CITRATE LYASE: MOLECULAR WEIGHT AND SUBUNIT STRUCTURE

S. P. Mahadik and C. SivaRaman
National Chemical Laboratory, Poona-8, India

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The purification of citrate lyase (citrate oxalacetate-lyase, EC 4.1.3.6) from Aerobacter aerogenes was described earlier by one of us (SivaRaman, 1961). In the present communication, the molecular weight of 314,000 reported by Bowen and Rogers (1963) has been shown to be inconsistent with the gel filtration behavior of the enzyme and a considerably higher value of 575,000 has been obtained on redetermination by the Archibald procedure. Data have been presented here which suggest that the enzyme is composed of eight subunits of identical size. The octameric form of the enzyme has been shown to dissociate reversibly in two stages, first to a tetramer and then to a dimer, on dialysis against EDTA solutions of low ionic strengths and pH 7.4. The enzyme molecule has also been shown to disaggregate in stages on reaction with p-chloromercuribenzoate (PCMB).

MATERIALS AND METHODS

Citrate lyase: For enzyme assay, aliquots equivalent to 10 - 15 μg of pure protein were added to 3.0 ml assay mixture consisting of 0.1 M citrate-0.01 M $MgSO_4$ - 0.05 M Tris-HCl buffer, pH 8.0; and the keto acid liberated in 2 min at 30° estimated by the method of Friedemann and Haugen (1943). The enzyme was purified from A.aerogenes, strain NCTC 418, by a procedure essentially similar to the one described earlier (SivaRaman, 1961), except that a filtration through a 110 cm X 1 cm column of Sephadex G-200 was carried out finally, using 1.6 mM $MgSO_4$ - 0.05 M potassium phosphate buffer, pH 7.4. The pure enzyme showed a specific activity of 36.5 μmoles/min/mg protein, on the

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basis of dry weight of protein. The corresponding value based on protein assayed by the method of Warburg and Christian (1941), relative to bovine serum albumin, was about 60, as reported earlier. The enzyme was homogeneous in the ultracentrifuge and on starch gel electrophoresis.

Ultracentrifugation: The Spinco Model E instrument equipped with phase plate was used and plates read on a microcomparator. Molecular weight determinations were made by the Archibald procedure as described by Schachman (1957), using a valve-type synthetic boundary cell. Only readings at the meniscus were taken for the calculation of molecular weights. Sedimentation coefficients were calculated in the usual manner from the sedimentation patterns at 59,780 rpm. The partial specific volume of the enzyme was assumed to be 0.735 (Bowen and Rogers, 1965) in all the solvent systems. Density and viscosity corrections were based on the values reported by Kawahara and Tanford (1966).

Sulfhydryl reagents: The reaction of -SH groups with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) was followed spectrophotometrically (Ellman, 1959). Titrations with PCMB were carried out by the method of Boyer (1954).

RESULTS AND DISCUSSION

Molecular weight data on the native enzyme and its subunits are summarized in Table 1.

Table 1. Sedimentation data on citrate lyase and its subunits

Buffers contained KH_2PO_4 - K_2HPO_4 .Temp. 20 - 25°.

Solvent system	Number of assays	Protein (mg/ml)	Molecular weight			$S^0_{20,w}$ (S)
			Rotor (rpm)	M_w		
1) 0.15 M NaCl-1.6 mM $MgSO_4$-0.03 M buffer, pH 7.0 (or pH 7.4)	12	5 - 10	4763	575,000 ±17,500		16.0
2) 6 M urea-0.15 M NaCl -0.03 M buffer, pH 7.0	6	6.0	12,340	73,800 ± 3,100	$S_{20,w}$	2.0
3) 6 M GuHCl-0.05 M buffer, pH 7.0	3	5.5	12,340	84,500	$S_{20,w}$	2.5

Molecular weight determinations on the native enzyme were carried out at four different protein concentrations and at two pH values, 7.0 and 7.4. The value of M_w was found to be independent of both protein concentration and pH in the ranges tested. The molecular weight of 575,000 is markedly higher than the value of 314,000 reported by Bowen and Rogers (1963) from Archibald runs carried out according to the procedure of Ehrenberg (1957), although the $S^0_{20,w}$ values are in agreement. Evidence in favour of the present value is the elution behavior of citrate lyase from a 110 cm X 1 cm column of Sephadex G-200 using Blue Dextran 2000 and freshly isolated, crystalline urease (jack beans) as markers. With an eluting system of 1.6 mM $MgSO_4$ - 0.05 M potassium phosphate buffer, pH 7.4, citrate lyase emerged after Blue Dextran but ahead of the main urease activity (M_w, 483,000; Sumner et al. 1937; Gorin et al., 1964) which would indicate a molecular weight greater than that of urease.

The molecular weight of 73,800 in 6 M urea solution indicates that citrate lyase is composed of eight subunits. The value obtained in 6 M guanidine hydrochloride (GuHCl) is essentially in agreement with this number. The subunits are evidently of identical size since sedimentation profiles of the enzyme in both urea and GuHCl solutions were single, symmetrical peaks of apparently homogeneous material. Attempts to reconstitute the octamer by reducing the urea or GuHCl concentration either by dilution or by dialysis always resulted in precipitation of the protein. The dissociation of octamer to monomer is evidently not reversible under these conditions. Studies on the effect of 2-mercaptoethanol on the structure of the enzyme and of its subunits in 6 M GuHCl are in progress and will be reported in a later communication.

Dissociation data on citrate lyase in EDTA solutions of low ionic strengths are summarized in Table 2.

The enzyme in 2 mM EDTA - 1 mM potassium phosphate buffer, pH 7.4, sedimented as a single, symmetrical peak ($S_{20,w}$, 10.3). The molecular weight

Table 2. Dissociation of citrate lyase in EDTA - buffers

Buffers contained KH_2PO_4 - K_2HPO_4, pH 7.4; temp. 5 - 8°.

Solvent system	Protein (mg/ml)	$S_{20,w}$ (S)	Molecular weight	
			Rotor (rpm)	M_w
1) 2 mM EDTA-1 mM buffer	6.1	10.3	7247	273,000
2) 1 mM EDTA-1 mM buffer	7.1	6.5,10.3(trace)		137,000
3) Enzyme treated as under (1) and redialysed against 0.05 M buffer	2.7	16.1		
4) Enzyme treated as under (1) and redialysed against 2 mM $MgSO_4$ - 1 mM buffer	2.7	16.1		

of 273,000 under these conditions indicates that the enzyme dissociates quantitatively to a tetramer. A slight lowering in EDTA concentration from 2 mM to 1 mM has a further marked effect on the enzyme molecule, causing most of the tetramer to dissociate to a 6.3 S component. This material was unstable and often precipitated out of solution on handling. The molecular weight of the 6.3 S component was derived from the approximate relationship, $\left[S_1/S_2\right]^{3/2} = M_{w,1}/M_{w,2}$ (Schachman, 1959), relative to the tetramer. The calculated value of 137,000 would indicate that the 6.3 S component is a dimer. The dissociation of the octamer to the tetramer is readily reversible, a 16.1 S component being reconstituted from the tetramer on dialysis either against 0.05 M potassium phosphate buffer, pH 7.4, or against 2 mM $MgSO_4$ - 1 mM potassium phosphate buffer, pH 7.4. Since in these studies the enzyme dissociates only in the absence of Mg^{++} and the enzyme is inactive under this condition, there was no way of testing whether or not the tetramer and dimer have enzymatic activity. However, material reconstituted from the tetramer by dialysis against 2 mM $MgSO_4$ - 1 mM phosphate buffer, showed 52% of initial activity; and aliquots of the dimer solution adjusted to 0.01 M $MgSO_4$ and 0.05 M

phosphate buffer, pH 7.4, 2 min before assay, showed 40% of initial activity.

The results obtained on treatment of citrate lyase with sulfhydryl reagents are shown in Fig. 1.

Reagent	DTNB	PCMB	PCMB
SH groups reacted per mole enzyme	8	8	16
Activity (% initial)	80	90	43

Reagent	PCMB	PCMB	PCMB
SH groups reacted per mole enzyme	26	34	68
Activity (% initial)	6	0	0

Figure 1. Effect of -SH reagents on citrate lyase

Buffer systems: 0.25 M Tris -HCl, pH 8.0, for DTNB; 0.05 M KH_2PO_4-K_2HPO_4, pH 7.4, for PCMB. Protein, 2.7 - 5.5 mg/ml. Speed, 59,780 rpm; temp. 3 - 7°; photographs taken at 32 min.

A total of only 8 -SH groups per mole enzyme reacted with DTNB in dilute buffer systems, while a total of approximately 72 were estimated by PCMB titration. It can be seen from Fig. 1 that the reaction of 8 - SH groups per mole enzyme, either with DTNB or with PCMB, has little effect on activity and sedimentation behavior. The titration of further -SH groups

with PCMB causes progressive inactivation with the appearance first of a slower sedimenting 12 S component. This change is nearly complete after the reaction of about 26 -SH groups. Further reaction with PCMB causes a shift from the 12 S to a 4 S component, till with the reaction of 68 -SH groups only the 4 S species is evident.

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