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# THE PRESENCE OF ESSENTIAL ARGININE RESIDUES AT THE ACTIVE SITES OF CITRATE LYASE COMPLEX FROM KLEBSIELLA AEROGENES

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The acyl-transferase and acyl-lyase activities of Klebsiella aerogenes citrate lyase complex are inactivated by the arginine specific reagents phenylglyoxal and 2,3-butanedione, the former reagent being the more potent inhibitor. Citrate and (3S)-citryl-CoA protect the transferase activity, while acetyl-CoA markedly enhances the rate of the inactivation. (3S)-Citryl-CoA protects the lyase subunit in the complex from inactivation. The kinetics of inactivation suggest the involvement of a single arginine residue at each of the active sites of the transferase and of the lyase subunits.

Citrate lyase (EC 4.1.3.6) from Klebsiella aerogenes is a multienzyme complex comprising three non-identical subunits of about 54 000 ($\alpha$), 32 000 ($\beta$) and 10 000 ($\gamma$) daltons respectively (1). The smallest subunit ($\gamma$) carries an essential acetyl group in thioester linkage with a CoA like prosthetic group and functions as an acyl-carrier protein (ACP) (2). In presence of citrate and EDTA, the $\alpha$ subunit functions as an acyl-transferase catalysing the formation of (3S)-citryl-ACP with liberation of acetate. The $\beta$ subunit acts as an acyl-lyase involved in the cleavage of the (3S)-citryl-ACP intermediate in presence of $Mg^{2+}$ (3). The CoA derivatives, acetyl-CoA and propionyl-CoA, serve as substrates for the transferase reaction, while (3S)-citryl-CoA functions as a substrate for the lyase reaction (4,5).

Abbreviations : ACP, acyl carrier protein; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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p-Azidobenzoyl-CoA has been shown recently by us to function as a photoaffinity reagent specifically inhibiting the lyase activity (6). Little is known, however, about the residues at the catalytic sites of the enzyme complex. The present report describes the involvement of arginine residues at the active sites of the transferase and lyase subunits.

## MATERIALS AND METHODS

Citrate lyase. Citrate lyase enzyme complex was purified from K. aerogenes (NCTC 418) as described earlier (6,7). Protein in the purified enzyme complex was estimated by its absorbance at 278 nm (8). Deacetyl citrate lyase was obtained as described by Buckel et al. (2).

Citrate lyase subunits. The catalytically active transferase ($\alpha$) and lyase ($\beta$) subunits were isolated by the procedure of Dimroth and Eggerer (3). Protein in the isolated subunits was estimated by the method of Lowry et al. (9) using bovine serum albumin as standard.

Enzyme assays. The overall citrate cleavage activity of the native enzyme complex and of the deacetyl citrate lyase as well as the individual acyl-transferase and acyl-lyase activities were determined as described earlier (6). Aliquots (10 to 20 µl) of enzyme samples incubated with the arginine modifying reagent, phenylglyoxal or 2,3-butanedione, were taken in a total volume of 1 ml of the assay systems containing 50 mM Tris-HCl buffer. Under the assay conditions, the modifying reagents were without effect on the coupling enzymes, malate dehydrogenase and citrate synthase.

CoA esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (10). (3S)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in presence of deacetyl citrate lyase and EDTA (5) and purified as described by Moffatt and Khorana (11).

Reaction with arginine modifying reagents. Citrate lyase (or the isolated subunit) was incubated at 30°C with either phenylglyoxal or 2,3-butanedione in 50 mM buffer solutions, pH 7.6. The buffers tested were potassium phosphate, Hepes and Tris-HCl. Borate buffer was not used as enzyme activities were markedly lowered in the presence of borate. The reaction mixture was shielded from light to avoid any photochemical effect when 2,3-butanedione was used as the probe (12). Enzyme incubated with the buffer alone served as the control. Aliquots were removed at different time intervals for assay of enzymatic activities. The effect of various ligands on inactivation by phenylglyoxal or 2,3-butanedione was studied after preincubating the enzyme with the compound for 5 min at 30°C followed by addition of the stated amounts of the inhibitor. Aliquots were removed thereafter at regular intervals of time and checked for loss in enzyme activity.

## RESULTS

Inactivation of transferase activity. The acetyl-CoA mediated acyl-transferase activity, both of deacetyl citrate lyase complex and of the isolated transferase subunit, was rapidly inactivated on incubation with phenylglyoxal or 2,3-butanedione at pH 7.6. The maximum inactivation was in potassium phosphate buffer, the inactivation being slightly less in Hepes and markedly lower in Tris buffer. Kinetic studies were therefore carried out in phosphate buffer.

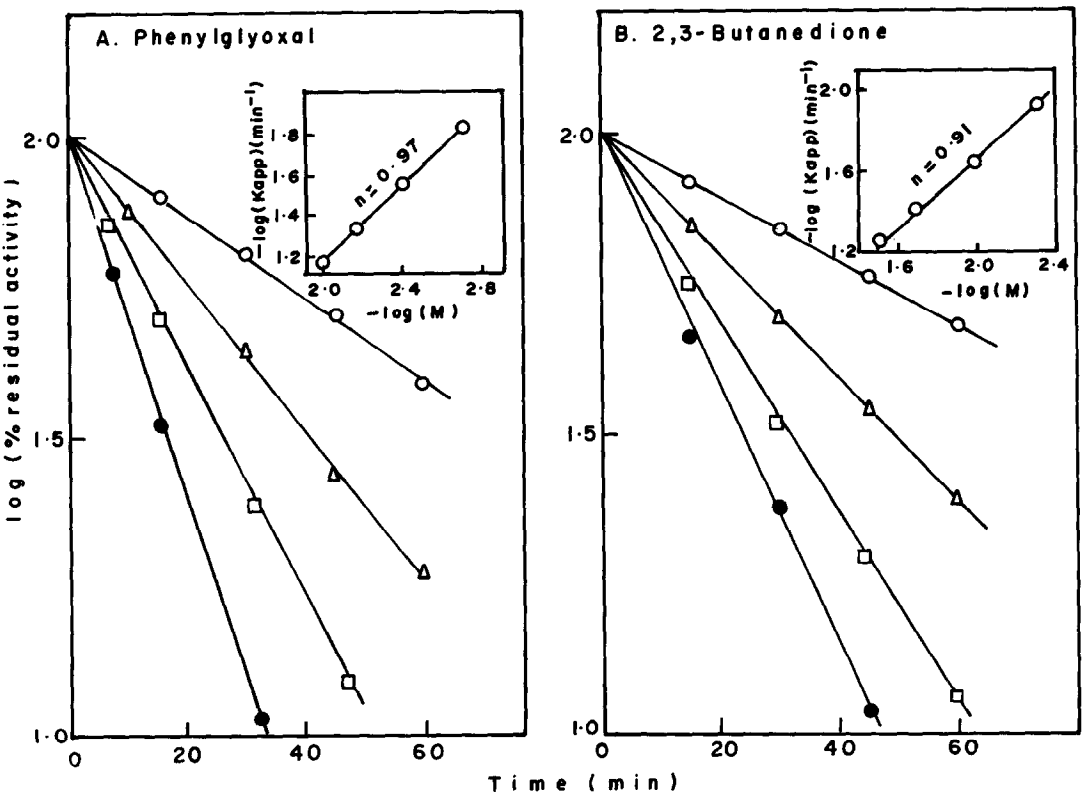


Figure 1 : Inactivation of the acyl-transferase activity of deacetyl citrate lyase by α-carbonyl reagents. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA and various concentrations of the reagents. (A) Phenylglyoxal 2 mM (○), 4 mM (△), 7 mM (□), and 10 mM (●). (B) 2,3-butanedione 5 mM (○), 10 mM (△), 20 mM (□), and 30 mM (●). At the indicated times, aliquots were assayed for transferase activity as described in the text. Insets : Determination of the order of the reaction with respect to phenylglyoxal (A) and 2,3-butanedione (B).

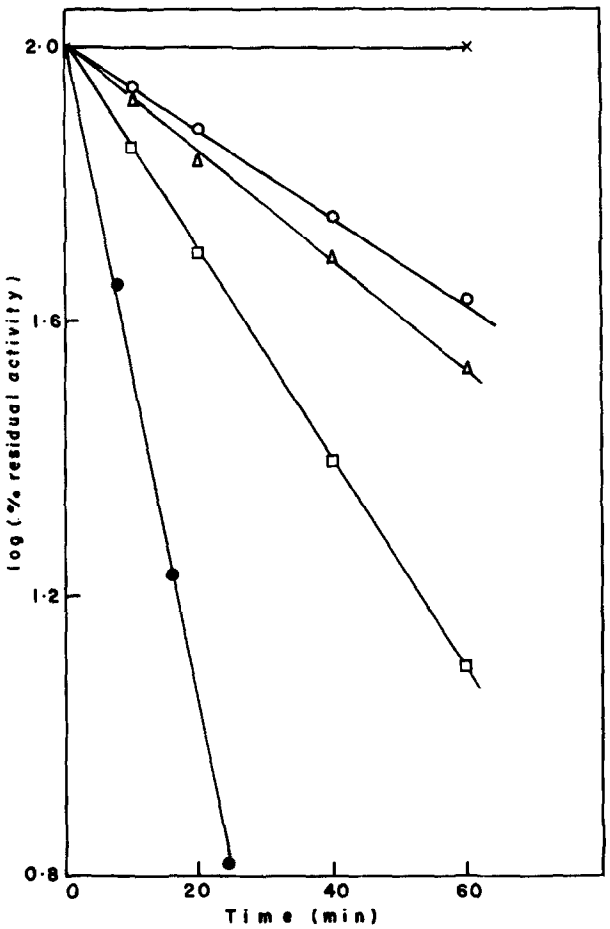


Figure 2 : Effect of ligands on the rate of inactivation of the acyl-transferase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with the ligands for 5 min at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA followed by incubation with 5 mM phenylglyoxal. Control without phenylglyoxal (×), no added ligand (□), 10 mM citrate (△), 0.5 mM (3S)-citryl-CoA (○), and 1 mM acetyl-CoA(●).Concentrations of ligands and modifier are final concentrations. Aliquots were assayed at specified time intervals for transferase activity.

The results obtained with deacetyl citrate lyase and various phenylglyoxal and 2,3-butanedione concentration are presented in Figure 1. The inactivation rates follow pseudo first-order kinetics, both plots being linear upto about 10% initial enzyme activity. The reaction order (n) with respect to the arginine reagent was determined from the plot of the logarithm of the

apparent first order rate constant, $K_{app}$, versus the logarithm of the reagent concentration (13) and the value of n = 0.97 for phenylglyoxal and of n = 0.91 for 2,3-butanedione suggest that loss of activity results from reaction of one arginine residue per acyl-transferase subunit (13 - 15). Similar results were obtained in case of the isolated $\alpha$-subunit where a value of n = 0.93 was obtained with phenylglyoxal.

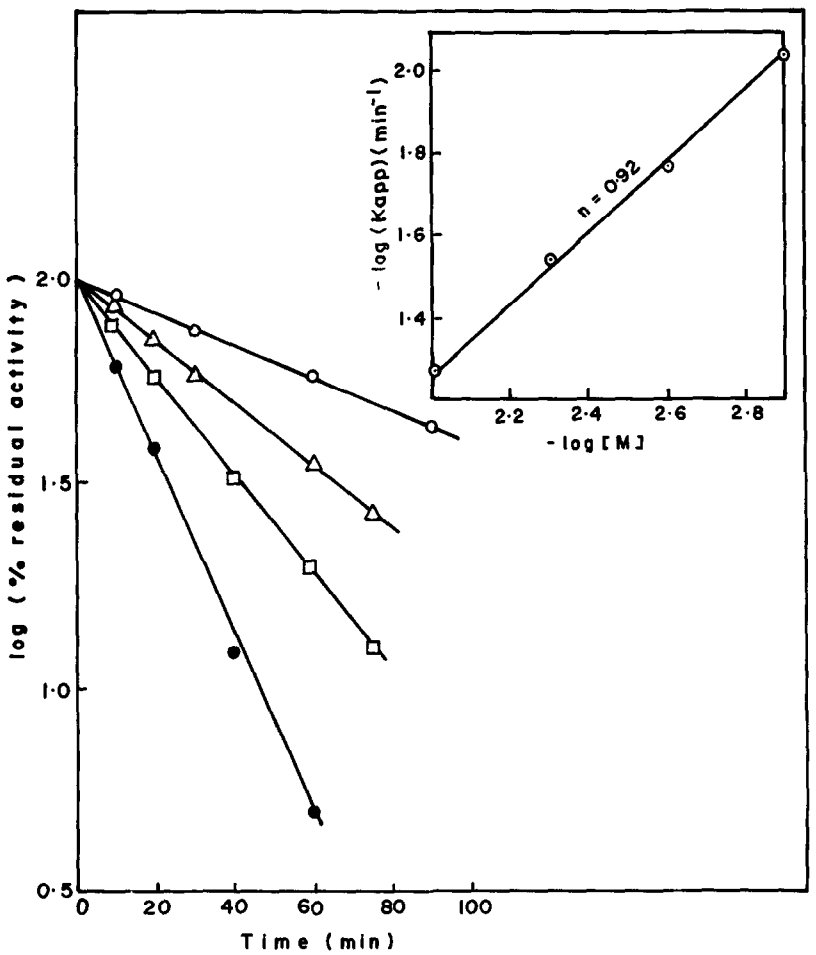


Figure 3 : Inactivation of the acyl-lyase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 3 mM $MgSO_4$ and phenylglyoxal 1.25 mM (○), 2.5 mM (△), 5 mM (□) or 10 mM (●). Aliquots were assayed for lyase activity at indicated times. Omission of $MgSO_4$ from incubation mixtures had no effect on the inactivation. The inset of the figure shows the determination of the order of the reaction with respect to phenylglyoxal.

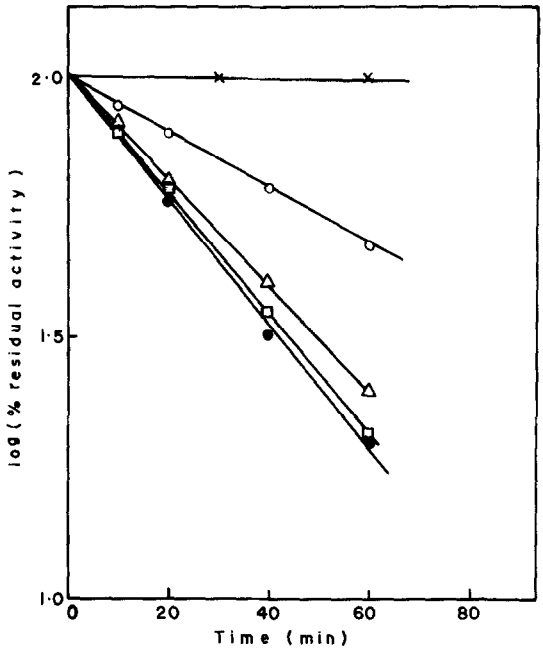


Figure 4 : Protection of lyase activity of deacetyl citrate lyase from inactivation by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with various ligands in presence of EDTA. Conditions were the same as described in Figure 2. Control (×), no added ligand (●), 10 mM citrate (□), 0.5 mM citryl-CoA (○), and 1.0 mM acetyl CoA (△).

The effect of various ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase by phenylglyoxal is shown in Figure 2. Citrate (10 mM) and (3S)-citryl-CoA (0.5 mM) decreased the rate of inactivation by 45% and 55% respectively; while acetyl-CoA (1 mM) increased the rate of inactivation 3-fold. Similar effects were observed with the isolated transferase ($\alpha$) subunit. The effects of 2,3-butanedione were similar to those of phenylglyoxal.

The reaction order for the inactivation was also determined in presence of acetyl-CoA (1 mM). A value of $n = 1.15$ with phenylglyoxal under these conditions would suggest that the rate of reaction of the essential arginine residue is enhanced without the involvement of any additional residues.

Inactivation of lyase activity. The lyase activity of deacetyl citrate lyase complex was also inactivated by the two reagents. The rate of inactivation followed pseudo first-order kinetics as shown in Figure 3. A value of n = 0.92 suggests the minimal requirement of one arginine residue per subunit. The isolated lyase subunit was not studied since it showed markedly lowered activity, probably due to denaturation during the process of isolation (3). The effect of ligands is shown in Figure 4, Citrate (10 mM) had no significant effect; acetyl-CoA (1 mM) reduced the inactivation slightly (15%); while the substrate (3S)-citryl-CoA (0.5 mM) more effectively protected against inactivation by phenylglyoxal.

## DISCUSSION

The presence of essential arginine residues has been reported in several enzymes acting upon anionic cofactors and substrates (16). Among the citrate enzymes, ATP-citrate lyase from rat liver has been shown recently to carry an essential arginine residue (13). The citrate lyase complex from K. aerogenes has been shown in the present study to carry an essential arginine residue in each subunit with enzymatic activity. Protection studies against inactivation by arginine specific reagents indicate that the arginine residue is present at the citrate binding site of the transferase subunit, one of the points of attachment of citrate to the transferase subunit presumably being through the guanidino group at the active site. Acetyl-CoA markedly increases the rate of inactivation of the transferase subunit probably through a conformational change at the citrate binding site making the essential arginine residue more accessible to $\alpha$-carbonyl reagents. In the case of the classical CoA transferases, a conformational change at the carboxylate binding site of succinyl-CoA: 3-oxoacid CoA-

transferase has been suggested to explain the enhanced reactivity of an essential thiol group on binding of acyl-CoA substrates (17).

The acyl-lyase activity of the deacetyl citrate lyase complex has also been shown in our studies to depend on an essential arginine residue; the acyl-CoA substrate, (3S)-citryl-CoA, in this case protecting against inactivation by arginine modifying reagents.

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