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Bacterial citrate lyase

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Abstract. Bacterial citrate lyase, the key enzyme in fermentation of citrate, has interesting structural features. The enzyme is a complex assembled from three non-identical subunits, two having distinct enzymatic activities and one functioning as an acyl-carrier protein. Bacterial citrate lyase, *si*-citrate synthase and ATP-citrate lyase have similar stereospecificities and show cofactor cross-reactions. On account of these common features, the citrate enzymes are promising markers in the study of evolutionary biology. The occurrence, function, regulation and structure of bacterial citrate lyase are reviewed in this article.

Keywords. Bacterial citrate lyase; anaerobic citrate utilization; citrate lyase autoinactivation; subunit structure and function; subunit stoichiometry; active sites; citrate lyase prosthetic group.

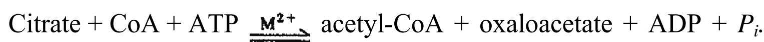
Citrate enzymes

Citrate occupies a central position in intermediary metabolism and the enzymes involved in its formation or breakdown have special importance. Four different groups of enzymes are known which catalyze the synthesis (or dissimilation) of citrate through an aldol- (or retroaldol-) type reaction involving an equilibrium between citrate on the one hand and oxaloacetate and an acetyl-moiety on the other. These are:

(i) Bacterial citrate lyase [citrate oxaloacetate-lyase (*pro*-3*S*-CH_2COO^- → acetate); EC 4.1.3.6] found as yet only in procaryotes, which catalyzes the cleavage of citrate into oxaloacetate and acetate in the presence of divalent metal ions such as Mg^{2+} or Mn^{2+}.

$$\text{Citrate} \xrightleftharpoons{M^{2+}} \text{oxaloacetate + acetate}$$

(ii) ATP-citrate lyase [ATP: . citrate oxaloacetate-lyase (*pro-3S*-CH2COO⁻ → acetyl-coenzyme A (CoA): ATP dephosphorylating); EC 4.1.3.8],a cytosolic enzyme responsible for the generation from citrate of 2-carbon moieties which enter a number of biosynthetic pathways. The enzyme, which is present generally in eucaryotes, requires ATP, CoA and a divalent metal ion such as Mg^{2+} for the cleavage of citrate.



$$\text{Citrate + CoA + ATP} \xrightleftharpoons{M^{2+}} \text{acetyl-CoA + oxaloacetate + ADP} + P_i.$$

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Abbreviations used: ACP, Acyl-carrier protein; DTNB, 5,5'-dithiobis(2-nitrobenzoate); DTE, dithioerythritol; *Mr,* molecular weight; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; CoA, coenzyme A.

(iii) *si*-Citrate synthase [citrate oxaloacetate-lyase (*pro*-3*S*-CH_2COO^-→ acetyl-CoA); EC 4.1.3.7], which catalyzes the formation of citrate by the overall reaction shown below. It is the most widely distributed of the citrate enzymes and is present in both procaryotes and eucaryotes.

$$\text{Citrate} + \text{CoA} + H^+ \rightleftharpoons \text{Acetyl-CoA} + \text{oxaloacetate} + H_2O.$$

(iv) *re*-Citrate synthase [citrate oxaloacetate-lyase (*pro*-3*R*-CH_2COO^- → acetyl-CoA); EC number yet to be assigned] found in some anaerobic bacteria. The enzyme exhibits a stereochemistry opposite to that of *si*-citrate synthase.

Citrate lyase, ATP-citrate lyase and *si*-citrate synthase show close resemblance both in the stereospecificity of the reactions they catalyze and in their cofactor requirements, CoA and the citrate lyase prosthetic group (or their acetyl derivatives) substituting for one another in mediating the three enzyme catalyzed reactions (Robinson *et al.,* 1976). On account of the occurrence of citrate lyase exclusively in primitive forms of life and from its simplest extraneous cofactor requirements, it has been suggested that citrate lyase is perhaps evolutionarily the earliest of these three citrate enzymes (Srere, 1972). Despite this speculation, no sequence data is available even of residues around the active sites of bacterial citrate lyase.

The present review summarizes the literature on bacterial citrate lyase. The earlier work on this enzyme has been reviewed by Srere (1965,1968,1972,1974,1975), Dagley (1969), Spector (1972) and Srere and Singh (1974).

Bacterial citrate lyase

Occurrence

From an analysis of the products of fermentation of citrate by *Klebsiella aerogenes** and by *Aerobacter indologenes,* Deffner and Franke (1939) and Brewer and Werkman (1939) concluded that oxaloacetate and acetate are the initial cleavage products. Citrate lyase, the enzyme responsible for this step in the anaerobic utilization of citrate, was characterized first by Dagley and Dawes (1953) in *K. aerogenes* and in *Escherichia coli* and independently by Gunsalus and his group in *E. coli* (Gillespie and Gunsalus, 1953) and in *Streptococcus faecalis* (Grunberg-Manago and Gunsalus, 1953).

Citrate lyase has been found to occur only in bacterial sources, mainly as a citrate-induced activity. The only source in which the enzyme has been found to occur constitutively is *Streptococcus diacetilactis* (Harvey and Collins, 1961). Some strains of *Streptococcus liquefaciens* and *Leuconostoc citrovorum* have also been shown to have citrate lyase activity (Harvey and Collins, 1961). Citrate lyase is present in *Rhodopseudomonas gelatinosa* (Weckesser *et al.*, 1969; Schaab *et al.*, 1972) and *Rhodopseudomonas palustris* (Giffhorn and Kuhn, 1980). *Salmonella typhiniurium* grown aerobically on citrate in presence of Na^+ has citrate lyase and oxaloacetate-decarboxylase activities in addition to the enzymes of the citric acid cycle (O'Brien *et al.*, 1969). Citrate lyase has also been reported in *Aerobacter cloacae* (O'Brien and Geisler,

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* Known earlier as *Aerobacter aerogenes*; also known as *Klebsiella pneumoniae* and *Enterobacter aerogenes.*

1974) and in *Proteus rettgeri* (Kröger, 1974) grown anaerobically on citrate. Out of 45 species of *Clostridium* studied: *C. sphenoides* (Walther *et al.*, 1977), *C. sporosphaeroides, C. symbiosum, C. rectum, C. indoles, C. subterminale* and *C. sporogenes* (Antranikian *et al.*,1984) have been shown to utilize citrate as carbon and energy source and to contain citrate lyase. The enzyme from *C. sphenoides* requires the presence of L-glutamate for its activity (Antranikian *et al.*, 1982a), while those from *C. sporosphaeroides* and *C. symbiosum* do not show this requirement (Antranikian *et al.*, 1984). The low enzyme levels in the other species could be detected only indirectly from immunological and [l,5-^{14}C]citrate experiments.

Role in metabolism

Citrate lyase functions as the key enzyme in initiating the anaerobic utilization of citrate by a number of bacteria, further catabolism of oxaloacetate formed taking place either by decarboxylation or by reduction. In organisms such as *K. aerogenes, S.faecalis* and *R. gelatinosa,* the oxaloacetate is decarboxylated to pyruvate by oxaloacetate decarboxylase, an enzyme which is also induced in these sources in the presence of citrate. The two enzymatic reactions, which occur sequentially, constitute the ‘citrate fermentation pathway’ (Dagley and Dawes, 1953; O’Brien and Stern, 1969). The subsequent oxidative decarboxylation of pyruvate to acetyl-CoA, a source for the generation of ATP and reducing power, is catalyzed by the pyruvate dehydrogenase complex.

Organisms such as *P. rettgeri* (Kröger, 1974) and *E. coli* (Lutgens and Gottschalk, 1980), however, do not have an oxaloacetate decarboxylase. In these the oxaloacetate formed through citrate cleavage catalyzed by citrate lyase is converted first to malate by the action of malate dehydrogenase and then to fumarate by fumarase. Both *P. rettgeri* and *E. coli* have membrane bound fumarate reductases which produce ATP during the reduction of fumarate to succinate (Hirsch *et al.,* 1963; Bernhard and Gottschalk, 1978). In *P. rettgeri,* the reducing power required for succinate formation is generated by the oxidation of a portion of the citrate through the tricarboxylic acid cycle, in addition to the reducing power one mol ATP being produced per mol of citrate oxidized (Kröger, 1974). In *E. coli,* the tricarboxylic acid cycle is repressed under anaerobic conditions (Amaransingham and Davis, 1965) and the presence of a co-substrate, such as glucose, is required for generation of reducing power. In contrast, *K. aerogenes* does not contain fumarate reductase and depends entirely on oxaloacetate decarboxylase for production of acetyl-CoA (Kulla, 1976).

Evans *et al.* (1966) proposed the reductive carboxylic acid cycle for CO_2 assimilation by photosynthetic anaerobic bacteria in which citrate lyase was assumed to function as a key enzyme. Beuscher and Gottschalk (1972), however, could not detect citrate lyase activity in *Chlorobium thiosulfatophilum* and *Rhodospirillum rubrum.* ATP-citrate lyase, which had been found earlier only in eucaryotes, has been shown to be involved in the cycle in *Chlorobium limicola* (Ivanovsky *et al.,* 1980; Antranikian *et al.,* 1982b).

Regulation of citrate lyase

Citrate lyase does not come under regulation in bacteria such as *S. diacetilactis* and *L. citrovorum* in which citrate synthase is not formed (Giffhorn and Gottschalk, 1975a).

However, in bacteria which produce both citrate lyase and citrate synthase, the simultaneous functioning of both enzymes results in energy loss through futile cycles. In such instances, stringent control of the antagonistic enzymes occurs through a variety of regulatory mechanisms. These are briefly reviewed here.

Regulation in K. aerogenes: O'Brien and Stern (1969) observed that *K. aerogenes* NCTC 418 grows anaerobically on citrate as the sole source of energy only in the presence of Na^+. The requirement was explained on the basis of the observation of Stern (1967) that the oxaloacetate decarboxylase of this organism is Na^+ -dependent. Sachan and Stern (1971a) have shown that the membrane-bound, Na^+-requiring oxaloacetate decarboxylase also functions as the carrier protein for the transport of citrate. More recently oxaloacetate decarboxylase from *K. aerogenes* has been shown to function in sodium transport converting the chemical energy of decarboxylation reactions into electrochemical gradients of Na^+ ions (Dimroth, 1980; 1982a,b). The citric acid cycle is turned off under anaerobic conditions as a result of repression of 2-oxoglutarate dehydrogenase activity (O'Brien, 1975; Wilkerson and Eagon, 1972). Under aerobic conditions of growth on citrate, citrate lyase and oxaloacetate decarboxylase are not induced in *K. aerogenes,* while all the enzymes of the citric acid cycle are formed and cells grown with or without Na^+ utilise citrate exclusively through this cycle. O'Brien *et al.* (1969) observed that both the pathways are operative when the cells are grown under controlled aerated conditions in which no oxygen tension is detected in the cultures. Under these conditions, citrate degradation proceeds mainly through the fermentation pathway in the presence of Na^+ and through the citric acid cycle in the absence of Na^+.

A mutant of *K. aerogenes* with an altered ionic requirement has been reported recently which unlike the parent strain utilises citrate anaerobically through the citrate fermentation pathway in the presence of K^+ and in the absence of Na^+ (Utting, 1983).

A mode of regulation of citrate lyase is through deacetylation of the active acetyl-S-enzyme to a deacetyl (HS)-form, the enzymatic reactivation being catalyzed by a deacetyl citrate lyase ligase in the presence of acetate and ATP. This has been dealt with in detail under 'essential acetyl groups'.

Kulla and Gottschalk (1977) performed a number of growth shift experiments to study the regulation of citrate lyase and citrate synthase activities in *K. aerogenes.* After an initial lag period, citrate lyase activity was found to increase rapidly during anaerobic growth on citrate. No deacetyl citrate lyase could be detected during the early growth phase but formation of the deacetylated enzyme was observed on substrate depletion. Citrate lyase was inactivated by deacetylation on shifting from anaerobic growth on citrate to aerobic growth on glucose or anaerobic growth on glucose in the presence of nitrate. Growth difficulties were noticed on shifting from anaerobic growth on citrate to anaerobic glucose due to the simultaneous functioning of both citrate synthase and citrate lyase. Citrate lyase inactivation was observed to be energy dependent. SivaRaman and SivaRaman (1979) have observed metal cofactor dependent conformational modulations of the enzyme and have suggested that the energy requirement for inactivation could be for the formation or utilization of metabolite(s) which change the conformation of the enzyme either directly or indirectly through complexing of Mg^{2+} .

Regulation in R. gelatinosa: Another mode of regulation of citrate metabolism, besides the induction and repression of enzymes of the two divergent pathways under different growth conditions, is through a loss of the essential acetyl moiety on the citrate lyase through enzymatic deacetylation. While *K. aerogenes* does not contain a citrate lyase deacetylase activity which can inactivate the enzyme (Kulla and Gottschalk, 1977), *R. gelatinosa* has a citrate lyase deacetylase which is regulated by the conditions of growth. *R. gelatinosa* utilises citrate rapidly under anaerobic conditions in the presence of light (Schaab *et al.*, 1972) and the regulation of the two antagonistic pathways has been shown to be through covalent modification and to follow a pattern distinct from that in *K. aerogenes.* The futile cycle is avoided by deacetylation of the enzyme after citrate is completely exhausted from the medium. When citrate is made available, citrate lyase is converted from the inactive deacetylated HS-form to the active acetyl-S-form (Gifflhorn and Gottschalk, 1975a, b). The deacetylase has been obtained pure and is a small protein of molecular weight (M_r) 14,300 (Giffhorn *et al.,* 1980). The deacetylase of *R. gelatinosa* is highly specific and does not inactivate citrate lyases from *K. aerogenes* and *S. diacetilactis.* The citrate lyase deacetylase is strongly inhibited by L-glutamate (Giffhorn and Gottschalk, 1975b). The intracellular glutamate concentration thus has a regulatory effect on citrate lyase activity. Giffhorn *et al.* (1980) have shown that the intracellular pool size of L-glutamate is in turn proportional to the levels of its precursor, citrate, in the medium. Thus a high concentration of citrate signals that it can be cleaved *via* the fermentation pathway, while at low concentrations, citrate lyase is inactivated by citrate lyase deacetylase and all the citrate is utilised for biosynthesis of L-glutamate. This organism also contains a citrate lyase ligase which activates deacetylated citrate lyase in the presence of ATP and acetate (Giffhorn and Gottschalk, 1975b). The citrate lyase ligase has been purified and shown to have a requirement for ADP for its stability (Antranikian and Gottschalk, 1982). The depletion of citrate from the medium results in inactivation of the ligase and of the citrate lyase activities, while addition of citrate leads to a rapid activation of the ligase which in turn activates citrate lyase (Antranikian *et al.,* 1978).

A similar mechanism of regulation was observed in *R. palustris* (Giffhorn and Kuhn, 1980). Both the enzymes, citrate lyase ligase and citrate lyase deacetylase are present in this organism also and these enzymes were found to cross-react with citrate lyase from *R. gelatinosa.*

Regulation in C. sphenoides: Antranikian *et al.* (1982a) have reported that the regulation of citrate lyase in *C. sphenoides* is by changes in the intracellular concentration of L-glutamate. The enzyme is active only in the presence of glutamate. While in the *Rhodospirillaceae* the regulatory action of glutamate is indirectly through its effect on the deacetylase, in the *Clostridium* glutamate interacts directly with citrate lyase. The lyase is not deacetylated in the absence of glutamate but apparently is modulated conformationally. Electron microscopy of this citrate lyase did indicate such an effect, the complex being present in undefined structures in the absence of glutamate and as 'star' and 'ring' forms in the presence of the ligand (Antranikian *et al.* 1982a).

Regulation in P. rettgeri: The oxaloacetate produced by the citrate lyase in this organism is reduced to succinate as oxaloacetate decarboxylase is not formed (Kröger,

1974). It was observed that citrate lyase is induced under anaerobic conditions only with citrate as the growth substrate, while citrate synthase activity is considerably lowered. Besides the induction and repression mechanism, other modes of regulation have not been studied in this organism.

Regulation in E. coli and S. typhimurium: In *E, coli* and *S. typhimurium,* the regulation of citrate lyase activity is through deacetylation. Citrate is utilised in both organisms in the presence of co-substrates and the mode of regulation under anaerobic conditions is by deacetylation of citrate lyase on removal or exhaustion of the co-substrate (Kulla, 1983). It was further shown that citrate lyase could not be deleted from mutants of the LT2 strain of *S. typhimurium* and based on this observation it has been suggested that citrate lyases in *E. coli* and *S. typhimurium* probably function protectively by scavenging citrate which might otherwise chelate divalent cations which are required for cell functions (Kulla, 1983).

Citrate transport

Citrate uptake in a number of citrate-utilising bacteria has been shown to be mediated by cation-dependent oxaloacetate decarboxylase which is induced in the presence of the substrate. *K. aerogenes* NCTC 418 cells require Na^+ specifically for anaerobic utilisation of citrate, a requirement which has been attributed to its Na^+-dependent oxaloacetate decarboxylase (Stern and Sachan, 1970; Sachan and Stern, 197 1a). Mutant strains have been isolated which are K^+-dependent, not requiring Na^+ for citrate uptake (Eagon and Wilkerson, 1972; Utting, 1983). In *S.faecalis,* citrate uptake as well as decarboxylase activity require Ca^{2+} and biotin (Sachan and Stern, 1971b). In *S. diacetilactis,* in which citrate lyase is constitutive, the transport system has been shown to be inducible (Harvey and Collins, 1962). Spontaneous cryptic *Cit*$^-$ mutants of the organism have been isolated which had lost the ability of citrate uptake while retaining citrate lyase activity (Collins and Harvey, 1968). These mutants have been shown either to have lost a plasmid or to have undergone a point mutation despite the presence of the particular plasmid (Kempler and McKay, 1979).

E. coli cells do not utilise citrate anaerobically as sole source of carbon and energy. However, the presence of another substrate like glucose, lactose, pyruvate or peptone leads to citrate utilisation under these conditions (Vaughn *et al.,* 1950). The need for a co-substrate such as glucose or lactose has been shown to be for the generation of reducing power from oxaloacetate, since oxaloacetate decarboxylase is not present in this organism (Lutgens and Gottschalk, 1980).

Under aerobic conditions, however, mutants of *E. coli* have been obtained with plasmid-encoded (Sato *et al.,* 1978; Ishiguro *et al.,* 1979; Ishiguro and Sato, 1980; Smith *et al.,* 1978) as well as chromosomal-encoded (Hall, 1982; Reynolds and Silver, 1983) abilities for citrate uptake.

Citrate transport in *S. typhimurium* is found to be inducible and is complex. Three separate transport systems are present in this source for tricarboxylic acids (Imai *et al.,* 1973).

Purification of citrate lyase

Citrate lyase from *K. aerogenes* has been studied the most since the enzyme was obtained pure for the first time from this source (SivaRaman, 1961).

The general properties of the enzyme purified from diverse sources are summarised in table 1.

Table 1. Properties of purified citrate lyases.

Source	pH optimum	Specific activity (μmol/min^{-1} mg^{-1})	Molecular properties $s_{20,w}$ (S)	M_r
K. aerogenes[a]	8·0	70–90	$s^o_{20,w}$ 17·8	575,000 540,000
S. diacetilactis[b]	7·0–7·3	200	16·8	585,000
R. gelatinosa[c]	7·2–7·4	360 (crys)	14·7	530,000– 560,000
S. faecalis[d]	8·0	90	$s^o_{20,w}$ 17·1	600,000
C. sphenoides[e]	6·5–7·0	60	16·3	515,000
E. coli[f]	8·0	120	(16·6)[g] polydispersive	(600,000)[h]

[a] SivaRaman (1961); Mahadik and SivaRaman (1968); Bowe and Mortimer (1971); Singh *et al.* (1976); SivaRaman and SivaRaman (1979); Tikare (1979).
[b] Singh and Srere (1975); Kümmel et al. (1975).
[c] Beuscher et al. (1974); Giffhorn and Gottschalk (1978).
[d] Hiremath et al. (1976).
[e] Antranikian et al. (1982a).
[f] Nilekani and SivaRaman (1983).
[g] Major component
[h] Computed from subunit composition; corresponds approximately to M_r of major component of $s_{20,w}$, 16·6.

Stability

Native citrate lyase carries essential acetyl groups and the loss of these by acyl transfer to water is a major mode of enzyme inactivation. That such a loss does occur on storage has been demonstrated by Buckel *et al.* (1971a) who showed that [^{14}C]-acetyl citrate lyase from *K. aerogenes* stored at 4°C for 4 days loses nearly a third of its activity with concomitant loss of an equivalent proportion of protein-bound radioactivity, implicating spontaneous deacetylation as the cause of inactivation. It was also shown that complete inactivation results on deacetylation with neutralized hydroxylamine or DTT/DTE. The inactive enzyme is reactivated chemically with acetic anhydride (Buckel *et al.*, 1971a) or enzymatically with citrate lyase ligase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974).

The stability of solutions of the pure *K. aerogenes* enzyme has been shown to be greater at higher protein concentrations (Bowen and Rogers, 1963a). The presence of Mg^{2+} or Ca^{2+} reduces the rate of inactivation of dilute solutions (Eisenthal *et al.*, 1966; Blair *et al.*, 1967), while added bovine serum albumin, sucrose and $MgSO_4$ greatly enhance storage stability of lyophilized enzyme preparations (Gruber and Moellering, 1966). The pure *S. diacetilactis* enzyme is stable to storage at – 90°C (Singh and Srere, 1975). The enzyme from this source was found to be stable at pH values upto 8·1,

dissociation and inactivation of the complex occurring at higher pH values. The purified enzyme from *S. faecalis* was stable when stored at 0°C or –20°C in the presence of ammonium sulphate. Repeated freezing and thawing of the enzyme led to its inactivation with formation of artifacts (Hiremath *et al.,* 1976). The crystalline enzyme from *R. gelatinosa* retains its activity for 8 months when stored at 4°C in the presence of 3 M ammonium sulphate (Giffihorn and Gottschalk, 1978). The purified *E. coli* enzyme is also stable at 0°C in the presence of saturated ammonium sulphate and $MgSO_4$ (Nilekani, 1980). The pure enzyme from *C. sphenoides* could be stored at 4°C for 4 days or at –20°C for 2 months without appreciable loss in activity, the stability being higher in the presence of L-glutamate (Antranikian *et al.,* 1982a). The loss of enzymatic activity in the absence of L-glutamate could be reversed by its addition. The presence of high concentrations of glycerol (30%), sucrose (40%) or bovine serum albumin (10 mg/ml) together with L-glutamate (7 mM) resulted in only a marginal loss in activity at 0°C. The reversible inactivation in the absence of glutamate was observed to follow a transition from 'ring' and 'star' shapes seen in electron micrographs to undefined shapes.

Reaction kinetics

The oxaloacetate formed on cleavage of citrate catalyzed by citrate lyase from *S. diacetilactis* had been characterized as the keto-form by spectral analysis (Harvey and Collins, 1963; Ward and Srere, 1965). The enzyme from *K. aerogenes* has also been shown to yield the keto-isomer in experiments carried out at 2°C and pH 7·4 when spontaneous equilibration of the oxaloacetate tautomers is slowed down (Tate and Datta, 1964). The enzyme thus resembles citrate synthase (Englard, 1959) in the involvement of the keto isomer in the aldol type reaction.

The overall reaction catalyzed by citrate lyase is towards citrate cleavage. The reaction goes virtually to completion in the presence of a large excess of the enzyme (Wheat and Ajl, 1955b; Bowen and Rogers, 1965). The reversibility of the reaction was demonstrated only through radioactive labelling of the citrate using [^{14}C]-acetate, oxaloacetate and Mg^{2+} in the presence of the enzymes from *S. faecalis* (Gillespie and Gunsalus, 1953), *E. coli* (Wheat and Ajl, 1955b) and *K. aerogenes* (Bowen and Rogers, 1963b).

The equilibrium constant calculated for the cleavage of citrate to oxaloacetate and acetate is about 1M (Burton, 1955). Several attempts had been made earlier to experimentally determine the equilibrium constant of the enzyme catalyzed cleavage (Smith, *et al.*, 1956; Tate and Datta, 1965; Harvey and Collins, 1963; Guynn *et al.*, 1973). These are of doubtful significance on account of the complexities of the reaction not realized at the time.

Product inhibition

Citrate lyases from *E. coli* and *K. aerogenes* have been shown to be irreversibly inhibited by oxaloacetate, a product of the reaction (Dagley and Dawes, 1955). Neither acetate nor pyruvate was found to inhibit the enzyme. The inhibition by oxaloacetate has been shown to be both concentration and time dependent and no changes in spectral or sedimentation profiles could be observed on inactivation (Bowen and

Rogers, 1963b). Malate was also shown to inactivate the enzyme irreversibly while oxalate, succinate, oxaloglutarate, tartarate, oxalosuccinate and isocitrate had no effect.

The inhibition has been shown not to be reversed by malate dehydrogenase and NADH and studies with oxaloacetate and its analogues indicated the requirement of divalent metal ions such as Mg^{2+} for the inactivation (Eisenthal *et al.,* 1966). In these studies the profile of pH dependence of oxaloacetate inhibition indicated involvement of Mg^{2+}-enolic oxaloacetate complex which was further supported by α,α-dimethyl oxaloacetate and ketomalonate, analogues which cannot enolise, not inhibiting the enzyme. The structural requirement for inhibition were a C_4 straight chain, 1,4-dicarboxylic acid with an ionisable α-hydroxyl group. Inhibition was also shown to depend on the nature of the divalent metal ion. The inhibitory effect decreased in the order Co^{2+}, Mg^{2+}, Ca^{2+}. Inhibition by oxaloacetate has been shown to be through the loss of essential acetyl groups with the formation of the inactive deacetyl citrate lyase (Buckel *et al.*, 1971a).

Stereochemistry

The stereospecificity of the citrate enzymes has been studied extensively (Eggerer *et al.*, 1970; Retey *et al.*, 1970; Lenz *et al.*, 1971; Wunderwald *et al.*, 1971; Srere, 1975). All citrate enzymes, except *re*-citrate synthase, exhibit similar stereochemistry in regard to the C-3 prochiral centre (Glusker and Srere, 1973) of citrate and the aldol reaction occurs at the *si*-face of oxaloacetate (Hanson and Rose, 1963; Gillespie and Gunsalus, 1953; Wheat and Ajl, 1955b; Buckel *et al.,* 1971a; Srere and Bhaduri, 1964). *re*-Citrate synthase is the only citrate enzyme with an opposite stereospecificity in which the C-1 and C-2 of citrate (*pro*-3*R*-CH_2COOH) are derived from the acetyl moiety of acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966). All citrate enzymes, including *re*-citrate synthase, however, show identical stereospecificity with respect to the prochiral centres involving the methylene groups on C-2 and C-4 of citrate, causing inversion at the methylene groups which become the methyl groups of acetate (Eggerer *et al.*, 1970; Retey *et al.*, 1970; Klinman and Rose, 1971; Buckel *et al.*, 197 1b).

Metal requirement

Citate lyases have an absolute requirement for divalent metal ions for catalyzing the cleavage reaction (Dagley and Dawes, 1955; Beuscher *et al.*, 1974; Hiremath *et al.*, 1976; Antranikian *et al.*, 1982a). In the early studies, partially purified enzymes from *K. aerogenes* and *E. coli* were found to be active in the presence of Mg^{2+} , Mn^{2+} ,Zn^{2+}, Fe^{2+}, Co^{2+} while Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+} and Hg^{2+} did not substitute for the active metal ions (Dagley and Dawes, 1955). Ca^{2+} was found to act as a competitive inhibitor of enzyme activation by Mg^{2+}, Mn^{2+} and other active metal ions. From these metal ion activation studies, Dagley and Dawes (1955) concluded that metals with a restricted ionic radius of 0·72 ± 0·08 A can activate the enzyme, while those such as Ca^{2+} of ionic radius (0·99 Å) outside this specific range have no such effect. The pH optimum of the enzymes was in the range of 7·0-8·2, which approximately parallels the stability of metal-citrate complexes (Bobtelsky and Jordan, 1945). This led to the suggestion that citrate lyase acts on a complex of citrate and divalent metal (Dagley and Dawes, 1955; Harvey and Collins, 1963). Later studies on Mn^{2+} binding by a partially purified *S.*

diacetilactis enzyme preparation using pulsed nuclear magnetic resonance as a probe, however, indicated the formation of a binary complex of metal-protein, no evidence being obtained for a ternary metal-citrate-enzyme complex (Ward and Srere, 1965). It was realized that this could have been due to the steady-state concentrations of such a complex being below detectable levels. The studies, however, confirmed the earlier finding based on kinetic data that Ca^{2+} competes with the active metal for its binding site on the enzyme.

Equilibrium dialysis studies have been carried out using radioactive $^{54}Mn^{2+}$ as a probe to characterize the nature and the stoichiometry of metal cofactor binding by pure citrate lyase from *K. aerogenes* (SivaRaman and SivaRaman, 1979). The metal binding was found to have no effect on the state of aggregation of the enzyme complex in buffers of adequate concentration. The binding isotherm, Scatchard and Hill plots reflected strong positive cooperativity in metal binding. The estimated number of Mn^{2+} binding sites per mol of enzyme complex was 18. More recent studies on the kinetics of inactivation of citrate lyase by 2-fluorocitrates and 2-hydroxycitrates in the presence of different metal ions have also indicated the possibility of metal dependent changes in the conformation of the enzyme (Rokita and Walsh, 1983).

Citrate lyase is a multienzyme complex having two separate enzyme activities, an acyl-transferase activity catalyzing the formation of a citryl-enzyme intermediate and an acyl-lyase activity catalyzing the subsequent cleavage of the citryl intermediate. Only the lyase reaction has been shown to have an absolute requirement for divalent metal ions, while the transferase reaction occurs even in the presence of EDTA (Buckel *et al.*, 1973; Dimroth and Eggerer, 1975b).

Essential acetyl groups

Buckel *et al.* (1971 a) were the first to observe that native citrate lyase from *K. aerogenes* is an acetyl-S-enzyme. Stereospecifically labelled [5-^{14}C]-(3*S*)citrate was cleaved by citrate lyase to [^{14}C]-labelled enzyme and unlabelled oxaloacetate, while [1-^{14}C]-(3*R*)citrate yielded unlabelled enzyme and [^{14}C]-oxaloacetate. This could be explained as a result of the turnover of acetate during the course of the reaction catalyzed by the enzyme. Thus the overall reaction was recognized to be the sum total of two partial reactions, an acyl exchange reaction:

acetyl-enzyme $\rightleftharpoons$ citrate citryl-enzyme + acetate and an acyl-lyase reaction with the cleavage at the *si*-face of oxaloacetate;

citryl-enzyme $\rightleftharpoons$ acetyl-enzyme + oxaloacetate. Further evidences provided by Buckel *et al.* (1971a) for the involvement of essential acetyl moieties in the reaction mechanism of citrate lyase were: (i) inactivation of [^{14}C]-acetyl labelled enzyme by hydroxylamine with formation of [^{14}C]-acetohydroxamic acid; (ii) the formation of low molecular weight radioactive products and unlabelled protein on treatment of [^{14}C]-acetyl-enzyme with cold citrate; (iii) the analogy between hydroxylaminolysis of standard thio-esters like N-succinyl-S-acetylcysteamine and enzyme inactivation by hydroxylamine; (iv) inactivation of citrate lyase by mercaptans like DTT and DTE; and (v) chemical reactivation with acetic anhydride of citrate lyase inactivated by hydroxylamine, mercaptans or oxaloacetate.

Srere *et al.* (1972) showed that citrate lyase inactivated by hydroxylamine or reaction

inactivated in the presence of DTNB could be reactivated only after incubation with DTT, The regeneration of active citrate lyases from the deacetyl form has also been reported for the enzymes from *S. diacetilactis* (Singh and Srere, 1975), *S. faecalis* (Hiremath *et al.*, 1976), *R. gelatinosa* and *L. citrovorum* (Kümmel *et al.*, 1975) and *E. coli* (Nilekani and SivaRaman, 1983), by treatment with acetic anhydride/1-acetyl imidazole.

Treatment of the deacetyl enzyme from *K. aerogenes* with acetic anhydride in the presence of excess of mercaptans results in affinity labelling and consequent reactivation (Dimroth and Eggerer, 1975a). All the sources of citrate lyase examined hitherto have also been shown to have a distinct enzyme, acetate: SH (acetyl carrier protein) citrate lyase ligase (AMP) which catalyzes the reactivation of deacetyl citrate lyase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974; Hiremath *et al.,* 1976; Bowien and Gottschalk, 1977; Antranikian *et al.,* 1978; Giffhorn and Kuhn, 1980; Antranikian and Gottschalk, 1982; Nilekani and SivaRaman, 1983). The ligases in most instances have been found to be specific for the lyase in the same sources. The *S. diacetilactis* ligase reactivates the lyase from the same source but not the lyases from *K. aerogenes* and *R. gelatinosa* (Bowien and Gottschalk, 1977). The *R. gelatinosa* ligase is active towards the lyases from *Rhodospirillaceae* species but do not cross-react with lyases from *S. diacetilactis, K. aerogenes* or *C. sphenoides* (Giffhorn and Kuhn, 1980; Antranikian and Gottschalk, 1982). The ligases from *S. faecalis* and *K. aerogenes,* however, cross-react with the lyases in the two sources (Hiremath *et al.,* 1976) and the *E. coli* lyase cross-reacts with the ligase from *K. aerogenes* (Nilekani and SivaRaman, 1983). The ligase from *K. aerogenes* has also been shown to reactivate deacetyl-citramalate lyase of *Clostridium tetanomorphum,* an enzyme which has close structural resemblance to citrate lyase (Buckel and Bobi, 1976).

Reaction-inactivation

A characteristic property of citrate lyases is that of autoinactivation during substrate turnover, by acyl transfer to water resulting in the formation of the catalytically inactive deacetyl enzyme. Among the earliest observations were those of Wheat and Ajl (1955b) on the cleavage of citrate by the partially purified *E. coli* enzyme, the reaction stopping rapidly after the initial activity. Inactivation was attributed at that time to inhibition by oxaloacetate. A study of the kinetics of the reaction-inactivation of *K. aerogenes* citrate lyase, however, clearly distinguished between the two modes of inactivation (Singh and Srere, 1971). Preincubation of the enzyme even with a 1000-fold excess of oxaloacetate over the amount formed during the reaction failed to inactivate to a comparable extent. Constant removal of oxaloacetate formed during reaction through coupling with malate dehydrogenase and NADH was also without affect on the inactivation rates. The inactivation rates followed first-order kinetics and was dependent on the nature of the divalent metal ion, Zn^{2+} causing the slowest rate and Mg^{2+} the highest among the metals tested. The rapid autoinactivation of the *K. aerogenes* enzyme was termed as a 'suicide' process (Srere and Singh, 1974).

Although the details of the mechanism of reaction-inactivation are not clearly understood, the process is known to be through deacetylation of the native enzyme to the deacetyl-form as the inactivated enzyme is reactivated chemically by acetic

anhydride (Srere *et al.*, 1972) or enzymatically by citrate lyase ligase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974). Suggested mechanisms for the inactivation are the labile nature of the citryl-S-enzyme intermediate from the observed behaviour of citryl-CoA at alkaline pHs (Buckel *et al.,* 1973); the hydrolysis of the citryl-enzyme intermediate by the acyl-transferase subunit of the enzyme complex (Dimroth and Eggerer, 1975b); and hydrolysis of the mixed anhydride intermediate of acetic and citric acids formed during the transacylation step (Rokita and Walsh, 1983).

Citrate lyases from diverse sources differ in their reaction-inactivation behaviour. The enzymes from sources such as *K. aerogenes* (Singh and Srere, 1971) and *R. gelatinosa* (Kümmel *et al.,* 1975) undergo severe reaction-inactivation, while enzymes from *S. diacetilactis* (Singh and Srere, 1975), *L. citrovorum* (Kümmel *et al.,* 1975) and *S. faecalis* (Hiremath *et al.,* 1976) inactivate at markedly slower rates. The first-order rate constant values for reaction-inactivation of the enzymes from *K. aerogenes, S. diacetilactis* (Singh and Srere, 1975), *S. faecalis* (Hiremath, 1977) and *E. coli* (Nilekani and SivaRaman, 1983) with Mg^{2+} at 25°C were determined to be 1·21, 0·06, 0·07 and 0·32 min^{-1} respectively. In the presence of Zn^{2+}, the values for the *K. aerogenes* and *S. diacetilactis* enzymes were 0·17 and 0·014 min^{-1}, respectively.

Citrate lyase autoinactivates also during the turnover of 2-hydroxy- and 2-fluorocitrates (Rokita and Walsh, 1983). While with citrate the frequency of inactivation of *K. aerogenes* citrate lyase was found to be about 1 in 1500 in the presence of 2 mM Mg^{2+} and 1 in 10,000 in presence of 2 mM Zn^{2+}, the frequencies were markedly higher with (2*R,* 3*R*)- and (2*S*, 3*S*)-2-fluorocitrates and the 2-hydroxycitrates, (2*S*, 3*R*)-2-hydroxycitrate being the most potent turnover-dependent inactivator deacetylating the enzyme without detectable cleavage of its carbon-skeleton.

Molecular weight

Citrate lyases have been obtained pure from several sources and shown to be complexes of about M_r 500,000–600,000 (table 1).

The purified *E. coli* enzyme differs from the others in not being monodisperse and exhibiting multiple states of subunit aggregation both in the ultracentrifuge and in Polyacrylamide gel electrophoresis under non-denaturing conditions. The sedimentation profile of the *E. coli* enzyme shows multiple peaks with $s_{20,w}$ values of 10·0,16·6, 20·4, 23·7 and 30·0 *S*. The polydisperse behaviour is not effected by a wide range of ionic strengths and pH of Tris and phosphate buffers, presence of divalent metals, mercaptans or EDTA (Nilekani and SivaRaman, 1983).

The enzyme from *K. aerogenes* was shown to dissociate reversibly in buffers of low ionic strength and in the absence of divalent metal ions (Mahadik and SivaRaman, 1968). A half-molecule of M_r 273,000 sedimenting at 10·3 *S* was observed in 1 mM potassium phosphate buffer, pH 7·4 containing 2 mM EDTA, while another species of 6·3 *S* was observed in 1 mM potassium phosphate buffer, pH 7·4, containing 1 mM EDTA. Increasing the buffer concentration (50 mM) or including Mg^{2+} (2mM) resulted in complete reconstitution of the native species. Treatment with *p*-hydroxymercuribenzoate has also been shown to result in dissociation in stages into three fragments (Mahadik and SivaRaman, 1968). The *S. faecalis* enzyme shows multiple states of aggregation on repeated freezing and thawing (Hiremath *et al.*, 1976) while the *S. diacetilactis* enzyme dissociates at pH values of 8·3 – 8·7 (Singh and Srere, 1975).

Subunit structure

The presence of a M_r 10,000 subunit in *K. aerogenes* citrate lyase which functions as an acyl-carrier protein (ACP) was first reported by Dimroth *et al.* (1973). Singh *et al.* (1974) detected by SDS-polyacrylamide gel electrophoresis the presence of three non-identical subunits of 55,000,33,800 and 11,900 M_r in the enzyme from this source. More precise determination of the molecular weights of the constituent subunits both by gel filtration and sedimentation equilibrium studies in the presence of 6M guanidinium hydrochloride established that the subunits were of M_r 54,000, 32,000 and 10,000 (table 2). Citrate lyases from *S. diacetilactis, S.faecalis, R. gelatinosa* and *C. sphenoides* were also shown later to be built up from three different polypeptide chains resembling in size those from the *K. aerogenes* complex. In marked contrast, the *E. coli* enzyme is built up of subunits of 85,000, 54,000 and 32,000 M_r (table 2).

Table 2. Subunit structure of citrate lyases.

Source	Subunits (M_r)			Holoenzyme structure
	Transferase (α)	Lyase (β)	ACP (γ)	
K. aerogenes[a]	54,000	32,000	10,000	$\alpha_6\beta_6\gamma_6$
S. diacetilactis[b]	54,000	35,000	12,000	$\alpha_6\beta_6\gamma_6$
S. faecalis[c]	54,000	37,000	14,000	$\alpha_6\beta_6\gamma_6$
R. gelatinosa[d]	55,600	31,600	11,400	$\alpha_6\beta_6\gamma_6$
C. sphenoides[e]	56,000	32,000	11,700	$\alpha_6\beta_6\gamma_6$
E. coli[f]	54,000	32,000	85,000	$\alpha_6\beta_6\gamma$

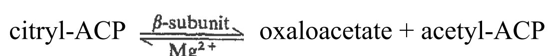
[a] Carpenter *et al.* (1975); Dimroth and Eggerer (1975a); Singh *et al.* (1976).
[b] Singh and Srere (1975).
[c] Hiremath *et al.* (1976); Hiremath (1977).
[d] Giffhorn and Gottschalk (1978).
[e] Antranikian *et al.* (1982a).
[f] Nilekani and SivaRaman (1983).

Subunit function

The 10,000 M_r subunits in *K. aerogenes* citrate lyase were shown to contain the covalently-bound 4′-phosphopantetheine moieties which had been detected by Srere *et al.* (1972) in purified holoenzyme from this source (Dimroth *et al.,* 1973). Further evidence for the ACP function of the subunit was provided by dissociation of [^{14}C]-acetyl-citrate lyase and detection of the radioactive label exclusively in the 10,000 M_r component after SDS-polyacrylamide gel electrophoresis (Dimroth *et al.,* 1973). The subsequent detection of three non-identical subunits in the enzyme complex (Singh *et al.,* 1974) and the sequence of acyl-exchange and acyl-lyase steps in the acetyl-CoA mediated cleavage of citrate by the inactive deacetylated complex (Buckel *et al.,* 1973) were suggestive of the presence of two enzymatically active subunits in addition to the ACP subunit. Unequivocal evidence for the multienzyme nature of the *K. aerogenes* citrate lyase complex was provided by the isolation of the three polypeptide chains in

pure and bilogically active states, characterization of their individual functions and reconstitution of the active enzyme from the isolated subunits (Dimroth and Eggerer, 1975b). The 54,000 M_r (α) subunit was shown to function as an acyl-transferase catalyzing the formation of the acyl-intermediate, citryl-ACP and acetate from citrate and the 10,000 M_r (*γ*) subunit which was earlier shown by Dimroth *et al.* (1973) to function as the ACP. The transferase reaction has no metal requirement and a dimer of the 54,000 (α) chain was reported to be the catalytically active form of the subunit. The 32,000 M_r (*β*) subunit in the presence of Mg^{2+} catalyzes the breakdown of citryl-ACP to oxaloacetate and acetyl-ACP, establishing the lyase function of the *β*-subunit. It was thus established that the overall breakdown of citrate by citrate lyase proceeds in the sequence:

$$\text{acetyl-ACP} + \text{citrate} \underset{\text{EDTA}}{\overset{\alpha\text{-subunit}}{\rightleftharpoons}} \text{citryl-ACP} + \text{acetate}$$



$$\text{citryl-ACP} \underset{Mg^{2+}}{\overset{\beta\text{-subunit}}{\rightleftharpoons}} \text{oxaloacetate} + \text{acetyl-ACP}$$

The active enzyme could be reconstituted only in the presence of all three subunits and was identical with the native enzyme in its specific activity and sedimentation behaviour.

Citrate lyases purified from *S. diacetilactis, R. gelatinosa, S.faecalis* and *C. sphenoides* have been found to resemble the *K. aerogenes* complex in containing subunits of about 55,000,30,000 and 10,000 M_r (table 2). The smallest subunit has been shown to contain the CoA-like prosthetic group. The largest subunit of about 55,000 M_r has been assumed to function as the acyl-transferase and the smaller subunit of about 32,000 M_r as the acyl-lyase in analogy with the *K. aerogenes* complex. In marked contrast, the citrate lyase complex from *E. coli* has been shown to diverge from the general subunit structure, containing subunits of 85,000, 54,000 and 32,000 M_r (Nilekani and SivaRaman, 1983). The 85,000 M_r subunit has been characterized as the ACP(γ) subunit both from the covalently-bound prosthetic group components present exclusively on the subunit as well as through reconstitution from the separated subunits. The 54,000 M_r subunit has been characterized as the acyl-transferase (α) subunit and the 32,000 M_r as the acyl-lyase (*β*) subunit both in acyl-CoA mediated and in acyl-ACP mediated reactions.

Citramalate lyase (EC 4.1.3.22) from *C. tetanomorphum* has been shown to resemble closely *K. aerogenes* citrate lyase despite their occurrence in unrelated bacteria (Buckel and Bobi, 1976). The relatedness between the enzyme complexes is further supported by hybridization experiments. The ACP of citramalate lyase forms an active citrate lyase hybrid complex with the acyl-transferase and acyl-lyase subunits of citrate lyase and the ACP of citrate lyase acts as a substrate for the transferase subunit of citramalate lyase, although other combinations are inactive (Dimroth *et al.,* 1977a).

Subunit stoichiometry

The estimates of subunit stoichiometry by scanning of Coomassie blue stained SDS-polyacrylamide gel electrophoretograms indicated equimolar amounts of the M_r 54,000 (α) and the M_r 32,000 (*β*) subunits in the citrate lyase complex from *K. aerogenes*

(Dimroth and Eggerer, 1975a; Carpenter *et al.,* 1975). The amount of the small ACP (γ) subunit estimated by this procedure failed to give consistent results, values varying with the development and staining procedures. This was presumed to be caused by losses due to diffusion from the gel of the small polypeptide chain. Singh *et al.* (1976) established a novel sequencing technique in which each of the subunits was sequenced to 10 residues from the amino terminus followed by a similar analysis of amino acids released sequentially at corresponding positions of the holoenzyme. The molar ratios of the amino acids released from the three subunits were shown to be 1:1:1, indicating the presence of equimolar amounts of the α-, β- and γ-subunits in the enzyme complex. The findings established that the enzyme from the source has a hexameric ($\alpha_6\beta_6\gamma_6$) structure. Estimates of subunits and holoenzyme molecular weights and subunit stoichiometry in the case of citrate lyase complexes from *S. diacetilactis, S.faecalis, R. gelatinosa* and *C. sphenoides* indicate that these enzymes resemble the *K. aerogenes* enzyme in their gross structural features (table 2). In contrast to the general subunit stoichiometry, the *E. coli* holoenzyme has been shown to have a ratio of $\alpha : \beta : \gamma$ subunits of 6:6:1.

Structural organisation

'Ring' shaped structures have been observed in electron micrographs of negatively stained *R. gelatinosa* citrate lyase (Beuscher *et al.,* 1974). These were composed of 6 large and 6 small subunits arranged hexagonally and lying face to face, each ring consisting of 3 large and 3 small subunits in alternating sequence. Similar structural features have been observed with *K. aerogenes* citrate lyase (Dimroth and Eggerer, 1975a; Schramm, 1981). Antranikian *et al.* (1982a) recognized 'star' and 'triangle' forms in addition to the 'ring' forms in electron micrographs of citrate lyases from *C. sphenoides, R. gelatinosa* and *S. diacetilactis.* The presence of citrate or its analogue, tricarballylate, altered the ratio in favour of the 'star' form in case of the enzyme from *S. diacetilactis.* The C. *sphenoides* enzyme, which has an absolute L-glutamate requirement for its activity, showed undefined shapes in the absence of glutamate while the presence of the activator caused the appearance of 'star' and 'ring' forms. The smallest subunit could not be observed in the electron micrographs of the native complexes, apparently because of its small size and the limited resolution of the technique. However, the ACP (γ) subunit could be located using immunoelectron microscopy and antibodies specific towards the γ-subunit. Under these conditions, the γ-subunit was located to be close to the two larger subunits both in 'stars' and 'rings' with a symmetrical location in the 'star' form and a polar attachment in the 'ring' form (Zimmermann *et al.*, 1982).

Mechanism of action

Acyl-transferase activity: The initial acyl-transferase reaction exhibited by bacterial citrate lyase has been shown to differ from that catalyzed by the well-known CoA transferases represented by succinyl CoA: 3-keto acid transferase in that a single displacement mechanism operates unlike the double displacement of the classical CoA transferases (Jencks, 1973). Kinetic evidences indicated that in citrate lyase an

anhydride intermediate between the substrate and the acyl moiety of the prosthetic group is generated without the formation of an enzyme-CoA intermediate in the acetyl-CoA mediated reaction or an acyl-transferase-carbonyl-S-ACP intermediate in the/acetyl-S-ACP mediated step (Dimroth *et al.*, 1977b).

With substrate analogues such as 3-fluoro-3-deoxycitrate, two different modes of reaction-mechanism based inactivation have been observed by Rokita *et al.* (1982). The enzyme was shown to be converted rapidly into equal proportions of two distinct inactive types, a deacetylated form and a 3-fluoro-3-deoxycitryl-S-enzyme. The behaviour of the citrate analogue as a 'suicide' substrate was explained on the basis of the initial formation of an anhydride of the analogue and acetic acid, which either hydrolyzes and leaves the enzyme in the deacetylated form or reacts with the enzyme to yield fluorocitryl-S-ACP, a derivative which structurally cannot undergo retroaldol cleavage but hydrolyzes slowly with the formation of the deacetyl enzyme.

The involvement of a single arginine residue at the active site of the acyl-transferase subunit of citrate lyase has been shown in studies using the arginine specific reagents, phenylglyoxal and 2,3-butanedione as inactivators, both with the complex as well as with the isolated subunit (Subramanian *et al.,* 1983). Citrate and (3*S*)-citryl-CoA protected the transferase activity while acetyl-CoA markedly enhanced the rate of inactivation, reflecting apparently a conformational change at the citrate binding site of the acyltransferase subunit in the presence of acetyl-CoA which makes the essential arginine residue more accessible to α-carbonyl reagents. An enhancement of reactivity has also been observed in the closely related classical CoA-transferases represented by succinyl-CoA: 3-ketoacid CoA-transferase, the presence of acyl-CoA substrates in this case enhancing the reactivity of an essential thiol group at the carboxylate binding site (White *et al.*, 1976). An 'alligator' model has been suggested for the CoA-transferases (White *et al.*, 1976). The effect of acetyl-CoA on the reactivity of the acyl-transferase α-subunit of citrate lyase is shown schematically in figure 1 on the basis of such a model.

The photoaffinity reagent *p*-azidobenzoyl-CoA, which specifically inactivates the acyl-lyase subunit of citrate lyase, has been shown to have no effect on the acyl-transferase activity both of the complex as well as of the isolated subunit (Basu *et al.,* 1982).

Acyl-lyase activity: The stereochemistry of citrate cleavage has been discussed earlier. The cleavage of the carbon skeleton of hydroxy- and fluoro-citrate isomers which are substrates for citrate lyase has been shown to follow the same regiospecificity, cleavage always occurring at the arm corresponding to the *pro-S-* arm of citrate. In detailed studies on these isomers, Sullivan *et al.* (1977) and Rokita and Walsh (1983) have shown that all isomers of 2-hydroxycitrate, except (2*S*, 3*R*)-2-hydroxycitrate serve as substrates for citrate lyase, while all 4 are linear competitive inhibitors for citrate in the cleavage reaction. Carbon skeletons of (2*R,* 3*R*)- and (2*S*, 3*S*)-2-fluorocitrates were also found to be cleaved catalytically by citrate lyase (Rokita and Walsh, 1983). The stereospecificity of cleavage in all cases was identical to that of citrate, (2*R*, 3*S*)- and (2*S*, 3*S*)-2-hydroxycitrates and (2*R,* 3*R*)-2-fluorocitrate giving oxaloacetate and the glycolyl- or fluoroacetyl form of the resting enzyme, while (2*R*, 3*R*)-2-hydroxycitrate and (2*S,* 3*S*)-2-fluorocitrate yielded *β*-hydroxyoxaloacetate and *β*-fluorooxaloacetate respectively, and the acetyl form of citrate lyase. The results were found to support the suggestion of

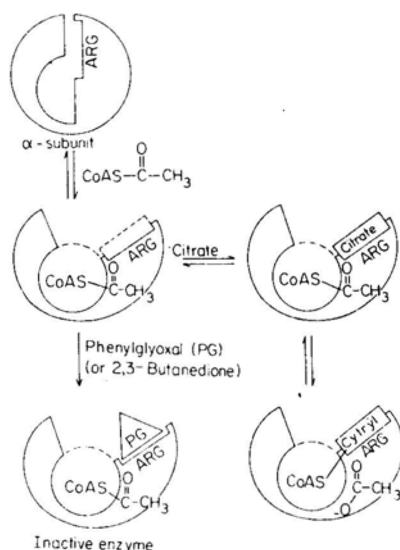


Figure 1. Schematic representation of modulation of activity of the acyl transferase (α) subunit of *Klebsiella aerogenes* citrate lyase by acetyl-CoA. The model is based on the alligator-type proposed by White *et al.* (1976) for classical CoA transferases.

Sullivan *et al.* (1977) that only the divalent metal chelate of the 2-hydroxycitrate which is similar to that of citrate leads to cleavage of the carbon skeleton, despite the many possible tridentate 2-hydroxycitrate chelation modes (Stalling *et al.*, 1979).

p-Azidobenzoyl-CoA has been shown to act as a potent competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA: oxaloacetate lyase reaction catalyzed by deacetyl citrate lyase complex (Basu *et al.,* 1982). The inactivation was irreversible on photolysis and Mg^{2+} which is required for the cleavage reaction had no effect on the binding of the inhibitor to the lyase subunit. It was suggested that (3*S*)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of Mg^{2+}. The stoichiometry of CoA-ester binding was investigated by the use of *p*-azido [^{14}C]-benzoyl-CoA. During photolysis a linear relationship was observed between incorporation of the [^{14}C]-label and the activity loss under limiting conditions of the reagent, the label being exclusively on the lyase subunit. Under such conditions, the substrate, (3*S*)-citryl-CoA, completely protected the enzyme and prevented incorporation of radioactivity on photolysis. An estimate of active sites per mol of the *K. aerogenes* complex made through this approach indicated that all 6 acyl-lyase subunits are accessible for the acyl-CoA mediated reaction. This would suggest that *in vivo* the cleavage of citrate can proceed both *via* the built-in prosthetic group and through the acyl-CoA mediated reaction.

The acyl-lyase subunit of *K. aerogenes* has also been shown from inactivation kinetics with phenylglyoxal and 2,3-butanedione to contain a single essential arginine residue at its active site (Subramaninan *et al.*, 1983). (3*S*)-Citryl-CoA was found to protect the lyase subunit in the complex from inactivation by the α-carbonyl reagents, while citrate and acetyl-CoA had no significant effect.

ACP activity: The ACP of citrate lyase from *K. aerogenes* (Dimroth *et al.,* 1973) resembles the ACP of fatty acid synthetase from *E. coli* (Vagelos, 1973) in its molecular weight and function. The two, however, have been shown to differ markedly in the nature of their prosthetic groups and in their amino acid compositions. The prosthetic group of fatty acid synthetase ACP is 4′-phosphopantetheine, whereas the ACP of the citrate lyase contains in addition adenine, phosphate and sugar residues (Dimroth, 1976; Robinson *et al.,* 1976). Dimroth *et al.* (1973) have shown that an ACP present in the crude extracts of *K. aerogenes,* but not the purified ACP subunit of its citrate lyase, could substitute for the ACP of the *E. coli* fatty acid synthetase system in the malonyl-CoA/CO_2 exchange reaction. This indicated the presence of two distinct ACPs in *K. aerogenes,* one for the fatty acid synthetase system and the other for its citrate lyase. Tryptic peptide maps of the carboxy-methylated ACPs of citrate lyase from *K. aerogenes* and of the ACPs of the fatty acid synthetases from *E. coli* and *K. aerogenes* showed the absence of any common peptide in the citrate lyase and fatty acid synthetase ACPs (Dimroth, 1975; Bayer and Eggerer, 1978). The ACPs of citrate lyase from *K. aerogenes* and of fatty acid synthetase from *E. coli* have also been shown to have no sequence homologies, except for two very short regions (Beyreuther *et al.*, 1978). Despite this unrelatedness, the two polypeptide chains have been found from CD measurements to have similar α-helical contents of about 40–50 % (Beyreuther *et al.*, 1978).

The ACP subunit of *K. aerogenes* citrate lyase has been shown to contain an acetylated cysteine residue in the polypeptide chain (Dimroth *et al.*, 1973). This has posed the problem of the biologically active acyl carrier in the ACP subunit. Indirect evidence that the prosthetic groups carry the essential acetyl moieties was obtained by affinity labelling of the deacetyl complex with low concentrations of iodoacetate in the presence of an excess of DTE when the cysteamine moiety was exclusively alkylated to yield a carboxymethylated complex inaccessible to reactivation either chemically with acetic anhydride or enzymatically with citrate lyase ligase (Dimroth and Eggerer, 1975b). Direct evidence that the cysteamine residue is the active acyl carrier was obtained when reaction-inactivated citrate lyase was shown to be devoid of acetyl-S-cysteamine while still retaining acetyl-S-cysteine residues (Basu *et al.*, 1983). The cysteamine and cysteine residues of *K. aerogenes* citrate lyase ACP have been shown to be located in close proximity. Oxidation of the deacetylated ACP subunit with Cu^{2+}-*o*-phenanthroline complex led exclusively to intrapeptide bridge formation, indicating that the sulphydryl residues are juxtaposed within 2Å (Basu *et al.*, 1983). Evidence was also obtained for the involvement of citrate lyase ligase in the post-translational modification of the cysteine residue in the ACP subunit. It was suggested that the acylation of the cysteine residue in the ACP polypeptide chain might be to protect against intrapeptide bridge formation with the closely located sulphydryl of the prosthetic group in the deacetylated enzyme.

Holoenzyme: Srere and Singh (1974) and Dimroth and Eggerer (1975b) have discussed a possible mechanism of citrate cleavage by the citrate lyase multienzyme complex. The cleavage mechanism has been postulated to be through the oscillation between the acyl-transferase and acyl-lyase subunits of the prosthetic group on the ACP subunit. An alternate mechanism proposed by Zimmermann *et al.* (1982) postulates the rotation of

the α-subunit around its vertical axis leading to a transition from the 'ring' form which represents the 'substrate' or citryl-enzyme to the 'star' form whereby the citryl residue on the ACP subunit is assumed to be brought close to the acyl-lyase subunit. On cleavage of the citryl moiety with release of oxaloacetate, the enzyme is assumed to revert to the 'ring' conformation. The latter mechanism is based on electron microscopic observations and cross-linking studies using bifunctional reagents. The working model assumes two layers of subunit molecules positioned one above the other. In each layer, the structural unit is assumed to consist of an α-subunit with a γ-subunit sitting as a polar cap and a *β*-subunit. 'Stars' and 'rings' are postulated to be transformed from one to the other by the rotation of the α-subunit around the vertical axis and opening followed by closing of contacts between *β*- and γ-component of neighbouring structural units. The 'triangle' form seen in electron micrographs are assumed to be a transition between the 'stars' and 'rings'. Contact with appropriate active sites is assumed to result from the rotation of the α-subunit.

Amino acid composition

The amino acid compositions of the holoenzyme and the separated subunits of *K. aerogenes* citrate lyase have been reported (Bowen and Rogers, 1965; Singh *et al.,* 1976). All the subunits of the enzyme from this source have been shown to have methionine as the amino terminal residue. In the *E. coli* complex, however, the acyl-transferase (α) subunit has been shown to have a methionine at the amino terminus while the acyl-lyase (*β*) subunit and the unusually large ACP (γ) subunit have isoleucine at the amino terminus (Nilekani and SivaRaman, 1983).

The amino acid compositions of the α - and *β* -subunits of *K. aerogenes* citrate lyase have been reported by Singh *et al.* (1976). The amino acid sequence upto 10 residues from the amino terminus of both subunits have also been reported. An unusual observation has been of microheterogeneity in the *β*-subunit, equal amounts of proline and arginine occurring at the fourth position from the amino terminus. Amino acid composition of the ACP (γ) subunit of *K. aerogenes* citrate lyase was reported by Dimroth *et al.* (1973). The sequence analysis of the subunit has been carried out by automated sequential Edman degradations (Beyreuther *et al.*, 1978).

Evidence of the repetitive structure of the unusually large M_r 85,000 ACP (γ) subunit of *E. coli* citrate lyase has been obtained from the almost quantitative recovery of the components of its four covalently-bound prosthetic groups in a single tryptic fragment. Only a single Pauly-positive fragment could be detected in the tryptic peptide map despite the presence of eight histidine residues in the polypeptide chain. The amino acid composition of the subunit was also characteristic of a protein with a highly repetitive structure. These evidences of sequence homologies in the large ACP subunit of *E. coli* citrate lyase probably reflect intragenic duplications (Subramanian *et al.*, 1984).

Nature of citrate lyase prosthetic group

The presence of covalently-bound pantothenate moieties in the holoenzyme from *K. aerogenes* was reported first by Srere *et al.* (1972). Since alkaline phosphatase treatment and alkaline hydrolysis were required for rendering the pantothenate accessible to microbial growth, it was suggested that the prosthetic group probably resembles 4′-

phosphopantetheine of the fatty acid synthetase ACP. It was later recognized that the citrate lyase prosthetic group was more complex and contained adenine, phosphate and sugar in addition to 4′-phosphopantetheine (Dimroth, 1975; 1976). The structure of the prosthetic group was elucidated as (1″→ 2′)-(5″-phosphoribosyl)dephospho-CoA from the characterization of several chemical and enzymatic degradation products and from nuclear magnetic resonance and mass spectrometric studies (Robinson *et al.*, 1976; Oppenheimer *et al.,* 1979). The attachment of the prosthetic group to the ACP polypeptide chain was shown to be through serine-14 (Beyreuther *et al.*, 1978). The prosthetic group of citramalate lyase from *C. tetanomorphum* has been shown to be similar to that of *K. aerogenes* (Buckel and Bobi, 1976).

The prosthetic group isolated from *K. aerogenes* citrate lyase after pronase digestion was shown to cross-react with rat liver ATP-citrate lyase, pig heart citrate synthase and HS-citrate lyase ligase (Robinson *et al.,* 1976).

Immunological behaviour

Citrate lyases from *K. aerogenes, E. coli* and *S. diacetilactis* have been shown to be immunologically distinct, cross-reacting only with antisera against the enzyme from the same source (Singh and Srere, 1975). Citrate lyases from the closely related *R. gelatinosa* and *R. palustris,* however, cross-react to a limited extent while being distinct immunologically from *K. aerogenes, S. diacetilactis* and *C. sphenoides* citrate lyases (Giffhorn *et al.*, 1981). Only the antibodies against purified α-subunit of *R. gelatinosa* citrate lyase complex formed precipitin bands with *R. palustris* citrate lyase, while antibodies to *β*- and γ-subunits showed no cross-reaction. Antibodies against citrate lyase from C. *sphenoides* react with the enzymes from other clostridial species but not with the enzymes from *R. gelatinosa, K. aerogenes* and *S. diacetilactis* (Antranikian *et al.*, 1984).

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