) using a VP-DSC from MicroCal., USA (Plotnikov et al. 1997), at a scan rate of 60°C/h and in the temperature range 20°–130°C.

Total protein estimation

Total protein concentrations were estimated by the method of Bradford (1974) using bovine serum albumin as standard.

Results

Citrate synthase purification

Citrate synthase was recovered from a cell extract prepared from an *R. marinus* stationary phase culture. Purification was performed in two steps (Table 1). After the Matrex Gel Red A affinity chromatography step, only two major bands (of approximately equal strength, and having M_r values of 42 and 45 kDa, respectively) were visible after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On subsequent gel filtration, two separate citrate synthases were eluted, with M_r values of approximately 100 kDa (V_e = 72.5 ml) and 280 kDa (V_e = 57.0 ml), assuming a spherical shape, suggesting that the two peaks represent native dimeric (n = 2.4) and hexameric (n = 6.2) forms of the enzyme. SDS-PAGE showed the hexameric protein to correspond to the upper electrophoretic band, and the dimeric protein to the lower band (Fig. 1).

N-terminal sequencing of citrate synthases

N-terminal sequencing and subsequent sequence comparisons were performed on both citrate synthases to establish whether the two native proteins were isoforms of the same monomer or if they were encoded by two separate genes. Phylogenetically, citrate synthases fit into three broad categories that correlate with the source organism: eukaryotes (length ≈430 amino acids; dimeric enzymes); Gram-negative bacteria (≈430 amino acids; includes both hexameric and dimeric proteins); and a group comprising the enzyme from Gram-positive bacteria and the Archaea (≈380 amino acids; dimeric enzymes). Thus the citrate synthases from the third group are all approximately 40–50 amino acids shorter at the N-terminus than those of the first two groups. Interestingly, the *E. coli* 2-methylcitrate synthase (Gerike et al. 1998), which also possesses citrate synthase activity and is of the "short type" (389 amino acids), falls within the third group.

Consistent with this pattern, the N-terminal amino acid sequence of the *R. marinus* hexameric citrate synthase aligns best with the N-termini of the Gram-negative bacterial enzymes, suggesting that it is of the longer type as seen on SDS-PAGE. The sequence from the dimeric citrate synthase aligns best with the N-termini of the third group, again consistent with its estimated M_r value by SDS-PAGE (Fig. 2). The results strongly suggest the existence of two separate gene products for the citrate synthases from *R. marinus*, and raise the possibility that the dimeric enzyme may be a 2-methylcitrate synthase. This is confirmed below.

Kinetic parameters

All enzyme assays were carried out at 55°C; this is approximately 10°C below the growth temperature optimum of the bacterium, but was chosen because of the thermolability of the substrate oxaloacetate. Whereas both hexameric and dimeric enzymes catalyzed the citrate synthase reaction using acetyl-CoA and oxaloacetate as substrates, the dimeric protein also exhibited 2-methylcitrate synthase activity, catalyzing the condensation of *n*-propionyl-CoA

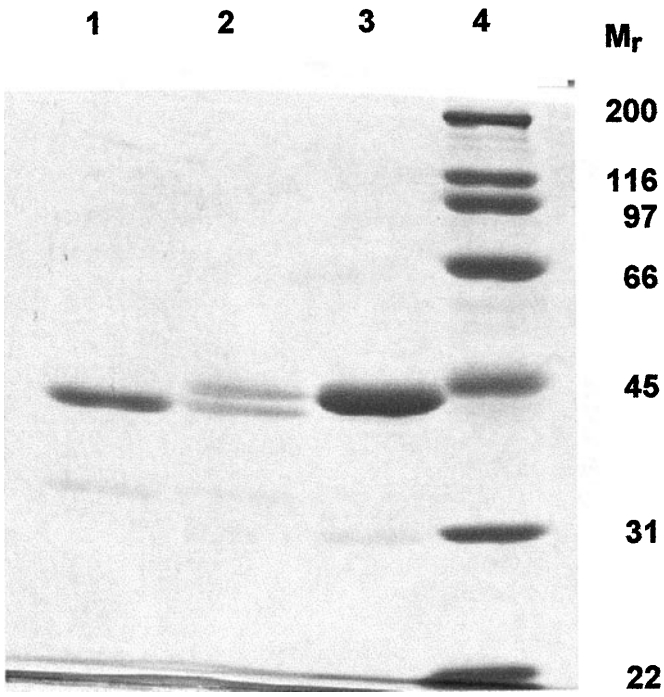

Fig. 1. SDS-PAGE of the purified citrate synthases from *Rhodothermus marinus*. *Lane 1* dimeric citrate synthase after gel filtration, *lane 2* the two citrate synthases after Matrex Gel Red A affinity chromatography, *lane 3* hexameric citrate synthase after gel filtration, *lane 4* marker proteins with their M_r values (kDa) indicated

Table 1. Purification of native citrate synthases from *Rhodothermus marinus*

Step	Enzyme	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)	Purification
Cell extract	Total CS	24	40	0.60	100	1
Affinity chromatography (Matrex Gel Red A)	Total CS	15	2.5	6	63	10
Gel filtration (Superdex 200)	Dimeric CS	6.7	0.24	28	28	47
	Hexameric CS	7.7	0.28	28	32	47

All enzyme activities were determined in *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propane sulfonic acid) (EPPS) buffer pH 8, at 55°C
CS, citrate synthase

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                        *        20         *        40         *        60
E.coli          -------madtkAkLtLngdTaVELdVLkGTLGqDVIDIrtL-gskGvFTFDPGFtSTASCESKI :  57
P.aeruginosa    -------madkkAqLiIegsapVELPVLsGTMGpDVVDVrgL-tatGhFTFDPGFmSTASCESKI :  57
A.aceti         msasqkegklstAtIsVdgkS-aEMPVLsGTLGpDVIDIrkLpaqlGvFTFDPGYgeTAaCnSKI :  64
R.tropici       -------mteqsAkLtwgekT-VdLPVktGTIGpsVIDIgaLykntstFTYDPGFtSTASCESsI :  57
C.glutamicum    mferdivatdnnkaVlhypggefEMdIIeaSeGnNgVvLgkMlsetGliTFDPGYvSTgStESKI :  65
R.marinus (6n)  ----------xtAkLiLddtT-VELPVIvG---------------------------------- :  18

A.DS2-3R        ---------------------------------------------mteptIhKGLagVtadvTaI :  20
M.smegmatis     ------------------------------------mttateseaprIhKGLagVvvdtTaI :  26
R.marinus (2n)  ---------------------------------------xxtteVkKGLagViadeSa- :  17
S.solfataricus  ------------------------------------------msvVsKGLenViikvTnL :  18
T.acidophilum   ---------------------------------------peteeIsKGLedVnikwTrL :  20
P.furiosus      ---------------------------------------ntekyLaKGLedVyidqTnI :  20
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Fig. 2. An alignment of the two N-terminal amino acid sequences obtained from the *Rhodothermus marinus* citrate synthases with sequences of known hexameric (*six upper sequences*) and dimeric (*six lower sequences*) citrate synthases (only the N-terminal parts of the citrate synthase sequences are shown). The hexameric citrate synthase sequences originate from *Escherichia coli*, *Pseudomonas aeruginosa*, *Acetobacter aceti*, *Rhizobium tropici*, *Corynebacterium glutamicum*, and *Rhodothermus marinus* (6n). The dimeric citrate synthase sequences originate from *Arthrobacter* strain DS2-3R, *Mycobacterium smegmatis*, *Rhodothermus marinus* (2n), *Sulfolobus solfataricus*, *Thermoplasma acidophilum*, and *Pyrococcus furiosus*. Identically conserved residues within the sequence group of the "long type" are shown in *normal uppercase*. Identities within the group of sequences of the "short type" are identified in *bold type*. Nonconserved residues are shown in *lowercase*. The letter *x* denotes positions where the amino acid could not be definitely assigned

Table 2. Kinetic parameters for the *Rhodothermus marinus* citrate synthases

Parameter	Dimeric citrate synthase	Hexameric citrate synthase
K_m acetyl-CoA (μM)	7 ± 1	12 ± 2
K_m oxaloacetic acid (μM)	3 ± 0.3	6 ± 2
V_{max} (μmol min^{-1} mg^{-1})	32 ± 1	28 ± 3
k_{cat} (s^{-1})	22 ± 1	21 ± 2
k_{cat}/K_m (μM^{-1} s^{-1})	3 ± 0.5	2 ± 0.3
K_m *n*-propionyl CoA (μM)	3 ± 1	No activity
K_m oxaloacetic acid (μM)	2 ± 0.5	No activity
V_{max} (μmol min^{-1} mg^{-1})	17 ± 2	No activity
k_{cat} (s^{-1})	12 ± 2	–
k_{cat}/K_m (μM^{-1} s^{-1})	4 ± 2	–

All enzyme activities were determined in EPPS buffer pH 8, at 55°C. In the calculation of k_{cat} values, it is assumed that there is one active site per citrate synthase monomer

and oxaloacetate to form 2-methylcitrate. The hexameric citrate synthase gave no detectable rate with *n*-propionyl CoA as substrate.

Both enzymes followed Michaelis–Menten kinetics, and the kinetic data for the two enzymes are summarized in Table 2. It is clear that the dimeric enzyme has very similar k_{cat}/K_m values with acetyl-CoA and *n*-propionyl-CoA, suggesting that it functions equally well with both substrates.

Production of citrate synthase and 2-methylcitrate synthase in *R. marinus*

In *E. coli*, the hexameric citrate synthase is produced constitutively, whereas the dimeric 2-methylcitrate synthase can be induced by growth on propionate as the sole carbon source; in the latter case, the catabolism of propionate to succinate and pyruvate via 2-methylcitrate has been proposed (Gerike et al. 1998).

The finding of these two enzymes in *R. marinus* led us to investigate whether their production was similar to those in *E. coli*. Consequently, to evaluate whether the dimeric 2-methylcitrate synthase enzyme was inducible, the growth of *R. marinus* in the presence and absence of propionate and the concomitant levels of citrate synthase and 2-methylcitrate synthase were investigated. The results are summarized in Fig. 3. No cell growth was observed using sodium propionate as the sole carbon source. Therefore, cultivations were performed using the complex medium [M162 + 2% (w/v) NaCl + 5 g/l xylan] with or without the addition of 10 mM sodium propionate. No difference in the specific activity or in the relative 2-methylcitrate synthase/citrate synthase activity could be observed when compared to the control cultivation without propionate. Furthermore, the specific activity for both substrates (acetyl-CoA and *n*-propionyl-CoA) remained nearly constant at different stages of the growth curve, with only a slightly higher level in the early logarithmic growth phase. These results strongly suggest a constitutive production of both enzymes.

Temperature dependence of enzyme activity

The temperature optima for the purified enzymes were determined from enzyme assays at differing temperatures between 40° and 90°C. Phosphate buffer was used due to its low temperature coefficient (dpH/dT), and therefore only minor adjustments to the pH value were needed in the temperature interval. A high concentration of oxaloacetate ($30 \times K_m$) was used in the assays, such that there would be no decrease in enzymic activity due to the substrate's thermolability over the time-course of the assay (1 min).

The activity profiles of the two enzymes were similar, with optima for activity around 85°C (Fig. 4), reaching a

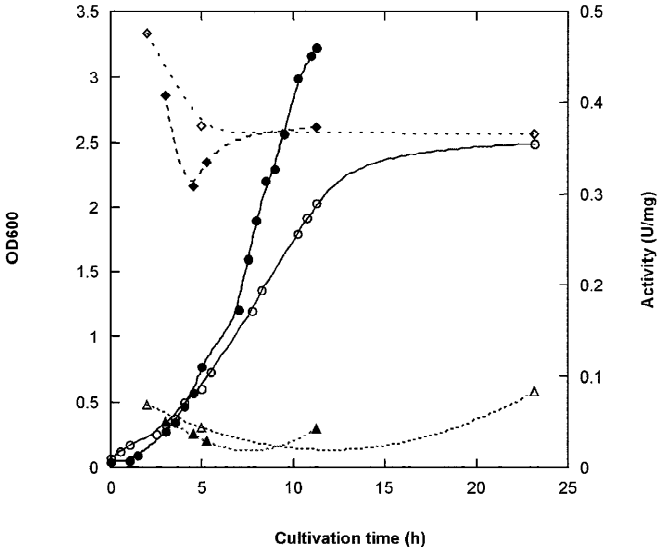

Fig. 3. Growth of *Rhodothermus marinus*, and citrate synthase and 2-methyl citrate synthase activity in unfractionated cell extracts. The cell growth is displayed as the optical density at 600 nm in the presence (*open circles*) and absence (*solid circles*) of propionate. Also indicated are citrate synthase activity in the presence (*open diamonds*) or absence (*solid diamonds*) of propionate, and 2-methyl citrate synthase activity in the presence (*open triangles*) and absence (*solid triangles*) of propionate

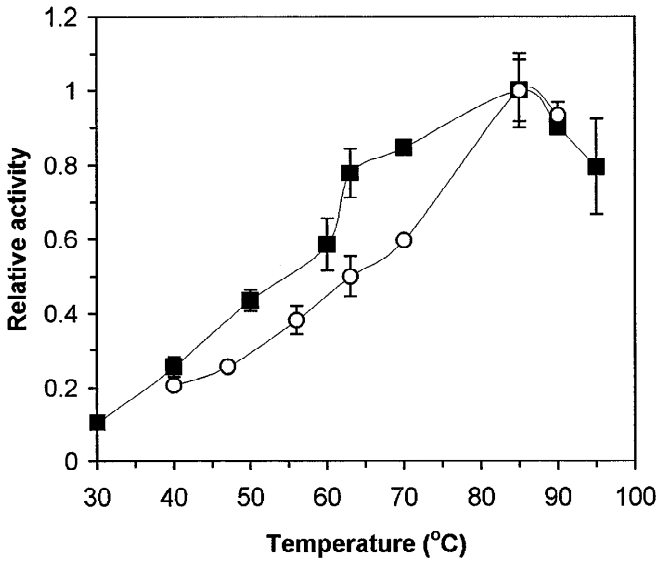

Fig. 4. The temperature dependence of enzyme activity for the hexameric (*solid squares*) and dimeric (*open circles*) citrate synthases from *Rhodothermus marinus*. Activity is shown as the ratio of the measured activity at each temperature to the maximum activity at the apparent T_{opt}. Assays were carried out using saturating concentrations of substrates (acetyl-CoA and oxaloacetate) in 50 mM sodium phosphate buffer, pH 8, 2 mM EDTA, 0.1 M KCl, at a protein concentration of 0.2 μg/ml in the cuvette

specific activity of approximately 60 U/mg. They are active even at mesophilic temperatures, and at 40°C the enzymes retain 20%–30% of the maximum activity.

Thermostability

In terms of thermostability, as measured by irreversible thermal inactivation, the hexameric enzyme appears to be the more stable of the two citrate synthases. The irreversible thermal inactivation of both dimeric and hexameric enzymes showed first-order kinetics at all temperatures tested. For example, the inactivation constants for the hexameric and dimeric enzymes at 80°C (slightly below the observed temperature optima) were 0.004 (±0.001) min^{-1} (resulting in a half-life of 2.9 h) and 0.025 (±0.004) min^{-1} (half-life of 28 min), respectively (using 2 μg protein/ml during the inactivation). At 90°C, which is slightly above the obtained activity optimum, the inactivation constants were 0.038 (±0.003) min^{-1} for the hexameric enzyme (half-life of 18 min) and 0.056 min^{-1} for the dimeric enzyme (half-life of 12 min). In the DSC analysis obtained for the hexameric enzyme, a single transition temperature (T_m) was observed at 94°C. However, two peaks were observed for the dimeric citrate synthase, at 85° and 95°C.

Discussion

In this article, we provide evidence that *R. marinus* possesses both a hexameric and a dimeric citrate synthase; the dimeric enzyme also exhibits 2-methylcitrate synthase activity and, from the relative k_{cat}/K_m values, appears to use both acetyl-CoA and propionyl-CoA equally well. To establish this, both enzymes were purified to apparent homogeneity and their kinetic and thermostability parameters determined. N-terminal sequence analyses and database comparisons are consistent with the two enzymes being the products of separate genes: the hexameric sequence aligns with the N-terminal sequences of other hexameric citrate synthases from the Gram-negative bacteria, whereas the dimeric enzyme aligns best with the group of shorter citrate synthases, all dimers, comprising the enzyme from Gram-positive bacteria, the Archaea, and the *E. coli* 2-methylcitrate synthase.

The situation in *R. marinus* thus reflects that found in *E. coli* (Textor et al. 1997; Gerike et al. 1998), except that in *E. coli* the 2-methylcitrate synthase is inducible by growth of the organism on propionate whereas in *R. marinus* it appears to be produced constitutively. Indeed, *R. marinus* could not be grown on propionate as the sole carbon source, and inclusion of this metabolite into a complex growth medium had no effect on the levels of 2-methylcitrate synthase produced. The role of this enzyme in *R. marinus*, therefore, might be to metabolize propionyl-CoA generated internally, for example, from the catabolism of odd-number fatty acids or the amino acids methionine, isoleucine, valine, and threonine.

As pointed out by Gerike et al. (1998), hexameric and dimeric "citrate synthases" have been seen in other Gram-negative bacteria, principally *Acinetobacter lwoffi* (Weitzman et al. 1978) and *Pseudomonas aeruginosa*

(Mitchell et al. 1995); however, there are no reported data on the 2-methylcitrate synthase activity of these enzymes. Nonetheless, our data on the enzymes from the Gram-negative *R. marinus* add strength to the argument that this is a common phenomenon within these bacteria, and highlight the need to define the possible enzymic activities carefully when more than one "citrate synthase" is found in a single organism, especially from genomic sequence comparisons.

A sequence comparison of the two enzymes from *E. coli* suggests that citrate synthase and 2-methylcitrate synthase probably share a common catalytic mechanism and result from the duplication and divergence of a single ancestral gene (Gerike et al. 1998). They share an identity of 30% (53% similarity) and many of the active site residues are conserved. Similar data from the recently sequenced genome of *Pseudomonas aeruginosa* support this idea, with the hexamer and dimer sharing a 32% identity (57% similarity). Between the two species, the hexameric citrate synthases are 69% identical (82% similarity), and the dimers are 52% identical (71% similarity). The available sequence data from presently known enzymes with and without 2-methylcitrate synthase activity do not, however, allow assignment of specific residues of importance for the different activity profiles. Sequence data from the more distantly related *R. marinus* will be important additions to these comparisons, and the isolation of the genes is now a high priority.

The thermostable nature of the enzymes from *R. marinus* adds a further dimension to the comparative study of citrate synthases, since the data in this paper describe the first reported thermostable hexameric citrate synthase. We are now in a position to explore the nature of subunit interactions with respect to protein thermostability by obtaining amino acid sequences and structural data on dimeric and hexameric forms of citrate synthase from the same organism. All our previous structural data are from dimeric citrate synthases from the Archaea (reviewed in Danson and Hough 1998), and the opportunity to bring a hyperstable hexameric homologue from a thermophilic bacterium into this study will be invaluable. The parallel evolution of a thermostable 2-methylcitrate synthase will also be important to this work, especially as the *E. coli* homologue is an unstable protein (Patton et al. 1993).

Finally, it is interesting to compare the T_{opt} values for activity, and the rates of thermal inactivation, of both *R. marinus* enzymes. That is, the two enzymes have optimal activity at approximately 85°C (assayed over a 1-min time interval), whereas even at 90°C their half-lives for thermal inactivation are 18 and 12 min for the hexamer and dimer, respectively. Clearly, factors other than stability to irreversible thermal inactivation determine the T_{opt} values, and these may include reversible conformational changes to the enzymes at these temperatures. We have recently formulated a quantitative model to explain this phenomenon, which we and others have noted (Daniel et al. 2001, and references therein), and *R. marinus* will no doubt prove to be a useful addition to our studies on the structural relationship between enzyme thermoactivity and thermostability.

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