PURIFICATION AND PROPERTIES OF CITRATE LYASE FROM STREPTOCOCCUS FAECALIS

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SUMMARY: Citrate lyase from Streptococcus faecalis has been purified to homogeneity. The enzyme has a molecular weight of about 580 000 and contains 3 non-identical subunits of about 53 000, 37 000 and 14 000 daltons. The subunits are not linked by interpeptide disulfide bridges. The enzyme does not dissociate in buffers of low ionic strength. The enzyme shows only a weak reaction inactivation and the enzyme complex is not associated with acetylating enzyme activity for reactivation of the HS-citrate lyase in presence of acetate and ATP.

Citrate lyase (EC 4.1.3.6) which catalyses the cleavage of citrate to oxalacetate and acetate has been obtained homogeneous from Klebsiella aerogenes (1), Streptococcus diacetilactis (2,3), and Rhodopseudomonas gelatinosa (4).

The enzyme from K.aerogenes, which has been studied the most, is a complex of 3 non-identical subunits of about 54 000, 32 000 and 10 000 daltons (5,6). The smallest subunit carries an acetyl group in thioester linkage with a cysteamine residue and functions as an acyl carrier protein (ACP) (6). The 54 000 and 32 000 daltons subunits have been shown to function as acyl transferase involved in citryl-ACP formation and in the subsequent lyase reaction, respectively(7). The enzyme undergoes reaction inactivation (8) through deacylation of the active enzyme to the desacetyl (HS-) enzyme (9). The inactive HS-citrate lyase is reactivated by acetylation either chemically or by an enzyme in the presence of acetate and ATP (9,10). Citrate lyase from S.diacetilactis shows only a weak reaction inactivation (2,3). This has been attributed to the association of the HS-enzyme acetylating activity in the enzyme complex which distinguishes it from the more rapidly inactivated enzymes from K.aerogenes and from R.gelatinosa (3).

The present communication describes the purification and properties of citrate lyase from S.faecalis. Gillespie and Gunsalus (11) first reported the presence of this enzyme as a substrate-induced activity in the organism.

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MATERIALS AND METHODS

Assay of Citrate lyase: The enzyme was assayed at 30 °C as reported earlier (12) except that the test medium contained 20 mM $MgSO_4$ instead of 10 mM as the higher concentration gave optimal activity. 1 U of enzyme is defined as the amount of enzyme required for catalyzing cleavage of 1 μmole citrate/min under assay conditions.

Protein determination: Protein was determined by the procedure of Lowry et al.(13). Samples containing streptomycin were dialysed before analysis.

Ultracentrifugation: Spinco model E instrument equipped with phase plate was used. Molecular weight and sedimentation coefficients were determined as described earlier (12). Partial specific volume of the enzyme was assumed to be 0.735.

Polyacrylamide gel electrophoresis: Disc electrophoresis was carried out in 5% acrylamide gel at pH 8.3 according to Davis (14). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out in 7% gels using the sodium phosphate buffer system described by Weber and Osborn (15). The gels were stained for protein with Amido black. For molecular weight estimates, bovine serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome c were used as marker proteins.

Organism: S.faecalis strain 10C1 was a gift from Dr. I.C.Gunsalus, University of Illinois. For enzyme production the organism was grown at 37 °C without aeration in a medium of the following composition: KH_2PO_4, 20 g; $(NH_4)_2SO_4$, 10 g; $MgSO_4.7H_2O$, 4 g; Yeast extract, 50 g; Peptone powder, 50 g; Na_3 citrate.$2H_2O$, 100 g; and water to make 10 L after adjustment of pH to 7.2 with 2N NaOH. Cells were harvested in a Sharples centrifuge after 30 h growth. Yield of cells was 15-20 g per 10 L medium.

Preparation of Cell-free extract: Cells were suspended in 3 volumes of 0.03M potassium phosphate buffer, pH 7.0, cooled in an ice bath and disrupted by sonic oscillation at 20 Kc (300 W) for 3 min. Cell debris was removed by centrifugation. This and all subsequent operations were carried out at about 4 °C.

Purification of Citrate Lyase: The cell-free extract (200 ml, 0.5% protein) was treated with sodium acetate (1 mM final concentration) and ATP (0.5 mM final concentration) and left for an hour to reactivate HS-citrate lyase by action of the acetylating enzyme present in the crude extract. The solution was then treated with streptomycin sulfate (2.8 g) and the precipitated nucleic acids removed by centrifuging. The clear supernatant was treated with 3 ml Alumina C_γ gel (dry weight 33 mg/ml) and centrifuged. The supernatant which contained the activity was treated with another 33 ml of the gel to adsorb the enzyme. The gel containing the activity was washed with 100 ml 0.01M potassium phosphate buffer pH 7.0. This buffer and those used in subsequent steps contained $MgSO_4$ (1.6 mM). The washing which contained no activity was discarded after centrifuging and the enzyme eluted from the gel with 100 ml of 0.05 M potassium phosphate buffer pH 7.0. The eluate was separated after centrifuging and treated with solid ammonium sulfate to 0.5 saturation and then centrifuged. The supernatant which contained the activity was separated and more solid ammonium sulfate added till 0.8 saturation to precipitate the enzyme. The precipitate was collected by centrifuging and dissolved in 0.05M potassium phosphate buffer pH 7.5 and dialysed against buffer of the same composition. The dialysate (4 ml) was chromatographed on a 30 x 1 cm column of DEAE-cellulose with gradual increase in buffer concentration over the range 0.05 M to 0.7 M potassium phosphate pH 7.5. The fractions containing the enzyme were pooled and precipitated with solid ammonium sulfate added to 0.8 saturation. The precipitate was collected after centrifuging and dissolved in 0.05M potassium phosphate buffer pH 7.5 and was subjected to gel filtration through a 110 cm x 1 cm column of Sepharose CL-6B previously equilibrated with the same buffer. The fractions containing activity were concentrated either by ultrafiltration or by precipitation with ammonium sulfate at 0.8 saturation.

Preparation of HS-citrate lyase: HS-citrate lyase was prepared using dithiothreitol according to the procedure described by Kümmel et al. (3).

RESULTS

Purification: A typical purification of the enzyme is summarized in Table 1.

Table 1. Purification of citrate lyase from Streptococcus faecalis

Step	Volume ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	200	2560	2.5	
Cell-free extract + acetate + ATP	201	5120	5.0	100
Streptomycin treatment	196	4403	5.9	86
0.05M buffer eluate of alumina C_γ gel	100	2652	13.0	52
Ammonium sulfate (0.5-0.8 sat.)	1.8	2371	40.0	46
DEAE-cellulose	1.5	1280	58.6	25
Sepharose CL-6B	0.8	563	90.0	11

The enzyme sedimented as a single symmetrical peak in 0.05M potassium phosphate buffer pH 7.5 containing 1.6 mM $MgSO_4$ (Fig. 1a). The enzyme was also homogeneous in disc electrophoresis (Fig. 2a). The $s_{20,w}$ value estimated at a protein concentration of 3.6 mg/ml was 15.5S and the molecular weight 580 000. Unlike the enzyme from K.aerogenes (12), citrate lyase from S.faecalis did not dissociate in buffer of low ionic strength (1 mM EDTA + 1 mM potassium phosphate buffer pH 7.5) as observed from sedimentation profiles.

Stability: The enzyme was stable to storage at 0 °C or at -20 °C in presence of ammonium sulfate. In the absence of ammonium sulfate the enzyme was less stable and such samples when frozen and thawed showed loss in activity and appearance of two faster sedimenting components as well as a slower sedimenting fraction (Fig. 1b). Interpolation from the $s_{20,w}$ values (16) of the faster moving components would suggest that these are probably dimeric and trimeric forms of the native enzyme; and the slower moving component a dissociated half molecule.

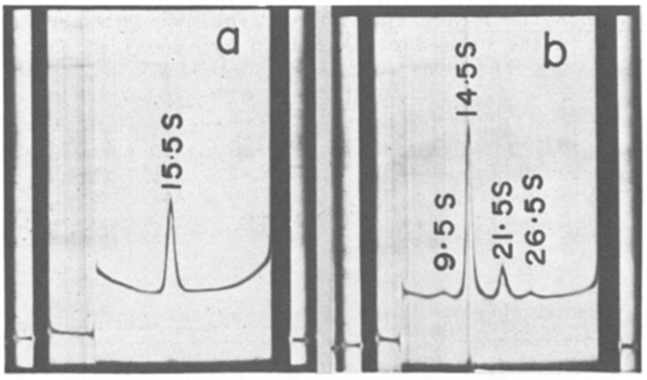

Fig. 1: Sedimentation profiles of citrate lyase from S.faecalis in 0.05 M potassium phosphate buffer + 1.6 mM $MgSO_4$ (pH 7.5). Speed 59 780 rpm. Phase plate 60°.
a) Native enzyme (3.6 mg/ml). Time 32 min.
b) Enzyme after freezing and thawing (6 mg/ml). Time 28 min.

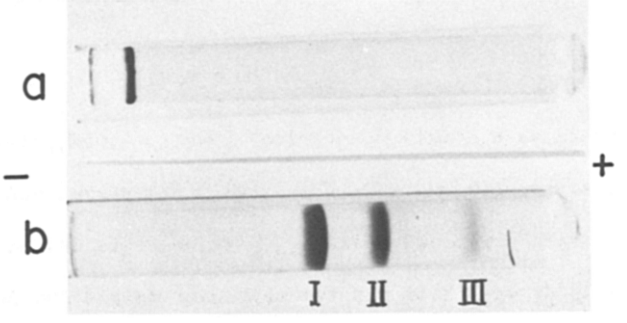

Fig. 2: Polyacrylamide gel electrophoresis
a) 5% gel. Tris-glycine buffer pH 8.3, 4mA/tube, 2.5 hrs, Protein loaded, 60 μg.
b) SDS polyacrylamide gel electrophoresis in Na-phosphate buffer pH 7.3, 8 mA/tube, 4.5 hrs. Total protein 100μg.

Subunit structure: Purified enzyme samples were dissociated either with 1% SDS + 1% 2-mercaptoethanol (2-ME) or plain 1% SDS by heating to 100°C for 3 min prior to electrophoresis. SDS-polyacrylamide gel electrophoresis indicated the presence of 3 non-identical subunits (Fig. 2b, Bands I, II & III). The estimated molecular

weights of subunits I, II and III were 53 000, 37 000 and 14 000 respectively. Separation into the 3 polypeptide chains was obtained whether 2-ME was used in the dissociating system or not indicating that the subunits are not linked by interpeptide disulfide bridges.

Reaction Inactivation: Progress curve of citrate cleavage with citrate lyase from S.faecalis is shown in Fig. 3 (Curve A). The corresponding curve obtained with the enzyme from K.aerogenes purified as described earlier (12) is included for comparison (Fig. 3, Curve B). The enzymes incubated in absence of citrate showed

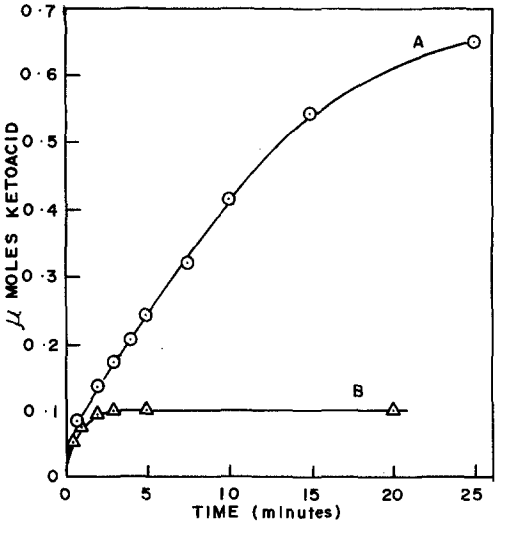

Fig. 3: Differences in the reaction inactivation of citrate lyases from S.faecalis (Curve A), and from K.aerogenes (Curve B), at 30°C. 12.5μg S.faecalis enzyme and 25.0μg K.aerogenes enzyme were taken separately in 15 ml reaction mixture containing 0.1M Na_3 citrate + 20 mM $MgSO_4$ + 0.05 M Tris HCl pH 8.0. 1 ml aliquots were taken out for assaying at different time intervals.

no measurable loss in activity. It will be apparent from Fig. 3 that the enzyme from S.faecalis shows only weak reaction inactivation. Measurable activity was observed with this enzyme even after a period of 90 min. In contrast the enzyme from K.aerogenes is completely inactivated after 2-3 min of reaction.

Reactivation of HS-citrate lyase: The HS-citrate lyase obtained from S.faecalis was reactivated by chemical treatment with acetic anhydride (Table 2). Treatment

Table 2. Reactivation of HS-citrate lyase from Streptococcus faecalis

HS-citrate lyase prepared by incubating 20 U enzyme (Sp. act. 90 U/mg) in 2 ml 0.1M K-phosphate buffer pH 7.8 + 0.2M KCl + 3 mM $MgSO_4$ + 4 mM DTT for 45 min at 30°C. 0.5 ml aliquots were treated separately with (i) acetic anhydride (final conc. 4.7 mM) for chemical activation and (ii) Na acetate (final conc. 5 mM) + ATP (final conc. 0.1 mM) to test for associated acetylating enzyme activity. (i) and (ii) were incubated at 25°C for 5 min and 30 min respectively. 0.2 ml aliquots were taken for assay of citrate lyase activity.

Treatment	% initial activity
DTT	0.0
DTT + Acetic anhydride	25.0
DTT + Acetate + ATP	0.0

of the desacetyl preparation with ATP and acetate produced no reactivation at all establishing the absence of acetylating activity in the enzyme complex. Acetylating enzyme activity checked in the samples at various steps in the purification procedure indicated that the acetylating activity originally present in cell-free extracts separates from citrate lyase during the second Alumina C_γ gel treatment, the acetylating activity being present in the supernatant while citrate lyase is adsorbed on the gel. The acetylating enzyme activity which was separated from citrate lyase by Alumina C_γ gel treatment completely reactivated HS-citrate lyases both from S.faecalis and K.aerogenes.

DISCUSSION

The isolation of citrate lyase from S.faecalis in ultracentrifugally and electrophoretically homogeneous form is reported here for the first time. The enzyme complex from this organism has a molecular weight of 580 000 which is in the range of values of 520 000 - 585 000 reported for the enzymes from K.aerogenes (5), S.diacetilactis (2) and R.gelatinosa (4). Like the enzymes from K.aerogenes and S.diacetilactis, citrate lyase from S.faecalis also contains 3 non-identical subunits of about the same molecular sizes. In contrast, the enzyme from R.gelatinosa is reported to contain probably only two subunits of 30 000 and 61 000 daltons, the smaller polypeptide chain acting as the ACP.

Citrate lyase from S.faecalis like the enzyme from S.diacetilactis shows

only a weak reaction inactivation compared to the enzyme from K.aerogenes. The enzyme complex from S.faecalis shows no acetylating enzyme activity for reactivation of the HS-citrate lyase in presence of acetate and ATP. The association of such acetylating activity in the enzyme complexes from S.diacetilactis and Leuconostoc citrovorum has been suggested as reason for their weak reaction inactivation as compared to the enzymes from K.aerogenes and from R.gelatinosa which have no associated acetylating activity and show strong reaction inactivation (3). The cause for the weak reaction inactivation, atleast in the case of the enzyme from S.faecalis, evidently lies elsewhere, since the enzyme complex from this source is devoid of any acetylating activity. In S.faecalis, like in K.aerogenes (10), the HS-citrate lyase ligase activity occurs as a separate enzyme distinct from the citrate lyase complex.

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