Anomalous Citrate Synthase from *Thermus aquaticus*

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**INTRODUCTION**

Our previous studies of citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] from a range of organisms have shown that there is a correlation between the molecular and regulatory properties of the enzyme and the taxonomic grouping of the organism (Weitzman & Jones, 1968, 1975; Weitzman & Dunmore, 1969; Weitzman & Danson, 1976). Citrate synthases from Gram-negative bacteria are 'large' (mol. wt approx. 250000) whereas those from Gram-positive bacteria and eukaryotic organisms are 'small' (mol. wt approx. 100000). Regulation through allosteric inhibition by NADH occurs only with the Gram-negative bacterial enzymes and appears to be connected with their more complex quaternary structure.

There has been much interest in thermophilic organisms and the stability of their enzymes. An enhanced thermostability was reported for citrate synthase from the Gram-positive moderate thermophile *Bacillus stearothermophilus* (Higa & Cazzulo, 1976) but it was otherwise very similar to that from mesophilic Gram-positive bacteria. The present communication describes some studies on the citrate synthase from the extreme thermophile *Thermus aquaticus*. This organism was first isolated by Brock & Freeze (1969) from both natural thermal springs and man-made hot water supplies. It has an optimum growth temperature of 70 °C and is Gram-negative. The results reported here show that citrate synthase of *T. aquaticus* is an exception to the general correlation between molecular size of the enzyme and Gram stain of the bacterium.

**METHODS**

*Growth of bacteria and preparation of extract.* A culture of *T. aquaticus* strain YT-1 (ATCC 25104) was kindly provided by Drs T. D. Brock (University of Wisconsin) and P. H. Ray (University of Kentucky). Bacteria were grown at 70 °C for about 24 h in 200 ml basal salts medium (Ramaley & Hixson, 1970) containing 0.2% (w/v) tryptone and 0.2% (w/v) yeast extract in 500 ml flasks shaken in a rotary waterbath shaker at 200 rev. min⁻¹. When the *A₄₅₀* value was approx. 1.6 (corresponding to about 2.5 × 10⁸ cells ml⁻¹; Freeze & Brock, 1970), the bacteria from two flasks were collected by centrifuging at 25000 g for 10 min, washed with buffer (20 mM-Tris/HCl, pH 8.0, 10 mM-MgCl₂, 1 mM-EDTA), resuspended in 5 ml buffer and disrupted ultrasonically in an MSE 100 W sonicator operated for 2 min at full power with cooling. Cell debris was removed by centrifuging at 25000 g for 10 min and the yellow supernatant solution was used for studies on citrate synthase. The protein content of this extract was 32 mg ml⁻¹ and the specific activity of citrate synthase was 0.04 μmol min⁻¹ (mg protein)⁻¹. *Thermus aquaticus* was also obtained as a frozen cell paste from the Microbiological Research Establishment, Porton Down, Wiltshire, and an ultrasonic extract was prepared as described above.

Purified pig heart citrate synthase was obtained from Boehringer.

*Enzyme studies.* Citrate synthase activity was assayed at 25 °C by either the spectrophotometric or the polarographic method (Weitzman & Jones, 1968). Unless otherwise stated, reaction mixtures contained 0.1 M-Tris/HCl, pH 8.0, 0.2 mM-oxaloacetate and 0.15 mM-acetyl-CoA; the spectrophotometric assay also required 0.1 mM-5,5'-dithiobis(2-nitrobenzoate).

Gel filtration was carried out at 4 °C on a column (2.5 × 35 cm) of Sephadex G-200 as previously described (Weitzman & Dunmore, 1969), using catalase (beef liver; Sigma) and lactate dehydrogenase (rabbit muscle;
Boehringer) as marker proteins. The eluting buffer was 20 mM-Tris/HCl, pH 8.0, containing 10 mM-MgCl₂ and 1 mM-EDTA.

 Thermal inactivation experiments were done by heating 0.2 ml portions of enzyme in pre-equilibrated glass tubes at various temperatures. After 5 min the tubes were cooled quickly in ice/water and the activity of the enzyme was assayed in the usual way.

RESULTS AND DISCUSSION

Citrate synthase was readily detected in crude extracts prepared as described in Methods. The polarographic assay was especially useful as the yellow colour of the extract produced no interference. There was no effect of NADH (0.5 mM) on enzymic activity; this result immediately differentiated the *T. aquaticus* citrate synthase from other Gram-negative bacterial citrate synthases. When the extract was subjected to gel filtration with catalase (mol. wt approx. 250000) and lactate dehydrogenase (mol. wt approx. 140000), citrate synthase was eluted after lactate dehydrogenase, indicating that *T. aquaticus* citrate synthase is of the ‘small’ type. The elution peak of *T. aquaticus* citrate synthase coincided precisely with that of the typical ‘small’ citrate synthase from pig heart, which has a mol. wt of 96000 to 100000 (Singh, Brooks & Srere, 1970; Wu & Yang, 1970). The molecular size of the enzyme thus also distinguishes it from other Gram-negative bacterial citrate synthases and indicates that the thermophile enzyme more closely resembles citrate synthases from Gram-positive bacteria and eukaryotic organisms (Weitzman & Dunmore, 1969). The enzyme eluted from the Sephadex column was not inhibited by NADH. These results were confirmed by similar experiments done on enzyme isolated from *T. aquaticus* obtained from the Microbiological Research Establishment.

The $K_m$ value of the enzyme for acetyl-CoA was 7 $\mu$M at pH 8.0 in 0.1 M-Tris/HCl in the presence of 0.2 mM-oxaloacetate. This low value is characteristic of ‘small’ citrate synthases and contrasts with the much higher $K_m$ values determined for the ‘large’, NADH-sensitive, Gram-negative bacterial citrate synthases (Weitzman & Danson, 1976).

Those citrate synthases which are insensitive to NADH are inhibited by ATP which, because of structural similarity, probably competes with acetyl-CoA for the active site (Harford & Weitzman, 1975; Weitzman & Danson, 1976). *Thermus aquaticus* citrate synthase was inhibited by 20, 40 and 60% in the presence of 1, 2 and 5 mM-ATP, respectively, and 15 $\mu$M-acetyl-CoA.

The regulatory properties of *T. aquaticus* citrate synthase reported above were observed at 25 °C, i.e. at a very much lower temperature than that for optimum growth of the organism. It was therefore conceivable that measurements made at a higher temperature might reveal different properties. However, assays done at 65 °C showed no inhibition by NADH, but sensitivity to ATP was similar to that observed at 25 °C.

The thermal stability of *T. aquaticus* citrate synthase was examined by heating pooled peak fractions of the enzyme from the gel filtration experiment at various temperatures (Fig. 1). No precipitation of protein occurred during heating. The enzyme was markedly resistant to thermal inactivation, a property shared by several other Thermus enzymes (Singleton & Amelunxen, 1973; Williams, 1975). The thermostability presumably results from a relatively rigid molecular conformation which might also be expected to resist other forms of denaturation. This was tested by examining the effect of urea on citrate synthases from *T. aquaticus* and pig heart, the latter serving as a mesophilic comparison. Various concentrations of urea were incorporated into the standard spectrophotometric assay mixture and the rates were recorded without prior incubation of the enzyme with urea. The pig heart enzyme activity was reduced to one-half by 1.5 M-urea, whereas *T. aquaticus* citrate synthase required 2.5 M-urea to produce the same fractional loss of activity.

The results reported here suggest that a 'small' form of citrate synthase is better able to achieve the extreme thermostability required by the *T. aquaticus* enzyme, even though this represents a marked abnormality for a Gram-negative bacterium. Our studies of a number of
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Fig. 1. Thermal inactivation of *T. aquaticus* citrate synthase. The enzyme was heated for 5 min at the temperatures indicated, cooled and assayed for activity by the spectrophotometric method.

mutants of *Acinetobacter* have revealed both ‘large’ and ‘small’ mutant forms of citrate synthase and have shown the ‘small’ enzymes to be more resistant to thermal inactivation than the ‘large’ forms (Weitzman *et al.*, 1978). Since sensitivity to allosteric regulation by NADH is exclusively a feature of ‘large’ citrate synthases, it would appear that *T. aquaticus* has sacrificed this general Gram-negative bacterial mode of control in order to acquire the requisite degree of enzymic thermostability. We have found that other Gram-negative Thermus-like bacteria isolated from hot-water supplies in this laboratory also produce thermostable ‘small’ citrate synthases which are insensitive to NADH (C. Lucas & P. D. J. Weitzman, unpublished results).

Brock (1967) has proposed that thermophiles may have sacrificed efficiency and control of enzyme function in order to grow at high temperatures, as their rigid, thermostable, proteins might not possess the flexibility required for allosteric interactions with small molecules. The properties of citrate synthase from *T. aquaticus* are consistent with this proposal. However, other studies have shown enzymes from thermophiles to exhibit allosteric regulatory phenomena similar to those observed with mesophiles (Singleton & Amelunxen, 1973; Williams, 1975), suggesting that Brock’s speculation cannot be generally applicable.

The only other exception to the general association of Gram-negative bacteria with ‘large’ citrate synthases is the citrate synthase from *Halobacterium* spp. (Cazzulo, 1973; Weitzman & Danson, 1976). The halobacteria contain extremely high intracellular salt concentrations – conditions which, like high temperature, may not be conducive to the stable existence of a ‘large’ citrate synthase. The production by these bacteria of the ‘small’ type of citrate synthase, with concomitant loss of NADH regulation, may reflect the evolutionary adaptation of these organisms to halophilic life (Cazzulo, 1973). It is certainly remarkable that the only naturally occurring Gram-negative bacteria so far found to produce ‘small’ citrate synthases are those which have adapted to an extreme mode of existence.

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REFERENCES


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