The Citrate Synthase from *Bacillus Stearothermophilus*

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Summary. Citrate synthase has been purified to apparent homogeneity from *Bacillus stearothermophilus*. Its kinetic and regulatory properties, and molecular weight, are similar to those of the enzymes from suitable mesophilic counterparts, but its thermal stability is considerably greater.

Citrate synthase (EC 4.1.3.7), a key regulatory enzyme for the control of the tricarboxylic acid cycle[2], has been purified from several Gram negative bacteria[3–6] and eucaryotes[7–9]; the instability of the enzyme from Gram positive bacteria[4, 10, 11] has so far prevented its obtention in a homogeneous or highly purified state. This paper describes the purification of the citrate synthase from the thermophile *Bacillus stearothermophilus* and some of its properties.

Methods. B. stearothermophilus strain 1503–4R[12] was grown at 65 °C in a New Brunswick G-76 shaker, in a medium containing 13 g of Nutrient Broth No. 1 (Oxoid), 3 g NaCl and 0.38 g $CaCl_2$ per l, adjusted to pH 7. The cells were harvested at the beginning of the stationary phase of growth ($E_{680\,nm}$ of about 0.8). The crude extract obtained after lysozyme digestion[13] was fractionated with ammonium sulfate; the 70–100% saturation fraction was dissolved in 20 m*M* phosphate buffer (pH 7.0) and 1 m*M* EDTA, dialyzed against the same buffer, and applied to a DEAE-cellulose column (12 × 1.6 cm), which was eluted stepwise with buffer solutions of similar composition and increasing concentrations of KCl. The active fractions, eluted by the buffer solution containing 0.13 *M* KCl, were pooled and dialyzed against 10 m*M* phosphate buffer (pH 7.0), 0.1 m*M* EDTA, 0.2 *M* KCl, and chromatographed on a hydroxylapatite column (1.6 × 1.1 cm) equilibrated with the same buffer. The column was eluted stepwise with buffer solutions of similar composition and increasing phosphate concentration. The enzyme eluted with 40 m*M* potassium phosphate was purified about 120-fold with a yield of 26% (Table I), and was homogeneous to polyacrylamide gel electrophoresis[14]. The dilute enzyme solutions from step 4 (10–20 µg of protein/ml) lost about 10% of their activity when kept for 40 days at 7 °C.

The approximate molecular weight of the thermophilic citrate synthase was determined by gel filtration[15] through a Sephadex G-200 column (32.5 × 1.2 cm). The elution volumes of malate dehydrogenase, pig heart citrate synthase, *B. stearothermophilus* citrate synthase, lactate dehydrogenase, catalase, pyruvate kinase and Blue Dextran 2000 were 26.0; 24.7; 24.3; 21.6; 19.5; 18.5 and 13.4 ml, respectively. The elution volumes of both citrate synthases were determined in different experiments. The data indicate that the molecular weight of the thermophilic enzyme, as in the case of the citrate synthases from other Gram positive bacteria[16], is similar to that of the mammalian enzyme, 100,000 (ref. [8]).

The enzyme activity was assayed spectrophotometrically at 30 °C, unless stated otherwise, as previously described[17], in the presence of 0.1 *M* KCl and 0.025 m*M* acetyl-CoA.

Results and discussion. The purified citrate synthase was slightly activated by KCl, NaCl or NH_4Cl, all of which caused a maximal activation of about 25% at 0.1 *M*. As demonstrated for other citrate synthases[4, 8, 9, 18, 19], the double-reciprocal plots obtained for each substrate at

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Table I. Purification of the citrate synthase from *Bacillus stearothermophilus*

Purification step	Volume (ml)	Total protein (mg)	Total enzyme activity (µmoles/min)	Specific activity (µmoles/min/mg of protein)	Purification	Yield (%)
Crude extract	20	188	58.2	0.31	1	100
Ammonium sulfate fractionation	1.9	24.3	24.9	1.02	3.3	42
DEAE-cellulose chromatography	23.1	2.1	20.6	9.81	32	35
Hydroxylapatite chromatography	32.6	0.42	15.1	35.95	116	26

Table II. Effect of temperature on the kinetic constants of the reaction catalyzed by the citrate synthase from *Bacillus stearothermophilus*.

Temperature (°C)	Apparent K_m for acetyl-CoA (μM)	Apparent K_m for oxaloacetate (μM)	Turnover number (min^{-1})
30	3.7	1.1	4000
37	4.4	1.3	7000
43.5	6.1	2.1	11100
50	6.9	2.9	21200
55.4	9.1	3.3	28200
62	13.5	3.3	38800

The reaction mixtures were as previously described[17], except that 20 mM potassium phosphate buffer (pH 7.6) was used instead of *Tris*-HCl, 0.1 M KCl was present, and the concentrations of acetyl-CoA or oxaloacetate were varied. When used as the fixed substrate, the concentrations of acetyl-CoA and oxaloacetate were 0.05 and 0.25 mM, respectively. The turnover number values were calculated from the V_{max} values obtained from the double-reciprocal plots for acetyl-CoA at a saturating concentration of oxaloacetate, employing the molecular weight of 100,000 estimated from gel filtration experiments.

several concentrations of the co-substrate intersected at the abcissa, showing that the K_m values were independent of the concentration of the co-substrate. As in the case of the citrate synthases isolated from other Gram positive bacteria[20,21], the thermophilic enzyme was not inhibited by NADH or α-oxoglutarate concentrations up to 0.5 mM or 5 mM, respectively, but was inhibited by ATP and less effectively by ADP; AMP was ineffective at concentrations up to 5 mM. The inhibition by 5 mM ATP or ADP (62% and 28%, respectively) was the same either in the presence or in the absence of KCl (0.1 M), at 30 °C or at 62 °C. As in the case of other bacterial citrate synthases, with the exception of the enzyme from *B. subtilis* HS 2A2[4], the inhibition by both nucleotides at 30 °C was strictly competitive towards acetyl-CoA, and non-competitive towards oxaloacetate. The apparent K_m for acetyl-CoA (27 μM oxaloacetate) was increased from 3.7 μM in the absence of inhibitors to 10.0 μM and 31.2 μM in the presence of 3 mM ADP or ATP, respectively, without change in the apparent V_{max}. The apparent K_m for oxaloacetate (50 μM acetyl-CoA), on the other hand, was not changed in the presence of 3 mM ADP or ATP, whereas the apparent V_{max} decreased by 19% and 45%, respectively.

The citrate synthase from *B. stearothermophilus* showed considerably greater thermal stability than the enzyme from pig heart, which has similar kinetic and regulatory properties. The latter, as a 20 μg/ml solution in 20 mM potassium phosphate buffer (pH 7.6) and 1 mM EDTA, was completely inactivated after heating for 2 min at 50 °C. A 10 μg/ml solution of the thermophilic enzyme in the same buffer, on the other hand, decayed only by 32% after heating for 20 min at 74 °C. Half-lives were not calculated, since the decay of the thermophilic enzyme did not follow first-order kinetics, being considerable within the first 3 min of incubation, and becoming much slower afterwards. A similar finding has been reported for the 5,10-methylenetetrahydrofolate dehydrogenase from *Clostridium tetanomorphum*[22], and has been interpreted as a possible conversion of the enzyme into a more stable, but less active, form during heating.

The apparent K_m values for both substrates increased about 3-fold when the temperature was raised from 30 °C to 62 °C (Table II). The turnover number of the thermophilic enzyme at 62 °C, 38,800 min^{-1} (Table II) was 3 times higher than the value reported for the rat heart enzyme at 28 °C, 13,000 min^{-1} (ref. [8]).

The Arrhenius plot of the natural logarithm of the turnover number values given in Table II as a function of the reciprocal absolute temperature values was linear from 30 °C to 62 °C; at higher temperatures (not shown), a decrease was observed. The activation energy calculated from the Arrhenius plot was 15 kcal/mol. The corresponding value for the pig heart enzyme was 9.7 kcal/mol at temperatures higher than 20 °C, and 13.7 kcal/mol at temperatures lower than 20 °C (ref. [23]).

The results described in this paper show that the citrate synthase from *B. stearothermophilus* shares the kinetic and regulatory properties and the molecular weight of the enzymes isolated from suitable mesophilic counterparts; only its thermal properties, particularly its considerable stability, seem to differ from those of the citrate synthases from mesophilic Gram positive bacteria and eucaryotes.

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Regional and Subcellular Distribution of Superoxide Dismutase in Brain

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Summary. Superoxide dismutase has been found to be widely distributed and of approximately the same specific activity in all regions of human brain examined. It is not reduced during degeneration of the basal ganglia in Huntington's Chorea. After subcellular fractionation of human and guinea-pig cerebral cortex, the highest specific activity of the enzyme was found in the soluble fraction.

Superoxide dismutase (SOD) has been demonstrated in a variety of tissues and cell types and appears to protect against the toxic effects of the O_2-radical[2]. Recent studies[3–6] indicate that very high levels of activity are present in liver, while the adrenals, kidney and red blood cells have intermediate activity and lower activities were found in most other tissues including brain. We have been interested in possible changes in SOD in nervous tissue in certain neurological diseases, and we report here the distribution of SOD in guinea-pig and human brain.