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Citrate synthase from a Gram-positive bacterium

Purification and characterization of the *Bacillus megaterium* enzyme

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Citrate synthase was purified to homogeneity from a Gram-positive bacterium (*Bacillus megaterium*) for the first time. The M_r of the native enzyme was determined to be 84000 (S.E.M. ± 5000). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration in guanidinium chloride revealed a single protein species of M_r 40300 (S.E.M. ± 4400), indicating a dimeric enzyme. This dimeric structure was confirmed by cross-linking the native enzyme with dimethyl suberimidate and with glutaraldehyde, followed by electrophoretic analysis. The enzyme follows Michaelis–Menten kinetics with respect to both substrates, acetyl-CoA and oxaloacetate, and is sensitive to non-specific inhibition by a range of adenine nucleotides. In both molecular and catalytic properties the citrate synthase closely resembles the enzyme from eukaryotic sources and contrasts markedly with the larger, hexameric, enzyme from Gram-negative bacteria.

Citrate synthase catalyses the reaction:

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \rightarrow \text{citrate} + \text{CoA}$$

This reaction effects the entry of carbon into the citric acid cycle, and citrate synthase is thus an important potential site for the control of this metabolic pathway. Accordingly, the enzyme is regulated by various metabolic effectors, which reflect the energetic and biosynthetic roles of the cycle. In turn, the patterns of regulation observed are correlated with the molecular structure of the citrate synthase and the taxonomic status of the source organism (Weitzman & Danson, 1976; Weitzman, 1981).

Only the 'large', probably hexameric (Tong & Duckworth, 1975; C. G. Mitchell & P. D. J. Weitzman, unpublished work), citrate synthases are allosterically inhibited by NADH, and these are found exclusively in Gram-negative bacteria (Weitzman & Jones, 1968). AMP relieves this inhibition only in the aerobic members of this group (Weitzman & Jones, 1968), whereas the enzyme from the facultative anaerobes is subject to additional inhibition by 2-oxoglutarate (Wright *et al.*, 1967; Weitzman & Dunmore, 1969). In contrast, Gram-positive bacteria and eukaryotic organisms possess 'small' citrate synthases (Weitzman & Danson, 1976; Weitzman, 1981), which, in the eukaryotes, have been shown to be dimeric (Wu & Yang, 1970; Singh *et al.*, 1970; Moriyama & Srere, 1971; Wiegand *et al.*, 1979). These enzymes are isosterically inhibited by ATP but are insensitive to NADH and 2-oxoglutarate (Harford & Weitzman, 1975; Weitzman & Danson, 1976; Weitzman, 1981).

The 'small' citrate synthase has been well characterized from a number of eukaryotic sources (Weitzman & Danson, 1976; Wiegand *et al.*, 1979; Bloxham *et al.*, 1980, 1981; Weitzman, 1981). However, the enzyme has not hitherto been purified to homogeneity from a Gram-positive bacterium, and its subunit composition therefore remains to be established. In the present paper we report the purification to homogeneity of citrate synthase from the Gram-positive bacterium *Bacillus megaterium*. Structural and regulatory properties of the pure enzyme are also reported, and comparisons are made with both the 'large' citrate synthases and the eukaryotic enzymes.

Experimental

Materials

The organism used was *Bacillus megaterium* strain D101 from the Culture Collection of the Department of Microbiology, University of Leicester, Leicester, U.K.

Chemicals used were analytical grade or the finest grade commercially available. Lysozyme (egg-white)

was from Miles Laboratories, Elkhart, IN, U.S.A. Protamine sulphate (salmon roe) was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. CoA, NADPH, oxaloacetic acid, fructose bisphosphate aldolase (rabbit muscle), catalase (bovine liver), citrate synthase (pig heart), glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart), phosphorylase *a* (rabbit muscle) and triose phosphate isomerase (yeast) were from Boehringer, Mannheim, Germany; ATP, ADP, AMP, $NADP^+$, NAD^+, NADH, 5,5′-dithiobis-(2-nitrobenzoic acid), glutaraldehyde (aqueous 25% solution), phenylmethanesulphonyl fluoride, albumin (bovine serum), cytochrome *c* (horse heart), β-galactosidase (*Escherichia coli*) and myoglobin (whale skeletal muscle) were from Sigma Chemical Co., Poole, Dorset, U.K.; dimethyl suberimidate and mercaptoethanesulphonic acid were from Pierce Chemical Co., Rockford, IL, U.S.A.; DEAE-Sephacel, Sephacryl S-200 (superfine grade), Sephacryl S-300 (superfine grade) and Blue Dextran 2000 were from Pharmacia; Matrex Gel Red A was from Amicon Corp., Lexington, MA, U.S.A.; iodo[2-^{14}C]acetic acid was from Amersham International, Amersham, Bucks., U.K.; guanidinium chloride was from Fluka, Buchs, Switzerland. Pyruvate dehydrogenase complex was prepared from *Escherichia coli* by the method of Danson *et al.* (1979*a*).

Assay of citrate synthase

Citrate synthase was assayed spectrophotometrically at 412nm at 25°C by the method of Srere *et al.* (1963). Unless otherwise stated, the assay mixture contained (final concentrations) 20mM-Tris/HCl buffer, pH8.0, 1mM-EDTA, 100mM-KCl, 20% (v/v) glycerol, 0.15mM-acetyl-CoA, 0.2mM-oxaloacetate and 0.1mM-5,5′-dithiobis-(2-nitrobenzoate).

The concentration of protein was determined in impure preparations by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and in purified preparations from the values of absorbance at 260nm and 280nm (Layne, 1957). Specific activity is expressed as μmol of CoA produced/min per mg of protein.

Purification of citrate synthase

B. megaterium was grown aerobically at 37°C in L-broth (Lennox, 1955). Cells from 10 litres of an overnight (16h) culture were harvested, washed in 200ml of 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 100mM-KCl, 20% (v/v) glycerol and 0.15mM-phenylmethanesulphonyl fluoride (to inhibit proteinase activity) and again collected by centrifugation. The pellet (35g) was resuspended in 100ml of the above buffer at 4°C containing lysozyme at a concentration of 25mg/ml. The suspension was stirred continuously on ice for 2h. The cells were then lysed by passing the suspension twice through a French press at a pressure of 62MPa (9000lbf/in^2). The debris was removed by centrifugation at 20000*g* ($r_{av.}$ 11.9cm).

An aqueous 2% (w/v) solution of protamine sulphate was added dropwise to the supernatant at 4°C (1mg of protamine sulphate/20mg of protein), and the suspension was stirred for 20min. The precipitate was removed by centrifugation at 20000*g* ($r_{av.}$ 11.9cm), and the supernatant was applied to a column (3cm × 30cm) of DEAE-Sephacel previously equilibrated with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 100mM-KCl, 20% (v/v) glycerol and 0.15mM-phenylmethanesulphonyl fluoride. The column was washed with a further 200ml of this buffer, and a gradient of 0.1–0.5M-KCl was applied in 500ml of the buffer. Fractions (5.2ml) were collected at a flow rate of 15ml/h. Fractions containing a specific activity of 1.9units/mg or more were pooled and dialysed at 4°C for 3h against 2 litres of the above buffer containing 50mM-KCl instead of 100mM-KCl. The product was applied to a column (1.2cm × 25cm) of Matrex Gel Red A that had been equilibrated with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 50mM-KCl, 20% (v/v) glycerol and 0.15mM-phenylmethanesulphonyl fluoride. A gradient of NaCl (0–0.5M) was applied in 100ml of this buffer, and 1ml fractions were collected at a flow rate of 10ml/h. Fractions containing a specific activity of 10.8units/mg or more were pooled and subjected to dialysis as before. The product was applied to a column (0.8cm × 8.0cm) of Matrex Gel Red A that had been equilibrated with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 100mM-KCl, 20% (v/v) glycerol and 0.15mM-phenylmethanesulphonyl fluoride. Citrate synthase was eluted by using this buffer containing 0.1mM-CoA and 0.1mM-oxaloacetate, 1ml fractions being collected at a flow rate of 10ml/h. Fractions containing enzyme at a specific activity of 20units/mg or more were tested for purity by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Gel filtration

(*a*) *Native enzyme.* Gel filtration was performed on a column (1.6cm × 70cm) of Sephacryl S-200 (superfine grade) in 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 100mM-KCl, 20% (v/v) glycerol and 0.15mM-phenylmethanesulphonyl fluoride. The flow rate was 10ml/h, and 1ml fractions were collected. Standard proteins were also run at the same time as the citrate synthase; these were bovine liver catalase (M_r 244000; Stokes radius $r = 5.05$nm), pig heart fumarase (M_r 194000; $r = 4.92$nm), pig heart lactate dehydrogenase (M_r 142000; $r = 4.47$nm), pig heart citrate

synthase (M_r 98000; $r = 3.88$ nm), pig heart malate dehydrogenase (M_r 67000; $r = 3.68$ nm) and whale skeletal-muscle myoglobin (M_r 17200; $r = 1.93$ nm). Catalase and myoglobin were detected spectrophotometrically at 410nm and 540nm respectively. Fumarase was assayed spectrophotometrically at 240nm (Bergmeyer, 1974*a*), and lactate dehydrogenase and malate dehydrogenase were assayed spectrophotometrically at 340nm (Bergmeyer, 1974*b*,*c*).

(*b*) *Dissociated enzyme.* Gel filtration in 6M-guanidinium chloride was performed by the method of Kresze *et al.* (1980) as described by Danson & Porteous (1981). All proteins were radiolabelled with iodo[2-^{14}C]acetic acid, and their elution volumes were detected by counting the radioactivity of samples of each fraction. For each protein, values of K_d (the distribution coefficient) were calculated as described by Belew *et al.* (1978):

$$K_d = (V_e - V_0)/(V_{re} - V_0)$$

where V_e, V_0 and V_{re} represent the elution volumes of the protein, Blue Dextran and 2-nitro-5-mercaptobenzoate respectively.

Polyacrylamide-gel electrophoresis

Disc gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate in phosphate-buffered 7.5% (w/v) polyacrylamide gels was performed as described by Shapiro *et al.* (1967).

Modification with cross-linking reagents

Cross-linking of *B. megaterium* citrate synthase by using dimethyl suberimidate was performed by the method of Davies & Stark (1970). A 40μg portion of enzyme was treated with 24mg of dimethyl suberimidate in 2ml of 0.2M-triethanolamine/HCl buffer, pH8.5, for 3h at 20°C.

Cross-linking with glutaraldehyde was performed by the method of Nucci *et al.* (1978). Protein (10μg/ml) was incubated for 3h at 20°C with 0.25% (v/v) glutaraldehyde in 0.05M-sodium phosphate buffer, pH7.5, containing 1mM-2-mercaptoethanol and 20% (v/v) glycerol.

Samples of cross-linked protein were dialysed against 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA before analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Analytical ultracentrifugation

Sedimentation-velocity analyses were performed in a Beckman L5-50B analytical ultracentrifuge at a rotor speed of 45000rev./min at or near 20°C. All sedimentations were in 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 0.1M-KCl and 20% (v/v) glycerol; migration of the boundary was followed at 280nm with a u.v. scanner, and the observed sedimentation coefficients were corrected to the density and viscosity of water ($s_{20,w}$ values). High-speed sedimentation-equilibrium studies (Yphantis, 1964) were also performed in the same buffer. The partial specific volume ($\bar{v}$) was calculated from the amino acid composition as described by Bowen (1970).

Amino acid analysis

Protein samples were reduced and *S*-carboxymethylated with iodoacetic acid (Gibbons & Perham, 1970). Samples were hydrolysed *in vacuo* in triplicate in 6M-HCl for 24, 48 and 72h. Values for tryptophan were obtained by hydrolysis for 24h in 3M-mercaptoethanesulphonic acid. Samples were analysed in a Rank Hilger Chromaspek J180 amino acid analyser. Values for threonine and serine were corrected for loss by extrapolation to zero time. For isoleucine and valine the 72h values were used.

Results

Purification of citrate synthase

A summary of the purification procedure is provided in Table 1. A 200-fold increase in the purity of the enzyme was achieved. The preparation was found to be homogeneous as judged by electrophoresis in sodium dodecyl sulphate/polyacrylamide gels, by gel filtration in 6M-guanidinium chloride and by sedimentation-velocity analysis.

In the absence of glycerol enzymic activity was rapidly lost in both crude and pure preparations. Sedimentation-velocity studies indicated that this inactivation was accompanied by dissociation of the enzyme into a monomeric form. KCl also afforded some protection against inactivation and, in the presence of both glycerol (20%, v/v) and KCl (0.1M), the pure citrate synthase was completely stable at 4°C for at least 6 months.

Determination of the M_r of the native enzyme

It proved impossible to determine the M_r of the enzyme by equilibrium ultracentrifugation; over the time required to reach equilibrium, dissociation of the enzyme was observed, even at 4°C and in the presence of glycerol. The M_r was therefore calculated from a combination of the Stokes radius and the sedimentation coefficient, as described by Danson *et al.* (1979*b*).

The Stokes radius was determined by zonal gel filtration on Sephacryl S-200 by the method of Andrews (1965). Filtration of the purified citrate synthase and a series of standard proteins of known Stokes radii was performed as described in the Experimental section. The *Bacillus* citrate synthase was eluted as a single symmetrical peak, and, from a plot of Stokes radius versus elution volume for the standard proteins (Fig. 1), the Stokes radius was calculated to be 4.0 (S.E.M. $\pm$0.2)nm. Sedimenta-

Table 1. *Purification of B. megaterium citrate synthase*
For experimental details see the text.

Step	Volume (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
1. French-press extract	110	461	4556	0.1	—
2. Supernatant from protamine sulphate treatment	110	441	3426	0.1	96
3. Pooled selected fractions from DEAE-Sephacel column	52	294	117	2.5	64
4. After dialysis	52	281	116	2.4	61
5. Pooled selected fractions from Matrex Gel Red A column (elution with salt)	13	117	10	11.7	25
6. After dialysis	13	109	10	10.9	24
7. Pooled selected fractions from Matrex Gel Red A column (elution with oxaloacetate plus CoA)	4	78	3.6	21.6	17

tion-velocity analysis of the citrate synthase again revealed a single protein species. The sedimentation coefficient ($s_{20,w}$) was determined to be 5.2 (S.E.M. $\pm$ 0.2) S. Combination of Stokes Law and the Svedberg equation gives the following relationship (Danson *et al.*, 1979*b*):

$$M_r = \frac{6\pi \cdot \eta \cdot N_A \cdot r \cdot s}{(1 - \bar{v}\rho)}$$

where M_r is the relative molecular mass, η is the solvent viscosity, N_A is Avogadro's number, r is the Stokes radius, s is the sedimentation coefficient, $\bar{v}$ is the partial specific volume and ρ is the solvent density. By taking $\bar{v}$ as 0.72 ml/g, from the amino acid composition (see Table 2), the M_r of *B. megaterium* citrate synthase was calculated to be 84000 (S.E.M. $\pm$ 5000).

Determination of the subunit M_r

Electrophoresis of the purified citrate synthase in sodium dodecyl sulphate/polyacrylamide gels gave a single protein band, confirming the homogeneity of the preparation. Comparison of the electrophoretic mobility with those of standard proteins run simultaneously (Fig. 2) gave a polypeptide-chain M_r of 41000 (S.E.M. $\pm$ 4000). Similarly, gel filtration on Sephacryl S-300 in the presence of 6 M-guanidinium chloride indicated a single species of protein. The data from the gel filtrations were plotted in the form $\ln(100\,K_d)$ versus $N^{\frac{1}{3}}$ (Fig. 3), where N is the number of amino acid residues in the polypeptide chain (Rydén, 1971). A good fit to a straight line is observed for the standard proteins. The K_d value obtained for citrate synthase [0.21 (S.E.M. $\pm$ 0.01)] corresponds to 377 (S.E.M. $\pm$ 18) amino acids per polypeptide chain; from the amino acid composition of the protein this leads to a value for the molecular weight of 39600 (S.E.M. $\pm$ 2000). This value is in excellent agreement with that obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and suggests that the citrate synthase is a dimeric enzyme composed of subunits of similar M_r.

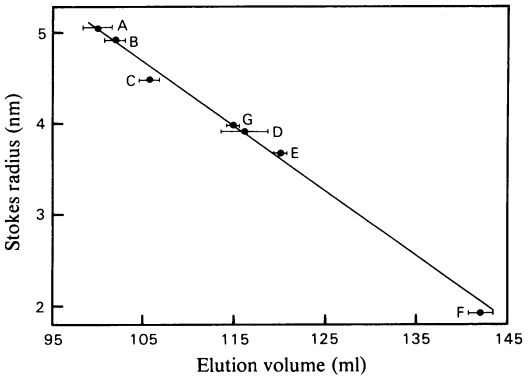

Fig. 1. *Gel filtration of B. megaterium citrate synthase and standard proteins on Sephacryl S-200: dependence of elution volume on Stokes radius*
Gel filtration on Sephacryl S-200 (superfine grade) was performed at 4°C in 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 100 mM-KCl, 0.15 mM-phenylmethanesulphonyl fluoride and 20% (v/v) glycerol as described in the text. Standard proteins: A, bovine liver catalase; B, pig heart fumarase; C, pig heart lactate dehydrogenase; D, pig heart citrate synthase; E, pig heart malate dehydrogenase; F, whale skeletal-muscle myoglobin; G, *B. megaterium* citrate synthase. The bars represent the S.E.M. of the volumes at which each protein was eluted in five separate filtration experiments.

Chemical modification with cross-linking agents

After treatment of the native enzyme with dimethyl suberimidate or glutaraldehyde, citrate synthase showed two protein bands on sodium dodecyl sulphate / polyacrylamide - gel electrophoresis. These two proteins corresponded to M_r values of 41000 and 89000, supporting the suggestion that the enzyme is dimeric. Modification with glutaraldehyde converted more than 70% of the protein into the dimeric form, but no species of higher molecular weight was observed.

Table 2. *Amino acid composition of B. megaterium citrate synthase*

Values for threonine and serine were obtained by extrapolation back to zero time. Values for valine and isoleucine are 72h values only. Values for tryptophan were determined by hydrolysis with 3M-mercaptoethanesulphonic acid at 105°C. All other values are means (±S.E.M.) from triplicate samples taken after 24h, 48h and 72h hydrolysis in 6M-HCl at 105°C.

Amino acid	Residues/molecule (M_r 84000)
CyS	8.0 (±0.9)
Asp	70.3 (±1.1)
Thr	46.1 (±3.0)
Ser	69.4 (±2.3)
Glu	106.9 (±1.5)
Pro	31.1 (±0.7)
Gly	76.6 (±2.1)
Ala	80.2 (±0.7)
Val	40.2 (±0.8)
Met	15.1 (±1.0)
Ile	34.0 (±1.6)
Leu	70.4 (±0.7)
Tyr	21.7 (±1.3)
Phe	20.0 (±0.5)
His	31.9 (±0.9)
Trp	1.8 (±0.2)
Lys	38.0 (±0.6)
Arg	38.6 (±0.8)

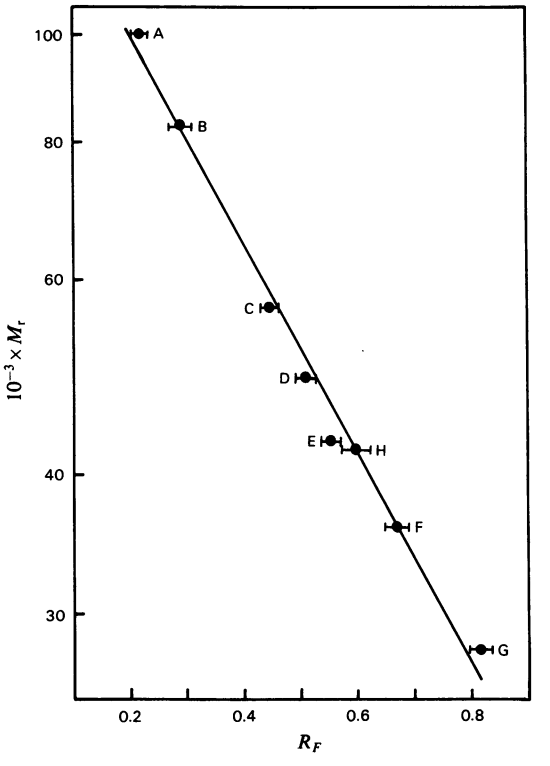

Fig. 2. *Relationship between molecular weight and relative electrophoretic mobility (R_F) of B. megaterium citrate synthase and standard proteins in sodium dodecyl sulphate/polyacrylamide gels*

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis was performed as described in the text. Standard proteins and their assumed polypeptide-chain molecular weights were: A, *E. coli* pyruvate decarboxylase (M_r 100000); B, *E. coli* lipoate acetyltransferase (M_r 83000); C, *E. coli* lipoamide dehydrogenase (M_r 56500); D, pig heart citrate synthase (M_r 49000); E, rabbit muscle fructose bisphosphate aldolase (M_r 40000); F, rabbit muscle lactate dehydrogenase (M_r 36000); G, yeast triose phosphate isomerase (M_r 28000). H represents *B. megaterium* citrate synthase.

Amino acid analysis

The amino acid composition of the citrate synthase is given in Table 2, and indicates a total of 800 residues for an M_r of 84000, i.e. 400 residues per polypeptide chain.

Kinetic and regulatory properties

The enzyme showed a hyperbolic dependence of rate on the concentration of each substrate. Analysis of the data by using the direct linear plot of Eisenthal & Cornish-Bowden (1974) gave apparent K_m values of 12 (S.E.M. ±3)μM and 76 (S.E.M. ±10)μM for oxaloacetate and acetyl-CoA respectively.

Low concentrations of NADH (e.g. 0.1mM), which produce specific allosteric inhibition of Gram-negative bacterial citrate synthases, were totally without effect on the *Bacillus* enzyme. However, some inhibition was observed at much higher concentrations (in the range 1–10mM). This inhibition appears to be non-specific, as similar inhibitions were produced by a range of related nucleotides, the order of effectiveness being ATP > ADP > AMP and NADPH > $NADP^+$ > NADH > NAD^+. Thus, for example, these nucleotides (at 10mM concentration) exerted the following inhibitions of enzymic activity: 79% (ATP), 64% (ADP), 36% (AMP), 57% (NADPH), 32% ($NADP^+$), 31% (NADH) and 9% (NAD^+). These inhibitions closely parallel, both qualitatively and quantitatively, the effect observed with other 'small' citrate synthases, both naturally occurring and mutationally generated (Weitzman & Danson, 1976; Weitzman *et al.*, 1978; Danson *et al.*, 1979*b*; Weitzman, 1981). All these non-specific inhibitors probably act isosterically (Harford & Weitzman, 1975) as a result of the similarities between the nucleotides and the substrate acetyl-CoA.

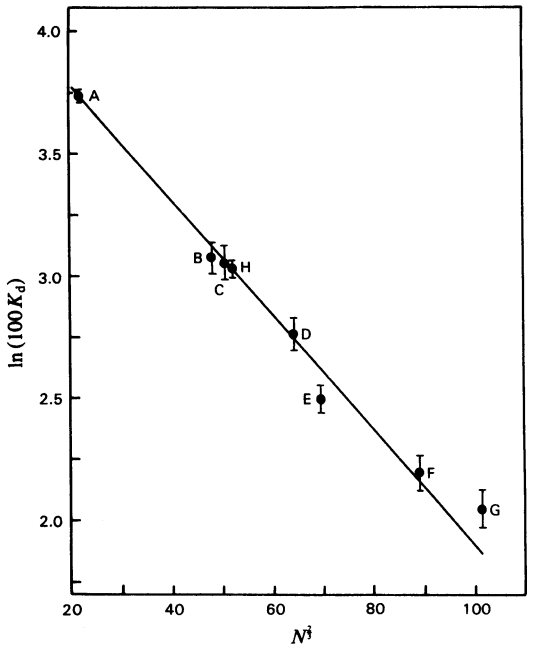

Fig. 3. *Gel filtration of B. megaterium citrate synthase and standard proteins on Sephacryl S-300 in guanidinium chloride*

Gel filtration on Sephacryl S-300 in 6 M-guanidinium chloride, pH 5.0, was performed as described in the text. N is the number of amino acids in the polypeptide chain, and K_d is the distribution coefficient, calculated as described in the Experimental section. Standard proteins: A, horse heart cytochrome *c* ($N = 104$); B, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase ($N = 330$); C, rabbit muscle fructose bisphosphate aldolase ($N = 358$); D, *E. coli* lipoamide dehydrogenase ($N = 511$); E, bovine serum albumin ($N = 579$); F, rabbit muscle phosphorylase *a* ($N = 841$); G, *E. coli* β-galactosidase ($N = 1021$); H, *B. megaterium* citrate synthase.

Discussion

The present paper represents the first report of the successful purification to homogeneity of citrate synthase from a Gram-positive bacterium. Its significance and potential stem from the established patterns of structural and regulatory diversity displayed by citrate synthases from diverse organisms (Weitzman & Danson, 1976; Weitzman, 1981). Whereas Gram-negative bacterial citrate synthases are 'large' hexameric molecules, the Gram-positive bacterial enzymes are 'small', and, in this respect, closely resemble the citrate synthases from eukaryotic sources. Some examples of the latter have been shown to be dimeric in structure, but the Gram-positive bacterial citrate synthases were hitherto only presumed to be dimeric on the basis of the similarity of their overall molecular size to that of the eukaryotic enzymes. It was clearly necessary to demonstrate this dimeric structure experimentally, and this we have now done.

A combination of gel filtration and sedimentation-velocity ultracentrifugation gave a value of 84000 for the M_r of the native *B. megaterium* citrate synthase. It is noteworthy that this value is rather lower than that calculated for the mammalian enzyme from its amino acid sequence (M_r 98000; Bloxham *et al.*, 1981). Two independent methods were used to determine the M_r of the enzyme subunits: sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration in the presence of 6 M-guanidinium chloride. Both techniques revealed a single subunit species of M_r approx. 40000, thereby indicating that the native enzyme is composed of two similar subunits. Again, this value is slightly less than that estimated for the mammalian enzyme analysed under identical conditions. Further confirmation of the dimeric structure of the *Bacillus* citrate synthase has been gained by cross-linking with dimethyl suberimidate and with glutaraldehyde. In each case the sodium dodecyl sulphate/polyacrylamide-gel electrophoretic analysis showed dissociated monomers and cross-linked dimers.

The absence of any indication of differences between subunits of the *Bacillus* enzyme is in keeping with all other previous studies on both eukaryotic and Gram-negative bacterial citrate synthases. In both the 'small' and the 'large' enzymes only one type of subunit has been suggested by the analytical studies (Weitzman & Danson, 1976; Weitzman, 1981). Indeed, there is a close similarity between the molecular sizes of subunits from all the citrate synthases examined, and it is therefore tempting to speculate that this similarity in size may be accompanied by more extensive molecular similarities, perhaps extending to significant amino acid sequence homologies. This remains to be investigated, and the availability of pure *Bacillus* citrate synthase will clearly assist such comparative studies. One objective of our work is to understand the molecular structural features that govern the extent of subunit association, i.e. to the dimeric or the hexameric state in the 'small' and the 'large' citrate synthases respectively. Association to the 'large' enzyme may be a crucial factor in the constitution of allosteric regulatory sites, and comparative studies on the subunits of both Gram-negative and Gram-positive bacterial citrate synthases should prove useful in exploring this aspect.

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