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The Quaternary Structure of Citrate Synthase from *Escherichia coli* K12†

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ABSTRACT: A combination of equilibrium ultracentrifugation and polyacrylamide gel electrophoresis techniques has been used to establish the quaternary structure of citrate synthase from acetate-grown *Escherichia coli* K12 3000. In polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), the pure enzyme showed one major band whose mobility was consistent with a molecular weight of 46,000 ± 2000 g/mol, and a little material of 87,000 ± 5000 g/mol. When first cross-linked with dimethyl suberimidate and then submitted to electrophoresis in SDS, citrate synthase showed six bands, in widely different amounts, whose apparent molecular weights were almost integral multiples of 47,000 g/mol. The dimer was the major product of the cross-linking procedure. In 6 M guanidine HCl at pH 7.0, citrate synthase behaved as a single component in high-speed sedimentation equilibrium experiments, with a weight average molecular weight of 43,400 ± 300 g/mol. The molecular weight of native citrate synthase was investigated by high-speed sedimentation equilibrium ultracentrifugation under different conditions of pH and KCl concentration. In 0.02 M Tris-Cl at pH 7.0 and 7.8, the enzyme was a mixture of oligomers, with species ranging from monomer (47,000 g/mol) to greater than decamer being present. At pH 9.0, only dimer was seen (94,000 g/mol). Large aggregates were present at pH 10.0. The addition of small amounts of KCl, a potent activator of the enzyme, simplified the mixture of oligomers considerably at pH 7.8. A detailed analysis of the data with 0.05 M KCl indicated that dimer and hexamer were the only species present, with marked nonideality. Increasing the KCl concentration to 0.10 M converted all the enzyme to hexamer. The amino acid composition of *E. coli* citrate synthase was presented. Taken together with peptide mapping experiments of others (J. A. Wright and B. D. Sanwal (1971), *J. Biol. Chem. 246* 1689), it indicates that the subunits have all the same or very similar amino acid sequences. The dansylation method revealed only methionine at the N-termini of the citrate synthase polypeptide chains. Citrate synthase from *E. coli* thus resembles the enzyme from eukaryotes in that it consists of subunits weighing just under 50,000 g/mol, although these subunits are more highly aggregated in the bacterial enzyme under most conditions. This conclusion is in disagreement with that of Wright and Sanwal (1971, see above), who reported a subunit size of 62,000 g/mol.

Citrate synthase catalyzes the reaction by which acetyl-CoA carbon enters the tricarboxylic acid cycle. The enzyme has been studied from a number of sources, and is often found to exhibit allosteric properties (Srere, 1972). The eukaryotic enzymes have molecular weights near 100,000 g/mol, and consist of two apparently identical subunits (Srere, 1972). Many bacterial enzymes are much larger (Weitzman and Dunmore, 1969), but there is little information about their subunit structures. Wright and Sanwal (1971) have reported that *Escherichia coli* citrate synthase is a mixture of octamers and tetramers of subunits the molecular weight of which is about 62,000 g/mol. Some of their results have been confirmed by Danson and Weitzman (1973). Our interest in the allosteric properties of the *E. coli* enzyme has prompted us to investigate its subunit structure in more detail. The results are presented in this paper, and they suggest that the molecule as isolated from *E. coli* K12 3000, grown on acetate, consists of subunits of size about 47,000 g/mol, in a state of aggregation which varies with pH and KCl concentration.

Experimental Section

Enzyme. Citrate synthase was purified from *Escherichia coli* K12 strain 3000 by a method like those published by Faloona and Srere (1969) and Wright and Sanwal (1971). We omitted the heat step used by the first group, and the reverse ammonium sulfate step of the second group, since neither step seemed to affect the final purity of the protein as judged by polyacrylamide gel electrophoresis, at pH 8.9 by the method of Davis (1964). Our citrate synthase shows three protein bands upon gel electrophoresis under these conditions, as did the preparations of the groups just cited. Various indications that our preparations are essentially homogeneous emerge in the Results section of this paper.

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Other Materials. Standard dansyl[1] amino acids and dansyl chloride, bovine serum albumin, ovalbumin, G3PD, and catalase were obtained from Sigma. Guanidine HCl was Mann Ultrapure grade. Oxaloacetic acid, α-ketoglutaric acid, and NADH were from Boehringer, and acetyl-CoA, trilithium salt, was from PL Biochemicals. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was from Aldrich Chemical Co. All other chemicals were reagent grade. Polyamide thin-layer sheets for chromatography of dansyl amino acids were obtained from Gallard-Schlesinger.

Enzyme Assay. Citrate synthase was assayed by the method of other workers (Srere *et al.*, 1963), involving the measurement with Ellman's reagent of the appearance of free sulfhydryl groups of coenzyme A. In our assay, we used 0.1 M KCl in 0.02 M Tris-Cl (pH 7.8) containing 1 mM EDTA, at a temperature of 21°. To conserve reagents, the concentrations of acetyl-CoA and oxaloacetate were chosen to be 0.1 mM each. Saturating concentrations of these substrates, such as those used by Faloona and Srere (1969), give about 2.5 times the rates observed in our assay.

Molecular Weight Determinations. Electrophoresis in SDS containing polyacrylamide gels and the preparation of the protein samples for this procedure were performed according to Weber and Osborn (1969), varying the percentage of cross-linking agent as needed. Proteins were reacted with dimethyl suberimidate and subjected to gel electrophoresis according to Davies and Stark (1970).

High-speed sedimentation equilibrium experiments were performed by the method of Yphantis (1964). The data were obtained at 20° in a Beckman Model E Analytical ultracentrifuge equipped with RTIC unit and interference optics; a six-channel Epon centerpiece and sapphire windows were used for all runs. The native enzyme was studied at 12,000 or 14,000 rpm, using the An-J head to provide added stability; the enzyme dissolved in guanidine HCl was studied in the An-H head at 36,000 rpm. Rayleigh interference patterns were photographed on Kodak Type 1-N spectroscopic plates, and fringe displacements (y) were read from the plates with a Nikon Comparator equipped with micrometer heads. Five fringes were read, and the readings averaged to give final values of y, at each of from 50 to 80 settings of the x coordinate, in every experiment. The computer program of Roark and Yphantis (1968), which uses the standard measurements of interference fringe displacement *vs.* radius to calculate a number of molecular weight averages and related quantities as a function of protein concentration, was routinely used in these studies.

For molecular weight calculations from the ultracentrifuge data, the partial specific volume of citrate synthase was taken to be 0.735 ml/g. This was calculated from the amino acid composition given in Table III, according to Cohn and Edsall (1943).

Other Methods. Amino acid analyses were performed with a Beckman 120 analyzer, using samples that had been hydrolyzed *in vacuo* at 110° for 24, 48, and 72 hr in 6 N HCl. Duplicate hydrolyses were run and analyzed for each time. Values for threonine and serine were corrected for loss by first-order extrapolation to zero time. Ammonia values were obtained by linear extrapolation to zero time. For valine and isoleucine, the 72-hr values were adopted. For the other amino acids, the averages of all six values were taken. Cysteine plus cystine were estimated together as cysteic acid after performic acid oxidation according to Moore (1963). Tryptophan was determined by the method of Edelhoch (1967), which also yielded an independent estimate for tyrosine.

Amino-terminal analysis by the qualitative dansylation method was performed according to the procedure for proteins given by Gray (1972).

The concentration of citrate synthase in solutions was determined from the optical density at 278 nm, using $E_{1cm}(1\%)$ 9.76 ± 0.20. This constant was determined from the absorption spectrum of a solution whose protein content had been measured by amino acid analysis. The value agrees with one of 9.8 obtained by Wright and Sanwal (1971), using dry weight analysis. Faloona and Srere (1969) measured citrate synthase concentration by the method of Warburg and Christian (1941). We find that for pure citrate synthase this method agrees with ours to within 0.6%, which is well within the error of our extinction coefficient.

Results

Purity of Enzyme. Our preparations of *E. coli* citrate synthase have a specific activity in our assay of 20–30 μmol coenzyme A formed per min per mg of protein. This corresponds to a maximal velocity at 21° of 50–75 μmol per min per mg (see Experimental Section). Specific activities as high as 130–150 have been reported (Faloona and Srere, 1969; Wright and Sanwal, 1971), but also considerably lower values (Wright, 1970). One of us has found that long-term incubation of the enzyme with 1 M KCl or more, either in the cold or at room temperature, will increase the assayed specific activity of the enzyme two- to threefold (E. K. Tong, unpublished observations), and this additional activity is not quickly lost upon dilution of the salt. Wright has recorded somewhat similar observations (Wright, 1970). It is of interest that Faloona and Srere (1969) employed in their purification a heating step in 2 M KCl, a treatment that almost certainly would activate citrate synthase significantly. In addition, their assays were performed at 27° rather than 21° as with us, so that their specific activities would be correspondingly higher.

In 0.02 M Tris-Cl–0.05 M KCl (pH 7.8) our preparations sediment as a single, apparently symmetric, boundary with $s_{20,w}$ in the range 10.0–10.6S, at protein concentrations of 6–8 mg/ml (not shown). Faloona and Srere (1969) reported a sedimentation coefficient of 8.0S for their preparation at 3.5 mg/ml, at pH 8.0 in 1 M KCl; corrected to $s_{20,w}$ this is 8.95S. As will be seen below, citrate synthase is not a single species at this pH, but a mixture of various oligomers, and even low levels of KCl significantly reduce the amounts of the largest components.

The sedimentation behavior of the preparations of Wright and Sanwal (1971) and Danson and Weitzman (1973) is rather different from that of ours, and we shall consider this difference in the Discussion.

Size of the Citrate Synthase Subunit. Figure 1 shows photographs of polyacrylamide gel electrophoresis patterns for citrate synthase in the presence of 0.1% SDS–0.1% 2-mercaptoethanol. Figure 1a is the result of an experiment according to the method of Weber and Osborn (1969). Citrate synthase shows two bands, the main one having the mobility expected for a polypeptide of molecular weight 46,000 ± 2000 g/mol, and the minor one that expected for 87,000 ± 5000 g/mol. The apparent molecular weights

[1] Abbreviations used are: G3PD, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; dansyl, 5-dimethylamino-1-naphthalenesulfonyl.

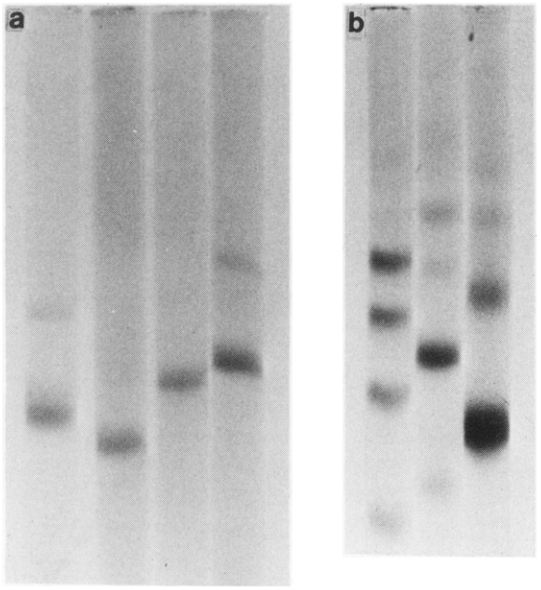

FIGURE 1: Photographs of polyacrylamide gel electrophoresis patterns for *E. coli* citrate synthase, in the presence of 0.1% SDS–0.1% 2-mercaptoethanol. (a) Samples prepared according to Weber and Osborn (1969), showing from left to right: citrate synthase, G3PD, catalase, and bovine serum albumin; 7% gels were used. (b) Samples cross-linked with dimethyl suberimidate according to Davies and Stark (1970), showing from left to right: G3PD, citrate synthase, and bovine serum albumin; 3.5% gels were used.

were determined from a standard curve of the usual kind (Weber and Osborn, 1969), employing as standards G3PD (subunit MW 36,000), ovalbumin (MW 44,000), catalase (subunit MW 60,000), and bovine serum albumin (MW 67,000). The minor citrate synthase band may be a dimer of the main species, not completely denatured by the procedure used. We have not attempted to remove this band by longer incubation with SDS.

Figure 1b shows the results of an experiment in which citrate synthase was cross-linked with dimethyl suberimidate according to Davies and Stark (1970), and then submitted to electrophoresis on SDS gels. The enzyme now yielded six polypeptide bands. The standard curve for this experiment was obtained using cross-linked preparations of G3PD (containing monomer, dimer, trimer, and tetramer), catalase (containing monomer, dimer, and tetramer), and bovine serum albumin (containing monomer, dimer, and trimer). The apparent molecular weights of the six citrate synthase bands, determined from the standard curve are: 49,000 ± 2100; 91,600 ± 5200; 146,000 ± 6000; 189,000 ± 8000; 247,000 ± 17,000; and 290,000 ± 19,000 g/mol. These values are almost integral multiples, from one to six, of a subunit weighing 47,000 ± 2000 g/mol. The sizes of the apparent pentamer and hexamer bands are subject to greater uncertainty than the others, not only because the bands were faint (though always present in several repeat experiments), but because no standard proteins were used which were larger than 200,000 g/mol. Although we have not tried to measure the relative amounts of polypeptide in each of the six bands, we have the impression than even-numbered bands are present in larger amounts than odd-numbered ones. The dimer is clearly the major product. By varying the pH and buffer composition used for cross-linking, one might expect to change the distribution among the six bands, in view of the results below, but we have not attempted this.

Citrate synthase in 6 M guanidine HCl appeared to be practically homogeneous by the method of high-speed equilibrium centrifugation. The usual plot of ln c *vs.* $r^2/2$, according to the method of Yphantis (1964), yielded a reduced weight average molecular weight, σ_w, of 4.03 ± 0.03, corresponding to a molecular weight of 43,400 ± 300 g/mol. This value is slightly smaller than the subunit size deduced from the polyacrylamide gel electrophoresis experiments in SDS.

Size of Citrate Synthase in Nondenaturing Media. Citrate synthase exhibits maximal activity at pH 8–9, with a

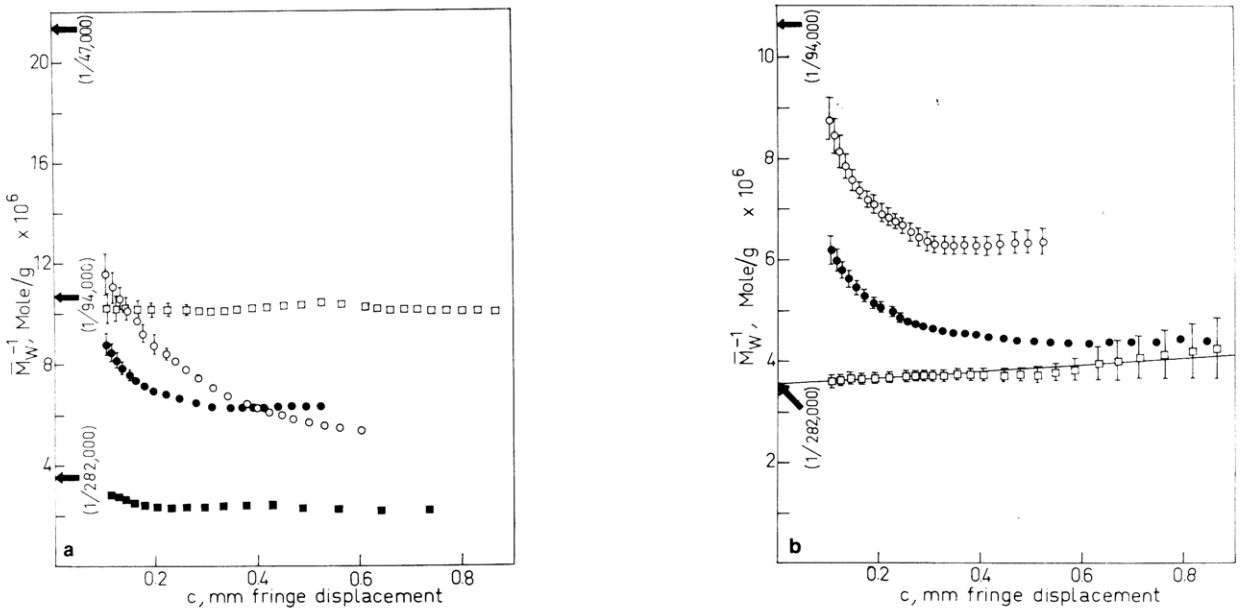

FIGURE 2: Sedimentation equilibrium of *E. coli* citrate synthase in nondenaturing media. Reciprocals of weight average molecular weights are plotted against protein concentration. (a) Effect of pH; buffers were 0.02 M Tris-Cl containing 1 mM EDTA. The curves are (○) pH 7.0; (●) pH 7.8; (□) pH 9.0; (■) pH 10.0. (b) Effect of KCl added to enzyme in 0.02 M Tris-Cl, (pH 7.8). The curves are (○) no KCl added (data taken from part a); (●) 0.05 M KCl; (□) 0.10 M KCl. Many points are omitted for clarity. All experiments were at 20°, 12,000, or 14,000 rpm, Beckman An-J head. Loading concentration of protein was 0.60 mg/ml in all cases.

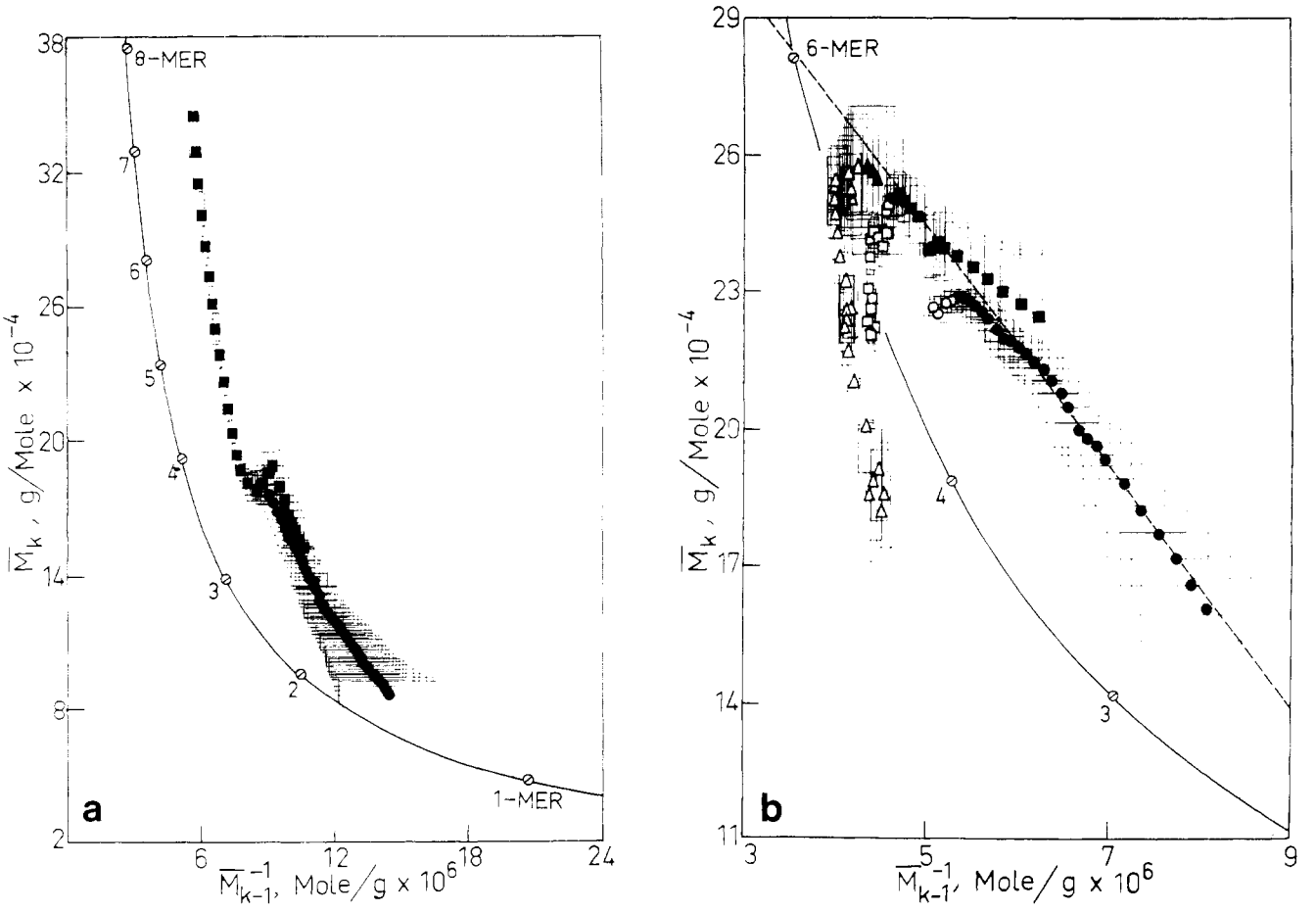

FIGURE 3: Sedimentation equilibrium of *E. coli* citrate synthase, two-species plots. (a) In 0.02 M Tris-Cl (pH 7.0). Symbols are (●) $\bar{M}_w$ *vs.* $\bar{M}_n^{-1}$; (■) $\bar{M}_z$ *vs.* $\bar{M}_w^{-1}$. The solid line is the hyperbola calculated for $\bar{M}_n = \bar{M}_w = \bar{M}_z = \bar{M}_{z+1}$. Points expected for particular homogeneous oligomers are marked on the hyperbola. The rectangles, centered about the points, have sides equal in length to the error bars in the two dimensions. These "error rectangles" were incorporated from diagrams drawn by the Calcomp Plotter at the University of Manitoba Computer Centre. (b) In 0.02 M Tris-Cl (pH 7.8) containing 0.05 M KCl. Symbols are (○) and (●) $\bar{M}_w$ *vs.* $\bar{M}_n^{-1}$; (□) and (■) $\bar{M}_z$ *vs.* $\bar{M}_w^{-1}$; (△) and (▲) $\bar{M}_{z+1}$ *vs.* $\bar{M}_z^{-1}$. The solid line and the rectangles are as for (a). The broken line is the least-squares line through all the *solid* points, which were taken as the linear parts of the plots.

rapid falling off on either side (Weitzman, 1966; Faloona and Srere, 1969). The enzyme tends to precipitate after a few hours at pH values below 7 (E. K. Tong and H. W. Duckworth, unpublished observations). We have obtained high-speed sedimentation equilibrium data for citrate synthase over the pH range 7–10. Plots of reciprocal weight-average molecular weight as a function of protein concentration, at the different pH values, are shown in Figure 2a. Considerable heterogeneity of molecular weight is observed at all pH values except 9. At pH 9, the molecular weight is independent of concentration throughout the range studied, and has a value of 98,400 ± 1300 g/mol. This is close to the value expected for a dimer of the subunit obtained in denaturing media.

The data at the other pH values can be analyzed further with the two-species plots of Roark and Yphantis (1968). In the absence of nonideal effects, such plots are straight lines if the system under study is a mixture of two different sizes of molecule. If more than two species are present, the plots curve upwards. The two-species plots for the citrate synthase system at pH 7 are shown in Figure 3a. Upward curvature is seen, and the total range of molecular weights covered by the plots is from about 450,000 (almost a decamer) to less than 90,000 g/mol. As an aid to interpretation, we have drawn the locus of points for homogeneous systems ($\bar{M}_n = \bar{M}_w = \bar{M}_z = \bar{M}_{z+1}$); the two-species plots will intersect this curve at two points, corresponding to the molecular weights of the highest (c very high) and lowest (c approaching zero) species present in the system (Roark and Yphantis, 1968). Since we can observe average molecular weights which are well below dimer, the smallest species must be monomer. The data do not permit a confident extrapolation to give the highest species present, although it may well contain at least 12 subunits.

The data at pH 7.8 and pH 10 have also been used to construct two-species plots (not shown). At pH 7.8, species from monomer up to greater than hexamer seem to be present. At pH 10, there is considerable nonideality evident in the plots, and species containing as many as 20 subunits may be involved (see Table I). More extensive measurements will be needed to define the equilibria which exist at the high pH values.

Except at pH 9, citrate synthase in dilute buffer is evidently involved in several association–dissociation equilibria, and shows no clear preference for any one oligomeric form in the concentration range we have used. Hoping to find conditions that simplify these equilibria, we have investigated the effects of small amounts of KCl, a powerful activator of the enzyme (Faloona and Srere, 1969). Figure 2b shows plots of reciprocal weight-average molecular weight as a function of concentration for citrate synthase in 0.02 M Tris-Cl (pH 7.8) containing 0.1 M, 0.05 M, and no added KCl, respectively.

The effect of 0.1 M KCl is very clear. The molecular

Table I: Molecular Weight Measurements on Native Citrate Synthase.

Conditions	Molecular Weights Present	Multiples of 47,000	Comments[a]
A. Effects of pH[b]			
7.0	Below 90,000 to above 450,000[c]	Less than 1.9 to more than 9.6	Nonideality, if present, is concealed by heterogeneity (see Figure 3A)
7.8	Below 90,000 to above 300,000[c]	Less than 1.9 to more than 6.4	
9.0	98,400 ± 1300[d]	2.09 ± 0.03	Homogeneous
10.0	Below 350,000 to above 700,000[c]	Less than 7.5 to more than 15	Strong nonideality
B. Effects of KCl[e]			
None added	Below 90,000 to above 300,000[c]	Less than 1.9 to more than 6.4	Taken from pH 7.8 entry in Part A
0.05 M	92,800 ± 1500[f] and 283,100 ± 2600[f]	1.97 ± 0.03 and 6.03 ± 0.06	Two species (see Figure 3B)
0.10 M	280,400 ± 2700[d]	5.97 ± 0.06	Homogeneous

[a] Comments regarding heterogeneity and nonideality are qualitative descriptions of the two-species plots. [b] Buffers were 0.02 M Tris-Cl containing 1 mM EDTA. [c] Estimated from the two-species plots. Values were chosen beyond which the plots must be extended to contact the $M_k = 1/M_{k-1}$ hyperbola. [d] Calculated by an unweighted least-squares linear fit of the line $(1/M_w)_c = \bar{M}_0 + ac$, as in Figure 2, where plots appeared to be straight lines. The values quoted are M_0 and its standard deviation. [e] Buffer was 0.02 M Tris-Cl containing 1 mM EDTA, at pH 7.8. [f] Values and standard deviations calculated from the plot in Figure 3B, as explained in Results.

weight data taken in this medium show a slight linear concentration dependence, and the least-squares line through the points yields a molecular weight of 280,400 ± 2700 g/mol at $c = 0$, close to that expected for a hexameric molecule. With less KCl present, 0.05 M, the enzyme is heterogeneous, and two-species plots of the data are shown in Figure 3b. There is a long straight section, followed by the downward curvature which is characteristic of nonideal behavior (Roark and Yphantis, 1968). The least-squares line through the linear parts of the plots has the equation

$$\overline{M}_k = (3.759 \pm 0.042) \times 10^5 - (2.627 \pm 0.069) \times 10^{10} \frac{1}{\overline{M}_{k-1}}$$

where the vertical intercept is $(M_1 + M_2)$ and the slope is $-(M_1 M_2)$, and M_1 and M_2 are the molecular weights of the two species present. The calculated values of M_1 and M_2, from the numerical values of the slope and intercept of the line, are thus 92,800 ± 1500 and 283,100 ± 2600 g/mol, suggesting that in 0.05 M KCl an equilibrium exists between dimers and hexamers of the subunit. The position of this equilibrium must shift when the KCl concentration is increased to 0.1 M, since under those conditions only hexamer is seen.

A summary of our molecular weight measurements for citrate synthase is given in Table I.

Amino Acid Composition of Citrate Synthase. The results of quantitative analyses of the amino acids present in acid hydrolysates of *E. coli* citrate synthase are given in Table II along with the results of separate determinations of half-cystine and tryptophan. The subunit size was taken as 47,000 g/mol for the purpose of calculating the values in the last two columns of the table.

The ammonia content of the hydrolysates, extrapolated to zero time of hydrolysis, implies that of 81 ± 1 aspartic and glutamic acid residues, 29 ± 1 are in the amide forms. The total of the lysine and arginine residues is 46. It has

Table II: Amino Acid Composition of *E. coli* Citrate Synthase.

Amino Acid	Average or Extrapolated Values ± SD	Moles per 47,000 g	Nearest Integer
Lysine	504 ± 3	23.7 ± 0.1	24
Histidine	260 ± 4	12.2 ± 0.2	12
Ammonia	627 ± 8[a]	29.5 ± 0.4	29–30
Arginine	469 ± 6	22.0 ± 0.3	22
Asparate	932 ± 19	43.8 ± 0.9	44
Threonine	528 ± 12[b]	24.8 ± 0.6	25
Serine	482 ± 11[b]	22.7 ± 0.5	23
Glutamate	789 ± 15	37.1 ± 0.7	37
Proline	400 ± 17	18.8 ± 0.8	19
Glycine	661 ± 13	31.1 ± 0.6	31
Alanine	831 ± 17	39.1 ± 0.8	39
Valine	478 ± 2[c]	22.5 ± 0.1	23
Methionine	347 ± 19	16.3 ± 0.9	16
Isoleucine	570 ± 2[c]	26.8 ± 0.1	27
Leucine	779 ± 17	36.6 ± 0.8	37
Tyrosine	321 ± 8 (312 ± 7)[d]	15.1 ± 0.4 (14.7 ± 0.3)	15
Phenylalanine	407 ± 13	19.1 ± 0.6	19
Tryptophan	82.5 ± 0.15[d]	3.88 ± 0.07	4
1/2-cystine	138 ● 11[e]	6.53 ● 0.52	6–7
Total			423–424[f]

[a] Linear extrapolation to zero hydrolysis time. [b] First-order extrapolation to zero hydrolysis time. [c] Values for 72-hr hydrolysis taken. [d] Method of Edelhoch (1967); average ± SD of two analyses. [e] As cysteic acid after performic acid oxidation. [f] Excluding ammonia.

been reported that when citrate synthase is denatured with SDS and reacted with Ellman's reagent, the number of sulfhydryl groups is 30 per 245,000 g (Wright and Sanwal, 1971), or 30 per 280,000 g (Faloona and Srere, 1969). These values correspond to 5.75 and 5.04 sulfhydryl groups per 47,000 g, respectively. Since we find 6 or 7 residues of cysteic acid per 47,000 g of enzyme which had been oxidized with performic acid (see Table I), it is rather unlikely that any of the cysteine residues in the enzyme are involved in disulfide bonds.

If the number of cysteine residues per subunit is taken as six, the subunit molecular weight implied by the amino acid composition in Table II is 47, 125 g/mol.

The amino acid composition of citrate synthase, from *E. coli* strain CA 244 grown on glycerol, was recently reported by Danson and Weitzman (1973). Our results are in excellent agreement with theirs, when differences in assumed molecular weight are taken into account, except that we find almost 4 residues of tryptophan per 47,000 g, whereas their results correspond to 5.1 residues in the same weight of protein. This difference may arise because of different methods used for measuring the amount of tryptophan in the protein. We used the method of Edelhoch (1967), while Danson and Weitzman (1973) used the method of Bencze and Schmid (1957).

Amino-Terminal Amino Acid. Methionine, sometimes accompanied by its sulfone, was the only amino-terminal amino acid which we could detect in *E. coli* citrate synthase, using the dansylation procedure as described in the Experimental Section.

Discussion

The evidence presented in this paper leads to the belief that citrate synthase from acetate-grown *Escherichia coli* K12 3000 is composed of subunits, the molecular weight of which is near 47,000 g/mol. This value is consistent with the results of the SDS polyacrylamide gel electrophoresis experiments, with and without prior cross-linking with dimethyl suberimidate (Figure 1b and 1a, respectively), and with the two molecular weight values for apparent dimers, and two for apparent hexamers observed in the equilibrium centrifugation experiments (Table I). It is of interest to note that citrate synthases isolated from eukaryotic sources all seem to be dimers of subunits weighing just under 50,000 g/mol (see Srere, 1972). The results in Figure 1b, which show a predominance of even-numbered subunits, especially dimers, in suberimidate-cross-linked *E. coli* citrate synthase, may indicate that a dimeric arrangement is especially stable. Our findings that dimer is the only species present in dilute buffer at pH 9.0, and one of two species present in 0.05 M KCl at pH 7.8, are also consistent with this conclusion. Such a dimer, at least superficially, would be equivalent to the eukaryotic enzymes.

Previous studies on the subunit structure of *E. coli* citrate synthase have led to the conclusion that it is a tetramer of subunits of about 60,000 g/mol. Wright and Sanwal (1971) reported a subunit molecular weight of 60,000–65,000, from the mobility on SDS gels; this result was based on the fact that their citrate synthase had a mobility slightly less than that of catalase (MW 60,000). Danson and Weitzman (1973) also employed SDS gel electrophoresis, but used a number of protein standards in the range molecular weight 36,000–68,000; in their hands citrate synthase had a mobility between those of fumarase (49,000) and pyruvate kinase (57,000), and interpolation gave a molecular weight of 55,000 ± 4000 for the subunit of citrate synthase. In a number of experiments of which those in Figure 1 are examples, we have always obtained a mobility for citrate synthase subunit corresponding to a molecular weight of 47,000 ± 2000 g/mol. The value given by Danson and Weitzman (1973) is not so much larger than this, and we prefer our value because it is more nearly consistent with the results of the sedimentation equilibrium experiments given in Table I of this paper.

The subunits of *E. coli* citrate synthase may all be of a single type. Wright and Sanwal (1971) have reported that tryptic hydrolysates of the enzyme contained 36–38 soluble peptides which could be resolved by the usual combination of paper electrophoresis and chromatography. Our amino acid analysis shows a total of 46 mol of arginine plus lysine per 47,000 g (Table II), so that the number of tryptic peptides should be 46 or 47 if all subunits are the same. Considerably more than 38 peptides should have been distinguishable in the experiments of Wright and Sanwal (1971) if more than one type of subunit were present. Our finding that a single type of amino acid, methionine, is present at the amino termini of the enzyme, is also consistent with the conclusion that there is one kind of subunit, although methionine is so common at the amino termini of *E. coli* polypeptides that this evidence is not strong. The citrate synthase of pig heart appears to consist of a single type of polypeptide chain (Singh *et al.*, 1970).

The quaternary structure of *E. coli* citrate synthase is highly dependent on the conditions chosen to study it. At pH 9 only the dimer is seen in the concentration range we studied, but as the pH is lowered the average molecular weight increases, and species ranging from smaller than dimer (*i. e.*, monomer) to greater than decamer become detectable. At pH 10, however, there is a dramatic increase in the degree of aggregation, involving species with molecular weights in the range 350,000–700,000 g/mol (Table I), and very considerable nonideality. Since there are 24 lysine residues in the protein, many of which would become deprotonated between pH 9 and pH 10, the net charge per subunit would become considerably more negative in this range, a fact which might well account for so abrupt a change in aggregation of the enzyme.

The addition of 0.1 M KCl to assays of *E. coli* citrate synthase activates the enzyme at least 15-fold at pH 7.8 under standard assay conditions; the concentration of KCl needed to produce half-maximal activation is near 0.02 M (Faloona and Srere, 1969). The results in Figure 2b indicate that one effect of KCl in this concentration range is to change the pattern of aggregation of the enzyme. In 0.05 M KCl at 7.8, an equilibrium mixture of dimer and hexamer is seen, with significant nonideality revealed in the two-species plots (Figure 3b). Increasing the concentration of KCl to 0.1 M converts the enzyme entirely to hexamer, at least at protein concentrations of 0.1 mg/ml and greater. This observation is in agreement with the report of Faloona and Srere (1969) that the enzyme had a molecular weight of 280,000 g/mol in 0.05 M Tris-Cl–0.1 M mercaptoethanol–0.1 M KCl, at pH 8.1, as determined by its volume of elution from Bio-Gel A 1.5m. These authors further reported that removal of KCl had no effect on the volume of elution of the enzyme from Bio-Gel P-200 (Faloona and Srere, 1969). It is difficult to decide whether these observations are inconsistent with ours, since the gel filtration technique reveals only changes in *average* molecular weight, rather

than changes in the detailed patterns of aggregation which are detectable by the methods we used.

Wright and Sanwal (1971) used sedimentation velocity analysis to study the effect of pH on the molecular weight of citrate synthase. The buffer employed was 0.05 M Tris-Cl, only slightly more concentrated than what we have used, and the protein concentration was 6–8.4 mg/ml, about 20 times as great as the median concentration in our sedimentation equilibrium experiments. Under these conditions, Wright and Sanwal (1971) found that citrate synthase consisted principally of a species having $s_{20,w}$ = 19.8S, with some smaller material, at pH 7 or 8. At pH 9.1 this species began to yield to two other boundaries, having $s_{20,w}$ = 13.2 and 5.3S. No results were reported for pH 10, but at pH 11 one rather asymmetric peak, sedimenting at a rate of 5.1S, was observed. Wright and Sanwal (1971) used the method of boundary spreading to measure the diffusion coefficients of the 19.8S (at pH 7) and 5.1S (at pH 11) species, and employed the Svedberg equation to calculate molecular weights of 470,000 and 61,000 g/mol, respectively. These observations were partly confirmed by Danson and Weitzman (1973), who found smaller sedimentation coefficients, and a somewhat lower degree of aggregation at pH 7.0.

In general these results imply a greater degree of polymerization than do ours, part of which may be explained by the higher protein concentrations used. All the experiments agree in demonstrating that the enzyme becomes more polymerized in going from pH 9 to pH 7, with the largest species at the lower pH being just under 500,000 g/mol. The small species observed in sedimentation velocity experiments at pH 11, having an apparent molecular weight above 60,000 g/mol, showed an asymmetric Schlieren boundary (see Wright and Sanwal, 1971, Figure 3D), and from our point of view could be regarded as an equilibrium mixture of monomer (47,000) and dimer (94,000 g/mol).

The results in this paper agree with those presented by Wright and Sanwal (1971) in suggesting that the quaternary structure of *E. coli* citrate synthase is highly dependent on such factors as pH and salt composition of the medium. We have been able to find conditions in which dimer, hexamer, or a mixture of the two are the only detectable species (Table II). It is interesting to note that conditions which favor high enzyme activity, such as pH values greater than 8 or moderate levels of KCl, tend to produce dimers and/or hexamers. Conditions that favor low activity and strong inhibition by the allosteric effector, NADH, on the other hand—that is, pH values less than 8, or the absence of KCl (see Weitzman, 1966)—also lead to a less definite pattern of polymerization, involving several species ranging from monomer to decamer or greater. It is not possible to say whether these changes in quaternary structure are a primary cause of allosteric inhibition, or lack of it. They may simply be one manifestation of conformational changes, induced by pH changes or by KCl, which have effects on many properties of the enzyme, including its catalytic activity.

Acknowledgments

The amino acid analyses were kindly performed by Mr. Ray Batenchuk of the Department of Plant Science, University of Manitoba, whom we thank. Mr. Michael Talgoy wrote the program for the Calcomp Plotter. We also thank Dr. I. Suzuki and the members of the Department of Microbiology, University of Manitoba, for unrestricted access to the Beckman Model E Analytical ultracentrifuge and to other equipment, Dr. J. A. Wright for discussions, and Dr. J. C. Jamieson and Ms. M. L. Hollier for useful comments on this manuscript.

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