On the Molecular Weights of the Three Nonidentical Subunits of Citrate Lyase from *Klebsiella aerogenes* 

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**SUMMARY**

The molecular weights of the three nonidentical subunits of citrate lyase of *Klebsiella aerogenes* have been determined by three methods: sedimentation equilibrium in 6 M guanidinium chloride, sodium dodecyl sulfate gel electrophoresis, and gel filtration on 6% agarose column in 6 M guanidinium chloride. The molecular weights of the subunits, named I, II, and III (or acyl carrier protein) in order of elution from the agarose column, were 54,500, 32,000, and 11,000, respectively. The agarose-guanidine column provided a nearly complete separation of the three subunits. The molecular weight of the native enzyme was found by sedimentation equilibrium to be 520,000 ± 10,000. The uncertainties in the molecular weights of the enzyme and its subunits did not permit a valid postulation of the subunit composition.

Several studies have been made on the size and subunit structure of citrate lyase (EC 4.1.3.6) from *Klebsiella aerogenes*. There has been a general agreement that the molecular weight of the native enzyme is in the range of 550,000 to 575,000 (1-4), but the subunit structure is not settled. Mahadik and SivaRaman (1) reported that the enzyme is composed of eight identical subunits of about 74,000 daltons, based on the results of sedimentation studies with denaturating solvents. Bowen and Mortimer (2) presented data which indicated that the enzyme contained 16 identical subunits of about 34,000 daltons. They pointed out that the omission of a reducing agent like β-mercaptoethanol from the denaturating solvents by Mahadik and SivaRaman (1) resulted in a higher molecular weight of the subunit, presumably due to interchain disulfide bond formation. These authors also stated that they observed two major protein bands on SDS gel electrophoresis with molecular weights of about 55,000 and 33,000. However, sedimentation equilibrium studies with denaturating solvents indicated a single species of 34,000 daltons, leading them to conclude that the protein band of 55,000 daltons was an artifact of the electrophoretic procedure. It has been shown in an earlier report (5) from this laboratory that the citrate lyase from *Klebsiella aerogenes* contains stoichiometric amounts of pantothenate. Dimroth et al. (3) confirmed this observation and demonstrated that the pantothenate is associated with a small subunit (MW ~ 10,000) of the enzyme. This subunit was not reported by Mahadik and SivaRaman (1) or Bowen and Mortimer (2). In analogy to the fatty acid synthase system, Dimroth et al. named the 10,000-dalton subunit as the acyl carrier protein of citrate lyase and postulated that citrate lyase is a multienzyme complex. Based on their results of SDS gel electrophoresis, they proposed that citrate lyase is composed of 16 identical subunits of 32,000 daltons along with four acyl carrier proteins of 10,000 daltons (3).

In a recent preliminary communication (4), we have shown that citrate lyase is composed of three nonidentical subunits. The three subunits were separated by SDS gel electrophoresis, and on subsequent re-electrophoresis, each of these protein bands was found to migrate in the same manner as in the original gel. This is a strong indication that none of the three bands were artifacts of the SDS treatment or the electrophoretic procedure. It was also shown that the three subunits can be separated from each other by gel filtration in 6 M urea.

In this paper, we present additional studies on the three polypeptide chains of citrate lyase. The purity of the preparation of each of the polypeptide chains was monitored for homogeneity by SDS gel electrophoresis.

The molecular weights of these subunits were determined by SDS gel electrophoresis, by analytical gel filtration on 6% agarose in 6 M guanidinium chloride, and by sedimentation equilibrium employing state-of-the-art experimental and evaluation procedures (6-9) that lead to results of greater reliability.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were of reagent grade and the commercial sources were as follows: Bio-Gel A-1.5m and A-5m (both 200 to 400 mesh) from Bio-Rad Laboratories; Sephadex G-15 (fine) and blue dextran from Pharmacia Fine Chemicals; DEAE-cellulose (DE20) from Reev-Angel; urea and guanidinium chloride from Heico Inc.; NADH, SDS, and crystalline urease from Sigma; dithiothreitol from Calbiochem; crystalline catalase from Worthington; N-dinitrophenyl-l-arginine, as well as the crystalline proteins ovalbumin, chymotrypsigenin, myoglobin, and lysozyme from Mann Research Laboratories; partially purified citrate lyase and crystalline enzymes malate dehydrogenase, lactate dehydrogenase, pyruvate kinase, citrate synthase, and aldolase from Boehringer Mannheim.

**Preparation and Assay of Citrate Lyase**—Citrate lyase was assayed by the malate dehydrogenase coupled procedure as described earlier (10). The commercially obtained enzyme was further purified by gel filtration on a Bio-Gel A-1.5m column (2.5 X
yielding an enzyme having a specific activity of about 70. Preparations were monitored for purity by SDS gel electrophoresis.

**SDS Gel Electrophoresis**—The procedure of Weber and Osborn (11) was used with a running buffer of 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS. The samples were usually prepared by putting the protein in 1% SDS containing β-mercaptoethanol in a boiling water bath for 5 min followed by dialysis against the running buffer.

**Gel Filtration in Denaturing Solvents**—Citrate lyase, dissociated in 6 M urea containing 10 mM dithiothreitol and 10 mM sodium phosphate, pH 7.0, was subjected to gel filtration on a Bio-Gel A-1.5 column (1.5 × 90 cm) equilibrated with the same solvent. The protein in each fraction was monitored by its fluorescence with a Farrand fluorometer, excitation at 280 nm, and emission at 345 nm as described earlier (4).

For analytical column chromatography, 6 M guanidinium chloride was the denaturant. The procedure of Fish et al. (12) was used. Eluant volume was obtained by weighing.

**Preparation of Acyl Carrier Protein of Citrate Lyase**—The acyl carrier protein from the urea column was diluted 10-fold with 0.01 M potassium phosphate, pH 7.0, prior to placing it on a 5-ml DEAE-cellulose column equilibrated with the solvent. The acyl carrier protein was removed by elution with 0.5 M ammonium acetate, lyophilized, and put through a Sephadex G-15 column equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol prior to sedimentation equilibrium studies.

**Sedimentation Equilibrium Studies**—High speed sedimentation equilibrium experiments (13) were performed with a Beckman model E ultracentrifuge equipped with Rayleigh optics and electronic speed control. The optical system was aligned according to the procedure of Richards et al. (14, 15). The rotor speed and solute concentration were calculated for a 3-mm column height and the V and molecular weight of the material of interest to give resolvable fringe patterns (about 7 to 10 fringes) at the cell bottom (6), ensuring that any material several times smaller or larger would be included in the molecular weight polydispersity analysis.

To increase the accuracy of the molecular weight determinations, the Rayleigh patterns were photographed on Kodak metallocraphic plates using the mask with 0.2-mm slits. At the conclusion of the run a base-line pattern was obtained after several hours of running at the same speed using the rinsed cell with both compartments filled to a slightly higher level with water. Fringe patterns were read with a Nikon Profile projector model 6C equipped with digital micrometers (IKL Inc., Newport Beach, Calif.). A 263 Multiplexer and 262 Teleprinter-System Interface (Princeton Applied Research, Princeton, N. J.) were used to interface the mierometers to a 700ASR data terminal (Texas Instruments Inc., Houston, Texas). Fringe centers for 80 to 100 radial positions were located with the aid of a light-difference detector placed on the projector screen. The averaging of five fringes for each radial position, following the procedure described in the preceding reference, resulted in an estimated average error for the fringe number J of 0.1 μm (0.003 fringe) after base-line correction. Before reading the patterns, a computer program was used to locate radial positions that would give approximately equal fringe increments for even spacing of molecular weight fringe-number plots along the horizontal axis. The averaged fringe versus radial position data were processed by a time-sharing program modified from one developed by R. D. Dyson. Plots were obtained on-line with a 7202A graphic plotter (Hewlett-Packard). Treatment of the data was similar to that performed by programs (6, 7) requiring batch operation, but results and plots were examined early in the processing and certain parameters were changed for optimum evaluation of the data. Since values of J obtained from the fringe patterns were measured relative to the unknown concentration at the meniscus (Jₘ), it was necessary to estimate this value to obtain the absolute values of J needed for evaluation of the In J versus Δ² plots (Δ² = r² − rₘ², where rₘ refers to the meniscus position). The first approximation was obtained from a linear extrapolation of In J versus Δ² by assuming Jₘ = 0 and performing a linear extrapolation to the meniscus for J values between 0.3 and values ranging from two to six fringes depending upon the curvature of the plots. The extrapolated value, usually less than 0.06 fringe, was then added at each point to give a new plot of In J versus Δ². This procedure was repeated until the plot was linear at low J values. Since some of the plots were not linear at high J values, indicative of non-ideality or polydispersity, the data were further processed by the computer program to give apparent number, weight, and Z-average molecular weights (Mn*, Mw*, and Mz*, respectively) at each point in the cell. The averages were obtained with the usual procedures (6, 7) involving the least-mean-squares quadratic fit of groups of points, usually 9 to 21. The molecular weight averages were plotted versus J. Since the values at the extremities of the plots are subject to greater error, excessive curvature in these regions was ignored in extrapolation to zero concentration (13).

Solution densities for 6 M guanidinium chloride were measured refractometrically with a Bausch and Lomb refractometer (16, 17). For dilute salt solutions the densities were measured. The V was assumed to be 0.73 ml/gm (2) for all molecular weight calculations.

### RESULTS

**Molecular Weight of Native Citrate Lyase**—The results of sedimentation equilibrium studies with the native citrate lyase are presented in Fig. 1. A is a plot of In J versus Δ² and B represents the plot of apparent molecular weight averages versus protein concentration. Linearity of the points in the first plot suggested that the enzyme is homogeneous. The slight positive slope observed in the more sensitive plot (B) showed a small amount of heterogeneity. From area measurements of the three subunits and trace components on densitometer tracings of SDS gels, the heterogeneity has been estimated to be less than 1%. The values obtained for Mₙ, Mₔ, and Mₕ, whether by averaging or extrapolation to zero concentration, differed by less than 0.5% from 527,000. Two other experiments on differ-

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1 R. D. Dyson, personal communication.
Fig. 2. Sedimentation equilibrium of unfractionated polypeptide chains at two rotor velocities. Solvent conditions: 6 M guanidinium chloride, 10 mM dithiothreitol, 0.01 M potassium phosphate, pH 7.0, room temperature. After reaching equilibrium for 25 hours at 30,000 rpm the speed was shifted to 36,000 rpm and equilibrium was reattained after 19 hours. A, In J versus Δr²; B, apparent weight average molecular weight versus concentration. One milligram per ml is about 2.87 fringes for protein in 6 M guanidinium chloride.

ent preparations gave molecular weight values of 520,000 ± 10,000 with evidence of somewhat greater heterogeneity.

Sedimentation Equilibrium of Citrate Lyase in 6 M Guanidinium Chloride—Our recent studies using SDS gel electrophoresis and gel filtration techniques have indicated that citrate lyase is composed of three nonidentical subunits (4) which is in contradiction with the earlier observation (2) that no heterogeneity could be detected when the enzyme was subjected to sedimentation equilibrium in denaturing solvents, such as 6 M urea, 6 M guanidinium chloride, or 0.1% SDS. This led us to re-examine the sedimentation equilibrium of citrate lyase in 6 M guanidinium chloride. The results of these studies at 2 different rotor velocities are shown in Fig. 2. Both the plots (A and B) indicate heterogeneity. The overall slope of the curved In J versus Δr² plot (A) gave 37,300 and 33,000 daltons, respectively, at the higher and lower speeds.

Separation of Nonidentical Subunits of Citrate Lyase—To determine the molecular weights of the subunits by sedimentation equilibrium, it was necessary to separate them into homogeneous polypeptide chains. Fig. 3A is an elution profile from a Bio-Gel A-5m in 6 M urea containing 10 mM dithiothreitol. A small leading peak, completely separated from the citrate lyase subunits, is a higher molecular weight polypeptide found as a contaminant in some of the preparations. The three distinct fractions representing the nonidentical subunits of the enzyme will hereafter be referred to as I, II, and III (or acyl carrier protein) in order of elution from the column. Each of the protein-containing fractions was analyzed by SDS gel electrophoresis, some of which are shown in Fig. 3B. Fractions 68, 73, 75, and 85 contained only subunits I, II, and III, respectively, with negligible contaminating protein.

Sedimentation Equilibrium of Subunits I and II in 6 M Guanidinium Chloride—If subunits I and II were not well separated on the Bio-Gel column, appropriate fractions were pooled and concentrated from a dialysis sack placed in dry Sephadex. Pure components were then obtained by another passage through the
same column. The absence of contaminants was verified from SDS gel analysis. Several sedimentation studies were performed with components I and II dialyzed into 6 M guanidinium chloride. Typical results are presented in Fig. 4. The plots of $\ln J$ versus $\Delta r^2$ are essentially linear for both the polypeptides (I and II). The molecular weight versus concentration plot for polypeptide II is representative of a homogeneous, nonideal solution. Extrapolation of the plots of $M_n$, $M_w$, and $M_z$ to zero concentration gives values for the molecular weight close to an average of 30,700. Two runs on samples obtained from other columns gave similar results. However, the shape of the plots of molecular weights versus $J$ for subunit I (Fig. 4B) suggests a heterogeneous, nonideal solution. The molecular weight was estimated to be 47,200 by extrapolation of $M_w$ to zero concentration (ignoring the curvature at low concentration). $M_w$ versus $J$ plots for two sedimentation equilibrium runs on different preparations, one involving a speed change from 28,000 to 32,000 rpm (Fig. 5), show similar contours. The point average molecular weights for the three curves were the same within 5%. Two other experiments of lesser reliability, one with a sample obtained from a guanidinium chloride column, gave lower values for the molecular weight.

Sedimentation Equilibrium of Subunit III (ACP)—High speed sedimentation equilibrium of this polypeptide in 6 M guanidinium chloride requires a prohibitively high speed because of its small size. Thus a sedimentation experiment in dilute salt solution was performed at a speed of 48,000 rpm. The overall slope of $\ln J$ versus $\Delta r^2$ (Fig. 6) gave a molecular weight of 10,400. Both the plots (A and B) indicate some heterogeneity.

SDS Gel Electrophoresis—Three bands were always present on SDS gel electrophoresis of citrate lyase as shown in Fig. 7A. Fig. 7B presents a typical calibration curve of a 7% gel with reference proteins. The molecular weights of the subunits obtained from averaging the results of 21 experiments with gels ranging from 7 to 12.5%, were 56,000, 32,000, and 13,000.

Analytical Gel Filtration in 6 M Guanidinium Chloride—When chromatographed on agarose in 6 M guanidinium chloride all three polypeptides were well separated from each other (Fig. 8, A and B). The calibration curve is shown in Fig. 8C. The chain weights were estimated to be 53,000, 32,000 and 10,800.

A summary of the molecular weight values obtained for the native enzyme and the subunits by the three methods is presented in Table I.
The experimental procedures for sedimentation equilibrium experiments described here enabled us to obtain more accurate results than was possible by the other workers on this enzyme. The absolute values for the molecular weights, of course, remain uncertain because of uncertainties in \( \bar{V} \) for the native enzyme and for the subunits in guanidinium chloride.

The molecular weight of native citrate lyase from three sedimentation equilibrium experiments reported here was 520,000 \( \pm \) 10,000, lower than earlier values of 550,000 to 575,000 (1, 2, 4). Values obtained by the Archibald method (1, 2) are subject to greater error and operator bias. The reported sedimentation equilibrium value of 547,000 (2) was obtained at speeds too high to give more than two to three fringes across the cell, resulting in a greater experimental error. More careful examination of our earlier data (4), which gave a value of 560,000, revealed a slight upward curvature in the \( J \) versus \( A^+ \) plots, indicative of heterogeneity. Based upon three experiments against one for the higher value, we favor the lower value of 520,000 from results presented here. We are currently examining the possibility that a variable loss of one or more of the subunits during purification of the enzyme may lead to variations in the molecular weight.

Sedimentation equilibrium of the enzyme in 6 M guanidinium chloride (without fractionation) yielded nonlinear plots for \( J \) versus \( A^+ \) (Fig. 2A) and these results are consistent with our earlier findings of three nonidentical subunits of this enzyme by SDS gel electrophoresis and gel filtration (4). But it does not support the results of Bowen and Mortimer (2) who indicated that no heterogeneity could be detected on sedimentation equilibrium of citrate lyase in denaturing solvents like 6 M urea or 6 M guanidinium chloride or 0.1% SDS. It is known that protein solutions in these solvents exhibit a high degree of non-ideality (8). Depending on the protein concentration and rotor speed, it might be possible to obtain conditions in which the effect of heterogeneity is exactly compensated by that of non-ideality.

In the present studies, we have carried out sedimentation of the unfractionated polypeptides in 6 M guanidinium chloride at two different rotor speeds and analysis of the data (Fig. 2, A and B) clearly shows the presence of more than one species, the higher speed giving a better indication of heterogeneity.

If a more concentrated sample had been used in this experiment, the increase in non-ideality at the bottom of the cell would have reduced the upward curvature perhaps sufficiently to have given an essentially straight plot. Bowen and Mortimer (2) did not report the actual data to allow for an evaluation of the results of their sedimentation equilibrium studies.

We have employed three independent methods for the determination of molecular weights of the subunits. For subunit I, the values obtained by gel electrophoresis and gel filtration are consistently higher than that by sedimentation equilibrium. Curvature suggestive of heterogeneity is seen in the molecular weight versus concentration plot (upper curve, Fig. 4B). It cannot be attributed to contaminating species since SDS gels of this sample gave only a single protein band. Moreover the same point-average molecular weights with different speeds and loading concentrations (Fig. 5) argue against heterogeneity. Such curvature at low \( J \) values has also been observed, but not explained, for other polypeptide chains in the same solvent (18, 19).

Bowen and Mortimer (2) reported a protein band with molecular weight of 55,000 on SDS gel electrophoresis (neither the gels nor densitometer tracings were shown), but discounted it as an artifact. The present findings are consistent with their observation regarding the size of this polypeptide; but there is no ground for considering it to be an artifact of the procedures employed in the present and earlier (4) studies.

For subunit II, the molecular weights obtained by the three methods are in good agreement and the analysis of the results of sedimentation equilibrium in 6 M guanidinium chloride (lower curve, Fig. 4B) is an excellent example of what is expected for a
homogeneous, non-ideal protein solution. Dimroth et al. (3) reported 32,000 as the molecular weight of the subunit of citrate lyase based on the results of SDS gel electrophoresis only. This corresponds to the size for subunit II, as determined by three independent methods in the present studies.

The acyl carrier protein (subunit III) was obtained free from other polypeptides by gel filtration in denaturing solvents. Due to the small size of this polypeptide, sedimentation equilibrium studies were not attempted in these solvents. The denaturing solvents were removed in an attempt to allow the polypeptide to regain its native structure in dilute buffers. The results of a single sedimentation equilibrium experiment of such a preparation of acyl carrier protein, however, indicated that a heterogeneous mixture of polypeptide chains was obtained (Fig. 6, A and B). Analysis of a two-species plot (7, 20) (not shown) showed the mixture to be quite complex and to contain more than two species. Since the sample was found to be homogeneous by SDS gel electrophoresis, the apparent heterogeneity revealed in the sedimentation equilibrium study is likely due to aggregation of the polypeptide chains. The molecular weight of the acyl carrier protein obtained in the present studies is in good agreement with the earlier value of 10,000, based on the results of SDS gel electrophoresis (3).

From a comparison of Figs. 3 and 8, it is evident that the separation of subunits I and II is more complete for the guanidinium chloride column. We do not know whether the better separation is due to the slower flow rate or a yet to be determined property of the two systems.

The results presented here establish that native citrate lyase with a molecular weight of 520,000 contains three nonidentical subunits. From the summary of molecular weights of the subunits presented in Table 1, we will select what we consider to be the most reasonable values. The sedimentation equilibrium values are uncertain because of lack of Y data. For subunits I and II the average of the last two methods gives 54,500 and 32,000 daltons, respectively. Owing to uncertainty in molecular

*Fig. 8. Analytical gel filtration of citrate lyase on 6% agarose in 6 M guanidinium chloride. A, Elution profile. Blue dextran (Δ—Δ) and N-dimethylphenylarginine (□—□) were used as internal standards for void volume (V0) and internal volume (V1), respectively. Citrate lyase is represented by ●. Fraction size was 0.85 ml and the flow rate was about 1.5 ml/hour. B, SDS gels for the peak protein fractions; a, b, and c represent the gels for Fractions 68, 79, and 101, respectively. C, calibration curve for molecular weight determination. The solid circles represent the following molecular weight markers: 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen; 4, myoglobin, and 4, cytochrome c (11,700). The solid triangles represent the polypeptides of citrate lyase. The distribution coefficient (Kd) was determined from the following relation: Kd = Vr - Ve/Vr - V0, where Vr is the elution volume of the polypeptide chain.*
weight estimates from SDS gels for polypeptide chains below 15,000 daltons (21), the gel filtration value of 11,000 is preferred.

In the light of the present findings, none of the earlier proposals for the subunit structure of citrate lyase (1-3) is acceptable since the existence of three nonidentical subunits was not known. Based solely on the molecular weight data reported here, one can consider a number of subunit compositions. If it is assumed that there are four or six of each subunit, the molecular weight of a citrate lyase would be 390,000 or 585,000, respectively. The former value is unreasonably low. The latter is near one of our values for the native enzyme, but too high for the three experiments giving 520,000. We have already mentioned the contribution of the uncertainty in V to molecular weight measurements by sedimentation equilibrium and the possibility of variable subunit composition in citrate lyase preparations. For example the removal of one subunit I or one subunit II and two acyl carrier proteins from the model with six of each subunit would give a value close to 530,000.

Preliminary results of integrating the areas under the peaks of the gel scans (Fig. 7A) give relative areas suggesting a subunit structure of 6 each of I and II with four acyl carrier proteins. The presence of four acyl carrier proteins per holoenzyme in this model agrees with the reported stoichiometry of pantothenate (3, 5). Based on the pantothenate content, a similar suggestion of four acyl carrier proteins per holoenzyme has also been made by Eggerer and his co-workers (3). But the amount of pantothenate present in citrate lyase has been determined only by microbiological procedures (3, 5). We feel that further substantiation of the stoichiometry of the pantothenate to enzyme, by independent lines of evidence is still necessary. Moreover, this model would give a molecular weight of 563,000 for the holoenzyme, which is higher than the observed value of 520,000.

We have applied here the most sophisticated and accurate ultracentrifugal techniques now available. The protein meets two standards set for homogeneity. Yet, it is still not possible to give an unequivocal answer concerning the subunit composition of citrate lyase.

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