Azotobacter vinelandii Citrate Synthase†

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ABSTRACT: We have purified the citrate synthase from *Azotobacter vinelandii* and have determined that the size of the subunit is 48 000 Da and the structure of the holoenzyme is a hexamer. This contrasts with earlier estimates that indicate a 58 000 Da subunit and a tetrameric structure. In addition, the enzyme is allosteric with a Hill coefficient of 1.5 and is inhibited by NADH. The Hill coefficient is changed to about 1 by high ionic strength and AMP. The enzyme is thus similar to the citrate synthases of many other Gram-negative, facultative, anaerobic organisms. In addition, the amino acid sequence of about 100 residues has been determined and found to be highly similar to the sequence of *Pseudomonas aeruginosa* citrate synthase.

Citrate synthase (CS)[1] is a ubiquitous enzyme with important cellular roles in energy production and in the biosynthesis of several cellular components (Beeckmans, 1984; Srere, 1972; Weitzman & Danson, 1976; Weitzman, 1981). On the basis of the identification of active site residues by both X-ray crystallography and site-directed mutagenesis, it has been shown that these residues are conserved in all 14 CSs which have been sequenced.

Weitzmann and his colleagues noted that CS from a variety of cell types can be classified according to size and kinetics (Weitzman & Danson, 1976). The small CSs are dimers of ~100 000 and exhibit hyperbolic Michaelis−Menten kinetics (Beeckmans & Kanarek, 1983; Löhlein-Werhahn *et al.*, 1988; Muir *et al.*, 1994; Srere, 1972; Smith *et al.*, 1987; Weitzman & Danson, 1976). The large CSs have an M_r of ~250 000 and for many years were considered to be tetramers (see below). The large CSs are inhibited by NADH and exhibit sigmoidal Michaelis−Menten kinetics (Johnson & Hanson, 1974; Weitzman & Danson, 1976; Weitzman, 1981). Many microorganisms can be roughly classified according to this system. Thus, the small CSs were found in Gram-positive bacteria, and the NADH sensitive, large CSs are found in Gram-negative species. This latter group is subdivided into strict aerobes in which the NADH inhibition of CS cannot be reversed by AMP and facultative anaerobes having a large CS in which the NADH inhibition is reversed by AMP (Flechtner & Hanson, 1970; Weitzman & Danson, 1976; Weitzman, 1981).

Azotobacter vinelandii (*A. vinelandii*) is a facultatively anaerobic Gram-negative bacterium whose CS has been purified and reported to be a tetramer of 58 000 monomers (Johnson & Hanson, 1974). Flechtner and Hanson (1970) reported sigmoid kinetics and AMP-reversible NADH inhibition but reported no data. Since many of the large CSs have been restudied and shown to be hexamers instead of tetramers (Tong & Duckworth, 1975; Weitzman, 1981), we decided to re-examine the molecular properties and kinetics of the *A. vinelandii* CS.

MATERIALS AND METHODS

Materials. NADH, AMP, OAA, CoA, DNTB, DEAE-Sepharose, and phenyl-Sepharose were purchased from Sigma Chemical Co. (St. Louis, MO). PD10 columns (Sephadex G25), Sephacryl S300, and Phast-gel gradient were purchased from Pharmacia (Alameda, CA), and DE-52 powder was from Whatman (Hillsboro, OR).

Bacterial Strains. CS was purified from *A. vinelandii* OP, which was grown at 30 °C in a Burk's modified medium, and generously provided by Dr. V. K. Shah (University of Wisconsin, Madison).

Isolation of CS. Frozen *A. vinelandii* cells (20 g) were thawed in 20 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 10% sucrose, 1 mM PMSF, and 0.5 mg/L leupeptin (9 g of wet cells/10 mL), frozen in liquid nitrogen, and thawed at room temperature. The cell suspension was lysed by addition of lysozyme (0.2 mg/mL suspension) and incubated for 45 min at 4 °C on a rocking table. The suspension was sonicated at 0 °C by alternating eight cycles of 30 s sonication with 1 min cooling intervals. All subsequent operations were carried out at 4 °C. The sonicated suspension was centrifuged at 45 000*g* for 45 min and the precipitate discarded. Nucleic acids were removed from the supernatant solution by precipitation with protamine sulfate (5 mg/g of wet cells) and centrifugation at 45 000*g* for 20 min. Solid ammonium sulfate was then added to the supernatant, and the fraction precipitating between 42% and 75% of saturation was collected, dissolved in a minimal amount of 2 mM KPO_4, and 50 mM KCl (pH 7.5), and dialyzed overnight against

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[1] Abbreviations: *Azotobacter vinelandii, A. vinelandii*; citrate synthase, CS.

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the same buffer. KCl (50 mM) was added to the dialysis buffer to stabilize the *A. vinelandii* CS. The dialysate was added to a 40 mL DE-52 column previously equilibrated with the same buffer. The column was washed with 50 mL of the buffer described above, and bound proteins were eluted with a 200 mL of 2−150 mM KPO_4 (pH 7.5) linear gradient. The CS was eluted at 35 mM KPO_4 as a single peak. The fractions (3 mL) containing CS activity were pooled and precipitated by addition of solid ammonium sulfate to 70% saturation, and the pellet was suspended in 2 mM KPO_4 (pH 7.5). The sample was then added to a PD10 column equilibrated in 2 mM KPO_4 (pH 7.5) to remove excess ammonium sulfate. The eluate was then placed on a 15 mL DEAE-Sepharose column pre-equilibrated with the same buffer. Proteins were eluted with a 150 mL, 0.0−0.25 M KCl gradient, and fractions (2 mL) containing CS activity were pooled and precipitated with ammonium sulfate to 70% saturation. The precipitate was suspended in 5 mM KPO_4, 50 mM KCl, and 70% NH_4SO_4 (pH 7.5) and added to a 10 mL phenyl-Sepharose column equilibrated with the same buffer. The column was washed with decreasing concentrations of ammonium sulfate, and the CS was eluted at 0% ammonium sulfate.

The purified *A. vinelandii* CS was kept at −20 °C in 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, and 10% glycerol. Under these conditions the CS activity was stable for several weeks.

Enzyme Assays. During purification, CS activity was determined at 25 °C using 0.2 mM acetyl-CoA, 0.5 mM OAA, and 1 mM DTNB in 100 mM Tris-HCl (pH 7.5) containing 0.1 M KCl (Srere *et al.*, 1963).

For kinetic analysis the enzyme was desalted as before on a PD10 column. Since the enzyme was very unstable at low ionic strength, the removal of salt was done just prior to the kinetic measurements. The buffer used was either 20 mM Tris-HCl (pH 7.5) or 100 mM Tris-HCl (pH 7.5) without any KCl. Further details are given in the text. The catalytic constant of the enzyme, k_{cat}, represents the maximum velocity per mole of catalytic site. $[E_0]$ is the total concentration of *A. vinelandii* CS's active site which was determined using the molecular weight of one subunit of enzyme (48 000).

Electrophoresis. SDS−PAGE was performed on a 10% polyacrylamide slab gel using Tris-HCl (pH 8.3) discontinuous buffer system according to Laemmli (1970). The M_r of the subunit of *A. vinelandii* CS was determined by comparing the relative mobility with those of the standard calibration (listed for Figure 1) proteins obtained from Pharmacia-LKB. Native gel electrophoresis was performed in a continuous gradient of 8−25% polyacrylamide by the standard procedure given for the Phast system (Pharmacia).

Determination of Molecular Weight by Column Chromatography. A Sephacryl HR300 column (0.7 × 49 cm) was used. The column was equilibrated with 5 mM KPO_4 and 0.1 M KCl (pH 7.5). The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), ferritin (440 kDa), bovine γ globulin (158 kDa), pig CS (100 kDa), and chicken ovalbumin (44 kDa). Elution was done with the same buffer, and 0.4 mL samples were collected and analyzed for CS activity.

Sequence Analysis of A. vinelandii CS. Amino acid sequence information was acquired by automated Edman degradation with a Model 477A sequencer from the Applied Biosystems Division of Perkin Elmer (Foster City, CA) using manufacturer's standard programming and chemicals. For N-terminal sequence analysis, 5 μg (110 pmol) of enzyme was repurified for sequencing by SDS electrophoresis followed by electroblotting to Immobilon-SQ paper (Millipore Corp., Bedford, MA). The protein band was located by staining with Coomassie Blue and excised according to the method of Matsudaira (1987). For acquisition of internal sequence information, 20 μg (440 pmol) was treated similarly, and the immobilized protein was digested according to the method of Fernandez *et al.* (1992) with endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN). The peptides released were purified by reverse phase chromatography on an Applied Biosystems Model 130A HPLC system equipped with a 2.1 × 100 mm Aquapore RP300 column. Chromatography was conducted at 50 μL/min in 0.1% trifluoroacetic acid in water, and elution effected with a 100 min gradient of 0−70% acetonitrile.

Amino Acid Analysis. Amino acid analyses were done on an Applied Biosystems Model 420A amino acid analyzer equipped with autohydrolysis and on-line PTC precolumn derivation. Samples were analyzed in triplicate, and results were expressed as the averages of these three determinations.

Analytical Ultracentrifugation. Equilibrium sedimentation measurements were made in a Beckman XL-A analytical ultracentrifuge. Samples (100 μL) of *A. vinelandii* CS at a concentration of 300 μg/mL in 5 mM potassium phosphate (pH 7.5), 100 mM KCl, and 10% glycerol were centrifuged at 6 000 and 7 000 rpm at 277 K in the An60T1 rotor. The cells were scanned at 280 nm. Each data point is the average of five readings taken at 0.001 cm intervals through the sample column. The sample was assumed to have reached equilibrium when multiple scans over a 4 h period did not change. The molecular weight was calculated using a partial specific volume of 0.728, which was calculated from the amino acid composition of the protein using standard values for the individual amino acids (Zamyatnin, 1972). The solution density was calculated to be 1.0215.

Sedimentation velocity measurements also were made in the Beckman XL-A analytical ultracentrifuge. Samples were run in a two-sector cell using 400 μL of sample and 410 μL of reference buffer. Buffer conditions were the same as used for sedimentation equilibrium. The experiment was performed at 20 °C at 25 000, 35 000, and 40 000 rpm. The cell was scanned at 280 nm, and data points represent the average of five measurements made at 0.005 cm intervals. A total of 15 scans were made at 6 min intervals. Values of solution density at 2 °C were calculated to be 1.0233 g/mL, and the viscosity relative to water was calculated to be 1.288.

RESULTS

Purification of the A. vinelandii CS. CS was purified according to the protocol described in Materials and Methods (Table 1), and the purification of the enzyme resulted in an overall increase in specific activity from 1.4 to 215 units/mg of protein. The SDS profile of proteins at each stage of purification is illustrated in Figure 1.

Molecular Weight of A. vinelandii CS. Upon SDS−PAGE the purified enzyme gave one major protein band of apparent molecular weight of ~48 000 (Figure 1).

To determine the molecular weight of the holoenzyme, *A. vinelandii* CS was examined by gel filtration chromatog-

Table 1: Purification of the CS from *A. vinelandii*[a]

steps	protein (mg)	CS act. (units)	SA_{CS} (units/mg)
sonication	1700	2380	1.4
protamine sulfate	1390	1970	1.4
NH_4SO_4 fractionation	430	940	2.2
DE-52	30	550	18.3
DEAE-Sepharose	7.0	405	57.8
phenyl-Sepharose	1.35	290	215

[a] Frozen cells (20 g) were used as described in Materials and Methods. The CS activity was determined in the presence of 0.1 M KCl. SA, specific activity.

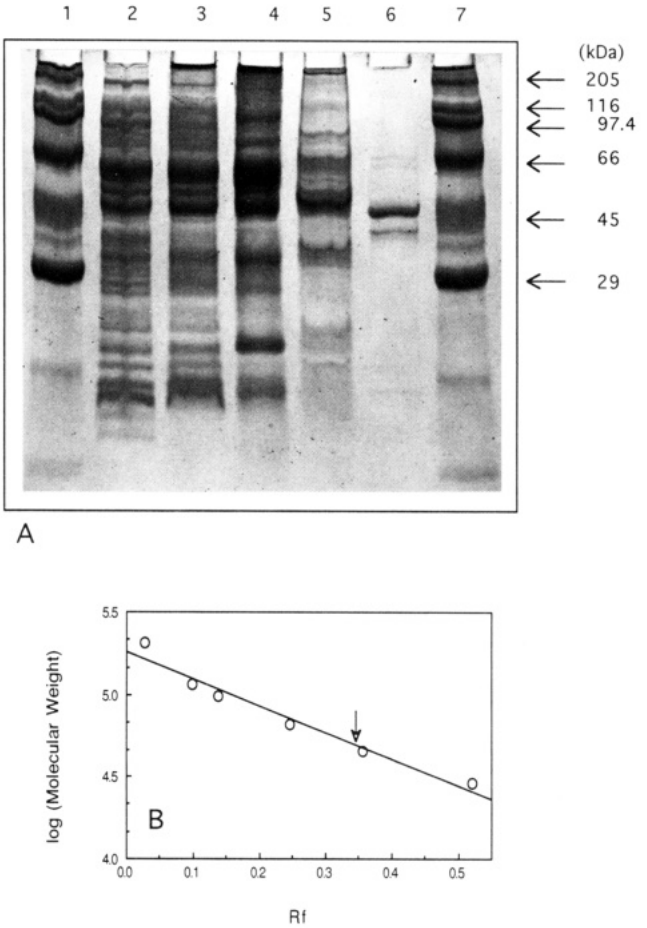

FIGURE 1: Purification of *A. vinelandii* CS. (A) SDS−PAGE. Protein samples obtained after each step in the purification procedure were electrophoresed on SDS−polyacrylamide gel and stained with Coomassie Blue. Lanes 1 and 7, 10 μg of standard proteins; lane 2, 20 μg of crude extract obtained after sonication of the sample; lane 3, 20 μg of proteins corresponding to the sample loaded on a DE-52 column; lane 4, 15 μg of proteins loaded on a DEAE-Sepharose column; lane 5, 8 μg of proteins loaded on a phenyl-Sepharose column; lane 6, 1 μg of purified *A. vinelandii* CS. (B) Calibration curve for the estimation of the molecular weight of the subunit for *A. vinelandii* CS. The continuous line is the best fit of the data corresponding to the following equation: low molecular weight = $5.2594 - 1.628R_f$, in which R_f is the distance traveled by a protein band in a polyacrylamide gel electrophoresis experiment relative to the dye front. The arrow indicates the relative mobility of the constitutive subunit for *A. vinelandii* CS corresponding to a molecular weight of ~48 000. Standard proteins were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

raphy (using an S-300 superfine gel) and by native gel electrophoresis. As shown in Figure 2, upon native gel

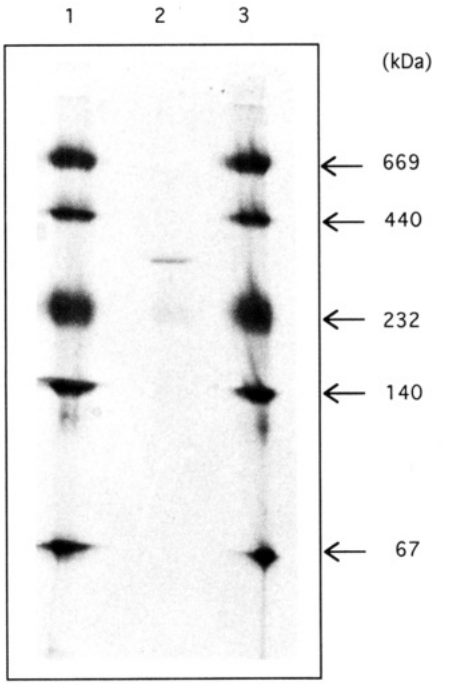

FIGURE 2: Native molecular weight of the *A. vinelandii* CS. Native gel electrophoresis. The purified *A. vinelandii* CS was electrophoresed on a 8−25% gradient polyacrylamide gel from Phast-system (Pharmacia) under nondenaturing conditions and stained with Coomassie Blue. Lanes 1 and 3, 2.5 μg of standard proteins; lane 2, 320 ng of purified *A. vinelandii* CS. The relative mobility of the native CS corresponds to a molecular weight of ~330 000.

electrophoresis, the purified enzyme gave one band. The analysis of the corresponding calibration curve indicates that the relative mobility of the native CS corresponds to a molecular weight of about 330 000. We have also checked the elution of the enzyme through a molecular sieving column, and in this case we found a molecular weight of 270 000. Since the M_r of the subunit is 48 000, these data indicated the enzyme exists as a hexamer constituted of six similar subunits of 48 000. This hexameric form was found in the presence of 0.1 M KCl which was in the elution buffer used in the gel filtration experiment.

Equilibrium Sedimentation. *A. vinelandii* CS was analyzed by equilibrium sedimentation as described in Materials and Methods. The equilibrium concentration gradients formed were initially analyzed using the program XLAEQ (Beckman Instruments). This program forces a best-fit line to a series of data points using a Nelder-Mead (Simplex) minimization method and displays the residuals for each point throughout the scan. Further modeling of the system as a single ideal solute, as nonideal solute, or as a self-associating system was done using the Optima XL-A data analysis software (Beckman Instruments), which utilizes MicroCal Origin nonlinear least-squares curve fitting to the equilibrium sedimentation functions. The amino acid composition for *A. vinelandii* CS is listed in Table 2. Figure 3 shows a representative scan of *A. vinelandii* CS at equilibrium. Using a partial specific volume of 0.728 cm^3/g, calculated from the amino acid composition, M_r of CS translated to between 277 000 (from the run at 5 000 rpm) and 249 000 (from the run at 6 000 rpm). Since the molecular weight was a weight average and the subunit molecular weight calculated from mobility in SDS−PAGE was 48 000, these results suggested that the native protein existed as a hexamer in solution. Comparison

Table 2: Amino Acid Analysis

amino acid	pmol/ sample	mol %	resi- dues	amino acid	pmol/ sample	mol %	resi- dues
Asp/Asn	1388.58	10.78	48	Tyr	345.27	2.68	12
Glu/Gln	1323.04	10.27	46	Val	697.31	5.41	24
Ser	702.33	5.45	24	Met	124.29	0.96	5
Gly	1243.37	9.65	43	Ile	613.23	4.76	21
His	291.85	2.26	10	Leu	1306.98	10.14	46
Arg	547.58	4.25	19	Phe	563.03	4.37	20
Thr	652.08	5.06	23	Lys	777.5	6.03	27
Ala	1469.22	11.40	51	Trp	ND	ND	ND
Pro	839.77	6.52	29	Cys	ND	ND	ND

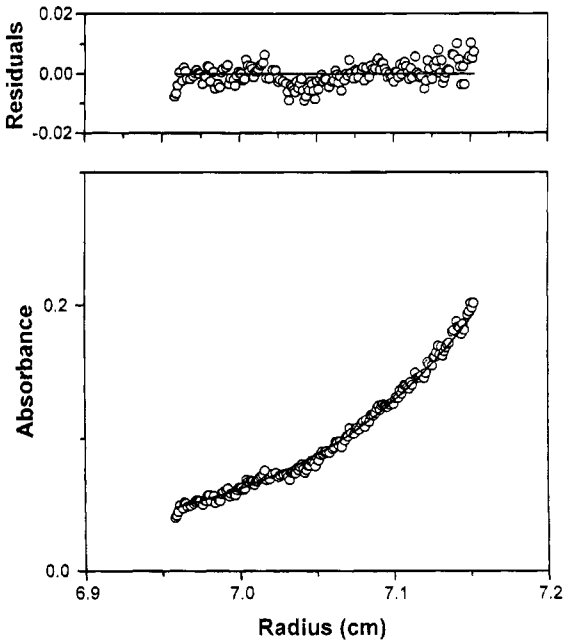

FIGURE 3: Sedimentation equilibrium studies (see Materials and Methods for details). The solid line is for a monomer–hexamer reaction.

of the experimentally determined data with a monomer–hexamer model (Figure 3) showed a reasonably good fit of the data to the calculated curve.

Sedimentation Velocity. Sedimentation velocity measurements were made as described in Materials and Methods, and the midpoint of solute boundary was plotted as a function of the rotor angular velocity and time, using the program XLAVEL (Beckman Instruments). Using the data obtained at 35 000 rpm and 40 000, the sedimentation coefficient of the molecule was calculated to be 7.26×10^{-13} and 7.17×10^{-13}, respectively. These can be corrected to $s_{20,w}$ values using the calculated partial specific volume of 0.728 cm^3/g and standard values for the viscosity and density of water and the solution used. These calculations yield sedimentation coefficients of 9.978 at 35 000 rpm and of 9.858 at 40 000 rpm. Assuming a molecular weight of 277 000 from the equilibrium sedimentation study, it is possible to calculate a frictional ratio (f/f_0) of 1.555. This indicates that the complex is a somewhat asymmetric globular protein.

Amino Acid Sequence Homology between CS of Gram-Negative Bacteria. The N-terminal region of the purified *A. vinelandii* CS was sequenced. The sequence of CSs from three Gram-negative bacteria (*Escherichia coli*, *Acinetobacter anitratum*, and *Pseudomonas aeruginosa*) have been reported (Bhayana & Duckworth, 1984; Donald & Duckworth, 1986; Donald *et al.*, 1989). The four bacterial partial sequences are aligned in Figure 4 along with the CS from pig heart (Bloxham *et al.*, 1982; Evans *et al.*, 1988), the only CS for which a three-dimensional structure is known (Remington *et al.*, 1982; Wiegand & Remington, 1986). On the basis of these partial sequences, the percentages of identical residues for all possible pairs of sequences evaluated with the alignment were determined. We found 91.2%, 71.05%, 74.5%, and 27.2% of identical amino acids between *A. vinelandii* CS compared to the CS from *P. aeruginosa*, *E. coli*, *A. anitratum*, and pig heart, respectively, although complete sequence data will be required to measure the overall similarity between these proteins. Thus, the *A. vinelandii* CS seems very closely related to CS from *P. aeruginosa*.

Kinetic Properties of A. vinelandii CS. The kinetic constants for the *A. vinelandii* CS are listed in Table 3. The k_{cat} for the enzyme (in KCl) was about twice that of the pig enzyme (Alter *et al.*, 1990). The allosteric properties of the enzyme were investigated, and the $[S]_{0.5}$ values for acetyl-CoA and the K_m values for OAA were determined in the presence or absence of allosteric effectors. As shown in Figure 5, the substrate velocity curves for acetyl-CoA in the absence of effector were sigmoidal, while for OAA normal Michaelis–Menten kinetics were observed. When 0.17 mM 5′-AMP or 0.1 M KCl was added to the reaction mixture, marked changes occurred in the kinetic behavior. The $[S]_{0.5}$ for acetyl-CoA decreased, the maximum velocity increased, and hyperbolic kinetics were observed. In the case of OAA, the maximum velocity increased without any change in K_m or Michaelis–Menten behavior (Table 3, Figure 5).

The Hill coefficient, n_H, was determined for acetyl-CoA saturation curves in the absence and presence of KCl or AMP at concentrations giving the maximal activation (Figure 6). In the absence of effectors in the reaction mixture the Hill parameter ($n_H = 1.5$) suggested cooperativity among reactive sites under these conditions. When 0.17 mM AMP was added to the reaction mixture, the results ($n_H = 1.2$) suggested that AMP acts as a positive effector, and in the presence of 0.1 M KCl the coefficient became $n_H = 1$.

Because the plot may be nonlinear over the entire range of the velocity, the Hill parameters are determined as the slope of the Hill plot at $v = 0.5V_m$ as recommended by Segel (1975). All determinations of this parameter were done using the substrate concentrations between $^1/_2[S]_{0.5}$ to $2[S]_{0.5}$ (Figure 5).

Effect of Cations on Enzyme Activity. The relative effect of potassium and other cations on the reaction velocity was investigated. The reaction mixture used as control contained 20 mM Tris-HCl (pH 7.5), and 100 mM cationic equivalent of salt was added. Potassium gave rise to the best activation (~4.8 times) compared to sodium chloride (~4.6 times), ammonium sulfate (~4.2 times), or Tris-HCl itself (~2.6 times).

Sensitivity of A. vinelandii CS to NADH Inhibition. As shown in Figure 7A, the enzyme was inhibited by adding NADH, and 70% of the activity was inhibited. The inhibition constant K_i changed, depending on the ionic strength of the buffer used for the activity measurements (Table 4). In both cases, the inhibition achieved by 1 mM NADH could be completely reversed by 0.17 mM 5′-AMP (Figure 7B). On the other hand, the presence of 0.1 M KCl or 0.17 mM AMP in the reaction mixture prevented inhibition of *A. vinelandii* CS by NADH (data not shown).

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A. vinelandii      1    ADKKAQLIIEGNAPVELPMLTGTVGPDVIDVRGLT  35
P. aeruginosa      1    .........▲.S......V▲S..M....V▲.....  35
E. coli            1    ..T..K.TLN.DTA...DV▲K..L.Q...▲I.T.G  35
A. anitratum       1  SEATG...V▲HLD.-KEI..▲IYS..L...▲...KDVL  37
Pig heart          1    ASSTN.KDILADLIPKEQARIKTFRQQHGNTVVG  34

A. vinelandii            GILLHRGYPIEQLAEK                LMGFGHRVYK
P. aeruginosa      64    .V....▲.........  79    300     ▲.........  309
E. coli            64    ......▲F..D...TD  79    300     ▲.........  309
A. anitratum       66    ....▲.....D...TQ  81    301     .........▲  310
Pig heart          62    ..RF-...S.PECQKM  77    315     VP.Y.▲A.LR   324
                               *                          ||||||*  *

A. vinelandii            QTCDEVLSELG-I-NDPQLELAMKL        ERNLYPNVDFYSGIILK
P. aeruginosa      320   ▲......Q..▲-.-.........▲..  342  354  .....▲.........▲  370
E. coli            320   E..H...K..▲-T-K.DL..V.▲E.  342  354  .KK...▲.........▲  370
A. anitratum       321   E......EA▲.-.-.....A.▲.E.  343  355  ..K..▲.........▲.  371
Pig heart          335   EFA---.KH.PHDPMFK-.-V.QLY  354  367  AKNPW....AH..VL.Q  383
                                                                      *

A. vinelandii            IGRPRQLYTGYEK
P. aeruginosa      405   .....▲....HTQ     417
E. coli            405   .A...▲.......     417
A. anitratum       406   ....▲.....EV.     418
Pig heart          419   LE..KSMS.DGLI     421
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FIGURE 4: Alignment of partial amino acid sequences for one eucaryotic and four bacterial CSs. The standard one-letter abbreviations are used. Sources of the sequence data: *A. vinelandii* (this paper); *P. aeruginosa* (Donald *et al.*, 1989); *E. coli* (Bhayana & Duckworth, 1984); *A. anitratum* (Donald & Duckworth, 1987); and pig heart (Bloxham *et al.*, 1982). Dots indicate residues which are identical to those in the *A. vinelandii* sequence, and dashes mark deletions introduced to improve the alignment. Below the pig heart CS sequence, symbols mark residues involved in substrate binding (Wiegand & Remington, 1986) both side-chain (*) and main-chain (■) interactions. Every 10th residue is underlined, and the number of the first and the last residue for each partial sequence is given at each extremity of the line.

Table 3: Kinetic Constants for the *A. vinelandii* CS[a]

	k_{cat} (s^{-1})	$[S]_{0.5}$(acetyl-CoA) (μM)	K_m(OAA) (μM)
−KCl	117 ± 4	130 ± 5	11.5 ± 0.8
+0.1 M KCl	174 ± 7	45 ± 2	14.2 ± 1.0
+0.17 mM AMP	174 ± 9	90 ± 5	

[a] All values were determined in 0.1 M Tris-HCl (pH 7.5). $[S]_{0.5}$(acetyl-CoA) and K_m (OAA) were determined in the presence of saturating amounts of the other substrate. The total concentration of *A. vinelandii* CS active site in the assay was 3.66 nM.

DISCUSSION

We have reported previously that the amounts of CS and other Krebs tricarboxylic acid cycle enzymes in cells are correlated to the energy requirements of the cells (Srere, 1969). *A. vinelandii* is a nitrogen-fixing species. It has been reported that 8 mol of ATP are required for each mole of N_2 fixed. Thus the Krebs TCA cycle is present in a high concentration. Only 150-fold purification was therefore necessary to obtain pure protein.

Since our structural results were different from those previously reported (Johnson & Hanson, 1974), we reinvestigated the kinetic properties of the enzyme and compared our results with those of CSs from other Gram-negative bacteria. The specific activity of the *A. vinelandii* CS (215 units/mg) was half the value previously reported (Flechtner & Hanson, 1970) for the putative tetrameric form. However, the earlier report measured activity at 30 °C, and all our studies were at 25 °C. Compared to other hexameric CSs, however, this value was 20 times higher than the one described for *P. aeruginosa* (Donald *et al.*, 1989). When comparing the corresponding k_{cat} values of *A. vinelandii CS* to other bacterial CSs, we found larger values than those reported for the *E. coli* CS and the *A. anitratum* CS (Anderson & Duckworth, 1988; Donald & Duckworth, 1987).

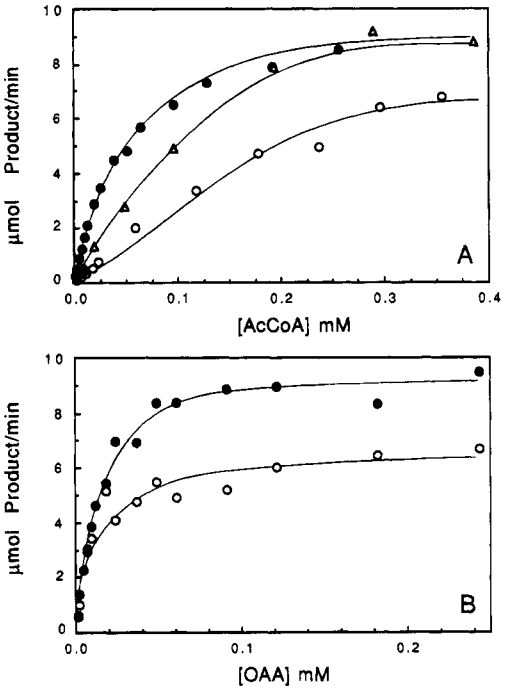

FIGURE 5: Kinetic behavior of *A. vinelandii* CS. (A) Saturation curve of acetyl-CoA substrate. The activity was determined at 25 °C in 0.1 M Tris-HCl (pH 7.5) containing 0.2 mM OAA (○) in the presence of 0.17 mM 5′-AMP (△), or in the presence of 0.1 M KCl (●). (B) Saturation curve for OAA substrate. The activity was determined in 0.1 M Tris-HCl (pH 7.5) containing 0.2 mM acetyl-CoA (○) in the presence of 0.1 M KCl (●). The concentration $[E_0]$ of the enzyme in the assay is 3.66 nM. The corresponding kinetic parameters are listed in Table 3.

All CSs thus far examined fall into two molecular mass classes (300 kD and 100 kDa). Large CSs (~300 kDa) are mainly found in Gram-negative bacteria. Earlier studies suggested that the enzyme from *A. vinelandii* was tetrameric with a molecular weight of ~250 000 and was composed of four subunits of M_r ~60 000 (Johnson & Hanson, 1974).

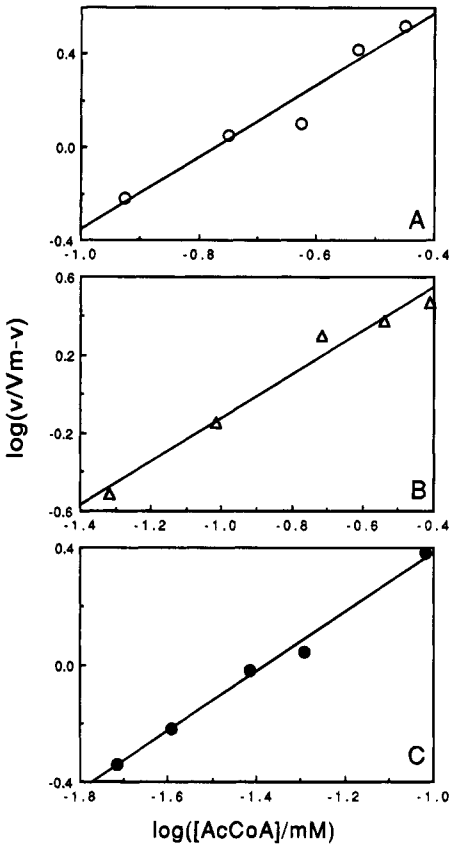

FIGURE 6: Hill plots for acetyl-CoA saturation of *A. vinelandii* CS. (A) Plot corresponding to the kinetic measurements determined in 0.1 M Tris-HCl (pH 7.5), $n_H = 1.53 \pm 0.08$. (B) Plot corresponding to the kinetic measurements determined in the presence of 0.17 mM 5′-AMP, $n_H = 1.2 \pm 0.02$. (C) Plot corresponding to the kinetic measurements determined in the presence of 0.1 M KCl, $n_H = 1.020 \pm 0.009$.

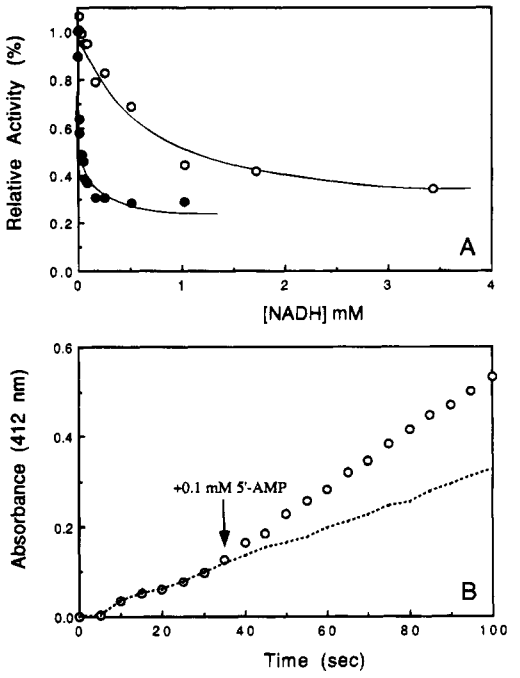

FIGURE 7: Inhibition of the *A. vinelandii* CS. (A) Dependence of NADH inhibition on ionic strength of the buffer. The reaction mixture contained 0.2 mM acetyl-CoA and 0.2 mM OAA in (○) 0.1 M Tris-HCl (pH 7.5) and (●) 20 mM Tris-HCl (pH 7.5). The concentration $[E_0]$ of *A. vinelandii* CS in the assay was 3.66 nM. The values of the initial velocity of the reaction are (○) 6.5 μmol of P min^{-1} and (●) 2.5 μmol of P min^{-1}. The corresponding values of k_{cat} are (○) 118 s^{-1} and (●) 45 s^{-1}. (B) Reactivation of *A. vinelandii* CS by 5′-AMP. The reaction cuvette contained 1 mM NADH, 0.2 mM acetyl-CoA, and 0.2 mM OAA in 0.1 M Tris-HCl (pH 7.5) (– – –). After 30 s of reaction, 0.17 mM 5′-AMP was introduced in the cuvette (○). The concentration of the enzyme in the assay was 3.66 nM. The values of the velocity of the reactions are (– – –) 3.6 μmol of P min^{-1} and (○) 7.5 μmol of P min^{-1}. The corresponding values of k_{cat} are (– – –) 95.5 s^{-1} and (○) 199 s^{-1}.

Table 4: NADH Inhibition Constants for the *A. vinelandii* CS[a]

Tris-HCl	K_i (NADH) (μM)	max inhibn (%)
20 mM	12.0 ± 1.5	70
0.1 M	660 ± 20	70

[a] The *A. vinelandii* CS was first desalted on a PD10 column (Sephadex G25). The enzyme concentration $[E_0]$ in the assay was 3.66 nM, and the activity was measured in the absence of KCl in the reaction mixture containing 0.2 mM acetyl-CoA and 0.2 mM OAA.

CS enzymes from other Gram-negative bacteria show a predominantly hexameric structure (Tong & Duckworth, 1975). In this report we presented evidence for a hexameric structure of the *A. vinelandii* CS. It consisted of six similar subunits of a molecular weight of ~48 000. These results agreed with the observation that all well-studied CSs are composed of a single type of subunit of M_r ~50 000 (Donald & Duckworth, 1987; Donald *et al.*, 1989; Ferea *et al.*, 1994; Heinzen *et al.*, 1991; Srere, 1972; Wood *et al.*, 1987). Moreover, the comparison of partial amino acid sequences of CSs shows a high degree of homology between *A. vinelandii* enzyme and those of three other Gram-negative bacteria, suggesting that all may possess similar subunit structure.

Weitzmann and Danson (1976) acknowledge that a number of exceptions exist in their bacterial classification of large and small CS. For each exception they argue either misclassification of the bacterial species or some special metabolic circumstance of the cell. In addition, the question of polymeric composition (tetrameric, hexameric) for all reported large CSs is not yet fully settled. A recent report (Kiriukhin *et al.*, 1993) indicates that CS from the obligate methylotroph, *Methylobacillus flagellatum*, is an octamer and moreover has the kinetics of a small (dimeric) CS. In another report (Takahashi *et al.*, 1994), it has been demonstrated that the CS for *Nitrobacter agilis* is reported to have an M_r of 250 000 that is a heterotetramer of 45 000 and 80 000 subunits with hyperbolic Michaelis–Menten kinetics. Moreover, it was recently shown that both forms (large and small) of CS could exist in the same organism as a result of two distinct genes (Anderson *et al.*, 1993; Patton *et al.*, 1993). It would appear that at this point one must maintain an open mind about the subunit structure of various CS enzymes and the relation of each structure to its kinetic behavior.

Another consideration is how the allosteric regulatory effects of *Azotobacter*'s (and other facultative Gram-negative organisms) CS are manifested *in situ*. It was demonstrated that the dimer is the functional unit of all types of CS and that the oligomeric association results in the acquisition of allosteric regulatory sensitivity. In this case the hexameric form appears to behave functionally as a trimer of the basic dimer (Else *et al.*, 1988). However, all of the allosteric regulatory behavior is eliminated in the presence of an ionic strength likely to exist *in situ* in these organisms (Luria, 1960). If there is still allosteric behavior *in situ*, a fact not

yet demonstrated experimentally, then one must imagine that the allosteric form of the enzyme is preserved *in situ* either by some unknown endogenous component, i.e., other proteins or metabolites, or by its binding to a structural element of the cell like the cell membrane.

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