Purification and Properties of Citrate Lyase from *Escherichia coli*†

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ABSTRACT: Citrate lyase (EC 4.1.3.6) has been purified from *Escherichia coli* and the homogeneity of the preparation established from the three-component subunits obtained on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The purified enzyme has a specific activity of 120 μmol min^{-1} mg^{-1} and requires optimally 10 mM Mg^{2+} and a pH of 8.0 for the cleavage reaction. The native enzyme is polydispersed in the ultracentrifuge and in polyacrylamide gel electrophoresis. The enzyme complex is composed of three different polypeptide chains of 85000, 54000, and 32000 daltons. An estimate of subunit stoichiometry indicates that 1 mol of the largest polypeptide chain is associated with 6 mol each of the smaller ones. The polypeptide subunits have been isolated in pure state and their biological functions characterized. The 54000-dalton subunit functions as the acyltransferase α subunit catalyzing the formation of citryl coenzyme A from citrate in the presence of acetyl coenzyme A and ethylenediaminetetraacetic acid. The 32000-dalton subunit functions as the acyllyase β subunit catalyzing the cleavage of (3*S*)-citryl coenzyme A to oxalacetate and acetyl coenzyme A. The 85000-dalton subunit, which carries exclusively the prosthetic group components, functions as the acyl-carrier protein γ subunit in the cleavage of citrate in the presence of Mg^{2+} and the α and β subunits. The presence of a large ACP subunit and the unusual stoichiometry of the different subunits distinguish the complex from other citrate lyases. A ligase which acetylates the deacetyl[citrate lyase] in the presence of acetate and ATP has been shown to be present in the organism. The deacetyl enzyme is also reactivated by the ligase from *Klebsiella aerogenes*.

Citrate lyase which catalyzes the cleavage of citrate to oxalacetate and acetate (Dagley & Dawes, 1955) through a two-step sequence of reactions (Dimroth & Eggerer, 1975a) has been obtained pure from several bacterial sources (SivaRaman, 1961; Singh & Srere, 1975; Kümmel et al., 1975; Hiremath et al., 1976; Giffhorn & Gottschalk, 1978; Antranikian et al., 1982) and shown to be a multienzyme complex built up from three different polypeptide chains of about 55000 (α), 30000 (β), and 10000 daltons (γ) (Dimroth & Eggerer, 1975b; Carpenter et al., 1975). The functions of these subunits have been characterized in the complex from *Klebsiella aerogenes*, a source from which the enzyme has been studied the most. The γ subunit acts as an acyl-carrier protein (ACP)[1] and carries an essential acetyl moiety in thio ester linkage (Dimroth et al., 1973; Buckel et al., 1971) on a CoA-like prosthetic group (Robinson et al., 1976). The α subunit functions as an acyltransferase catalyzing the formation of (3*S*)-citryl-ACP[1] in the presence of citrate with the elimination of acetate (Dimroth & Eggerer, 1975a). The β subunit acts as an acyllyase catalyzing the cleavage of the citryl intermediate in the presence of Mg^{2+} with release of oxalacetate and regeneration of the acetyl-ACP[1] (Dimroth & Eggerer, 1975a). The complex from this source has a hexameric structure (Singh et al., 1976). Estimates of subunit structure and stoichiometry in the complexes isolated hitherto from other sources have also shown similar structural relationships.

This paper reports the isolation and properties of citrate lyase from *Escherichia coli*. The enzyme complex from this source has charactertistic structural features which distinguish it from the other enzymes of this group.

Experimental Procedures

Products. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were from Whatman, Sepharose CL-6B was from Pharmacia, alumina C_γ was from Calbiochem, and polyamide paper was from Cheng Chin Trading Co.

Malate dehydrogenase, citrate synthase, alkaline phosphatase, bovine serum albumin, ovalbumin, myoglobin, muscle cytochrome *c*, NADH, acetyl-CoA, $NaDodSO_4$,[1] dithiothreitol, dansyl[1] chloride, and dansyl amino acids were obtained from Sigma, and salmine sulfate was from BDH. Analytical grade urea was recrystallized from aqueous ethanol, and solutions were prepared fresh before use.

(3*S*)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate and was purified and assayed as described earlier (Basu et al., 1982). All other chemicals used were of the highest purity.

Escherichia coli (ATCC 8739), *Klebsiella aerogenes* (NCTC 418), and *Lactobacillus plantarum* (ATCC 8014) were obtained from the National Collection of Industrial Microorganisms (India).

Enzyme Assay. The citrate cleavage activity of the native enzyme was determined as described earlier (Basu et al., 1982). Protein was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as standard, except in samples containing streptomycin sulfate which were dialyzed before analysis by the method of Warburg & Christian (1941).

Ultracentrifugation. Analytical runs were carried out on a Spinco Model E instrument and sedimentation coefficients estimated as described by Schachman (1957).

Polyacrylamide Gel Electrophoresis. Disc electrophoresis was carried out in 5% acrylamide gel at pH 8.2 according to Davis (1964).

Molecular weights of the citrate lyase subunits were determined by $NaDodSO_4$/polyacrylamide gel electrophoresis on 7.5% gels (Weber & Osborn, 1969). Samples were prepared by heating aliquots (100 μL) containing about 100 μg of protein with 2% $NaDodSO_4$ and 1% 2-mercaptoethanol in

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[1] Abbreviations: ACP, acyl-carrier protein; acetyl-ACP, acetylacyl-carrier protein; (3*S*)-citryl-ACP, (3*S*)-citrylacyl-carrier protein; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CoA, coenzyme A.

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a boiling water bath for 5 min. Gels were stained with Coomassie brilliant blue R-250 and the protein bands visualized by destaining with 7.5% acetic acid solution containing 5% methanol. Protein standards used were the following: cytochrome *c*, M_r 11 700; myoglobin, M_r 17 200; ovalbumin, M_r 43 000 and 86 000; bovine serum albumin, M_r 68 000 and 136 000.

Quantitative gel scans were made at 600 nm on a Gilford Model 250 spectrophotometer.

Isolation and Purification of E. coli Citrate Lyase. Step I: Cell-Free Extracts. E coli cells were grown in the medium described by Dagley & Dawes (1955) and harvested after 18–22 h. The yield of packed cells was 18–20 g from 10 L of medium.

Cells were suspended in cold 30 mM potassium phosphate buffer, pH 7.0 (3.5 mL/g of packed cells), and sonicated in an ice bath at 20 000 Hz (300 W) for 6 min. Cell debris was removed by centrifugation at 50000*g* for 45 min and discarded. This and subsequent steps were carried out at 0–4 °C without storage at any stage. All buffers used in the subsequent steps contained 1.6 mM $MgSO_4$ to stabilize the enzyme. Batches of 100–120 g of packed cells were worked up at a time.

Step II: Treatment with Streptomycin Sulfate. The cell-free extract was treated with streptomycin sulfate to a final concentration of 1.4% (w/v), and the precipitated nucleic acids were removed by centrifugation at 50000*g* for 45 min.

Step III: Treatment with Salmine Sulfate. The supernatant solution from the streptomycin sulfate step was treated with 0.5 volume of cold 0.5% (w/v) salmine sulfate in 50 mM potassium phosphate buffer, pH 7.4, to precipitate citrate lyase activity and the suspension centrifuged at 50000*g* for 40 min. The inactive supernatant was discarded. The citrate lyase activity was extracted by homogenizing the pellet with 50 mM potassium phosphate buffer, pH 7.4, containing 0.5 M NaCl (about 0.15 of initial volume) for 20 min in a glass tube with a close-fitting ground glass pestle. The homogenate was centrifuged at 50000*g* for 45 min. The supernatant was separated and the citrate lyase activity precipitated by the addition of 50 mM potassium phosphate buffer, pH 7.4, until the NaCl concentration was lowered to 0.15 M.

Step IV: Treatment with CM-cellulose. The suspension obtained on dilution of the extract of the salmine sulfate precipitate was treated with CM-cellulose which had previously been equilibrated with 50 mM potassium phosphate buffer containing 0.15 M NaCl until a clear solution was obtained (7 g of wet-packed CM-cellulose/100 mL of suspension). CM-cellulose was then removed by centrifugation at 3000*g* for 20 min and the supernatant containing the citrate lyase activity worked up further.

Step V: Treatment with Alumina C_γ Gel. Alumina C_γ gel (32.5 mg dry weight/mL) was added to the supernatant from the CM-cellulose step under constant stirring (30 mL of gel suspension/100 mL of enzyme solution). The suspension was stirred for 15 min and centrifuged at 3000*g* for 25 min. The supernatant containing the citrate lyase activity was processed further.

Step VI: Precipitation with Ammonium Sulfate. Citrate lyase activity was precipitated from the supernatant by the addition of powdered solid ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation at 50000*g* for 30 min, dissolved in 50 mM potassium phosphate buffer, pH 7.4 (4–5 mL), and dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.4.

Step VII: DEAE-cellulose Column Chromatography. The dialysate was centrifuged to remove turbidity, and the clear protein solution (8–9 mL) was applied on a DEAE-cellulose column (1.5 × 35 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The enzyme was eluted with a linear concentration gradient of 10–120 mM potassium phosphate buffer, pH 7.4. Fractions containing the enzyme were pooled, stirred, and precipitated by dropwise addition of an equal volume of neutralized, cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 50000*g* for 30 min and dissolved in 50 mM potassium phosphate buffer, pH 7.4 (1.5 mL).

Step VIII: Sepharose Column Chromatography. The enzyme solution was then filtered through a column of Sepharose CL-6B (2.5 × 110 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.4. The column was eluted with buffer of the same composition, and all fractions containing enzyme activity which were spread over an eluate volume of about 20 mL were pooled and precipitated with neutralized, cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 50000*g* for 30 min and dissolved in 50 mM potassium phosphate buffer, pH 7.4 (1–2 mL).

Isolation of E. coli Citrate Lyase Subunits. Biologically active subunits were isolated from the complex by gel filtration in the presence of urea. The enzyme was treated with 6 M urea in the presence of 50 mM potassium phosphate buffer, pH 7.4, containing 10 mM dithiothreitol. The dissociated subunits were separated by filtration through a Sepharose CL-6B column (1.5 × 100 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 6 M urea and 10 mM 2-mercaptoethanol. Buffer of the same composition was used for elution, and 1-mL fractions were collected. The fractions were assayed for protein at 280 nm and aliquots subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Pooled fractions comprising protein peaks were dialyzed immediately against buffers without urea as described by Dimroth & Eggerer (1975a).

Subunits required for chemical and microbiological assays were obtained from the complex (0.5–1.0 mg) after dissociation at 100 °C for 5 min in the presence of 50 mM sodium phosphate buffer, pH 7.2, containing 2% $NaDodSO_4$ and 1% 2-mercaptoethanol. The subunits were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (Weber & Osborn, 1969). Sets of six gel tubes (7.5%; 1 × 10 cm each) were run at a time at 8 mA/tube. A representative section of the gel was stained with Coomassie blue to visualize protein bands. Gel sections containing subunits were pooled separately, and the protein was recovered by electrophoresis through a 7.5% polyacrylamide gel containing 6 M urea (Hanaoka et al., 1979). The eluted protein was dialyzed exhaustively against 50 mM potassium phosphate buffer, pH 7.4, to remove urea. The dialyzed fractions were concentrated separately by ultrafiltration through PM-10 membrane. The preparations were homogeneous in $NaDodSO_4$/polyacrylamide gel electrophoresis.

Characterization of Functions of Isolated Subunits. The acyltransferase α subunit was identified by the formation of citryl-CoA from acetyl-CoA and citrate as described by Dimroth et al. (1977), except that the reaction was carried out in the presence of 1 mM EDTA and monitored at 412 nm. Individual subunits were tested separately for initiation of the reaction.

The acyllyase β subunit was characterized from oxalacetate formation on cleavage of (3*S*)-citryl-CoA in the presence of Mg^{2+}, oxalacetate being assayed with malate dehydrogenase (Buckel et al., 1973). The lyase activity was further checked

Table I: Summary of Purification of Citrate Lyase from *Escherichia coli*

purification step	volume (mL)	protein (mg/mL)	total act. (μmol/min)	sp act. (μmol min^{-1} mg^{-1})	yield (%)
(I) cell-free extract	380	14.5	8246	1.5	(100)
(II) streptomycin sulfate	375	8.5	7866	2.4	95
(III) salmine sulfate	65	13.0	5038	6.0	61
(IV) CM-cellulose	236	1.9	4970	11.2	60
(V) alumina C_γ gel	260	1.1	4446	15.6	54
(VI) ammonium sulfate	5	22.5	4210	37.4	51
(VII) DEAE-cellulose/ ammonium sulfate	1.5	24.3	2588	71.2	31
(VIII) Sepharose CL-6B/ ammonium sulfate	1.2	11.0	1584	120	19

from citrate cleavage in the presence of Mg^{2+}, acetyl-CoA, and the acyltransferase α subunit by enzyme-coupled assay of the oxalacetate formed (Buckel et al., 1973).

The ACP γ subunit was characterized by reconstituting the active enzyme complex from the individual subunits in the presence of dithiothreitol and acetic anhydride (Dimroth & Eggerer, 1975a).

Prosthetic Group Components. The pantothenate content of the citrate lyase complex was determined microbiologically with *Lactobacillus plantarum* (ATCC 8014). Alkaline hydrolysates of the protein (0.25–0.5 mg) were treated with alkaline phosphatase (Srere et al., 1972), and growth of the organism was measured by alkali titration. The detection of pantothenate in individual subunits was done after extraction from unstained $NaDodSO_4$/polyacrylamide gels, protein bands being located by staining of separate gels run under identical conditions.

β-Alanine was determined by amino acid analysis on a Beckman Model 120 C analyzer after hydrolysis of samples (2 mg of holoenzyme; 0.1–1.0 mg of isolated subunits) with 6 N HCl at 110 °C for 24 h (Moore & Stein, 1963).

The cysteamine residues in the enzyme complex and in individual subunits were determined as taurine after oxidation of the protein with performic acid followed by acid hydrolysis (Hirs, 1967; Singh et al., 1976).

Sulfhydryl Groups. The total sulfhydryl groups in the citrate lyase complex was estimated by DTNB titrations in the presence of hydroxylamine (0.1 M) and $NaDodSO_4$ (0.9%) according to the procedure described by Ellman (1959). Total half-cystine residues in the complex and subunits were estimated as cysteic acid after performic acid oxidation (Hirs, 1967).

End-Group Analysis. The N-terminal amino acids in individual subunits were detected by dansylation of the protein (100–250 μg) followed by acid hydrolysis and identification of the dansylated amino acid by thin-layer chromatography on silica gel (Gros & Labouesse, 1968). Hydrolysates of the dansylated protein were subjected to two-dimensional chromatography on polyamide paper for quantitative end-group analysis (Woods & Wang, 1967; Gray, 1972). Fluorescence measurements were made on an Aminco Model SPF 125 spectrofluorometer.

Deacetyl[citrate lyase]. The enzyme was deacetylated with dithiothreitol (Kümmel et al., 1975). The deacetylated enzyme was reactivated chemically with acetic anhydride (Buckel et al., 1971) and enzymatically with citrate lyase ligase in the presence of ATP and acetate (Kümmel et al., 1975).

K. aerogenes citrate lyase ligase was prepared as described earlier (Hiremath et al., 1976). Ligase activity is defined in terms of citrate lyase units formed. The ligase preparation had a specific activity of 8 units/mg and was free from citrate lyase activity.

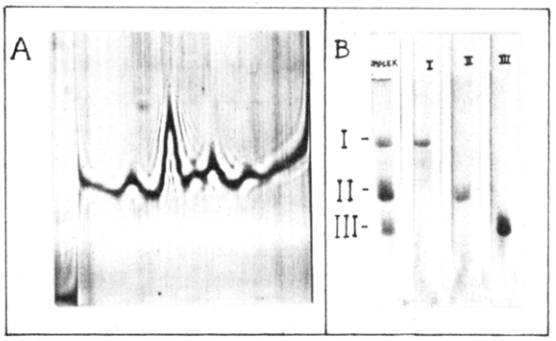

FIGURE 1: (A) Sedimentation profile of *E. coli* citrate lyase (specific activity 120 μmol min^{-1} mg^{-1}). Enzyme (4 mg/mL) in 50 mM potassium phosphate buffer (pH 7.4)/1.6 mM $MgSO_4$; 26 min at 59 780 rpm and 4.2 °C. (B) $NaDodSO_4$/polyacrylamide gel electrophoresis of *E. coli* citrate lyase (lane 1) and of isolated subunits (lanes 2–4).

Results

Isolation of Citrate Lyase from E. coli. The results obtained in a typical experiment are summarized in Table I.

The purification procedure was worked up continuously until the first ammonium sulfate fractionation step. At this stage the undialyzed enzyme solution in 50 mM potassium phosphate buffer, pH 7.4, containing 1.6 mM $MgSO_4$ was stable to storage at 0 or −20 °C for at least 2–3 days.

The procedure results in an 80-fold purification with approximately 20% recovery of activity. The final gel filtration step yields enzyme with a specific activity of about 120 μmol min^{-1} mg^{-1}.

Freshly isolated enzyme preparations with specific activity of 100–120 μmol min^{-1} mg^{-1} were used in all the studies.

Citrate Cleavage. The enzyme showed a pH optimum around 8 for the cleavage of citrate similar to that reported earlier with relatively crude preparations from this source (Dagley & Dawes, 1955; Wheat & Ajl, 1955). The maximum activity was exhibited in the presence of Mg^{2+} followed by Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, and Cu^{2+}. The optimum concentration of Mg^{2+} for the cleavage of citrate was 10 mM. Ca^{2+}, Ba^{2+}, Sr^{2+}, Hg^{2+}, and Ni^{2+} did not activate the enzyme.

Sedimentation Behavior. The sedimentation profile of the purified enzyme preparation shows multiple peaks with $s_{20,w}$ values of 10.0, 16.6, 20.4, 23.7, and 30.0 S (Figure 1A). The preparation was fully active when remixed and recovered from the centrifuge cell. The apparent heterogeneity was found to be independent of buffer composition (50 mM Tris-HCl, pH 7.4–8.0; 50 mM potassium phosphate buffer, pH 7.0–7.5), concentration of buffer (30–200 mM potassium phosphate buffer, pH 7.0–7.5), the presence of EDTA (2–15 mM) with or without dithiothreitol (4 mM), the presence of divalent metal ions (Mg^{2+}, 1.6–10 mM in 50 mM potassium buffer, pH 7.4, or in 50 mM Tris-HCl buffer, pH 7.4; Mn^{2+}, 10 mM

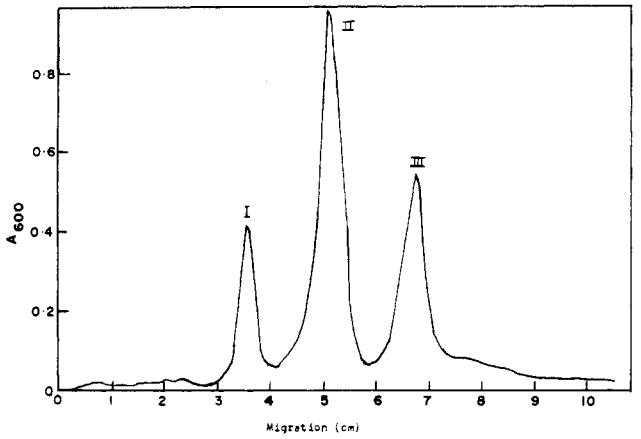

FIGURE 2: Analysis of *E. coli* citrate lyase subunits on $NaDodSO_4$ gels. The enzyme (40–60 μg) was treated as described under Experimental Procedures. A typical scanner trace of Coomassie blue stained gel is shown.

in 50 mM Tris-HCl buffer, pH 7.4, and the presence of mercaptans (2-mercaptoethanol, 5 mM, with or without dithiothreitol, 8 mM).

Electrophoretic Behavior. Five protein bands were also observed on polyacrylamide gel electrophoresis of the native enzyme under nondenaturing conditions.

Reaction–Inactivation. The reaction–inactivation behavior of the *E. coli* enzyme was determined at 25 °C in the presence of 10 mM Mg^{2+} by using the malate dehydrogenase coupled assay procedure as described by Singh & Srere (1971, 1975). The rate constant of the inactivation calculated from the strictly linear first-order plot was 0.32 min^{-1}, 50% activity being lost in 2.16 min. The rate constant values for *K. aerogenes* citrate lyase, which undergoes severe reaction–inactivation, and the *Streptococcus diacetilactis* enzyme, which is only weakly inactivated, are 1.21 and 0.06 min^{-1}, respectively (Singh & Srere, 1975).

Subunit Structure and Stoichiometry. The electrophoretic pattern obtained with purified *E. coli* citrate lyase in $NaDodSO_4$/polyacrylamide gel electrophoresis is shown in Figure 1B. Under the denaturing conditions the enzyme shows the presence of three distinct polypeptide chains, a faint protein band of a slow moving component (I), and two relatively more intense bands of faster moving components (II and III). The enzyme treated with 2% $NaDodSO_4$ in the absence of 2-mercaptoethanol showed no difference in the banding pattern, indicating the absence of interchain disulfide bridges.

The molecular weights of the subunits were determined by $NaDodSO_4$ gel electrophoresis as described under Experimental Procedures. The values from triplicate determinations were in good agreement, and the average estimates were the following: subunit I, M_r 85 000; subunit II, M_r 54 000; subunit III, M_r 32 000.

An approximate estimate of subunit stoichiometry was obtained from gel scans. A scan of the $NaDodSO_4$/polyacrylamide gel is reproduced in Figure 2. The molar ratios of the three subunits were calculated from the integrated areas under the peaks of the gel scan and the estimated molecular weights of the individual subunits. Subunit stoichiometry estimates made in triplicate were in agreement with the ratios of I, II, and III of 1:6:6.

Isolation of Biologically Active E. coli Citrate Lyase Subunits. Elution profile of the subunits separated from 15–20 mg of protein by filtration through a Sepharose CL-6B column in the presence of 6 M urea is shown in Figure 3. Subunit I was eluted completely separate from the other components.

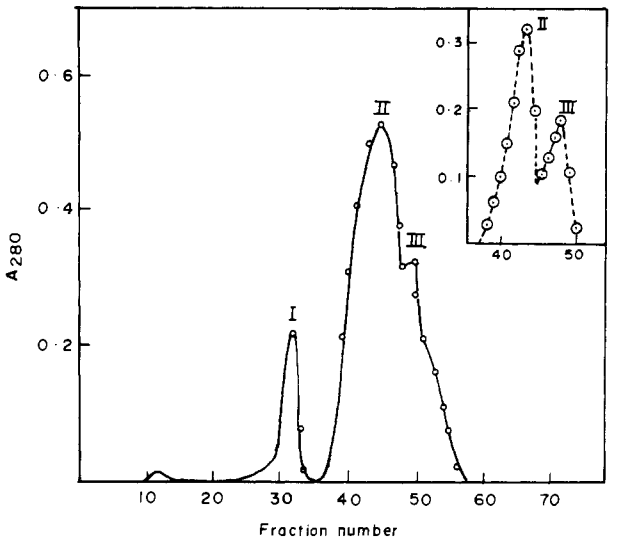

FIGURE 3: Separation of *E. coli* citrate lyase subunits on a Sepharose CL-6B column in the presence of 6 M urea. Gel filtration was carried out as described under Experimental Procedures. Loads of 15–20 mg of total protein were applied to the gel permeation column for separation of subunit I. (Inset) Lower loads (7–10 mg) used for separation of subunits II and III.

In case of the run represented in Figure 3, fractions 30–33, both inclusive, were pooled for isolation of the subunit. Subunits II and III eluted with considerable overlap when such loads were taken. All fractions from this region showed the presence of both subunits II and III. The separation of subunits II and III was possible with lower loads of protein (7–10 mg) as shown in the elution profile in the inset of Figure 3. The trailing fractions of subunit II (38–44) and the leading fractions of subunit III (47–50) when separately pooled gave samples of the pure subunits without contamination from overlap. The homogeneity of the subunit preparations was established by analytical $NaDodSO_4$ gel electrophoresis (Figure 1B).

Characterization of Subunit Function. (1) Acyltransferase α Subunit. The 54 000-dalton subunit (II) was catalytically active in the formation of citryl-CoA from acetyl-CoA and citrate, while the 85 000-dalton subunit (I) and the 32 000-dalton subunit (III) were completely inactive. This establishes the function of the 54 000-dalton subunit (II) as an acetyl-CoA:citrate-CoA transferase α subunit of the *E. coli* citrate lyase complex.

(2) Acyllyase β Subunit. The 32 000-dalton subunit (III) catalyzed the cleavage of (3*S*)-citryl-CoA, while the other two subunits were inactive. This establishes the role of subunit III as the (3*S*)-citryl-CoA:oxalacetate lyase β subunit in the *E. coli* citrate lyase complex. This was confirmed by the acetyl-CoA-dependent cleavage of citrate in the presence of subunit II (acyltransferase) and Mg^{2+}. Citrate cleavage to oxalacetate under these conditions was observed only with subunit III in combination with subunit II, individual subunits as well as other combinations being inactive. The progress curve with the active combination was characterized by an initial lag phase followed by a steady-state phase (Buckel et al., 1973).

(3) ACP Subunit. An active citrate lyase was generated only with all the three subunits in combination and after activation with acetic anhydride. Omission of the 85 000-dalton subunit I resulted in complete loss of catalytic activity. Activity obtained with the combination of all three subunits was low, probably due to denaturation during the isolation procedure and consequent lowering in the activities. The

results, however, establish the ACP function of subunit I from *E. coli* enzyme, since subunits II and III function as the acyltransferase and the acyllyase subunits, respectively, in acyl-CoA-mediated reactions.

The citrate lyase activity shown by the reconstituted enzyme would also confirm that subunits II (54 000 dalton) and III (32 000 dalton), which have been shown in the experiments described earlier to function as acyltransferase and acyllyase in acyl-CoA-dependent reactions, also act as acetyl-ACP:citrate ACP transferase and citryl-ACP:oxalacetate acetyl-ACP lyase, respectively.

(*4*) *Prosthetic Group Components.* Microbiological assay showed the presence of 3.5 mol of pantothenate in *E. coli* citrate lyase complex (computed M_r 600 000 for the $\alpha_6\beta_6\gamma$ complex). The content was consistent with the values of about four residues each of β-alanine and cysteamine in the complex obtained by chemical analysis.

Pantothenate was detected only in the 85 000-dalton ACP subunit after extraction and hydrolysis of the separated subunits from $NaDodSO_4$ gels. β-Alanine and cysteamine residues were also present exclusively in the separated ACP subunit, the contents of 3.6 and 3.7 residues, respectively, in the 85 000-dalton subunit accounting for the entire pantothenate components in the enzyme and being consistent with its role as the ACP in the complex.

(*5*) *End-Group Analysis.* N-Terminal residues detected by dansylation of the separated subunits showed methionine at the N terminus of the 54 000-dalton acyltransferase α subunit and isoleucine at the amino ends of the 32 000-dalton acyllyase (β) and the 85 000-dalton ACP subunits. All subunits of the citrate lyase complex from *K. aerogenes* have been shown to have methionine at the N terminus (Singh et al., 1976).

Estimation of the N-terminal isoleucine residue in the ACP subunit by derivatization with dansyl chloride followed by hydrolysis and chromatographic separation indicated the presence of 0.9 N-terminal residue/mol of ACP (M_r 85 000). The estimate provides further evidence for the subunit being a single 85 000-dalton polypeptide chain.

(*6*) *Sulfhydryl Content.* The total sulfhydryl content of *E. coli* citrate lyase was estimated by DTNB titration in the presence of 0.1 M hydroxylamine and 0.9% $NaDodSO_4$. Replicate determinations gave values in the range 70–75 total SH for the computed M_r 600 000 of the complex. The SH groups exposed on treatment with hydroxylamine and $NaDodSO_4$ would include those from the four cysteamine residues on the prosthetic groups of the ACP subunit. The total half-cystine residues estimated after performic acid oxidation indicated the presence of 64 residues in the complex. The half-cystines in individual subunits were the following: α, 6 residues; β, 2 residues; γ, 11 residues. The titration data would indicate that all these arise from cysteine residues and none apparently from cystines.

Sodium borohydride reduction in the presence of 8 M urea (Cavallini et al., 1966) was also carried out to exclude the presence of any disulfide-bridged polypeptide chains, particularly in the large ACP subunit. The electrophoretic profiles of the treated enzyme showed that the 54 000- and 32 000-dalton subunits were apparently intact, while in the region of 70 000–85 000 daltons a cluster of three faint but distinct bands was observed. The banding pattern would indicate that in the case of *E. coli* citrate lyase the largest 85 000-dalton ACP subunit is not converted to fragments of significantly smaller polypeptides. Under the denaturing and reducing conditions used, the ACP subunit apparently does not break down to half or lower fractional fragments which might have been expected if the subunit had been built up of two to six polypeptide chains linked by disulfide bridges.

Table II: Reactivation of Deacetyl[citrate lyase] from *Escherichia coli*[a]

E. coli citrate lyase fraction[b]	treatment	enzyme activity (% control)
(VIII) Sepharose column chromatography	none (control)	(100)
	dithiothreitol	0
	dithiothreitol + ATP + acetate	0
	dithiothreitol + acetic anhydride	26.0
	dithiothreitol + ATP + acetate + *K. aerogenes* citrate lyase ligase	90.7
(VII) DEAE-cellulose column chromatography	none (control)	(100)
	dithiothreitol	0
	dithiothreitol + ATP + acetate	91.0

[a] Deacetyl[citrate lyase] was prepared by incubating citrate lyase with 10 mM dithiothreitol in 100 mM potassium phosphate buffer, pH 7.8, containing 100 mM KCl and 3 mM $MgCl_2$ at 30 °C for 45 min. Chemical reactivation with acetic anhydride and enzymatic reactivation with deacetyl[citrate lyase] ligase from *K. aerogenes* were carried out as described under Experimental Procedures. [b] Described in Table I.

(*7*) *Deacetyl[citrate lyase].* The citrate lyase from *E. coli* was completely inactivated with dithiothreitol at a final concentration of 10 mM (Table II). *E. coli* citrate lyase thus behaves like the other citrate lyases in being inactivated by mercaptans.

The results on the chemical and enzymatic reactivation of deacetyl[citrate lyase] obtained by treatment with dithiothreitol are included in Table II. An aliquot of the enzyme from the Sepharose CL-6B filtration step of purification is not reactivated on treatment with ATP and acetate, establishing the absence of the ligase in the citrate lyase fraction. Reactivation (90.7%) was obtained, however, on addition of a ligase preparation from *K. aerogenes*. Treatment of the deacetylated enzyme with acetic anhydride (4.7 mM) in the presence of dithiothreitol reactivates the enzyme partially (26%).

A test for ligase activity carried out at various stages of purification of citrate lyase from *E. coli* showed that the citrate lyase ligase separates from citrate lyase only after filtration through Sepharose CL-6B. An aliquot taken from the DEAE-cellulose step of the purification is inactivated on incubation with dithiothreitol but is reactivated (91%) on mere addition of ATP and acetate, indicating the presence of citrate lyase ligase in the DEAE-cellulose fraction.

From Table II it will be apparent that *E. coli* citrate lyase is an acetyl-enzyme, the deacetylated enzyme being reactivated either chemically by acetic anhydride or enzymatically by the ligase in the presence of ATP and acetate. The ligase present in *E. coli* is distinct and separable from the citrate lyase complex.

Discussion

Purified *E. coli* citrate lyase exhibits multiple states of subunit aggregation in the ultracentrifuge and in polyacrylamide gel electrophoresis. Purified citrate lyases obtained hitherto from other sources show the presence of a monodisperse species with a unique state of aggregation. The *K. aerogenes* enzyme has been shown to dissociate into half-molecules and a 6.5S species in the absence of divalent metal and under conditions of low buffer ionic strengths (Mahadik & SivaRaman, 1968), while the *S. diacetilactis* enzyme dis-

sociates at pH values above 8 (Singh & Srere, 1975). The enzyme from *S. faecalis* is inactivated by repeated freezing and thawing with concurrent formation of multimeric forms (Hiremath et al., 1976). In marked contrast, the native citrate lyase complex from *E. coli* exhibits polydispersity.

Previous work on citrate lyase from *K. aerogenes* and other sources has established the close structural relationships between the enzyme complexes from various bacterial sources. The *K. aerogenes* enzyme has been shown to contain three different subunits of 54000 (α), 32000 (β), and 10000 daltons (γ) (Dimroth & Eggerer, 1975b; Carpenter et al., 1975). In this case the α subunit has been shown to function as an acyltransferase involved in citryl-ACP formation with release of acetate and the β subunit to catalyze the cleavage of the citryl-ACP intermediate to oxalacetate and acetyl-ACP (Dimroth & Eggerer, 1975a). The γ subunit functions as an ACP and carries the essential acetyl moiety (Dimroth et al., 1973). Enzymes obtained hitherto from other sources such as *S. diacetilactis* (Singh & Srere, 1975), *Streptococcus faecalis* (Hiremath et al., 1976), and *Rhodopseudomonas gelatinosa* (Giffhorn & Gottschalk, 1978) have all been shown to be complexes of three different subunits resembling in size those from the *K. aerogenes* complex. In all cases the smallest subunit of 10000–14000 daltons has been shown to function as the ACP with the CoA-like prosthetic group. The largest subunit of about 54000 daltons has been assumed to function as an acyltransferase subunit and the smaller 32000-dalton subunit as the acyllyase from analogy with the *K. aerogenes* complex.

The data obtained in the present investigation indicate a marked divergence from this general subunit structure in the case of the *E. coli* enzyme. The enzyme complex from this source contains subunits of 85000, 54000, and 32000 daltons. The 85000-dalton subunit has been shown in the present study to function as the ACP from data obtained in reconstitution studies. This observation has been confirmed by the exclusive presence of the prosthetic group components: pantothenate, cysteamine, and β-alanine in this subunit. The 54000-dalton subunit of *E. coli* enzyme has been shown to function as acyltransferase α subunit both in the acetyl-CoA-mediated reaction and in the reconstituted enzyme. The 32000-dalton subunit of the complex has been demonstrated to function as the acyllyase β subunit in the acetyl-CoA as well as the acetyl-ACP mediated reactions. Besides the *K. aerogenes* enzyme, these are the only reports confirming unambiguously the functions of all three component subunits of citrate lyase complexes.

Citrate lyase from *K. aerogenes* has been shown to contain equimolar amounts of the three subunits α, β, and γ from the molecular weight estimates of the complex and determination of molar ratios of the component subunits (Singh et al., 1976) and from molecular profiles obtained in electron microscopic studies (Dimroth & Eggerer, 1975b). The findings establish that the enzyme from this source is of hexameric structure $\alpha_6\beta_6\gamma_6$. Estimates of subunits and holoenzyme molecular weights in the case of citrate lyase complexes from *S. diacetilactis* (Singh & Srere, 1975), *S. faecalis* (Hiremath et al., 1976), *R. gelatinosa* (Giffhorn & Gottschalk, 1978), and *Clostridium sphenoides* (Antranikian et al., 1982) indicate that these enzymes resemble the *K. aerogenes* complex in their gross structural features. In contrast to this general subunit stoichiometry, the enzyme from *E. coli* has a ratio of α:β:γ subunits of 6:6:1.

Citrate lyase from *E. coli*, like citrate lyases from other sources, contains pantothenate moieties. The *E. coli* enzyme has 3.5 mol of pantothenate in the $\alpha_6\beta_6\gamma$ aggregate. This value is in close agreement with the values obtained from β-alanine and cysteamine residues estimated by amino acid analysis. Estimations of the prosthetic group components in *K. aerogenes*, *S. diacetilactis*, and *S. faecalis* citrate lyases have also revealed the presence of only four pantothenate moieties in their hexameric $\alpha_6\beta_6\gamma_6$ structures (Singh et al., 1976; Singh & Srere, 1975; Hiremath, 1977). The data obtained in the present investigation show that even in the *E. coli* system which apparently has six copies each of the transferase α and lyase β subunits associated with a single ACP subunit of 85000 daltons, only four prosthetic groups are present on the single ACP subunit. Determination of the stoichiometry of CoA ester binding sites in *K. aerogenes* citrate lyase by the use of *p*-azido[^{14}C]benzoyl-CoA as a photoaffinity reagent has indicated the possibility that citrate cleavage can proceed through both the acyl-ACP-mediated and the acyl-CoA-mediated reactions (Basu et al., 1982).

The generation of catalytically active citrate lyase from the deacetyl enzyme by treatment with acetic anhydride or 1-acetylimidazole has been reported for the enzyme complexes from *K. aerogenes* (Buckel et al., 1971), *S. diacetilactis* (Singh & Srere, 1975), *S. faecalis* (Hiremath et al., 1976), *R. gelatinosa*, and *Lactobacillus citrovorum* (Kümmel et al., 1975). Affinity labeling of the deacetyl enzyme in the presence of excess mercaptans has been reported for the *K. aerogenes* enzyme (Dimroth & Eggerer, 1975b). The enzyme from *E. coli* resembles the other citrate lyases in this behavior. Citrate lyases have been shown to undergo reaction–inactivation through spontaneous hydrolysis of the citryl intermediate to citrate and deacetyl enzyme (Dimroth & Eggerer, 1975a; Singh & Srere, 1975). A distinct enzyme has been characterized in all the sources examined, hitherto, which catalyzes reactivation of the deacetyl[citrate lyase], the enzyme acetate:deacetyl[citrate lyase] ligase (AMP) bringing about the acetylation of inactive enzyme in the presence of ATP and acetate (Bowien & Gottschalk, 1977; Hiremath et al., 1976; Schmellenkamp & Eggerer, 1974; Antranikian et al., 1982). The ligases in some instances have been shown to be specific for the lyases from the same sources. Thus the homogeneous citrate lyase ligase obtained from *S. diacetilactis* has been shown to be specific for the lyase from the same organism, not acetylating the deacetyl[citrate lyases] of *K aerogenes* or *R. gelatinosa*. The citrate lyase ligase from *R. gelatinosa* is active toward deacetyl[citrate lyases] from *R. gelatinosa* and *Rhodopseudomonas palustris*, not cross-reacting with enzymes from *S. diacetilactis*, *K. aerogenes*, or *C. sphenoides* (Giffhorn & Kuhn, 1980; Antranikian & Gottschalk, 1982). However, Hiremath et al. (1976) have shown that the deacetyl[citrate lyases] from both *S. faecalis* and *K. aerogenes* are substrates for the ligases present in the two sources. The present study extends this observation to the *E. coli* lyase which cross-reacts with the ligase from *K. aerogenes*.

Registry No. Citrate lyase, 9012-83-3; citrate lyase ligase, 52660-22-7.

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