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BACTERIAL CITRATE SYNTHASES: PURIFICATION, MOLECULAR WEIGHT AND KINETIC MECHANISM

DONOVAN E. JOHNSON* and RICHARD S. HANSON
Department of Bacteriology, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)
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SUMMARY

Citrate synthase (citrate oxaloacetate-lyase, CoA-acetylating, EC 4.1.3.7) from cells of *Azotobacter vinelandii* and *Acinetobacter anitratum* was purified to apparent homogeneity. The molecular weight of native citrate synthase from *A. anitratum* was determined to be 242 000, and the enzyme from *A. vinelandii* had a molecular weight of 250 000. The subunit molecular weights of *A. vinelandii* and *A. anitratum* citrate synthases appeared identical. Each had a monomeric molecular weight, determined by sedimentation equilibrium studies, of 58 500–59 000. Purification of citrate synthase from *Bacillus subtilis* was hampered by low specific activity levels and poor enzyme stability and was not purified to homogeneity. The molecular weight of the active enzyme was determined to be 60 000–95 000 by acrylamide gel electrophoresis.

The patterns observed from initial velocity and product inhibition studies of citrate synthase catalysis are consistent with those predicted for a random sequential reaction order mechanism. Extensively purified citrate synthase from each of the three genera studied gave similar kinetic patterns, despite variations in enzyme size and regulatory complexity.

INTRODUCTION

The tricarboxylic acid cycle serves both catabolic and anabolic needs for energy production and biosynthesis in many procaryotic and euracyotic cells. The entry of acetyl-coenzyme A (acetyl-CoA) into this pathway is catalyzed by the enzyme citrate synthase (citrate oxaloacetate-lyase, CoA-acetylating, EC 4.1.3.7). Regulation of the synthesis and activity of this enzyme in bacteria have been reported previously [1–5], but little is known about its physical properties or its mechanism of catalysis.

Citrate synthase appears to be widely distributed among animal and plant tissues, as well as microorganisms. The enzyme was first obtained in impure crystalline form from pig heart [6], and has since been examined in various degrees of purity

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

* Present address: Northern Regional Research Laboratory, A.R.S., U.S.D.A., Peoria, Ill. 61604, U.S.A.

from both eucaryotic and procaryotic sources. Citrate synthase has been purified to homogeneity from pig heart [7, 8], rat liver and rat heart [9], *Escherichia coli* [10], and baker's yeast [11]. Good agreement exists between the molecular weights of mammalian native citrate synthase (96 000–100 000) and subunit composition (two identical units, of 50 000 molecular weight). The enzyme isolated from *E. coli* has a molecular weight of $2.1 \cdot 10^5$–$2.8 \cdot 10^5$ [12].

Few substrate or product inhibition studies have been performed with purified citrate synthase although Michaelis constants (K_m values) and ATP inhibition constants (K_i (ATP)) have been established for citrate synthase from a variety of organisms [12]. Moriyama and Srere [9] performed initial velocity studies on rat liver and rat heart citrate synthase and observed similar intersecting patterns in double reciprocal plots for each enzyme. Similar kinetic analysis have been reported for other citrate synthases by Shepard and Garland [13] and Srere [12].

Studies with ATP and/or NADH as inhibitors gave competitive inhibition patterns with respect to acetyl-CoA and non-competitive or mixed inhibition patterns with respect to oxaloacetate [3, 4, 9, 11, 13–15].

In the present study, initial velocity patterns and product inhibition kinetics were used to determine the reaction order mechanism of citrate synthase catalysis from three bacterial sources. Molecular weight data are presented for each native enzyme, together with suggested subunit compositions and molecular weights.

MATERIALS AND METHODS

Organisms and media

Bacillus subtilis HS2A2 was isolated by Carls and Hanson [16] from *B. subtilis* Marburg 168, a tryptophan auxotroph. *B. subtilis* HS2A2 is an isocitrate dehydrogenase mutant (IDH^-), but has normal levels of all the remaining tricarboxylic acid cycle enzymes. Citrate synthase was purified from this mutant rather than the wild type strain in order to eliminate the high levels of isocitrate dehydrogenase that co-purified with citrate synthase. Growth of all liquid cultures was accomplished in 1.5% Heart Infusion Broth (Difco), and routine storage was on purification medium containing Nutrient Agar (Difco), 0.2% glucose, trace metals, and 0.00015% bromcresol purple [16]. For the growth of *B. subtilis* HS2A2 in fermentors, the medium was supplemented with a glucose feed (100 mg/l per h, started approx. 2 h after inoculation) in order to maintain the culture in the logarithmic growth phase at low glucose concentrations. High glucose levels repress the synthesis of citrate synthase [3].

Azotobacter vinelandii OP was grown in a modified Burk's nitrogen-free medium [17] and was kindly provided by Dr W. J. Brill (University of Wisconsin, Madison, Wisconsin).

Acinetobacter anitratum was obtained from the culture collection of the Department of Bacteriology, University of Wisconsin. The organism was grown on a mineral salts medium which contained 14 g K_2HPO_4; 6 g KH_2PO_4; 5 g sodium acetate; 0.2 g $MgCl_2 \cdot 6H_2O$; 6.8 mg $ZnCl_2$; 9.9 mg $MnCl_2 \cdot 4H_2O$; 0.16 mg $FeCl_3$; 10 mg $CaCl_2 \cdot 2H_2O$; and distilled water to 1000 ml.

All cells grown for enzyme purification were harvested during the middle to late logarithmic growth phase in a refrigerated Sharples continuous flow centrifuge. Wet, packed cells were divided into 50-g quantities and stored frozen at −20 °C.

Reagents

The buffer used for all purification procedures and assays was 10 mM Tris–EDTA–citrate buffer. It contained 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.1 mM EDTA, 1.0 mM sodium citrate, and was adjusted to pH 7.8 with 1 M acetic acid. Oxaloacetate was prepared daily in 10 mM Tris–EDTA–citrate buffer and pH was adjusted to 6.8 with 1 M $KHCO_3$. Acetyl-CoA was prepared by the method of Stadtman [18] and stored in 1.0-ml aliquots at −20 °C. 5,5′-dithiobis-(2-nitrobenzoic acid)(DTNB) was prepared daily as needed in 10 mM Tris–EDTA–citrate buffer, pH 7.8.

Fibrous DEAE-cellulose and Whatman microgranular DEAE-cellulose (DE-52) were purchased from Sigma Chemical Co., St. Louis, and Reeve Angel Co., Clifton, N.J., respectively. DEAE-Sephadex A-50 was purchased from Pharmacia Chemicals, New Market, N.J., and Bio-Gel A-5M was purchased from Bio-Rad Laboratories, Richmond, Calif. Enzyme grade $(NH_4)_2SO_4$ and Tris buffer were purchased from Schwartz/Mann Chemicals, Orangeburg, N.Y. All other chemicals were reagent grade compounds readily available from commercial sources.

Enzyme assays

Citrate synthase activity was measured at 412 nm according to the method of Srere et al. [19], or by following the decrease in absorbance at 233 nm according to the method of Srere and Kosicki [20]. All spectrophotometric assays were performed at 30 °C with a Gilford Model 2000 recording spectrophotometer equipped with a Beckman DU monochromotor.

The reaction velocity was measured in 1-ml glass (412-nm assay) or quartz cuvettes (233-nm assay). Unless otherwise indicated, the reaction mixture contained 10 μmoles Tris–EDTA–citrate buffer, 0.1 μmole DTNB, 0.3 μmole oxaloacetate, 0.12 μmole acetyl-CoA, and enzyme in a total volume of 1.0 ml. All assays during enzyme purification were performed in the presence of 0.1 M KCl.

Product inhibition studies with free CoA could not be accomplished by the method using DTNB. The 233-nm assay which measures the hydrolysis of acetyl-CoA was used for these experiments. The assay mixture included 10 μmoles Tris–EDTA–citrate buffer, 100 μmoles KCl and enzyme. Citrate, Coenzyme A (CoA), acetyl-CoA, and oxaloacetate were added in the concentrations indicated in each experiment.

Units of enzyme activity are expressed in micromoles of CoA produced per minute. Specific activity is expressed as micromoles CoA produced per minute per mg protein.

Purification of enzymes

The enzymes from *B. subtilis*, *A. vinelandii*, and *A. anitratum* were treated similarly during purification. A more detailed description of the purification procedures has been published [21]. Minor variations from the following scheme are noted where necessary. All of the purification steps were performed at 4 °C. Steps which required concentration were accomplished by precipitation during dialysis against 80% saturated $(NH_4)_2SO_4$ and the precipitate was dissolved in a smaller volume or by ultrafiltration using Amicon stirred cells with XM50 Diaflo Ultrafilters (Amicon Corp., Cambridge, Mass.) at a pressure of 30 lb/inch^2 nitrogen. Protein was measured by the method of Lowry et al. [22], or by absorbance at 280 nm when nucleic acid content of the preparation was 5% or less.

Crude cell-free extracts were prepared from 100 to 200 g washed cells of *B. subtilis* and *A. anitratum* suspended in twice their weight of 50 mM Tris–EDTA–citrate buffer, pH 8.0, by passage through a french pressure cell at 5000–6000 lb/inch^2. Glycerol (20%) was added to the buffer of *B. subtilis* suspensions to promote enzyme stability during purification. The viscous extract was subjected to a short burst of ultrasonic waves (15–30 s duration) in order to reduce viscosity and it was centrifuged at 27 000 $\times$ *g* for 20 min in a Sorval RC2-B centrifuge.

Citrate synthase could be solubilized from *A. vinelandii* cells by osmotic shock. Packed cells, 300–500 g, were suspended in 5 vol. of 4 M glycerol in 0.025 M Tris–HCl, pH 7.4, and incubated for 30 min at 20 °C. The suspension was then centrifuged (16 300 $\times$ *g*, 20 min), and the supernatant fraction was discarded. The cells were re-suspended in 3 to 4 vol. of 0.025 M Tris–HCl, pH 7.4, and were shaken vigorously with glass beads at room temperature. A small amount of DNAase was added and the suspension was incubated for 10 min at 20 °C. The suspension was then centrifuged (16 300 $\times$ *g*, 60 min, 4 °C), and the supernatant fraction was recovered.

The crude extract from either method was diluted to approx. 10 mg protein/ml, and was dialyzed overnight against 50 mM Tris–EDTA–citrate buffer, pH 7.2. The enzyme solution was then applied to a column (2.5 cm $\times$ 40 cm) packed with DEAE-cellulose (either fibrous or microgranular DE-52) and the column was washed with several column volumes of 50 mM Tris–EDTA–citrate buffer, pH 7.2 to elute the bulk of the 260-nm absorbing material. When the initial absorption peak was eluted, the remaining protein was batch eluted from the column with 0.6 M potassium acetate in 50 mM Tris–EDTA–citrate buffer. This procedure increased the 280 nm/260 nm absorption ratio of the crude enzyme preparation from 0.55 to 0.90 or higher and improved the reproducibility of subsequent purification steps.

The active enzyme eluted from the first DEAE column was dialyzed overnight against 50 mM Tris–EDTA–citrate buffer and was applied to an identical DEAE-cellulose column (previously re-equilibrated with 50 mM Tris–EDTA–citrate buffer). The protein was eluted with a linear potassium acetate gradient of 0–0.6 M. Citrate synthase activity was eluted when the potassium acetate concentration reached 0.30–0.35 M. The fractions containing citrate synthase activity were combined and precipitated overnight by dialysis against 4.24 M $(NH_4)_2SO_4$.

The precipitated protein fractions from *B. subtilis* and *A. vinelandii* were recovered by centrifugation at 27 000 $\times$ *g* for 20 min and suspended in a volume of 3.25 M $(NH_4)_2SO_4$ to give a protein concentration of 10 mg/ml or less. The suspension was stirred for 2 h at 4 °C and centrifuged. The pellet was suspended in sufficient 2.36 or 2.65 M $(NH_4)_2SO_4$ to give 3–5 mg protein/ml. After stirring for an additional 2 h at 4 °C, the mixture was centrifuged and the protein in the supernatant fraction was precipitated by dialysis against 4.24 M $(NH_4)_2SO_4$. The precipitate contained only that protein which was soluble in $(NH_4)_2SO_4$ solutions between 2.65 and 3.25 M. This protein contained the bulk of the citrate synthase activity. It was found necessary to assay the enzyme in each fraction during this step of the purification before proceeding. The active enzyme was dissolved in 2.65–3.26 M $(NH_4)_2SO_4$ in some purification attempts and in the 2.36–3.26 M $(NH_4)_2SO_4$ fraction in others.

The enzyme preparation was dialyzed against 0.1 M potassium acetate in 50 mM Tris–EDTA–citrate buffer, pH 8.5, and was applied (in a concentration not exceeding 5 mg protein/ml) to a 1.2 cm $\times$ 30 cm column of DEAE-Sephadex A-50.

The protein was eluted with a linear gradient of 0.1–0.6 M potassium acetate in 50 mM Tris–EDTA–citrate buffer, pH 8.5. The fractions containing the majority of the citrate synthase activity were combined and stored at −20 °C. Minor variations in this scheme were applied to the purification of the three different enzymes.

Additional purification procedures were used for the enzymes from *A. vinelandii* and *A. anitratum*. Molecular seiving through a Bio-gel A-5M column and/or preparative electrophoresis proved to be most useful.

A 1 cm × 90 cm column was routinely used for citrate synthase purification on Bio-Gel A-5M. The enzyme was eluted by continuous addition of the buffer, 50 mM Tris–EDTA–citrate with 50 mM KCl, pH 7.8.

Preparative electrophoresis was performed with a Buchler Fractophorator, a Buchler Model 3-1008 power supply and a Buchler Fractomat refrigerated fraction collector. A polyacrylamide gel containing 8% monomer with a stacking gel of 2.5% monomer (total length 6 cm) was cast as described by Davis [23]. The separating gel and the stacking gel were both polymerized by the addition of 1 ml of a 1% solution of $(NH_4)_2S_2O_8$ and 0.02 ml *N,N,N′,N′*-tetramethylethylenediamine for each 20 ml of gel solutions. An asparagine reservoir buffer described by Hedrick and Smith [24] was used for both the cathode and anode chambers. The elution reservoir buffer contained 25 mM Tris–HCl and 50 mM KCl, pH 8.0. Partially purified citrate synthase preparations were concentrated by ultrafiltration and were dialyzed against 0.06 M imidazole–HCl buffer, pH 7.0, containing 20% glycerol. 2 ml of sample containing a maximum of 20 mg protein could be layered onto the stacking gel without a significant loss of resolution. The current was adjusted to 10 mA during the run. Both upper and lower reservoir buffers were pumped through their respective chambers at a flow rate of 20 ml/h with separate Sigmamotor model A1-2-E pumps (Sigmamotors, Inc., Middleport, N.Y.).

Electrophoresis

A Buchler Polyanalyst apparatus and power supply were employed for all disc gel electrophoretic procedures. The disc gel electrophoretic technique devised by Hedrick and Smith [24] involving a molecular weight–slope relationship was used with the minor variations noted below for the determination of the molecular weight of native citrate synthase. $(NH_4)_2S_2O_8$ was used as the catalyst for the polymerization of the stacking and the separating gels. After electrophoresis, the gels were immersed in 20% sulfosalicylic acid for 30 min to fix the protein bands prior to staining.

The active enzyme was assayed in the gels by the following procedure. Identical gels were prepared as described above, one was stained for protein and the other was sliced into sections 2 mm thick. Each section was cut in half and one-half of each slice was placed in a separate well of a white porcelain spot plate. A mixture containing 15 μmoles Tris–EDTA–citrate buffer, 0.1 μmole DTNB, 0.3 μmole oxaloacetate, and 0.12 μmole acetyl-CoA in 1 ml was added to each well. The development of a yellow color indicated the presence of citrate synthase. The location of active enzyme in the gel was compared with the stained gel pattern. No color developed when the remaining half of each slice was incubated in a reaction mixture without oxaloacetate.

Ultracentrifugation

The apparent molecular weight of purified citrate synthase was measured by

sedimentation equilibrium employing the meniscus depletion method of Yphantis [25]. A Spinco Model E ultracentrifuge equipped with Rayleigh interference optics was used for all runs. The protein concentration was 0.5 mg/ml in 0.025 M Tris, pH 8.0, with 0.1 M KCl and each sample was extensively dialyzed against additional buffer to ensure homogeneity between sample buffer and blank. A picture was taken after 20 h at 10 000 rev./min and a temperature of 4 °C. The fringe displacement was measured with a two-coordinate comparator.

Sedimentation equilibrium studies to determine subunit molecular weight were performed in the presence of 6 M guanidine·HCl and 1 mM dithiothreitol and the enzyme solutions were dialyzed against the reagents for 4 days to ensure complete dissociation of the enzyme complex and to provide for homogeneity between the sample and the blank. A picture was taken after 20 h at room temperature. The rotor speed was 30 000 rev./min.

The molecular weights of the native and denatured enzymes were calculated using partial specific volumes estimated from the amino acid compositions of the homogeneous enzyme preparation [21] by the method of Schachman [26]. The calculated partial specific volumes were 0.743 ml/g for the *A. vinelandii* enzyme and 0.723 ml/g for the *A. anitratum* enzyme.

RESULTS

Stability and purification

The specific activity of partially purified citrate synthase from *B. subtilis* decreased during handling and storage. Inactivation occurred in crude and partially purified preparations and there was no apparent protection by high concentrations of protein or by increasing the ionic strength up to 0.5 M potassium acetate. Glycerol, in concentrations of 20 % (v/v) or more, stabilized the enzyme (Fig. 1). Glycerol (20 %, v/v) was added to all storage buffers and was used in each purification step involving *B. subtilis* citrate synthase, with the exception of the $(NH_4)_2SO_4$ fractionation. The *A. vinelandii* and *A. anitratum* citrate synthases did not require glycerol for stability

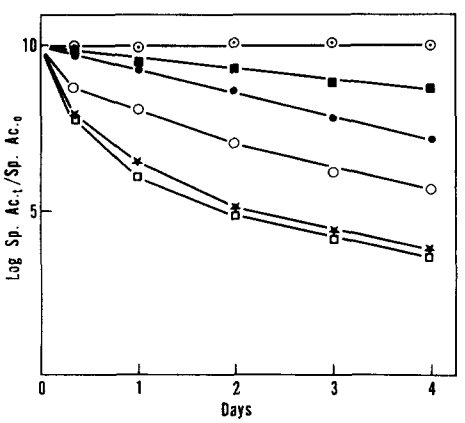

Fig. 1. The effect of glycerol concentration on the stability of *B. subtilis* HS2A2 citrate synthase. The enzyme solutions were stored on ice in solutions containing different amounts of glycerol. Glycerol (%): 30, ○—○; 20, ■—■; 15, ●—●; 10, ○—○; 5, ★—★; and 0, □—□.

during purification but glycerol was beneficial in maintaining activity during storage of the enzymes.

We were unable to obtain purified preparations of *B. subtilis* citrate synthase with a specific activity greater than 2.6 units/mg protein (Table I) in several attempts.

TABLE I

PURIFICATION OF *BACILLUS SUBTILIS* HS2A2 CITRATE SYNTHASE

Step	Volume (ml)	Protein (mg/ml)	Total units	Recovery (%)	Specific activity (units/mg protein)	Purification
1. Crude	85	40.0	448	100	0.13	—
2. DEAE batch	89	9.2	347	76	0.42	3.2
3. DEAE gradient	92	1.0	59	13	0.65	5.0
4. $(NH_4)_2SO_4$ (2.65–3.25 M)	22	3.0	53	12	0.81	6.2
5. DEAE-Sephadex	33.5	0.5	43	10	2.6	20

All attempts to improve the purification procedures resulted in severe losses of enzym atic activity.

The purification of *A. vinelandii* citrate synthase resulted in a maximum specific activity of 523 units/mg protein (Table II). Crude extracts were prepared by osmotic shock because the specific activity of crude extract obtained in this way was 10 times greater than that obtained from cells broken in the french pressure cell. The enzyme

TABLE II

PURIFICATION OF *AZOTOBACTER VINELANDII* CITRATE SYNTHASE

Step	Volume (ml)	Protein (mg/ml)	Total units	Recovery (%)	Specific activity (units/mg protein)	Purification
1. Crude shockate	3000	5.8	180 000	100	10	—
2. DE-52 batch	1310	3.0	188 400	104.7	47	4.6
3. DE-52 gradient	80	2.0	33 488	18.6	209	20
4. Bio-Gel A-5M	40	1.8	17 520	9.7	243	24
5. DEAE-Sephadex	24	1.0	12 560	7.0	523	51

was purified on a column of DEAE-Sephadex A-50 twice. Preparative electrophoresis was not used to purify the *A. vinelandii* enzyme because of its instability in buffers with low ionic strength. Disc gel electrophoresis of the purified enzyme revealed a single major band, though surrounded by a region of less densely stained protein (Fig. 2). Sedimentation equilibrium studies of the *A. vinelandii* citrate synthase at protein concentrations of 0.5 mg/ml, indicated a completely homogeneous preparation (Fig. 3). The diffuse region that appeared upon electrophoresis could represent dissociation of the active complex in an environment of low ionic strength. Similar problems have been encountered in demonstrating the purity of the enzyme from *E. coli* [10].

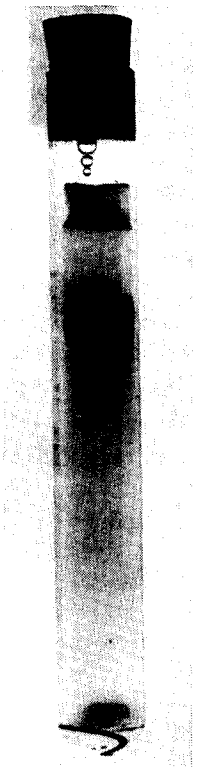

Fig. 2. Disc gel electrophoresis of purified *A. vinelandii* citrate synthase. The gel contained 50 μg of enzyme.

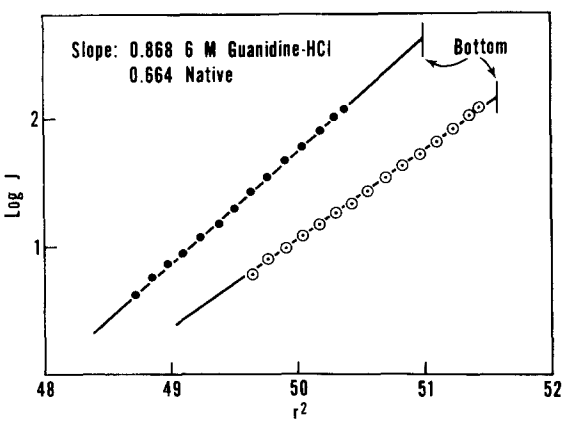

Fig. 3. Sedimentation equilibrium of *A. vinelandii* citrate synthase. The Rayleigh fringe displacement (log J, in μm) is plotted against the square of the distance (r^2, in cm) from the center of rotation. Citrate synthase: native (○—○) in 50 mM Tris–EDTA–citrate buffer and 100 mM KCl; and subunits, (●—●) in 6 M guanidine·HCl and 10^{-3} M dithiothreitol.

TABLE III

PURIFICATION OF *ACINETOBACTER ANITRATUM* CITRATE SYNTHASE

Step	Volume (ml)	Protein (mg/ml)	Total units	Recovery (%)	Specific activity (units/mg protein)	Purification
1. Crude	340	44.0	10 040	100	0.67	—
2. DE-52 batch	650	11.5	7 600	76	1.0	1.5
3. $(NH_4)_2SO_4$ (2.36–3.25 M)	113	11.4	6 000	60	4.7	7.0
4. DE-52 gradient	20	3.2	5 600	55	87	130
5. DEAE-Sephadex	24.5	1.1	4 300	43	161	239
6. Bio-Gel A-5M	10.5	1.4	3 500	34	235	351

A. anitratum citrate synthase was purified according to scheme presented in Table III. A maximum specific activity of 235 units/mg protein was achieved. Polyacrylamide electrophoresis revealed this preparation to be contaminated by small amounts of two separable proteins. Preparative electrophoresis was used to obtain a single, active protein band (Fig. 4). The specific activity decreased to one-fourth of

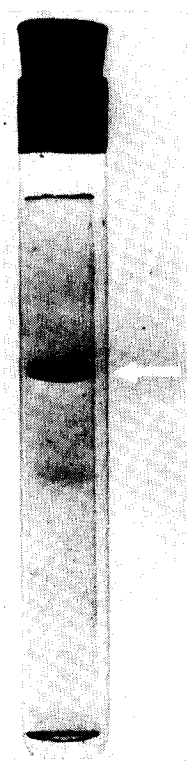

Fig. 4. Disc gel electrophoresis of purified *A. anitratum* citrate synthase. Enzyme preparation (50 μg) after preparative electrophoresis. The active protein band assayed in gel slices as described in Materials and Methods, is indicated by an arrow.

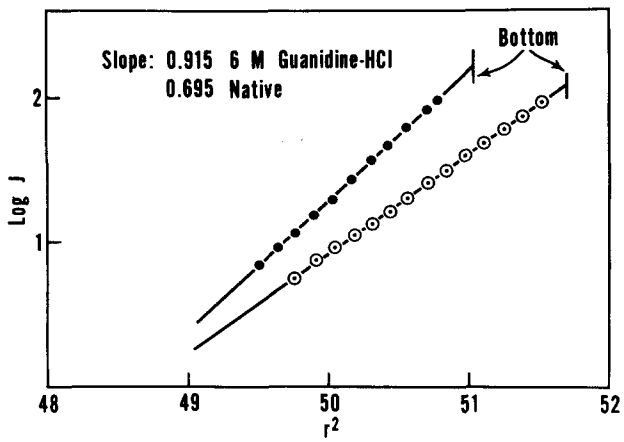

Fig. 5. Sedimentation equilibrium of *A. anitratum* citrate synthase. All experimental conditions are the same as in Fig. 3. Citrate synthase: native, ○—○; and subunits, ●—●.

that observed in the most active preparation because of the absence of cations required for optimal stability during preparative electrophoresis. Evidence for the purity of the *A. anitratum* citrate synthase is shown in Fig. 5. The sedimentation equilibrium data revealed a linear distribution of protein throughout the cell.

Molecular weights

The molecular weight of the purified native citrate synthase complex was estimated by the techniques of disc gel electrophoresis and sedimentation equilibrium centrifugation. The slope–molecular weight relationship of Hedrick and Smith [24] was determined using a variety of protein standards. According to this method, the *A. vinelandii* enzyme had a molecular weight of 260 000 (Fig. 6). The enzyme from *A.*

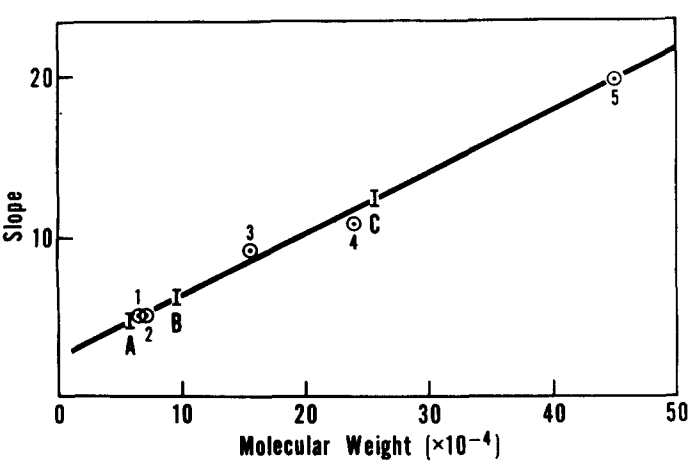

Fig. 6. Native protein molecular weight determination by acrylamide gel electrophoresis. The standard proteins and their molecular weights were: 1, bovine serum albumin (65 000); 2, pepsin (70 000, dimer); 3, bovine serum albumin (130 000, dimer); 4, catalase (240 000); and 5, apoferritin (450 000). The citrate synthase molecular weight determinations by this method were: *B. subtilis*, range A to B; and *A. vinelandii*, C.

anitratum showed variable heterogeneity on acrylamide gels. This has also been reported for the *E. coli* enzyme [10]. The molecular weight of the *B. subtilis* enzyme, because of its impure state, was estimated using a gel slicing procedure to detect the enzymatic activity. The molecular weight of the *B. subtilis* citrate synthase was estimated to be between 60 000 and 95 000 by this technique (Fig. 6). This data should be considered preliminary because of the inaccuracies in the technique although it is obvious that the enzyme is considerably smaller than the enzyme from *A. vinelandii.*

The molecular weight of citrate synthase from *A. anitratum* was calculated as 242 000 by sedimentation equilibrium studies from the data in Fig. 5. Sedimentation equilibrium studies with the *A. vinelandii* citrate synthase (Fig. 3) indicated an apparent molecular weight of 250 000.

Subunits

Sedimentation equilibrium studies of the *A. anitratum* enzyme in 6 M guanidine·HCl and 10^{-3} M dithiothreitol indicated an apparently homogeneous protein with a molecular weight of 69 000 (Fig. 5). Similar studies with the *A. vinelandii* enzyme in 6 M guanidine·HCl and dithiothreitol also indicated a single homogeneous protein with a molecular weight of 58 500 (Fig. 3).

Kinetics

Double reciprocal plots of enzyme velocity versus acetyl-CoA or oxaloacetate concentration were linear for all three citrate synthase preparations. KCl increased the reaction velocity in all three instances by lowering the K_m for acetyl-CoA. However, the K_m for oxaloacetate was generally unaffected by 100 mM KCl (Table IV).

TABLE IV

SUMMARY OF KINETIC DATA FOR CITRATE SYNTHASE

Experimental determinations	*B. subtilis* HS2A2	*A. anitratum*	*A. vinelandii*
K_m acetyl-CoA	0.06 mM	0.10 mM	0.08 mM
K_m acetyl-CoA (with 100 mM KCl)	0.04 mM	0.03 mM	0.03 mM
K_m oxaloacetate	0.03 mM	0.10 mM	0.02 mM
K_m oxaloacetate (with 100 mM KCl)	0.03 mM	0.10 mM	0.01 mM
ATP inhibition patterns			
varying acetyl-CoA	non-competitive	—	competitive
varying oxaloacetate	non-competitive	—	non-competitive
K_i (ATP) acetyl-CoA varied	1.0 mM	—	0.75 mM
K_i (ATP) oxaloacetate varied	—	—	2.00 mM
Initial velocity patterns			
varying acetyl-CoA	intersecting	intersecting	intersecting
varying oxaloacetate	intersecting	intersecting	intersecting
Product inhibition patterns			
1. Citrate			
varying acetyl-CoA	competitive	competitive	competitive
varying oxaloacetate	non-competitive	non-competitive	non-competitive
2. CoA			
varying acetyl-CoA	competitive	competitive	competitive
varying oxaloacetate	non-competitive	non-competitive	non-competitive

Inhibition of *B. subtilis* HS 2A2 citrate synthase by ATP was non-competitive with respect to either acetyl-CoA or oxaloacetate (Table IV). These results are atypical, for citrate synthases from *B. subtilis* 168 [3] and *B. subtilis* HS 1A17 [21] exhibit competitive ATP inhibition patterns with respect to acetyl-CoA. The reason for this discrepancy is not known. Adenosine triphosphate inhibition of *A. vinelandii* citrate synthase was competitive with respect to acetyl-CoA and non-competitive with respect to oxaloacetate (Table IV). Conversely, *A. anitratum* citrate synthase was unaffected by ATP (Table V). ATP did not inhibit this enzyme when the acetyl-CoA

TABLE V

EFFECT OF VARIOUS REGULATORY EFFECTORS UPON *ACINETOBACTER ANITRATUM* CITRATE SYNTHASE

Additions, in the concentrations noted, were added to the standard assay system (Materials and Methods). Appropriate controls were performed where necessary to eliminate stimulation by counter ions.

Additions	Concentration	Activity	Percent of control
None	–	0.031	100
ATP	10 mM	0.030	99
AMP	5 mM	0.037	117
KCl	50 mM	0.057	181
NADH	1 mM	0.027	88
NADPH	0.25 mM	0.022	71
α-Ketoglutarate	7 mM	0.038	123

concentration was decreased to 0.10 mM (K_m concentration) or in the absence of KCl. The effect of other known modulators of procaryotic and eucaryotic citrate synthase except KCl is shown in Table V. Adenosine monophosphate shows slight stimulation while NADH and NADPH are slightly inhibitory. α-Ketoglutarate did not inhibit this enzyme but caused some stimulation. KCl stimulated enzyme activity.

Without exception, all the patterns observed with initial velocity studies were intersecting (Table IV). The intersecting patterns are convincing evidence for a sequential reaction order mechanism, and all three enzymes gave the same patterns. The double reciprocal plots for *A. vinelandii* citrate synthase are shown in Fig. 7.

The initial velocity patterns for *B. subtilis* HS2A2 citrate synthase, although intersecting, revealed a non-competitive substrate inhibition with oxaloacetate as the variable substrate and acetyl-CoA as the changing fixed substrate (Fig. 8). Inhibition was observed at high levels of acetyl-CoA and its presence affected the intercepts and the slopes of the double reciprocal plot when oxaloacetate was varied. The replot of the intercepts versus 1/acetyl-CoA gave a hyperbola. The characteristic pattern for an ordered sequential reaction order mechanism is an uncompetitive substrate inhibition, where the slope replot appears normal but the intercept replot is a hyperbola [27]. A random sequential mechanism would not be expected to show substrate inhibition unless one substrate has some affinity for the part of the absorption pocket that binds the other substrate [27]. Substrate inhibition was not observed with citrate synthase from *A. vinelandii* and *A. anitratum*.

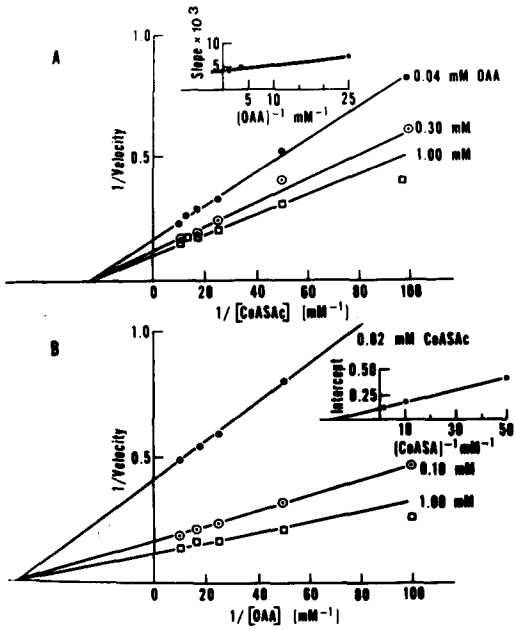

Fig. 7. Initial velocity patterns of *A. vinelandii* citrate synthase. (A) Varying concentrations of acetyl-CoA. (B) Varying concentrations of oxaloacetate (OAA). Replots of the slope and intercept versus the variable substrate concentration are shown in the insets. 0.1 M KCl was added to the reaction mixture. The enzyme concentration was 0.02 μg/ml.

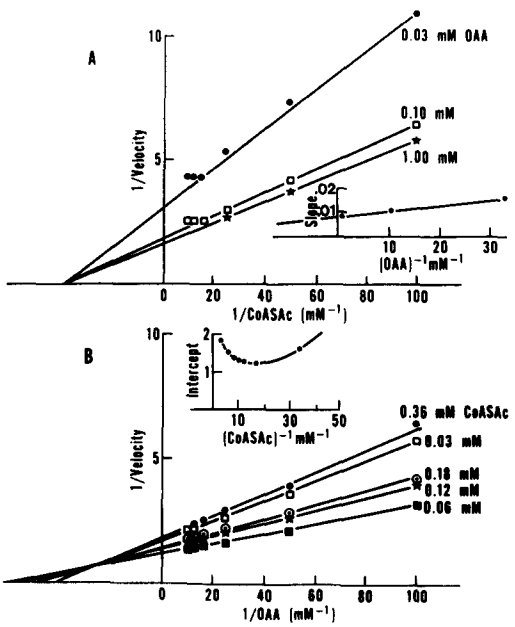

Fig. 8. Initial velocity patterns of *B. subtilis* HS2A2 citrate synthase. (A) Varying concentrations of acetyl-CoA. (B) Varying concentrations of oxaloacetate (OAA). The replots of the slope and intercept versus the variable substrate concentration are shown in the insets. The reaction mixtures except for oxaloacetate and acetyl-CoA concentrations were identical to those in Fig. 7.

The results of the product inhibition studies with citrate and CoA gave two competitive and two non-competitive (or uncompetitive) inhibition patterns. The patterns observed for *A. vinelandii* are presented (Figs 9 and 10). Other plots [21] have been omitted to conserve space.

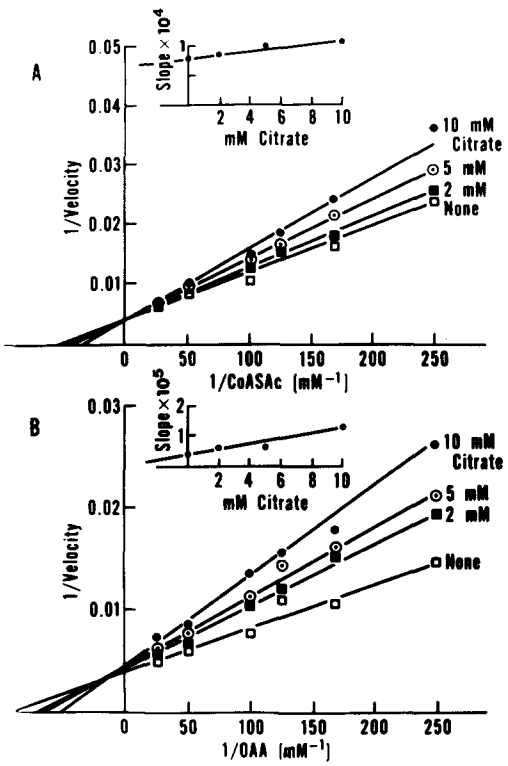

Fig. 9. Inhibition of *A. vinelandii* citrate synthase by citrate. (A) Varying concentrations of acetyl-CoA, 0.3 mM oxaloacetate (OAA). (B) Varying concentrations of oxaloacetate, 0.12 mM acetyl-CoA. The enzyme velocity is in units/ml. Replots of the slope and intercept versus mM citrate are shown in the insets. The reaction mixtures except for citrate, oxaloacetate and acetyl-CoA concentrations were identical to those in Fig. 8.

DISCUSSION

According to Cleland [27], all product inhibition patterns for random sequential mechanisms are competitive except for cases in which inhibitors form dead-end complexes with the enzyme, and those are non-competitive. In this study, a non-competitive or uncompetitive pattern was observed when oxaloacetate was varied at different levels of CoA for all three citrate synthases examined. This evidence suggests a random mechanism, because the two reactants lacking the acetyl moiety that is transferred during the reaction, oxaloacetate and CoA, would tend to form a dead-end complex with the enzyme. Thus, the kinetic patterns observed in the initial velocity and product inhibition studies provided evidence of a completely random sequential mechanism for citrate synthase catalysis. The kinetics of the reverse reaction and dead-

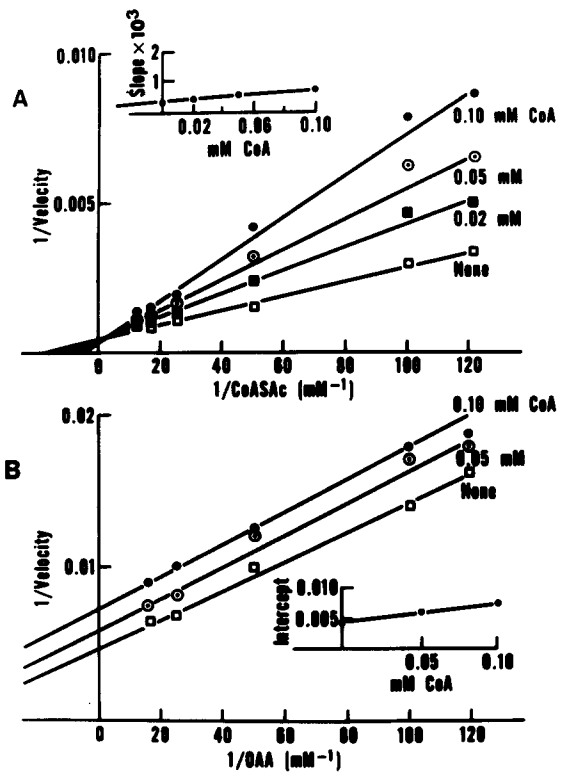

Fig. 10. Inhibition of *A. vinelandii* citrate synthase by coenzyme A. (A) Varying concentrations of acetyl-CoA, 0.3 mM oxaloacetate. (B) Varying concentrations of oxaloacetate, 0.08 mM acetyl-CoA. The replots of the slope and intercept versus CoA (mM) are shown in the insets. The reaction mixture (233-nm assay) contained 0.05 μg of enzyme per ml.

end inhibitor effects are needed before the reaction order mechanism can be established conclusively.

Though the three enzymes exhibit an identical reaction order mechanism, each is unique in other ways. The molecular weights of the citrate synthases from the two gram negative bacteria, *A. vinelandii* and *A. anitratum*, were calculated to be between 240 000 and 260 000 in sedimentation equilibrium studies. Each is apparently composed of identical subunits with a molecular weight of approx. 60 000. Our results suggest that the enzymes from *A. anitratum* and *A. vinelandii* have four subunits and are similar in molecular weight to the enzyme from *E. coli* which has a molecular weight reported to be from $2.1 \cdot 10^5$ to $2.8 \cdot 10^5$ [12]. The molecular weights of the enzymes from all three gram negative bacteria are therefore similar. Srere [12] has reported the molecular weight of the enzyme from *Azotobacter* to be $3 \cdot 10^5$ by sedimentation equilibrium and $5 \cdot 10^5$ by gel filtration but the data have not been published. Our results gave molecular weights of $2.5 \cdot 10^5$, by sedimentation equilibrium and $2.6 \cdot 10^5$ by gel electrophoresis and are in better agreement. Srere (unpublished observations cited in ref. 12) has concluded that the *Azotobacter* enzyme has six subunits, while our data suggest a smaller number, probably four.

The data on the molecular weight of the active enzyme from *B. subtilis* indicate

that it is approximately the same molecular weight as the subunits from the gram negative organisms. The data on this enzyme must be considered tentative, but our experiments clearly show that it is smaller than the enzymes from the gram negative bacteria studied to date. Its subunit composition could not be determined because we were unable to purify this enzyme to homogeneity.

The turnover numbers calculated from the enzyme preparations with the highest specific activities were $5.65 \cdot 10^4$ and $1.26 \cdot 10^5$ moles of citrate/min per mole of enzyme at 30 °C for *A. anitratum* and *A. vinelandii*, respectively. These values are higher than those reported for three crystalline citrate synthase preparations from pig heart [7], rat heart and rat liver [9]. The specific activities reported in this paper are the highest yet obtained for any reported procaryotic or eucaryotic citrate synthase.

It is apparent that there is a complex regulatory mechanism for citrate synthase, which differs among organisms of various physiologies and nutritional requirements [4, 29–31]. All gram positive bacterial citrate synthases appear to be sensitive to ATP, and most gram negative bacterial citrate synthases are insensitive to ATP with the exception of those from *A. vinelandii* and one strain of *Rhodopseudomonas rubrum* [4]. Jangaard et al. [28] have shown that ATP inhibits citrate synthase from *E. coli*, but only at pH values below the optimal pH. The enzyme from *S. typhimurium*, a related bacterium, was not inhibited by ATP [4]. Published reports indicate that inhibition of citrate synthase by α-ketoglutarate is restricted to the facultatively anaerobic enterobacteria [29], an anaerobic bacterium [30], some chemolithotrophic bacteria [29], and the facultative gram positive bacteria, *Bacillus polymyxa*, and *Bacillus macerans* [32].

The sensitivity of citrate synthase from gram negative organisms to NADH is well established. Recently, Weitzman and Dunmore [33] attempted to correlate enzyme size with regulatory behavior. They grouped "large" and "small" citrate synthases according to their sensitivity to NADH. While this pattern may be true for NADH inhibition and AMP reactivation in gram negative organisms, no pattern indicative of regulatory complexity which was dependent only upon molecular size was observed in this study. The citrate synthase from *A. anitratum*, a large enzyme with a molecular weight of 240 000, was insensitive to every known modifier of citrate synthase including NADH, ATP, and α-ketoglutarate under all conditions tested. This finding is in contrast to the results of Weitzman and co-workers [31, 33], who reported the citrate synthase from *Acinetobacter lwoffi* resembled that of the *E. coli* enzyme in regard to NADH sensitivity and AMP reactivation. The *A. vinelandii* enzyme, which is sensitive to ATP and NADH, and stimulated by AMP [3], is shown in this paper to be as large as the citrate synthase from *A. anitratum*. The *B. subtilis* enzyme was found to be smaller and was sensitive to ATP but not NADH. The *B. polymyxa* citrate synthase has a molecular weight of 60 000 and is sensitive to α-ketoglutarate and ATP. Adenosine monophosphate stimulated the citrate synthase from *B. subtilis*.

The inhibition of the *A. vinelandii* and *B. subtilis* enzymes by ATP and stimulation by AMP at physiological levels suggest a possible control mechanism regulated by variations in the in vivo molar ratio of ATP/AMP. The energy charge concept for regulation of the key enzymes of amphibolic pathways has been proposed by Atkinson [34] and is implicated as a control parameter for citrate synthase from yeast [35]. Current studies (Johnson, D. E. and Hanson, R. S., unpublished) reveal that citrate synthases from *A. vinelandii* and *B. subtilis* respond negatively to an adenylate energy charge ratio greater than 0.80 and positively at lower ratios. Additional modu-

lation of citrate synthase activity can occur through catabolite repression and feedback repression of citrate synthase [3, 4].

The lack of response of citrate synthase from *A. anitratum* to normal regulatory effectors could have reflected a loosely controlled tricarboxylic acid cycle as was found to be true for *Corynebacterium lilium* [4], an organism that excretes glutamic acid.

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