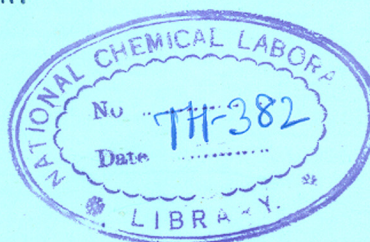


MICROBIAL ENZYMES :
STUDIES ON STRUCTURE - FUNCTION RELATIONSHIPS
OF Klebsiella aerogenes CITRATE LYASE (EC 4.1.3.6)

A THESIS
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BY
AMARESH BASU
M. Sc.

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BAS

DIVISION OF BIOCHEMISTRY
NATIONAL CHEMICAL LABORATORY
PUNE - 411008 (INDIA)

1983

Certified that the work incorporated in the thesis "Microbial enzymes : Structure-function relationships in Klebsiella aerogenes citrate lyase" submitted by Shri Amaresh Basu was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in this thesis.


C. Sivaraman
(Research Guide)

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Amaresh Basu

ABBREVIATIONS

List of abbreviations used other than the accepted ones.

ACP	-	Acyl carrier protein
DTNB	-	5,5'-Dithiobis (2-nitrobenzoate)
DTT	-	Dithiothreitol
$E_{1\text{ cm}}^{1\%}$	-	Absorbance index (1% w/v protein)
MDH	-	Malate dehydrogenase (EC 1.1.1.37)
OAA	-	Oxaloacetic acid
SDS	-	Sodium dodecyl sulfate
Tris-HCl	-	Tris(hydroxymethyl)aminomethane hydrochloride

CONTENTS

	Page No.
ACKNOWLEDGEMENTS	
ABBREVIATIONS	
<u>GENERAL INTRODUCTION</u>	1 – 64
CITRATE ENYMES	1
CITRATE LYASE (EC 4.1.3.6)	3
Occurrence	3
Metabolic significance	5
Regulation of citrate lyases	7 – 17
(i) Regulation in <u>K. aerogenes</u>	7
(ii) Regulation in <u>R. gelatinosa</u>	11
(iii) Regulation in <u>C. Sphenoides</u>	13
(iv) Regulation in <u>P. rettgeri</u>	14
(v) Regulation in <u>E. coli</u>	14
(vi) Regulation in other organisms	15
Bacterial citrate transport	17
Purification of citrate lyase	20 – 24
(i) Citrate lyase from <u>K. aerogenes</u>	20
(ii) Citrate lyase from <u>S. diacetilactis</u>	21
(iii) Citrate lyase from <u>R. gelatinosa</u>	22
(iv) Citrate lyase from <u>S. faecalis</u>	22
(v) Citrate lyase from <u>C. sphenoides</u>	23
(vi) Citrate lyase from <u>E. coli</u>	23
Properties of citrate lyase	24 – 64
(i) Stability	24
(ii) pH optimum	27
(iii) Metal requirements	28

(iv)	Reaction kinetics	30
(v)	Stereospecificity	33
(vi)	Inhibition by oxaloacetate	36
(vii)	Essential acetyl group	37
(viii)	Reaction inactivation	41
(ix)	Molecular weight	44
(x)	Subunit structure	46
(xi)	Subunit stoichiometry	49
(xii)	Subunit organization	51
(xiii)	Multienzyme nature of citrate lyase	54
(xiv)	Effect of citrate analogues	56
(xv)	Amino acid composition	59
(xvi)	Prosthetic group of citrate lyase	61
(xvii)	Immunological behaviour	64
 <u>PRESENT INVESTIGATION</u>		 65 – 69
 <u>PART I : PHOTOAFFINITY LABELING OF CITRATE LYASE BY p-AZIDOBENZOYL-COA</u>		 70 – 123
	Summary	70
	Introduction	71
	Materials and Methods	73
	Results	100
	Discussion	120
 <u>PART II : REACTION MECHANISM BASED INHIBITORS OF CITRATE LYASE (EC 4.1.3.6)</u>		 124 – 147
	Summary	124
	Introduction	126
	Materials and Methods	128
	Results	133
	Discussion	143
 <u>PART III : S-ACYLATED RESIDUES OF THE ACYL-CARRIER PROTEIN SUBUNIT OF KILBESIELLA AEROGENES CITRATE LYASE</u>		 148 - 170
	Summary	148

Introduction	149
Materials and Methods	150
Results	158
Discussion	168
<u>REFERENCES</u>	171 – 182
<u>PUBLICATIONS</u>	

GENERAL INTRODUCTION

GENERAL INTRODUCTION

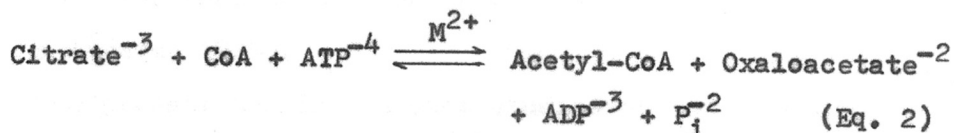
CITRATE ENZYMES

Citrate functions as a key intermediate in diverse metabolic pathways. Four different groups of enzymes are known which catalyze the synthesis or dissimilation of citrate through a lyase reaction involving an equilibrium between citrate on the one hand and oxaloacetate and an acetyl-moiety on the other. These are :

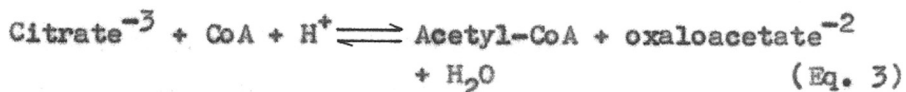
(a) Citrate lyase [citrate oxaloacetate-lyase (pre-3S-CH₂COO⁻ $\longrightarrow$ acetate); EC 4.1.3.6], an enzyme found only in procaryotes, which catalyzes the dissimilation of citrate into oxaloacetate and acetate in presence of divalent metal ions such as Mg²⁺ or Mn²⁺ (Eq. 1).



(b) ATP-citrate lyase [ATP: citrate oxaloacetate-lyase (pre-3S-CH₂COO⁻ $\longrightarrow$ acetyl-CoA and ATP dephosphorylating); EC 4.1.3.8], which is a cytosolic enzyme found mainly in eucaryotes and responsible for the generation from citrate of 2-carbon moieties which enter a number of biosynthetic pathways. Its occurrence in Chlorobium limicola has been reported recently (Ivanovsky et al., 1980; Antranikian et al., 1982). The enzyme requires ATP, CoA and a divalent metal ion such as Mg²⁺, Mn²⁺ or Co²⁺ for the cleavage of citrate (Eq. 2).



(c) si-Citrate synthase [citrate-oxaloacetate lyase (pro-3S-CH₂COO⁻ → acetyl-CoA); EC 4.1.3.7] (si-Citrate synthase), which catalyzes the formation of citrate by the overall reaction shown in eq. 3. It is the most widely distributed of the three citrate enzymes being present in both procaryotes and eucaryotes.



(d) re-Citrate synthase [citrate oxaloacetate-lyase (pro-3R-CH₂COO⁻ → acetyl-CoA)] (re-citrate synthase, EC not yet been assigned) which has been found in some anaerobic microorganisms. The enzyme exhibits a stereochemistry opposite to that of si-citrate synthase as well as the other enzymes.

The three citrate enzymes, citrate lyase, ATP-citrate lyase and si-citrate synthase show close resemblances in the stereospecificity of the reaction they catalyze as well as in their cofactor requirements, coenzyme A and the citrate lyase prosthetic group (and 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 cofactor supplement requirements, it has been suggested that citrate lyase may represent the earliest of the three citrate enzymes to evolve (Srere, 1974).

The present thesis embodies studies on some aspects of the structure and function of citrate lyases. The

literature on this group of enzymes is therefore reviewed in the rest of this chapter. Although recent reviews have not appeared, earlier work has been discussed extensively in reviews by Dagley, 1969; Lowenstein, 1969; Spector, 1972; Srere, 1965; 1968; 1972; 1974; 1975; Srere and Singh, 1974.

II CITRATE LYASE (EC 4.1.3.6)

II.1 OCCURRENCE : From an analysis of the products of fermentation of citrate by Klebsiella aerogenes and Aerobacter indologenes Defner and Franke (1939) and Brewer and Werkman (1939) suggested that oxaloacetate and acetate are the initial cleavage products. Citrate lyase, the enzyme responsible for the initiation of the fermentation of citrate was first characterized by Dagley and Dawes (1953) in K. aerogenes and 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. Table I summarizes the various sources in which the activity has been detected. The only source in which citrate lyase has been shown to be present constitutively is Streptococcus diacetylactis (Harvey and Collins, 1961). Detectable levels of citrate lyase have been reported in the organism grown on a lactose medium without citrate, the level increasing considerably with citrate supplements.

Citrate lyase is present in the photosynthetic bacteria Rhodospseudomonas gelatinosa and Rhodospseudomonas palustris

TABLE I.1: SOURCE OF CITRATE LYASE

Nature of activity	Bacteria	Reference
1. Constitutive	<u>Streptococcus diacetylactis</u>	Harvey and Collins (1961)
2. Induced	<u>Klebsiella aerogenes</u> *	Dagley and Dawes (1953b)
	<u>Escherichia coli</u>	Grunberg-Manago and Gunsalus (1953); Dagley (1954)
	<u>Streptococcus faecalis</u>	Gillespie and Gunsalus (1953)
	<u>Aerobacter cloacae</u>	O'Brien (1975c)
	<u>Leuconostoc citrovorum</u>	Harvey and Collins (1961)
	<u>Streptococcus liquefaciens</u>	Harvey and Collins (1961)
	<u>Salmonella typhimurium</u>	O'Brien <u>et al.</u> (1969)
	<u>Rhodopseudomonas gelatinosa</u>	Weckesser <u>et al.</u> (1969); Schaab <u>et al.</u> (1972)
	<u>Rhodopseudomonas palustris</u>	Giffhorn and Kuhn (1980)
	<u>Proteus rettgeri</u>	Kroger <u>et al.</u> (1974)
	<u>Clostridium sphenoides</u>	Walther <u>et al.</u> (1977)

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

grown anaerobically on citrate under illuminated conditions. Clostridium sphenoides, an anaerobic spore-former, is characterized by its ability to grow on a medium containing citrate as the carbon and energy source. Walther et al. (1977) have shown that citrate lyase from this source is highly unstable except in the presence of glutamate. The presence of glutamate is also required for the citrate cleavage activity of the C. sphenoides enzyme (Antranikian et al., 1982).

II.2 METABOLIC SIGNIFICANCE : The anaerobic metabolism of citrate by K. aerogenes, A. indologenes and S. faecalis is through a sequence of reactions which has been termed the citrate fermentation pathway (Dagley and Dawes, 1953; O'Brien and Stern, 1969). The pathway is initiated by the cleavage of citrate catalyzed by citrate lyase to yield oxaloacetate and acetate. The oxaloacetate formed is then degraded further by oxaloacetate decarboxylase to pyruvate and CO₂. There is no involvement of hydrogen transfer in this pathway and ATP production is only from acetyl-CoA which is formed from pyruvate.

An alternate pathway for anaerobic breakdown of citrate has been shown to occur in Proteus rettgeri, since oxaloacetate decarboxylase is not formed by this organism (Kroger et al., 1974). In the modified pathway, a part of the citrate is cleaved by citrate lyase to generate oxaloacetate, while the rest of the citrate is simultaneously oxidized through the tricarboxylic acid cycle to generate

reducing power which is utilized for the reduction of oxaloacetate to succinate via malate and fumarate, generating a mol of ATP. Yet another modification of this pathway occurs in E. coli. This organism does not contain oxaloacetate decarboxylase (Luttgens and Gottschalk, 1980) and the tricarboxylic acid cycle is repressed under anaerobic growth conditions (Amaransingham and Davis, 1965). Thus the organism has to catabolize a second substrate like glucose, lactose or pyruvate, in order to gain reducing power for succinate formation from oxaloacetate (Luttgens and Gottschalk, 1980).

In studies on S. typhimurium which is capable of growth on citrate, both aerobically and anaerobically Kulla (1983) failed to obtain mutants lacking citrate lyase activity. It was therefore suggested that citrate lyase in E. coli and in the strain of S. typhimurium probably serves as a protective agent by breaking down excess intracellular citrate which might otherwise interfere with vital cell functions by chelating divalent metal ions.

The diverse pathways involving citrate lyase as the key enzyme for the anaerobic metabolism of citrate are described schematically in Figure 1.

Anaerobic phototropic bacteria Chlorobium thiosulfatophilum and Rhodospirillum rubrum, have been shown to contain enzyme systems catalyzing the formation of ketoacids from acyl-CoA esters by ferredoxin-dependent carboxylations (Evans and Buchanan, 1965). Citrate lyase has been postulated

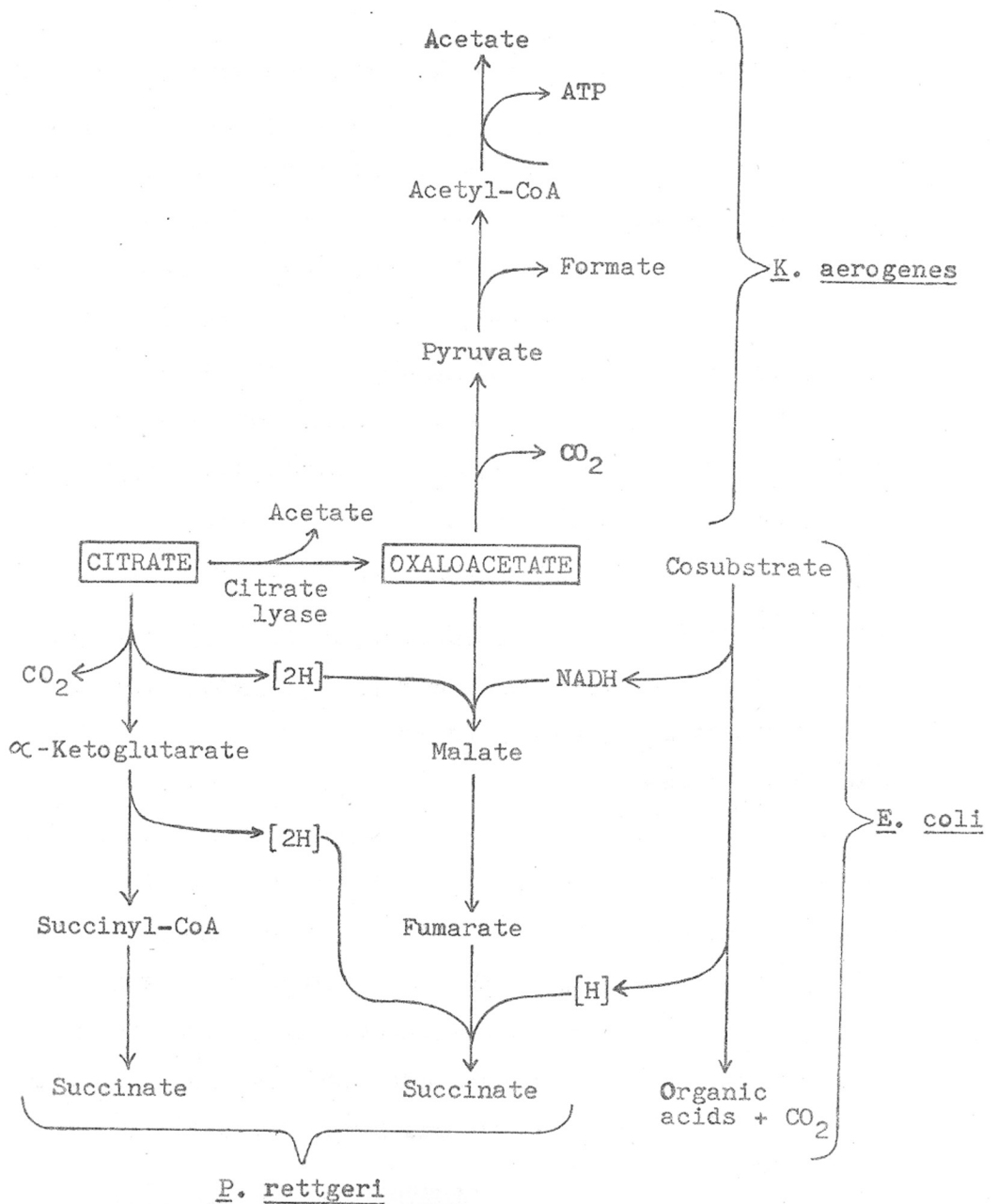


Figure 1. Metabolic pathways for the fermentation of citrate (Kröger, 1977 ; Lütgens and Gottschalk, 1980)

as a key enzyme for this reductive carboxylic acid cycle—a proposed new autotrophic pathway of CO_2 assimilation for green sulfur bacteria (Evans et al., 1966), but Beuscher and Gottschalk (1972) failed to detect citrate lyase activity in the extracts of these organisms. Recently Ivanovsky et al. (1980) have reported the presence of ATP-citrate lyase activity in two strains of C. limicola forma thiosulfato-phylum. The ATP-citrate lyase of C. limicola has been partially purified by Antranikian et al. (1982). These are the only reports on the occurrence of ATP-citrate lyase in procaryotes.

II.3 REGULATION OF CITRATE LYASES : In microorganisms, such as S. diacetilactis and L. citrovorum in which citrate synthase is not formed, citrate serves as a substrate for citrate lyase only and the enzyme is not under regulation (Giffhorn and Gottschalk, 1975a). Many bacteria, however, also contain citrate synthase, an antagonistic enzyme to citrate lyase. Citrate lyase in these organisms has to be under stringent control to prevent the simultaneous functioning of both the enzymes and consequent energy loss through futile cycles. The diverse regulatory mechanisms which have been observed are reviewed briefly.

(i) Regulation in K. aerogenes :

O'Brien and Stern (1969) demonstrated that Na^+ is specifically required for the growth of K. aerogenes (NCTC 418) under anaerobic conditions on citrate as sole source of energy. This requirement was shown to be for the

oxaloacetate decarboxylase from this source, the second enzyme of the citrate fermentation pathway. The organism has no other pathway for citrate catabolism and its oxaloacetate decarboxylase has an absolute requirement of Na^+ ions for activity (Stern, 196). Under anaerobic conditions, the citric acid cycle is turned off as a result of repression of 2-oxoglutarate dehydrogenase activity (O'Brien, 1975a; Wilkerson and Eagon, 1972). Under aerobic conditions of growth on citrate, citrate lyase and oxaloacetate decarboxylase are not induced while all the enzymes of the citric acid cycle are formed and cells grown with or without Na^+ utilize 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^+ .

Citrate lyase from K. aerogenes undergoes rapid reaction inactivation in the course of the reaction it catalyzes in vitro (Singh and Srere, 1971) through deacetylation (Srere et al., 1972). The presence in K. aerogenes of a distinct citrate lyase ligase, an enzyme which reacetylates the inactive deacetyl citrate lyase in the presence of ATP and acetate or acetyl adenylate has been shown by Schmellenkamp and Eggerer (1974). The citrate

lyase ligases from S. diacetilactis (Bowien and Gottschalk, 1977) and R. gelatinosa (Antranikian and Gottschalk, 1982) have been obtained pure.

Kulla and Gottschalk (1977) performed a number of growth shift experiments to study the mode of regulation of citrate lyase and citrate synthase activities in K. aerogenes. In cells grown anaerobically on citrate, the citrate lyase activity increases rapidly after the initial lag period until the end of growth phase (12 h). During the early growth phase no deacetyl citrate lyase could be detected, but after about 15 h there was a steady increase in the amount of the deacetylated enzyme and at the end of 24 h incubation, a considerable amount of inactive enzyme was present. Growth shift experiments were carried out after the organism was grown in continuous culture with ammonia as the growth-limiting substrate. Specific activities of citrate synthase and citrate lyase before and after treating with acetic anhydride, were monitored after growth shifts from anaerobic growth on citrate to aerobic growth on citrate, aerobic growth on glucose, anaerobic growth on glucose and anaerobic growth on glucose plus nitrate. Growth shifts from "anaerobic citrate" to "aerobic citrate" resulted in the induction of citrate synthase while the synthesis of citrate lyase increased considerably and the active citrate lyase was slowly inactivated by deacetylation. A shift from "anaerobic citrate" to "aerobic glucose" resulted in a rapid inactivation of citrate lyase and induction of citrate synthase, the steady state level being considerably lower than

that observed in a shift to "aerobic citrate". This was due to the catabolic repression of citric acid cycle enzymes by glucose (Gray et al., 1966). However, there was no rapid inactivation of citrate lyase when the culture was shifted to "anaerobic glucose" and growth difficulties were observed in the organism with a drop in cell population. This was attributed to the simultaneous functioning of the two antagonistic enzymes resulting in futile cycles. The cells apparently suffered from a shortage of glutamate since the decrease in cell population was partially overcome by supplementing the medium with 2-oxoglutarate. These results indicate that citrate lyase is inactivated only under aerobic conditions, however, no deacetylation activity could be detected in the cells incubated with glucose under aerobic conditions. The citrate lyase of K. aerogenes is subjected to reaction-inactivation in vitro but this does not play a role in the regulation of citrate lyase activity in vivo, as it does not become inactive under anaerobic conditions in the presence of glucose. Thus the inactivation process somehow involves oxygen or the respiratory chain. Oxygen does not play a direct role since a shift from "anaerobic citrate" to "anaerobic glucose plus nitrate" also resulted in the inactivation of citrate lyase. Cells grown in batch cultures with citrate under anaerobic conditions, when suspended in "aerobic glucose" medium, were found to lose citrate lyase activity completely within 90 min. Chloroamphenicol delayed this inactivation process and 2,4-dinitrophenol prevented it. It was

concluded from this result that the inactivation of K. aerogenes citrate lyase is energy dependent and electron transport to oxygen or nitrate was required for inactivation. Some other examples of "energy-dependent inactivation" are of nitrate reductase in K. aerogenes, Proteus mirabilis and E. coli (Van'T Reit et al., 1968; deGroot and Stouthamer, 1969; 1970a,b,c; Showe and De Moss, 1968) and of aspartate transcarbamylase of Bacillus subtilis (Waindle and Switzer, 1973) which are inactivated by O₂-dependent electron transport.

SivaRaman and SivaRaman (1979) have shown that the binding of divalent metal cofactor brings about a conformational change in the enzyme complex from K. aerogenes, the metal-free conformation being catalytically inactive in the cleavage of citrate. They have suggested that conformational modulations could be a possible mode of regulation. The energy requirement could be for the formation or utilization of metabolite(s) acting either directly as modulators or indirectly through complexing of Mg²⁺. Blair et al. (1967) had speculated earlier that the inactivation of citrate lyase by ATP by its ability to chelate metal ions may play a role in its regulation.

ii) 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 any 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 utilizes citrate rapidly under anaerobic conditions in 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 SH form to the active S-acetyl form (Giffhorn and Gottschalk, 1975a,b). The deacetylase is a small protein of M_r 14 300 (Giffhorn et al., 1982) and has been obtained pure by 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 in the medium of citrate, its precursor. Thus a high concentration of citrate, signals that it can be cleaved via the fermentation pathway, while at low

concentrations citrate lyase is activated by citrate lyase deacetylase and all the citrate is utilized for biosynthesis of L-glutamate. This organism also contains a citrate lyase ligase (Giffhorn and Gottschalk, 1975a) which activates deacetylated citrate lyase in presence of ATP and acetate. The citrate lyase ligase has been purified and has been shown to have a requirement for ADP for its stability (Antranikian and Gottschalk, 1982). Antranikian et al. (1978) have shown that depletion of citrate from the medium results in inactivation of the ligase and of the citrate lyase activities, while addition of citrate leads to rapid activation of the ligase which in turn activates citrate lyase.

Thus the regulation of citrate lyase activity in R. gelatinosa is by deacetylation and acetylation brought about by two enzymes, citrate lyase deacetylase which in turn is regulated by intracellular L-glutamate concentration and citrate lyase ligase whose activity is controlled by citrate levels in the medium.

A similar mechanism of regulation of citrate lyase 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.

(111) Regulation in C. sphenoides :

Antranikian et al. (1982) have studied the regulation of citrate lyase in C. sphenoides. In this organism the

activity of citrate lyase is not regulated by acetylation and deacetylation. The enzyme requires high concentrations of L-glutamate for its activity. Thus the intracellular concentrations of L-glutamate directly regulates the citrate lyase activity. The intracellular concentration of glutamate is in turn directly proportional to the citrate in the medium, decreasing with the depletion of citrate resulting in decreased citrate lyase activity. While in the Rhodospirillaceae the regulation of citrate lyase is indirectly through the action of glutamate on the deacetylase and the ligase, in the Clostridium glutamate directly interacts with citrate lyase to regulate its activity.

(iv) Regulation in P. rettgeri :

The oxaloacetate produced by the citrate lyase in this organism is reduced to succinate as oxaloacetate decarboxylase is not formed (Kroger et al., 1974). It was observed that citrate lyase was 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.

(v) Regulation in E. coli :

E. coli has been characterized by its inability to grow on citrate as sole substrate (Koser, 1923). Under aerobic conditions its inability to grow on citrate has been attributed to the permeability barriers of its membrane (Lara and Stokes, 1952). However, under anaerobic conditions

it can utilize citrate if the medium is supplemented with peptone or carbohydrates (Vaughn et al., 1956) and citrate lyase was shown to be responsible for its catabolism (Dagley, 1954; Wheat and Ajl, 1955a). Luttgens and Gottschalk (1980) later found that the cells grown on citrate plus glucose under anaerobic conditions have citrate lyase and all the enzymes required for the reduction of oxaloacetate to succinate, while oxaloacetate decarboxylase is not detectable. This indicated that in E. coli cells, further catabolism of oxaloacetate occurs only through its reduction to succinate. Thus E. coli requires reducing power for succinate formation from oxaloacetate and this is achieved by catabolism of a second substrate. Citrate lyase was shown to exist in the active acetylated form as long as the second substrate was present and was deacetylated rapidly as soon as the second substrate was exhausted (Dagley, 1954; Kulla, 1976). It has been suggested that citrate lyase in E. coli has a protective rather than a catabolic role (Kulla, 1983). The function of the enzyme according to this hypothesis is to reduce the intracellular citrate pool, which otherwise may chelate divalent cations and interfere with important cell functions. Under aerobic conditions this problem is assumed not to arise since the cells are impermeable to citrate.

Recently, however, it has been reported that certain plasmids help E. coli to grow on citrate under aerobic conditions (Smith et al., 1978; Ishiguro et al., 1981).

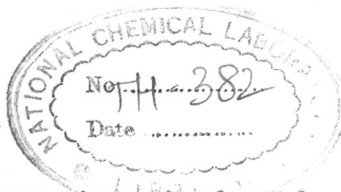
(vi) Regulation in other organisms :

In A. cloacae, growth on citrate is unaffected by the presence or absence of Na^+ under both aerobic and anaerobic

conditions (O'Brien and Geisley, 1974). Under anaerobic growth conditions, citrate metabolism proceeds mainly through the fermentation pathway, the tricarboxylic acid cycle being repressed by the lack of synthesis of 2-oxoglutarate dehydrogenase. Under aerobic conditions, however, citrate lyase was repressed while 2-oxoglutarate dehydrogenase activity was induced. The presence of Na^+ in the medium did not lead to a repression of 2-oxoglutarate dehydrogenase as in the case of K. aerogenes. Under aerated conditions, when oxygen tension in the medium is zero, citrate lyase activity was induced while oxaloacetate decarboxylase activity was lowered rendering the fermentation pathway inoperative beyond the initial citrate cleavage. Under such conditions of controlled aeration, futile cycles lead to energy loss and cell depletion.

S. typhimurium requires Na^+ specifically for the aerobic growth on citrate (O'Brien et al., 1969). Cells grown on citrate under aerobic conditions contained the enzymes of the citrate fermentation pathway and all the enzymes of the citric acid cycle. Thus, two separate routes of citrate catabolism are operational in the cells, but the mode of regulation of the two antagonistic pathway is not known. Recently Kulla (1983) obtained a mutant of S. typhimurium in which the mode of regulation of citrate lyase under anaerobic conditions resembled the E. coli system, the citrate lyase being deacetylated as soon as the cosubstrate is exhausted or removed.

The growth of S. faecalis on citrate under anaerobic



conditions has been shown to depend on the presence of Ca^{2+} and biotin in the medium which are required for its oxaloacetate decarboxylase activity (Sachan and Stern, 1971b).

Lactic acid bacteria such as S. diacetilactis and L. citrovorum lack citrate synthase activity and citrate is metabolized only through the fermentation pathway. Thus there is no need for regulation of citrate lyase activity in these organisms (Giffhorn and Gottschalk, 1975a). In S. diacetilactis citrate utilization was found to be dependent on a plasmid DNA as cells cured with acridine orange were unable to grow on citrate (Kempner and McKay, 1979). A plasmid of 5.5 megadalton was found to be missing in Cit^- mutants. It was shown by Kempner and McKay (1979) that the loss of the plasmid results in loss of citrate permease activity, the cells still retaining citrate lyase activity although less than in the parent cells.

II.4 BACTERIAL CITRATE TRANSPORT :

Citrate transport in a number of citrate utilizing bacteria is mediated apparently by the membrane bound ^{de}oxaloacetate/carboxylase which is induced only in the presence of the substrate. K. aerogenes (NCTC 418) cells require Na^+ specifically to grow anaerobically on citrate (Stern and Sachan, 1970). These authors (Sachan and Stern, 1971a) also showed that oxaloacetate decarboxylase specifically requires Na^+ for its activity and that citrate binds to this protein and competitively inhibits

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the decarboxylase reaction. Johnson et al. (1975) confirmed the findings in studies on vesicles prepared from K. aerogenes cells grown anaerobically on citrate.

However, in some strains of K. aerogenes, differences in the metal requirement for citrate transport has been observed. Eagon and Wilkerson (1972) have shown that citrate transport in K. aerogenes UGa-I is K^+ -dependent. Na^+ inhibits the uptake of citrate by strains UGa-I and ATCC 12658 and has no effect on citrate uptake by strain PRL-RB (Wilkerson and Eagon, 1974).

Sachan and Stern (1971b) have shown that S. faecalis requires Ca^{2+} and biotin for citrate uptake, this was explained on the basis of the specific requirement by the membrane bound OAA-decarboxylase of S. faecalis for Ca^{2+} and biotin for decarboxylation.

Transport system in S. diacetylactis has been shown to be an energy requiring process (Harvey and Collins, 1962). Collins and Harvey (1968) observed that spontaneous mutation in S. diacetylactis results in Cit^- mutants which have lost the ability to transport citrate while retaining citrate lyase activity. Later, Kempler and McKay (1979) showed that the cryptic Cit^- mutants lack citrate permease activity which is associated with a 5.5 megadalton plasmid.

E. coli cells cannot utilize extracellular citrate under anaerobic conditions and it was generally believed that there is no transport system for the substrate (Gunsalus, 1958). However, under anaerobic conditions

E. coli can utilize citrate if another substrate like glucose, lactate or pyruvate is present in the medium (Vaughn et al., 1950). Thus E. coli is able to form a transport system under anaerobic conditions but the nature and specific requirements of this system are not known.

It was reported by Smith et al. (1978) that certain thermosensitive H₁ plasmids from some strains of S. typhimurium can mediate citrate utilization by E. coli under aerobic conditions. A number of isolates of naturally occurring Cit⁺ E. coli strains have been obtained from several birds and mammals, but the Cit⁺ phenotype was always found to be associated with plasmids (Ishiguro et al., 1978; Sato et al., 1978; Ishiguro et al., 1981). Recently Hall (1982) has reported the isolation of a spontaneous mutant of E. coli K-12 which is able to utilize citrate aerobically as a sole source of carbon. The Cit⁺ phenotype resulted from two mutations in two genes and these two together have been found to specify a citrate transport system.

The citrate transport system in S. typhimurium has been found to be inducible and is complex. The presence of three separate transport systems has been reported in this source for tricarboxylic acids by Imai et al. (1973).

PURIFICATION OF CITRATE LYASES

Citrate lyase has been obtained pure from K. aerogenes, S. diacetilactis, R. gelatinosa, S. faecalis, C. spenoides and E. coli. The purification procedures are described briefly below :

(1) Citrate lyase from Klebsiella aerogenes:

The enzyme from this source was the earliest to be obtained pure. An eight-fold partial purification was reported by Dagley and Dawes (1955). The enzyme was obtained pure from K. aerogenes (N TC 418) by SivaRaman (1961). The steps involved streptomycin sulfate treatment, adsorption on alumina C gel followed by selective elution, DEAE-cellulose chromatography and ammonium sulfate fractionation. Modifications of the original procedure have been described, including a final filtration through Sephadex G-200 (Mahadik and SivaRaman, 1968) or Biogel A 1.5 m (Singh and Srere, 1975), treatment with salmine sulfate and CM-cellulose replacing alumina C gel and DEAE-cellulose chromatography (Bowen and Mortimer, 1971).

The purity of the enzyme preparation has been established by ultracentrifugation (SivaRaman, 1961), electrophoresis in free solution (Bowen and Rogers, 1963) and polyacrylamide gel electrophoresis (Singh and Srere, 1971). The pure enzyme from this source has an absorbance index $E_{1\text{cm}}^{1\%}$ of 6.2 at 278 nm and a specific activity 70 $\mu\text{mol min}^{-1}\text{mg}^{-1}$ at pH 7.4, 28°C and 10 mM MgCl_2 (Singh et al., 1976).

A procedure suitable for preparation on large scale has been reported by Dimroth et al. (1977). This involves the precipitation of the alumina C eluate with polymin HS (ethyleneimine polymer), followed by elution and gel filtration through Sephadex G-200.

(ii) Citrate lyase from Streptococcus diacetylactis:

The purification of citrate lyase from this source was reported independently by Singh and Srere (1975) and Kummel et al. (1975). The procedure of Singh and Srere (1975) involves protamine sulfate treatment, ammonium sulfate fractionation, gel filtration through Biogel A 0.5 m and DEAE-cellulose chromatography. The final preparation was homogeneous as judged by sedimentation velocity and sedimentation equilibrium experiments and has a specific activity of $200 \text{ } \mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH 7.2 in presence of 2 mM MgCl_2 at 25°C .

Kummel et al. (1975) obtained a 38-fold purification of citrate lyase from this source. The procedure involved cetyltrimethylammonium bromide treatment, treatment with alumina C gel, Sepharose 6B and DEAE-Sephadex A-50 column chromatography. The enzyme preparation was homogeneous in the ultracentrifuge and on polyacrylamide gel electrophoresis and had a specific activity of $125 \text{ } \mu\text{mol.min}^{-1} \text{mg}^{-1}$ at pH 7.2 in presence of 2 mM MgCl_2 . Citrate lyase ligase activity was found to be associated with the preparation of citrate lyase (Kummel et al., 1975). This was shown by Bowien and Gottschalk, (1977) to be an

impurity which could be completely separated from the lyase complex after alumina C gel treatment.

(iii) Citrate lyase from Rhodopseudomonas gelatinosa:

The enzyme from this source was purified by Beuscher et al. (1974). The procedure involved protamine sulfate treatment, DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B. The purified enzyme had a specific activity of $138.8 \text{ umol.min}^{-1}\text{mg}^{-1}$ at pH 7.2, 30°C in the presence of 3 mM MgCl_2 .

Giffhorn and Gottschalk (1978) were later able to crystallize the enzyme from this source. The steps involved were treatment with cetyltrimethylammonium bromide, alumina C gel treatment and DEAE-cellulose chromatography. The active fraction from the DEAE-cellulose column was concentrated by ultrafiltration till turbidity^{and}/was allowed to crystallize. The enzyme crystals had a specific activity of $358 \text{ umol.min}^{-1}\text{mg}^{-1}$. The crystals suspended in 3 M ammonium sulfate were stable for several months at 4°C .

(iv) Citrate lyase from Streptococcus faecalis:

Hiremath et al. (1976) obtained pure citrate lyase from S. faecalis. The method involved the initial reactivation of deacetyl citrate lyase with sodium acetate and ATP. The subsequent steps were streptomycin sulfate treatment, adsorption and elution from alumina C gel, ammonium sulfate fractionation, DEAE-cellulose

chromatography and gel filtration through Sepharose CL 6B. The enzyme which was homogeneous by ultracentrifugation and gel electrophoresis had a specific activity of 90 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 8.0, 30°C in the presence of 20 mM MgSO_4 .

(v) Citrate lyase from *Clostridium sphenoides*:

A 72-fold purification of citrate lyase from *C. sphenoides* was obtained by Antranikian *et al.* (1982). In contrast to citrate lyases from other sources the activity of this enzyme was strictly dependent on the presence of L-glutamate. All the buffers used in purification contained 150 mM L-glutamate for stabilization of the enzyme. The procedure involved protamine sulfate treatment, ammonium sulfate fractionation and Sepharose 6B chromatography. At this stage the protein solution containing ligase activity was treated with ATP and acetate to reacetylate any deacetylated enzyme. The activated enzyme was then fractionated by ultracentrifugation in a sucrose density gradient and gel filtration on Sephadex G-200. The pure enzyme had a specific activity of 60 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

(vi) Citrate lyase from *Escherichia coli* :

Partial purification of citrate lyase from *E. coli* had been reported by Dagley and Dawes (1955); Wheat and Ajl (1955) and Bowen and SivaRaman (1960).

Nilekani and SivaRaman (1983) purified the enzyme by a procedure which involves treatment with streptomycin sulfate, precipitation with salmine sulfate followed by elution, alumina C gel treatment, ammonium sulfate

fractionation, DEAE-cellulose and Sepharose CL-6B chromatography. The enzyme was polydisperse in gel electrophoresis and showed multiple states of aggregation on analytical ultracentrifugation. The purity was established from the three component subunits obtained on SDS-polyacrylamide gel electrophoresis. The pure enzyme had a specific activity of $125 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

PROPERTIES OF CITRATE LYASE

(1) Stability:

Bowen and Rogers (1963) studied the stability of pure *K. aerogenes* citrate lyase. The half life of a 0.3% protein solution at -14°C is about 4 weeks, dilute solutions being even less stable. At 20°C , 20% of the activity is lost within 5.5 h at a protein concentration of 0.3%, compared to only 5% at 4°C . Crude extracts were found to be relatively unstable, losing 70% of activity in 4 days at -20°C (Dimroth and Eggerer, 1975a). At higher temperature, however, the crude enzyme was relatively more stable than the purified enzyme. Cysteine did not stabilize the enzyme from heat inactivation.

Presence of Mg^{2+} increases the stability of pure citrate lyase from *K. aerogenes* (Eissenthal et al., 1966). Inactivation observed at low Mg^{2+} concentrations (0.025 - 1.0 mM) was partially reversed by raising the Mg^{2+} concentration to 2.5 mM (Blair et al., 1967). At higher protein and Mg^{2+} concentrations, the rates of both the reversible and irreversible inactivation were reduced.

EDTA and adenine nucleotides enhanced inactivation at low Mg^{2+} concentrations by chelation of metal ions. Though Ca^{2+} competitively inhibits the enzyme in presence of Mg^{2+} it was shown to protect the enzyme from inactivation in dilute solutions (Blair et al., 1967). Partially purified enzyme from K. aerogenes has been shown to be stable for long periods when freeze-dried in presence of bovine serum albumin, sucrose and $MgSO_4$, the loss in activity being only 10% after a month (Gruber and Mollering, 1966).

Active citrate lyase was shown in later studies to carry an essential acetyl moiety linked by thioester linkage to a sulfhydryl group on the enzyme (Buckel et al., 1971). Treatment with neutral hydroxylamine or with oxaloacetate in presence of Mg^{2+} resulted in deacetylation and formation of catalytically inactive HS-citrate lyase. Citrate lyase labeled with $|^{14}C|$ -acetate was found to lose one third of its activity within 4 days at $4^{\circ}C$ and this correlated with the loss in radioactivity indicating that spontaneous deacetylation of the active acetyl enzyme causes irreversible inactivation on storage. The inactive HS-citrate lyase can be reactivated either chemically by acetic anhydride (Buckel et al., 1971) or enzymatically by HS-citrate lyase ligase in presence of ATP and acetate (Schmellenkamp and Eggerer, 1974).

Citrate lyase preparations obtained from R. gelatinosa were reported by Beuscher et al. (1974) to be largely deacetylated and to contain considerable amounts of the oxidized enzyme. The enzyme could be reactivated only

after reducing with DTT or DTE, followed by reactivation with acetic anhydride. Citrate lyase crystallized from R. gelatinosa was found to be stable for 8 months at 4°C when suspended in 3 M ammonium sulfate. (Giffhorn and Gottschalk, 1978).

Singh and Srere (1975) observed that the purified enzyme from S. diacetilactis could be stored frozen at -90°C for weeks without appreciable loss in activity. They also observed that the enzyme loses activity above pH 8.1 through a dissociation process.

The stability of pure citrate lyase from S. faecalis was studied by Hiremath et al. (1976). The enzyme is stable when stored at 0° to -20°C in presence of ammonium sulfate, removal of ammonium sulfate decreasing the stability. Repeated freezing and thawing of the enzyme solution resulted in loss of activity and appearance of multiple peaks in sedimentation profiles.

Partially purified citrate lyase from E. coli rapidly loses activity particularly in dilute solutions or on prolonged dialysis (Wheat and Ajl, 1955). Nilekani (1980) has reported the stability of pure citrate lyase from E. coli. The enzyme is stable at 0°C in 0.05 M potassium phosphate buffer, pH 7.4 containing 1.6 mM MgSO₄ and ammonium sulfate, but loses activity rapidly if ammonium sulfate is removed from the solution. Freezing and thawing of the enzyme also leads to loss of activity.

Antranikian et al. (1982) have examined the stability of pure citrate lyase from C. sphenoides. L-Glutamate was

found to have a stabilizing effect on the purified enzyme. The enzyme was stable in presence of high concentrations (150 mM) of L-glutamate but at low concentration (7 mM) the enzyme was completely inactivated within 3 min at 0°C. In presence of high levels of glutamate the enzyme could be stored for 4 days at 4°C or for 2 months at -20°C without measurable loss in activity. But at 30°C the enzyme loses activity rapidly. The inactivation in absence of glutamate is reversible and not a result of deacetylation. The inactivation was correlated to a transition from star and ring forms to an undefined form seen in electron micrographs.

pH optimum:

The optimum pH of citrate cleavage by citrate lyases from K. aerogenes (Dagley and Dawes, 1955), E. coli (Dagley and Dawes, 1955; Wheat and Ayl, 1955b) and S. faecalis (Hiremath et al., 1976) is around 8.0. The pH optimum reported for the enzymes from S. diacetylactis (Harvey and Collins, 1963) and R. gelatinosa (Beuscher et al., 1974) is around 7.0 to 7.3. The enzyme from S. diacetylactis has been shown to lose activity above pH 8.1 and at pH 8.7 to undergo dissociation giving rise to two species with sedimentation coefficients of 5.3 S and 3.5 S (Singh and Srere, 1975). The pH optimum for the C. sphenoides enzyme has been reported to be between 6.5 and 7.0 (Antranikian et al., 1982).

(iii) Metal requirements:

Citrate lyases have an absolute requirement for divalent metal ions for the cleavage reaction. Dagley and Dawes (1955) showed that partially purified enzymes from K. aerogenes and E. coli are active in presence of Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} or Co^{2+} . Ca^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} and Hg^{2+} could not substitute for the active metal ions. Ca^{2+} was found to act as a linear competitive inhibitor of enzyme activation by Mg^{2+} , Mn^{2+} and other active metal ions. Dagley and Dawes (1955) postulated from the metal ion activation studies, that only those metal ions which have a restricted ionic radius of $0.72 \pm 0.08 \text{ \AA}$ can activate citrate lyase, while those such as Ca^{2+} ($0.99 \text{ \AA}$) with ionic radius outside this specific range have no activation effect.

The pH optimum of citrate lyase is in the range of 7.0 - 8.2, which approximately parallels the stability of metal-citrate complexes (Bobtelski and Jordan, 1945). On this basis Dagley and Dawes (1955) assumed that the substrate for citrate lyase is a metal-citrate complex that binds to the enzyme through the metal. The findings of Harvey and Collins (1963) that a 1:1 complex of citrate and divalent metal ion probably acts as a substrate for the S. diacetilactis citrate lyase was in agreement with the suggestion. Free Mg^{2+} was stated to act as a competitive inhibitor, probably by binding at the active sites of the enzyme. Ca^{2+} ions do not activate the enzyme, but do act as a competitive inhibitor to magnesium.

Later, however, Ward and Srere (1965) suggested from pulsed nuclear magnetic resonance studies on the partially purified citrate lyase of S. diacetilactis using Mn^{2+} as the activating metal, that a binary complex of metal-enzyme probably acts upon citrate rather than the enzyme acting upon a metal-citrate complex. Although they failed to observe a ternary complex of metal-citrate-enzyme using the pulsed technique, they recognized that the formation of such a complex could not be ruled out, since on addition of citrate to the binary metal-enzymes complex the reaction proceeds immediately and the steady-state concentration of a ternary complex could be quite low. They also obtained evidence in these studies that Ca^{2+} competes for the binding site of Mn^{2+} on the enzyme.

Recently SivaRaman and SivaRaman (1979) have reported on the nature and stoichiometry of metal cofactor binding by pure citrate lyase from K. aerogenes. Ultracentrifugal studies showed that in buffers of adequate ionic strength there was no change in the subunit structure of the enzyme on binding of divalent metal ions. The nature of metal binding was studied by using radioactive $^{54}MnCl_2$ and equilibrium dialysis. The binding isotherm, Scatchard and Hill plots indicated a significant extent of positive cooperativity in metal binding. The total number of binding sites were shown to be 18/mol of enzyme. These studies indicated that metal binding brings about conformational changes. Regulation of citrate lyase activity through such a process has been discussed

by these authors.

Metal requirements of pure citrate lyase from R. gelatinosa was reported by Beuscher et al. (1974). The metal ions indescending order of effectiveness in activating the enzyme were Mg^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} and Ni^{2+} . Cu^{2+} and Fe^{2+} could not activate the enzyme at all. Hiremath (1977) studied the effect of various metals on S. faecalis citrate lyase and observed that maximum activity is obtained with Mg^{2+} followed by Mn^{2+} , Zn^{2+} and Fe^{2+} . Co^{2+} and Cu^{2+} gave very low activity while Ca^{2+} , Ba^{2+} , Hg^{2+} and Ni^{2+} showed none. The enzyme from C. sphenoides has been shown by Antranikian et al. (1982) to require divalent metal ions, Mg^{2+} being the most effective. Mn^{2+} and Co^{2+} could partially replace Mg^{2+} while Zn^{2+} , Ni^{2+} , Cu^{2+} and Ca^{2+} did not activate the enzyme.

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 presence of EDTA (Buckel et al., 1973; Dimroth and Eggerer, 1975).

(iv) Reaction kinetics :

The kinetics of the citrate lyase catalysed reaction

are complex for the following reasons:

- (1) Citrate lyase multienzyme complex has two separate enzymatic activities associated with an acyl-carrier protein and the cleavage of citrate proceeds through a two step reaction (Dimroth and Eggerer, 1975b).
- (b) The enzyme shows an unique behaviour of reaction-inactivation (Singh and Srere, 1971) through the spontaneous hydrolysis of the unstable citryl-intermediate to citrate and inactive enzyme (Dimroth and Eggerer, 1975b).
- (c) The existence of the substrate, citrate, in various forms of chelates with divalent metal ions, such as Mg^{2+} .
- (d) Oxaloacetate, one of the reaction product, existing in both keto and enol forms, both of which are capable of chelating divalent metal ions.

Some of these factors were not recognised during the earlier studies on the kinetics of the citrate cleavage reaction. Consequently, these observations are of limited significance. The earlier work on the kinetics is summarized here in brief.

One mol of citrate is stoichiometrically broken down into one mol each of oxaloacetate and acetate by citrate lyase from *K. aerogenes* (Dagley and Dawes, 1973a and b; Dagley and Dawes, 1955) and *E. coli* (Dagley and Dawes, 1955; Wheat and Ajl, 1955b). That the reaction does not require coenzyme A was shown by removing the cofactor from crude extracts of *K. aerogenes* and *E. coli* with Dowex-1 (Dagley and Dawes, 1955). Harvey and Collins (1963) showed for the first time by spectral analysis

that the keto isomer of oxaloacetate is produced during the reaction catalyzed by the enzyme from S. diacetilactis and this was later confirmed by Ward and Srere (1965). That K. aerogenes citrate lyase also yields the keto-isomer was demonstrated by Tate and Datta (1964) by carrying out the reaction at 2°C and pH 7.4 to decrease the spontaneous equilibration of oxaloacetate isomers. Citrate lyase resembles citrate synthase in this respect since the reaction catalyzed by the latter enzyme also involves the keto-form of oxaloacetate (Englard, 1959).

The equilibrium of the reaction catalyzed by citrate lyase under optimal conditions favours breakdown of citrate. By using large excess of the enzyme from E. coli (Wheat and Ajl, 1955b) or from K. aerogenes (Bowen and Rogers, 1963b) it was shown that the reaction goes virtually to completion. The reversibility of the reaction could only be shown by radioactive exchange between ^{14}C -labeled acetate and citrate in presence of oxaloacetate, Mg^{2+} and citrate lyase from S. faecalis (Gillespie and Gunsalus, 1953) and from K. aerogenes (Bowen and Rogers, 1963b).

The equilibrium constant calculated for the cleavage of citrate to oxaloacetate and acetate is about 1 M (Burton, 1955). Several attempts were made to determine the equilibrium constant of the enzyme catalysed citrate cleavage. Earlier studies by Smith et al. (1956) and Harvey and Collins (1963) are of doubtful significance, since they did not take into account the complexities of

the reaction.

The equilibrium constant K , $(\text{citrate}^{-3})/(\text{OAA}^{-2} \text{ keto})$ (acetate^{-1}) was calculated by Tate and Datta (1965) who obtained a value of $3.08 \pm 0.72 \text{ M}^{-1}$ at a limiting concentration of citrate (2 mM) in presence of excess MgCl_2 at 25°C and using triethanolamine-HCl buffer, pH 8.4, which has no complexing effect on Mg^{2+} . The value obtained by Guynn *et al.* (1973) is $2.22 \pm 0.16 \text{ M}^{-1}$ at pH 7.0 in 0.025 M potassium phosphate buffer at 38°C and 10^{-3} M free Mg^{2+} . It was also observed that the value of K_{obs} varies with the change in Mg^{2+} concentration. When $(\text{Mg}^{2+})_{\text{free}} = 0$ the K_{obs} was $1.0 \pm 0.07 \text{ M}^{-1}$ while at $(\text{Mg}^{2+})_{\text{free}} = 10^{-2} \text{ M}$ the K_{obs} was 9. The effect of oxaloacetate-inhibition in these cases was minimised by reducing the substrate concentration.

(v) Stereospecificity of citrate enzymes:

Ogston In enzyme catalyzed lyase reactions, the two $-\text{CH}_2\text{COOH}$ groups of citrate are distinguished from each other (Ogston, 1948). Although there are no stereoisomers of citric acid, modifications of one of the two methylene groups will give rise to a number of assymetric isomers. Thus there are potential prochiral centres in citric acid. The two methylene groups for example can be represented by an extension of the standard R (rectus)/S (sinister) convention (Cohn *et al.*, 1966). This mode of representation is pictorially shown in Fig. 2. Here citric acid is represented in a Fischer projection with R/S convention labeling of the molecule. Here C-3 is one of the

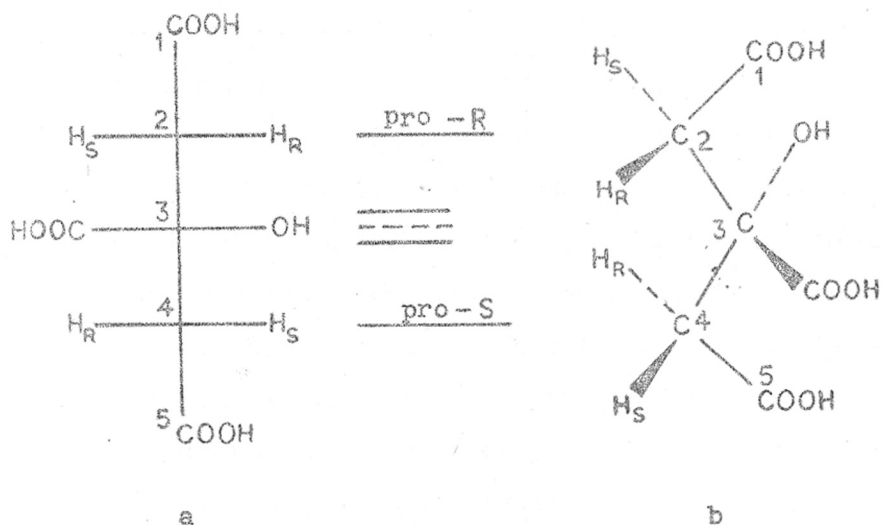


Figure 2. DEPICTIONS OF CITRIC ACID

a - As a pentane derivative

b - Perspective representation according to
(pro-R / pro-S) nomenclature.

(Adapted from Glusker and Srere, 1973)

pro-chiral centres. Fig. 2a shows citric acid depicted as a pentane derivative with the backbone carbon atoms numbered 1 to 5. Fig. 2b shows the perspective depiction with the central carbonyl group above the plane of the paper and the hydroxyl group below the plane, both being on the right of the carbon backbone. The two $-\text{CH}_2\text{COOH}$ groups are distinguished by the pro-R and pro-S system (Hirschmann and Hanson, 1971) and according to convention the pro-S part of the molecule is designated with higher numbers (Fig. 2b). On the same basis the hydrogen atoms of the methylene groups at C_2 and C_4 , which represent the other two pro-chiral centres, of citric acid, can also be distinguished. Thus the H atom on C-2 in Fig. 2b can be represented as the pro (2S, 3R) hydrogen atom of the citric acid molecule.

A number of investigations have led to the understanding of the stereospecificity of the different enzymes involved in the synthesis and breakdown of citrate (Eggerer *et al.*, 1970; Retery, 1970; Lenz *et al.*, 1971; Wunderwald *et al.*, 1971; Srere, 1975). From these investigations it has been well established that in the reaction catalyzed by the citrate synthase of the citric acid cycle, the acetyl moiety of acetyl-CoA attacks on the si-face of oxaloacetate (Hanson and Rose, 1963) and the resulting citrate has C-4 and C-5 derived from this acetyl moiety. Citrate lyase and ATP-citrate lyase have been shown to have the same stereospecificity. Using crude

preparation of citrate lyase from S. faecalis, Gillespie and Gunsalus (1953) showed that the enzyme removes the pro-3S-CH₂COOH group as acetate, which is the acetate moiety put on by si-citrate synthase. Similar results were obtained by Wheat and Ajl (1955) using a partially purified preparation of E. coli citrate lyase. Buckel et al. (1971) have studied the stereospecific reaction of citrate lyase using ¹⁴C-labeled citrate and purified citrate lyase from K. aerogenes. They observed that (5 - ¹⁴C) (3S)-citrate gives ¹⁴C-acetyl-citrate lyase and unlabeled oxaloacetate while |1 - ¹⁴C| (3R) citrate yields unlabeled citrate lyase and ¹⁴C-oxaloacetate. Srere and Bhaduri (1964) have shown that ATP citrate lyase also removes the pro-3S-CH₂COOH group of citrate as acetyl-CoA.

The only exception among the citrate enzymes to the si-attack stereospecificity is shown by re-citrate syntheses found in certain anaerobic bacteria. This enzyme catalyzes the synthesis of citrate in which the pro-(3R)-CH₂COOH group of citrate (C-1 and C-2) is derived exclusively from the acetyl-moiety of acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966).

Stereospecificity of all citrate enzymes including the re-citrate synthase has been shown to be similar with respect to the two pro-chiral centres of citrate involving the C-2 and C-4 methylene groups. Using the chiral acetates (R) (²H, ³H) acetate and (S) (²H, ³H) acetate it was found that an inversion of configuration of the protons on the methylene group of acetyl moiety

takes place during the reaction catalysed by all the citrate enzymes (Eggerer et al., 1970; Klinman and Rose, 1971; Reter et al., 1970; Buckel et al., 1971b).

(vi) Inhibition by oxaloacetate:

Citrate lyase from K. aerogenes and E. coli comes under product inhibition by oxaloacetate (Dagley and Dawes, 1955; Wheat and Ajl, 1955b). Acetate and pyruvate have no inhibitory effect. Later Bowen and Rogers (1963a) observed that the inhibition was time dependent and did not result in any change in absorption spectra or sedimentation behaviour of the enzyme. They observed that malate also causes irreversible inactivation of the enzyme while other carboxylic acids like oxalate, succinate, oxaloglutarate, tartarate, oxalosuccinate and isocitrate have no inhibitory effect.

Eisenthal et al. (1966) investigated the structural features of the inhibition by using different analogues of oxaloacetate. The inhibition was found to be irreversible and removal of oxaloacetate by malate dehydrogenase and NADH did not reverse the inhibition. The inhibition by oxaloacetate and its analogues was also found to be dependent on the presence and the nature of divalent metal ions. Inhibition in presence of Co^{2+} was more severe than that with Mg^{2+} , while Ca^{2+} was less effective than Mg^{2+} . The pH profile of the inhibition indicated the possible involvement of the enolic form of oxaloacetate and probably of two different

Mg^{2+} -oxaloacetate (enolic) complexes. The hypothesis was supported by the fact that analogues such as dimethyl-oxaloacetate and ketomalonate, which cannot enolise, do not show any inhibitory effect in presence of Mg^{2+} , while the derivatives which could yield enolic forms, inhibited the enzyme. From these results the authors postulated that the structural requirement for an inhibitor is a C_4 straight chain, 1 : 4 dicarboxylic acid with an ionizable -hydroxyl group.

Buckel et al. (1971a) showed later that the mechanism of inactivation by oxaloacetate is through the loss of essential acetyl groups of the enzyme to give the inactive deacetylated citrate lyase. The inactivated enzyme can be reactivated by chemical or enzymatic reacetylation.

(vii) Essential acetyl group:

Buckel et al. (1971a) provided the earliest evidence for the involvement of protein bound essential acetyl groups in the reaction mechanism of *K. aerogenes* citrate lyase. The evidences in support of this were the following:

(a) Active citrate lyase was inactivated irreversibly on treatment with hydroxylamine. This inactivation was apparently caused by deacetylation since ^{14}C -aceto-hydroxamic acid was produced on treatment of ^{14}C -labeled enzyme with hydroxylamine.

(b) Treatment of ^{14}C -labeled enzyme with oxaloacetate or cold citrate in presence of Mg^{2+} resulted in the loss of the ^{14}C -labeled as acetate which could be separated

from the enzyme protein by gel filtration. This observation led to the conclusion that an acyl exchange takes place during the reaction catalyzed by the enzyme.

(c) The kinetics of hydroxylaminolysis of standard thioesters like N-succinyl-S-acetyl cysteamine were found to be similar to the hydroxylamine inactivation of the enzyme. The mercaptans, dithiothreitol or dithioerythreitol were also able to inactivate citrate lyase. These data clearly showed that the linkage of the essential acetyl moiety was through thioester bonds.

(d) Citrate lyase inactivated by hydroxylamine, mercaptans, oxaloacetate in presence of Mg^{2+} or by reaction-inactivation, could be reactivated chemically with acetic anhydride.

Further evidence for acetyl exchange was obtained in experiments with stereospecifically labeled ^{14}C -citrate (Buckel *et al.*, 1971a). Use of $[5-^{14}C]-(3S)$ -citrate as a substrate for citrate lyase reaction results in ^{14}C -labeled enzyme and non-radioactive oxaloacetate, while $[1-^{14}C]-(3R)$ -citrate yields ^{14}C -labeled oxaloacetate and unlabeled enzyme. This data could be explained only on the basis of the turn over of the acetyl moiety during the course of citrate cleavage through a two step reaction: (1) an acyl-exchange reaction with the formation of a citryl-enzyme intermediate and (2) an acyl-lyase reaction, the citryl-intermediate being cleaved at the *si*-face of oxaloacetate.

These findings were substantiated and extended to citrate lyases from other sources. Srere et al. (1972) observed that after deacetylation, the oxidation of the liberated thiol groups prevented acetylation by acetic anhydride. This was confirmed by inactivating the enzyme, either by reaction-inactivation or by hydroxylamine treatment, in the presence of DTNB. Under these conditions, the enzyme could be reactivated only after prior incubation with dithiothreitol. Deacetyl citrate lyase from several other sources like S. diacetilactis, K. aerogenes, R. gelatinosa and L. citrovorum could be reactivated chemically with 1-acetyl imidazole (Kummel et al., 1975). It was shown by Buckel et al. (1973) that only the deacetyl citrate lyase could be reactivated with acetic anhydride, while the carboxy-methylated derivative of the enzyme could not be reactivated.

In addition to chemical reactivation, deacetyl citrate lyase is also reactivated by a specific enzyme, acetate:SH (acyl-carrier protein) citrate lyase ligase (AMP). The enzyme was first characterized by Schemellenkamp and Eggerer (1974) in cell extracts of K. aerogenes and was shown to catalyze acetylation of deacetyl citrate lyase in presence of ATP and acetate. The ligase was partially purified from S. faecalis (Hiremath et al., 1976) and was found to acetylate the deacetyl enzyme from K. aerogenes also. In contrast the homogeneous ligase preparation from S. diacetilactis was specific for the

citrate lyase from only this source, having no action on citrate lyase from R. gelatinosa or K. aerogenes (Bowien and Gottschalk, 1977). The ligase purified from R. gelatinosa acetylates the deacetyl citrate lyase from R. palustris in addition to the one from R. gelatinosa but has no effect on the enzymes from K. aerogenes, S. diacetilactis and C. sphenoides (Antranikian and Gottschalk, 1982).

An interesting observation made by Buckel et al. (1973) was that the inactive deacetyl citrate lyase cleaves citrate catalytically in presence of Mg^{2+} and acyl-CoA derivatives such as acetyl-CoA or (3S)-citryl-CoA. In presence of acetyl-CoA, citrate is cleaved to oxaloacetate and acetate, while (3S)-citryl-CoA itself is broken down to acetyl-CoA and oxaloacetate in presence of Mg^{2+} . Using ^{14}C -labeled citrate it was shown that during the acyl-CoA mediated citrate cleavage, the acetyl-moiety of the acetyl-CoA is liberated as free acetate while the pro-(3S)- CH_2COOH group of citrate is transformed to acetyl-CoA. In the presence of EDTA, (3S)-citryl-CoA was shown to accumulate in the acetyl-CoA mediated reaction. These results clearly established the multienzyme nature of citrate lyase involving two reactions; an acyl exchange which is independent of Mg^{2+} and an acyl lyase reaction, which is Mg^{2+} dependent, in which the citryl-intermediate is cleaved. The formation of the postulated citryl intermediate was established by isolation of citryl-CoA which accumulates in presence of HS-citrate lyases; citrate,

acetyl-CoA and EDTA. Buckel et al. (1973) also observed that acetyl-dephospho-CoA and propionyl-CoA could replace acetyl-CoA in the mediated citrate cleavage reaction. The sequence of the reaction catalyzed by propionyl-CoA is



Citrate lyases from *S. diacetilactis* (Singh and Srere, 1975), *R. gelatinosa* (Kummel et al., 1975), *S. faecalis* (Hiremath et al., 1976) and *E. coli* (Nilekani and SivaRaman, 1983) have also been shown to be acetyl-enzymes.

(viii) Reaction inactivation:

Citrate lyase from *K. aerogenes* undergoes rapid, irreversible inactivation in vitro during the course of citrate cleavage. This unique phenomenon of reaction-inactivation has been termed as "suicide" (Singh and Srere, 1971).

Earlier work by Wheat and Ajl (1955b) with partially purified *E. coli* citrate lyase had shown that the rate of citrate cleavage decreased to zero during the course of the reaction. They attributed this inactivation to the reaction product oxaloacetate, which was known to inhibit the enzyme irreversibly. It was, however, shown later by Singh and Srere (1971) that the irreversible reaction inactivation could not be due to oxaloacetate since oxaloacetate (both keto and enol forms) at a concentration level 1000 fold more than that produced during the citrate lyase

catalyzed reaction, failed to inactivate the K. aerogenes enzyme completely. Even under conditions where the oxaloacetate formed was removed continuously from the reaction system by coupling the reaction with malate dehydrogenase and NADH, there was no decrease in the rate of inactivation. The process of reaction-inactivation was found to follow simple first order kinetics. The nature of divalent metal cofactor was shown to effect the rate of reaction-inactivation. Amongst the several metals tested Zn^{2+} gave the slowest rate while Mg^{2+} gave the highest rate of inactivation (Singh and Srere, 1971). The initial rate of citrate cleavage was however, much less in the presence of Zn^{2+} than in the presence of Mg^{2+} . Although the mechanism of reaction-inactivation is not well understood, it is known that the reaction-inactivation process leads to the loss of the essential acetyl moiety of the enzyme. This was concluded from the fact that reaction inactivated enzyme could be reactivated chemically by acetic anhydride (Srere et al., 1972). A possible mechanism of reaction-inactivation was suggested by Buckel et al. (1973) on the basis of the labile nature of the citryl-intermediate from analogy with the spontaneous hydrolysis of Citryl-CoA observed at alkaline pH. Dimroth and Eggerer (1975b) suggested that this process is possibly due to the hydrolysis of the citryl-intermediate to citrate and inactive deacetyl enzyme catalysed by the transferase () subunit of the citrate lyase complex, since such as effect on $|1,5-^{14}\text{C}|$ -citryl-ACP was observed in presence of the isolated

-subunit.

On the basis of their reaction-inactivation behaviour, citrate lyases from different sources can be classified under two groups; ones which undergoes rapid reaction-inactivation such as the enzymes from K. aerogenes (Singh and Srere, 1971) and R. gelatinosa (Kummel et al. 1975) and others which show only a weak reaction-inactivation like the enzymes from S. diacetylactis (Singh and Srere, 1975), L. citrovorum (Kummel et al., 1975) and S. faecalis (Hiremath et al., 1975). The process of reaction-inactivation of the enzymes follows first order kinetics and the rate constants (K_1) for the enzymes from K. aerogenes (Singh and Srere, 1975), E. coli (Nilekani and SivaRaman, 1983), S. diacetylactis (Singh and Srere, 1975) and S. faecalis (Hiremath, 1977) at 25°C in presence of Mg^{2+} were found to be 1.21, 0.32, 0.06 and 0.07 min^{-1} respectively. The corresponding value of K_1 in presence of Zn^{2+} for the enzymes from K. aerogenes and S. diacetylactis are 0.17 and 0.014 min^{-1} .

The weak reaction-inactivation observed was throu to be due to the presence of an HS-citrate lyase acetylating activity associated with the native citrate lyase complex (Kummel et al., 1975), since such an activity could be detected in the citrate lyase preparations from S. diacetylactis and L. citrovorum but not in enzymes from K. aerogenes or R. gelatinosa. However, Hiremath et al. (1976) showed that the enzyme complex from S. faecalis,

which also undergoes weak reaction-inactivation does not contain any acetylating activity. Bowien and Gottschalk (1977) later found that the acetylating activity observed earlier in the enzyme complex from S. diacetilactis was actually an impurity which could be separated from the citrate lyase complex.

(ix) Molecular weight:

Citrate lyases in crude cell extracts of E. coli and K. aerogenes were shown to sediment with a distinct 12S - 16S component seen only in citrate grown cells (Bowen and Dagley, 1955; Dagley and Sykes, 1956). Later, using a homogeneous preparation of K. aerogenes citrate lyase SivaRaman (1961) obtained a $S_{20,w}$ value of 16. The molecular weight of citrate lyase from K. aerogenes which was earlier reported as 314 000 - 318 000 (Bowen and Rogers, 1963a) was shown by Mahadik and SivaRaman (1968) to be considerably higher and a value of 575 000 was obtained from transient state sedimentation equilibrium ultracentrifugation. The higher value was confirmed by Bowen and Mortimer (1971) and obtained a value of 566 000 by transient state method and 550 000 by high speed sedimentation method. Carpenter et al. (1975) and Singh et al. (1976) also reported similar values.

Citrate lyases from all other sources have been found to resemble the K. aerogenes enzyme in their molecular weights. R. gelatinosa citrate lyase has a molecular weight of 530 000 - 560 000 (Beuscher et al.,

1974). Pure citrate lyase from S. diacetilactis has a molecular weight of 585 000 and a $S_{20,w}$ value of 16.8. Hiremath et al. (1976) reported the molecular weight of S. faecalis citrate lyase as 600 000 with a $S_{20,w}$ value of 17.1 S. The molecular weight of the enzyme from C. sphenoides has been shown recently by Antranikian et al. (1982) to be around 515 000 using gel filtration and sucrose density gradient. The native enzyme from E. coli has been shown to exist in multimeric states of aggregation. In addition to the major 16.6 S component, 10 S, 20.4 S, 23.7 S and 30 S species have been observed in sedimentation profiles (Nilekani and SivaRaman, 1983).

Citrate lyase from K. aerogenes dissociates reversibly in buffers of low ionic strength and in the absence of divalent metal ions (Mahadik and SivaRaman, 1968). In 1 mM potassium phosphate buffer, pH 7.5 containing 2 mM EDTA an half-molecule of 273 000 daltons sedimenting at 10.3 S was obtained while in 1 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA an additional component sedimenting at 6.5 S was observed. Mg^{2+} at a concentration of 2 mM or an increase in the ionic strength of the buffer completely reversed the dissociation of the enzyme complex. Bowen and Mortimer (1971) and Dimroth et al. (1973) also observed similar behaviour. No such dissociation was shown by the enzyme complex from S. faecalis (Hiremath, 1977).

(x) Subunit structure :

Early investigations on the subunit structure of citrate lyase from K. aerogenes were based on analytical ultracentrifugation under denaturing conditions (Mahadik and SivaRaman, 1968; Bowen and Mortimer, 1971). The heterogeneous nature of the dissociated system and the low resolution of the technique were probably responsible for the erroneous conclusions drawn from these. Bowen and Mortimer (1971) observed the presence of two components of 33 000 and 54 000 daltons on SDS-polyacrylamide gel electrophoresis, but the 54 000 dalton component was believed to be an artifact, since a value of 33 000 dalton was calculated from sedimentation equilibrium runs and no heterogeneity was apparent from the sedimentation profiles.

The presence in the enzyme from K. aerogenes of a small subunit having a molecular weight of about 10 000 daltons was first reported by Dimroth et al. (1973) and this component was shown to function as an acyl-carrier protein (ACP). The presence of two other non-identical subunits of 55 800, 33 800 in addition to an 11 900 dalton subunit was reported by Singh et al. (1974) from SDS-polyacrylamide gel electrophoresis analysis. The molecular weights of the three different subunits were determined precisely by Carpenter et al. (1975) using various techniques such as sedimentation equilibrium in 6 M guanidinium-HCl, SDS-polyacrylamide gel electrophoresis and filtration through 6% agarose beads in presence of

6 M guanidinium-HCl. The values reported by them for the three subunits of K. aerogenes citrate lyase were 54 000 (), 32 000 () and 11 000 () daltons. Dimroth and Eggerer (1975a) obtained similar estimates for the molecular weights of the three subunits. The exact molecular weight of the -subunit(ACP) computed from its amino acid sequence analysis is 9378 (Beyreuther et al., 1978). The molecular weights of the subunits in the citrate lyases from K. aerogenes and other sources is summarized in Table II.

The 54 000 dalton subunits in K. aerogenes (Dimroth and Eggerer, 1975a) and in E. coli (Nilekani and SivaRaman, 1983) have been shown to function as the acyl-transferase () subunit and the 32 000 dalton subunit as the acyl-lyase, while in other sources the functions have been attributed to the two subunits through analogy with those of corresponding size in the K. aerogenes complex. The smallest subunit in all cases except in the E. coli enzyme functions as the ACP () subunit and has been shown to contain the pantothenate moiety. E. coli citrate lyase differs markedly from the enzyme from other sources in the unusually large size of the ACP subunit which carries 4 pantothenate moieties on a single polypeptide chain of 85 000 daltons (Nilekani and SivaRaman, 1983) unlike the 10 000 dalton K. aerogenes citrate lyase ACP with a single prosthetic group (Dimroth, 1975).

Table II. Molecular weights of subunits of citrate lyase complex from different sources

Subunit	Source					
	<u>Klebsiella</u> <u>aerogenes</u> ^a	<u>Streptococcus</u> <u>diacetylactis</u> ^b	<u>Streptococcus</u> <u>faecalis</u> ^c	<u>Rhodopseudomonas</u> <u>gelatinosa</u> ^d	<u>Clostridium</u> <u>sphenoides</u> ^e	<u>Escherichia</u> <u>coli</u> ^f
Transferase ()	54 000	54 000	54 000	55 600	56 000	54 000
Lyase ()	32 000	32 000	37 000	31 600	32 000	32 000
ACP ()	10 000	12 000	14 000	11 400	11 700	85 000

^aCarpenter et al. (1975)^bSingh and Srere (1975)^cHiremath et al. (1976)^dGiffhorn and Gottschalk (1978)^eAntranikian et al. (1982)^fNilekani and Sivaraman (1983)

(xi) Subunit stoichiometry :

The presence of a low molecular weight (ACP) subunit in the citrate lyase enzyme complex of K. aerogenes has posed difficulties in the estimation of the stoichiometry of this subunit in the complex from gel scans after separation by SDS-polyacrylamide gel electrophoresis and staining with dye. Such gel scans of K. aerogenes enzyme showed the presence of the and subunits in equimolar amounts with a computed content of 6 copies of each in the complex (Dimroth and Eggerer, 1975a; Carpenter et al., 1975). Dimroth and Eggerer (1975a) observed, however, that the value for the -subunit determined by this procedure varied with the period of staining. This was attributed to the loss of the small subunit by diffusion from the gel. SDS-gel scans yielded an approximate ratio of 1:1:0.7 for the and subunits of K. aerogenes citrate lyase (Dimroth and Eggerer, 1975a; Carpenter et al., 1975). The three subunits were, however, assumed to occur in equimolar proportions on the considerations of symmetry and the known losses of the -subunit in the assay process.

Attempts were also made to estimate the stoichiometry of the ACP subunit in the complex by chemical and microbiological assay of the prosthetic group component which is exclusively associated with the ACP () subunit. The values obtained by this approach were in the range of 3 - 5 ACPs/mol of the enzyme complex (Srere et al., 1972; Dimroth, 1975). A value of 3 - 4 mol pantothenate

per mol enzyme was also obtained by Singh et al. (1975) by using ^{14}C -labeled pantothenate and a pantothenate-less mutant of K. aerogenes. The ^{14}C -pantothenate-labeled enzyme when doubly labeled with ^3H -citrate yielded in ^3H -acetyl- ^{14}C -pantothenate complex which showed on SDS-polyacrylamide gel electrophoresis that the labeled acetyl is also exclusively associated with the ACP subunit.

Srere et al. (1976) used a novel technique of amino acid sequencing to establish the presence of equimolar amounts of the three subunits in the holoenzyme from K. aerogenes. Each of the separated subunits were sequenced upto 10 residues from the N-terminus. Subsequently the holoenzyme was sequenced and the ratios of the amino acids released at various positions were quantitated. From these values the ratio of the three subunits in citrate lyase was calculated to be 1:1:1. It was concluded from the determined stoichiometry and the earlier estimates of the prosthetic group that in the holoenzyme two chains of the acyl carrier protein lack pantothenate moieties.

The subunit stoichiometry of citrate lyase complex from S. faecalis was determined by Hiremath (1977) using gel scans of Coomassie Brilliant blue stained SDS-polyacrylamide gels. As in the case of the K. aerogenes enzyme the molar ratio of and subunits was found to be 1:1:0.7. Pantothenate assay in both S. faecalis (Hiremath, 1977) and S. diacetilactis (Singh and Srere,

1975) indicated the presence of only 4 mols of pantothenate in the holoenzyme. Electron microscopic studies carried out on the enzyme supports the hexameric structure of citrate lyase and these are reviewed in the following sections.

The subunit stoichiometry of citrate lyase complex from E. coli has been recently reported by Nilekani and SivaRaman (1983) and have shown from SDS-gel scans that the enzyme complex consists of six copies each of the α - and β -subunits and a single copy of the γ -subunit. This is the first report of a citrate lyase having a subunit stoichiometry which differs from the general hexameric structure of $\alpha_6 \beta_6 \gamma_6$ observed in the enzyme complexes from K. aerogenes, S. faecalis, S. diacetilactis and R. gelatinosa.

(xii) Subunit organization :

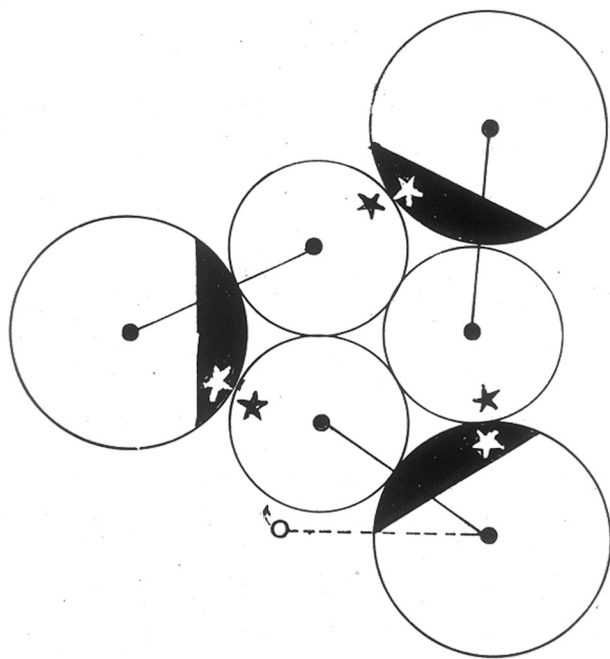
Preliminary electron microscopic studies carried out on R. gelatinosa citrate lyase using negative staining techniques (Beuscher et al., 1974) revealed two types of projections, a ring like structure (160 A diameter) assumed to be the face on view and a sandwich pattern (100 A thick) consisting of two parallel rows of density which was assumed to be the side on view. The ring structure was shown to be made up of three large ellipsoidal structures (axes of 58° and 49A) and three small spheres (diameter 43° A) arranged in alternate sequence. Two such rings were shown to face each other in a hexameric

structure. At the time these studies were carried out, R. gelatinosa citrate lyase was thought to be made up of only two types of subunits and the location of the 10 000 dalton subunit was not considered. Citrate lyase from K. aerogenes has also been reported to exhibit similar profiles in electron micrographs (Dimroth and Eggerer, 1975a; Schramm, 1981).

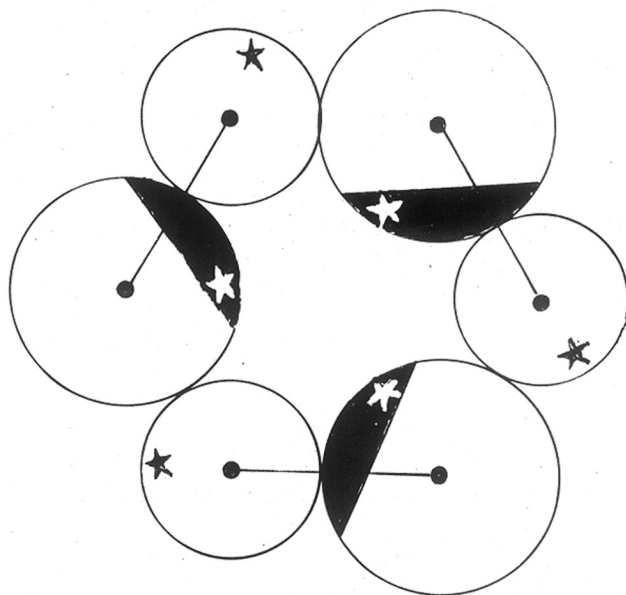
Antranikian et al. (1982) observed the presence of three distinct configurational projections in electro-micrographs of citrate lyases from R. gelatinosa, S. diacetylactis and C. sphenoides. These forms were designated as "ring", "star" and "triangle" forms. It was assumed that the intensity maxima represent the large () subunits and the minor ones () subunits. The smallest subunit () was not resolved. The relative proportion of "triangles" was very small, representing only about 1% of all molecules and was thus assumed to be a transition between the other two forms. In citrate lyases from R. gelatinosa and S. diacetylactis the "ring" form accounted for about 90% and the "star" form about 8-9% of the total molecules. In the C. sphenoides enzyme, which requires glutamate for activity, the star form predominated over ring forms in presence of glutamate. Removal of glutamate resulted in the appearance of undefined particles. In solutions containing citrate lyase from S. diacetylactis, it was observed that the proportion of stars increased with inclusion of

citrate or its analogue, tricarballylate, indicating that the two forms are interconvertible .

The arrangement of all the 18 subunits in the multi-enzyme complex from *R. gelatinosa* and their cooperation during the enzyme reaction was studied in detail by using cross-linking reagents and immunoelectron microscopy with antibodies directed against native citrate lyase and the isolated subunits (Zimmerman *et al.*, 1982). From results obtained in these studies working model has been proposed based on the observed star and ring forms of citrate lyase as shown in Figure 3. In the face on view, the ring form is made up of alternating large () subunits and middle sized () subunits with each large () subunit carrying one of the small subunit () as a polar cap. Each layer comprising three copies of each subunit represents a structural unit. According to the model, the ring form (Figure 3a) represents the configuration of the enzyme where acyl-transfer occurs on the -subunit (ACP) forming a citryl-S-citrate lyase intermediate. At this stage a rotation occurs around the vertical axis of the -subunit resulting in the star form (Figure 3b) where the citryl residue on the -subunit (ACP) is assumed would be brought close to the active site of -subunit (lyase). In the star form the citryl intermediate is cleaved into free oxaloacetate and acetyl-S-citrate lyase and the enzyme returns to the ring form by the reversal of the postulated rotation. The triangles are assumed to be a transition form.



STAR FORM



RING FORM

FIG. 3 Proposed structure-function model for citrate lyase from R. gelatinosa.

(Adapted from Zimmermann et al., 1982)

(Xiii) Multienzyme nature of citrate lyase :

The following evidences led to the recognition of the multienzyme nature of the citrate lyase.

(a) Buckel et al. (1971a) showed using specifically labeled ^{14}C -citrate that the K. aerogenes enzyme catalyzed citrate cleavage through a two step reaction, with the formation of a citryl intermediate which is cleaved in the next step to oxaloacetate with the regeneration of active acetyl enzyme.

(b) Buckel et al. (1973) found that inactive deacetyl K. aerogenes citrate lyase can catalyze the cleavage of citrate in presence of acetyl-CoA or citryl-CoA. Using these two CoA derivatives they showed conclusively that the cleavage reaction proceeds via two distinct enzymatic steps.

(c) Dimroth et al. (1973) showed the presence of a small molecular weight (10 000 daltons) subunit in the K. aerogenes citrate lyase which functions as an acyl-carrier protein. The subunit carries the covalently bound CoA-like prosthetic group (Srere et al., 1972; Dimroth, 1975; 1976; Robinson et al., 1976). The presence of two other non-identical subunits having molecular weights of 54 000 () and 32 000 () daltons in addition to the ACP () subunit was shown by Singh et al. (1974).

(d) Dimroth and Eggerer (1975b) established unequivocally the multienzyme nature of citrate lyase from K. aerogenes by isolating and characterising the function of each subunit.

The 54 000 daltons () subunit was shown to catalyze the formation of $|1,5-^{14}\text{C}|-$ citryl-ACP and acetate in presence of 10 000 daltons acetyl-ACP and $|1,5-^{14}\text{C}|-$ citrate. Thus the subunit was shown to be an acyl-transferase.



The catalytically active form of the isolated subunit was found to be as a dimer () and the reaction was shown to occur in presence of EDTA, being independent of the presence of divalent metal cofactors such as Mg^{2+} . The 32 000-dalton () subunit was shown to function as an lyase subunit catalyzing the cleavage of $|1,5-^{14}\text{C}|-$ citryl-ACP to $|1-^{14}\text{C}|-$ oxaloacetate and the acetyl-ACP.



This subunit has an absolute requirement of divalent metal ions like Mg^{2+} for activity.

Further, the active complex, which was identical to the native enzyme in its sedimentation behaviour as well as its specific activity, was reconstituted from the three separated subunits.

Citramalate lyase from Clostridium tetanomorphum has been shown to resemble citrate lyase in being a built-in multienzyme complex with subunits of similar molecular weights, functions, stoichiometric proportion and similar prosthetic group composition indicating similar mechanisms of action (Buckel and Bobl, 1976).

Citrate lyase from E. coli has recently been shown to have similar enzyme subunits as the K. aerogenes enzyme, although the associated acyl-carrier protein () subunit is a single large polypeptide chain carrying 4 covalently bound prosthetic groups (Nilekani and SivaRaman, 1983).

(xiv) Effect of citrate analogues :

Sullivan et al. (1977) and Stallings et al. (1979) have studied the effects of hydroxycitrates on citrate enzymes. All the four isomers of hydroxycitrate were found to function as linear competitive inhibitors for citrate in the reaction catalyzed by citrate lyases from K. aerogenes and S. diacetilactis. The kinetic constants are reproduced in Table III. It is evident from the K_1 values that (2S, 3S)-2-hydroxycitrate is the strongest inhibitor and the (2R, 3S)-2-hydroxycitrate isomer the weakest for the K. aerogenes citrate lyase, while for the S. diacetilactis enzyme (2R, 3R)-2-hydroxycitrate is the strongest inhibitor and the (2R, 3S) isomer the weakest.

Except (2S, 3R)-2-hydroxycitrate, all the other isomers were found to serve as a substrate for the citrate lyase reaction, (2R, 3R)-2-hydroxycitrate undergoing the fastest reaction in presence of citrate lyase. The inactivation by hydroxycitrate isomers was shown to be due to the loss of the essential acetyl moiety from the enzyme complex through a process of reaction-inactivation similar to that in presence of citrate.

From the varying abilities of the four isomers to

inactivate citrate lyase and to undergo reaction in presence of citrate lyase and from the postulated conformations of metal citrate chelates, interesting speculations were made regarding the geometry of substrate binding to the enzyme (Stallings et al., 1979). It was postulated that if hydroxycitrate binds in a "citrate like" manner, then the $C_3 - C_4$ bond of the substrate is oriented in a position favourable for cleavage. However, in alternate modes of binding, in which the terminal hydroxyl and the central hydroxyl groups are involved, the enzyme is inhibited probably as a result of nonproductive binding.

Recently Rokita et al. (1982) have reported the use of a novel fluorinated analogue of citrate, 3-fluoro-3-deoxycitrate (3-fluorocitrate), as a mechanistic probe for the citrate utilizing enzymes. Making use of differential abilities of acetic anhydride and acetate to reactivate inactive citrate lyase, the authors have shown that 3-fluorocitrate inactivates at two points during the reaction of acetyl-citrate lyase. First the inhibitor can form a mixed acetic acid-3-fluorocitric acid anhydride intermediate which diffuse away from the active site leaving the enzyme in the deacetylated, inactive form, which can be reactivated by acetic anhydride. It can also inactivate the enzyme by forming the 3-fluorocitryl-S-enzyme intermediate which renders the enzyme inactive, since 3-fluorocitrate cannot be cleaved between the C_2 and C_3 positions. Then 3-fluorocitryl-S-

enzyme is not reactivated by acetic anhydride but the 3-fluorocitrate is displaced by nucleophilic acetate ion or treatment with sodium acetate. Experimental data indicated a 1:1 distribution of inactive enzymes initially between the two types of inactivation.

Table III

Enzyme	Inhibitor	K_m (citrate) uM	K_i uM
<u>Klebsiella</u> <u>aerogenes</u> citrate lyase	(2 <u>S</u> ,3 <u>S</u>)-2-hydroxycitrate	57	8
	(2 <u>R</u> ,3 <u>R</u>)-2-hydroxycitrate	52	31
	(2 <u>R</u> ,3 <u>S</u>)-2-hydroxycitrate	59	178
	(2 <u>S</u> ,3 <u>R</u>)-2-hydroxycitrate	36	51
	Tricarballoylate*	160	1090
	3-fluorocitrate*	160	43
<u>Streptococcus</u> <u>diacetylactis</u> citrate lyase	(2 <u>S</u> ,3 <u>S</u>)-2-hydroxycitrate	245	279
	(2 <u>R</u> ,3 <u>R</u>)-2-hydroxycitrate	253	168
	(2 <u>R</u> ,3 <u>S</u>)-2-hydroxycitrate	232	419
	(2 <u>S</u> ,3 <u>R</u>)-2-hydroxycitrate	254	248

Reproduced from Sullivan et al. (1977)

*Values taken from Rokita et al. (1982)

(xv) Amino acid composition:

Holoenzyme : The amino acid composition of K. aerogenes citrate lyase holoenzyme was estimated by Bowen and Rogers (1965) using high voltage paper electrophoresis for separation of the component amino acids in the acid hydrolysate. Singh et al. (1976) used the more standard method of ion exchange chromatography for the automated analysis of the amino acids and observed considerable differences in the contents of several residues reported by Bowen and Rogers (1965). Singh et al. (1976) also reported that all the three subunits of the enzyme complex contain methionine at the N-terminus and not lysine as reported by Bowen and Mortimer (1971).

The amino acid composition of the enzyme complex from S. faecalis was shown to be markedly different from the K. aerogenes enzyme (Hiremath, 1977). The partial specific volume of the enzyme from S. faecalis was calculated from the amino acid data and a value of 0.742 ml.g⁻¹ was obtained. The corresponding value for the K. aerogenes enzyme is 0.737 ml.g⁻¹ (Singh et al., 1976). The amino acid composition of purified citrate lyase from E. coli was reported by Nilekani (1980) and was found to resemble that of the K. aerogenes enzyme.

Subunits : Singh et al. (1976) determined the amino acid composition of the isolated (M_r 54 000) and (M_r 32 000) subunits of K. aerogenes citrate lyase. The amino acid sequence for ten residues from the N-terminus was also determined for each of the subunits. All had

methionine as N-terminal residue and leucine at the 8th position. Microheterogeneity was observed in the subunit sequence, proline and arginine occurring in equal proportions at the 4th position.

The amino acid composition of the isolated ACP () subunit of K. aerogenes citrate lyase was first reported by Dimroth et al. (1973). The ACP subunit contains a cysteamine and a cysteine residue, both acylated. This was shown by treating the ACP first with unlabeled iodoacetate followed by deacetylation and subsequent treatment with iodo[2-¹⁴C]acetate. Analysis of the acid hydrolysate of the ¹⁴C-labeled ACP showed that the radioactive label was equally distributed in both cysteamine and cysteine.

Dimroth and Eggerer (1975a) showed by affinity labeling of deacetyl citrate lyase by iodoacetate in presence of excess dithioerythritol that the cysteamine residue of the ACP subunit is apparently the biologically active acyl-carrier.

Beyreuther et al. (1978) determined the complete amino acid sequence of the ACP subunit of K. aerogenes enzyme complex. The ACP subunit is made up of 78 residues and the total absence of tyrosine and histidine residue were unique features. The molecular weight of ACP subunit computed from the amino acid sequence and the prosthetic group was 9378. Circular dichroism studies indicated a high degree of helical content.

The acyl-carrier proteins of citrate lyase and

citramalate lyase (Dimroth and Loyal, 1977; Buckel and Bobi, 1976) were found to be similar in their amino acid compositions. The structure of prosthetic groups of the two enzymes also appeared to be identical. However, the ACP of citrate lyase differs markedly from the ACP of fatty acid synthetase from E. coli (Dimroth et al., 1973; Vagelos, 1973). The prosthetic group of fatty acid synthetase contains 4-phosphopantetheine while the lyase ACP contains adenine, phosphate and sugar residues in addition to phosphopantetheine.

Hiremath (1977) has reported the amino acid compositions of the isolated subunits of the enzyme from S. faecalis. The ACP subunit of S. faecalis enzyme contains four histidine residues while that from K. aerogenes has none. ACPs of both these enzyme contain a single cysteine residue which is acylated in the native enzyme. The amino acid composition of E. coli subunits (Nilekani, 1980) resembles that of K. aerogenes subunits more than that of S. faecalis. The ACP subunit of E. coli however, showed the presence of tyrosine which is not present in the ACPs of K. aerogenes and S. faecalis enzyme.

(xvi) Prosthetic group of citrate lyase :

Srere et al. (1972) observed for the first time that citrate lyase from K. aerogenes contains covalently attached pantothenate moieties. Microbiological assays showed that about 3 mols of protein-bound pantothenate are present in a mol of the pure enzyme. Since pantothenate

could be detected only after alkali and alkaline phosphatase treatments it was assumed from analogy with the fatty acid synthetase system, that the prosthetic group was 4-phosphopantetheine covalently bound to the enzyme. Since earlier work by Buckel et al. (1971a) had shown that citrate lyase contains essential acetyl groups in a thioester linkage it was assumed that these groups are on the 4-phosphopantetheine moieties. The prosthetic group was later shown to be associated with the 10 000 dalton -subunit of the *K. aerogenes* citrate lyase (Dimroth et al., 1973). This established the acyl-carrier protein (ACP) function of the -subunit. Later work indicated that the prosthetic group of citrate lyase is more complex than the 4-phosphopantetheine in the fatty acid synthetase system. Dimroth (1975; 1976) showed that the prosthetic group of citrate lyase contains adenine, ribose, phosphate,

-alanine, cysteamine and pantoic acid. By analysis of the products of chemical and enzymic degradation Robinson et al. (1976) proposed the structure 3'(or 2') 2" (5"-phosphoribosyl) dephospho-CoA for the prosthetic group. That the site of attachment of the second ribose moiety to the dephospho-CoA is at the 2' position has been indicated from NMR and mass spectrometry studies. (Oppenheimer et al., 1979). The proposed structure for the prosthetic group is (1"-—— 2')-(5"-phosphoribosyl) dephospho-CoA.

Dimroth (1975; 1976) showed that the prosthetic group of the ACP is bound through a phosphodiester linkage

to a specific serine residue. Beyreuther et al. (1978) sequenced the ACP subunit and showed that the prosthetic group is linked through the 5' phosphoribosyl moiety to serine-14 of the polypeptide chain. This was shown by treating the ACP with alkali to convert the serine residue carrying the prosthetic group to dehydroalanine by

-elimination, followed by NaB^3H_4 reduction of dehydroalanine to ^3H alanine. The position of the modified serine residue was then located by Edman degradation.

Robinson et al. (1976) reported that the isolated prosthetic group of citrate lyase could substitute for coenzyme A in the citrate synthase and ATP-citrate lyase catalyzed reactions. The prosthetic group is the first compound which has been found to substitute for CoA in the ATP-citrate lyase catalyzed reaction. The isolated prosthetic group can also serve as a substrate for acetate: SH (acyl-carrier protein) enzyme ligase (AMP) from K. aerogenes. Acetyl-CoA and citryl-CoA in turn substitute for the prosthetic group in the reactions catalyzed by deacetyl citrate lyase from K. aerogenes (Buckel et al., 1973) and by the separated and subunits (Dimroth et al., 1977b). The close similarity in the mechanism of action of the three citrate enzymes which catalyzes the same bond making and breaking reaction has led to the suggestion that these enzymes have possibly evolved from a common ancestral enzyme (Robinson et al., 1976).

(xvii) Immunological behaviour :

Citrate lyase from K. aerogenes, E. coli and S. diacetilactis were found to be immunologically distinct (Singh and Srere, 1975). In double diffusion studies it was shown that pure citrate lyase from S. diacetilactis and E. coli does not cross react with rabbit antisera against the purified citrate lyase from K. aerogenes. The precipitation bands were observed with rabbit antisera against purified S. diacetilactis citrate lyase only with its own antigen.

Giffhorn (1981) found that the antibodies to native citrate lyase from R. gelatinosa cross reacts only with the citrate lyase from R. palustris, but did not form any precipitation bands against citrate lyases from K. aerogenes, S. diacetilactis or C. sphenoides. Another interesting observation made by them was that only the antibody against purified α -subunit of R. gelatinosa citrate lyase complex formed precipitin bands with citrate lyase from R. palustris while anti- β and anti- γ subunit antibodies did not cross-react. This suggested that the common antigenic determinants were located only on the large (α) subunit of the enzyme complexes from R. gelatinosa and R. palustris.

PRESENT INVESTIGATION

PRESENT INVESTIGATION

INTRODUCTION

Citrate lyase (citrate oxaloacetate lyase, EC 4.1.3.6) which catalyzes the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal ions like Mg^{2+} and Mn^{2+} is a key enzyme in the anaerobic fermentation of citrate. Much attention has been focused on this enzyme on account of its built-in multienzyme structure and its phylogenetic significance in the evolution of other citrate enzymes. The earlier studies on this enzyme has been reviewed in the Introductory Chapter of this thesis.

Although the subunit structure of citrate lyase complexes from several sources has been well documented, the nature of the substrate binding sites and details of the mechanism of its action are not understood. An attempt was therefore made to study some aspects of the nature of the active sites of the enzyme subunits of the complexes from K. aerogenes, S. faecalis and E. coli; the mechanism of inhibition by some substrate for the acyl-transfer reaction and the structural features of the acyl-carrier protein subunit. The results of these investigations are reported in the present thesis under three sections and the significant findings are briefly enumerated in the following General Summary.

GENERAL SUMMARYPart I. Photoaffinity labeling of citrate lyase (EC 4.1.3.6)
by p-azidobenzoyl-CoA

p-Azidobenzoyl-CoA, which carries a photoreactive azido group, was synthesized and used as a photoaffinity reagent for probing the acyl-CoA binding sites of citrate lyase from *K. aerogenes*, *S. faecalis* and *E. coli*.

p-Azidobenzoyl-CoA was found to have no inhibitory effect on the acyl-transferase activity of the purified, native citrate lyase complex from *K. aerogenes* as well as of the isolated acyl-transferase () subunit. The substrate binding sites of the acyl-lyase () subunit, however, have been shown to accept the aryl-CoA ester. p-Azidobenzoyl-CoA was shown by kinetic data to function in the dark as a linear competitive inhibitor for (3S)-citryl-CoA in the citrate lyase reaction, having an inhibitor constant, $K_i = 80 \text{ } \mu\text{M}$. In these experiments the K_m value for (3S)-citryl-CoA was found to be $67 \text{ } \mu\text{M}$. Irreversible inactivation occurs on photolysis of the reagent in the presence of deacetyl-citrate lyase complex. The binding of p-azidobenzoyl-CoA to the (3S)-citryl-CoA binding site of the lyase subunit on photolysis has been shown to occur both in the presence of Mg^{2+} as well as in the presence of excess of EDTA. These results would suggest that (3S)-citryl-CoA binding to the lyase subunit is independent of divalent metals, while its cleavage requires the presence of Mg^{2+} .

That the binbeling is exclusively on the lyase () subunit of the multienzyme complex was shown by using p-azido- ^{14}C -benzoyl-CoA. The stoichiometry of the CoA-ester binding was also investigated by the use of p-azido- ^{14}C -benzoyl-CoA, and a titre of about seven sites per mol enzyme complex was obtained. This establishes for the first time that all the active sites on the six lyase subunits present in the hexameric complex are indeed accessible to (3S)-citryl-CoA.

Similar results have been obtained with the purified citrate lyase complex from Streptococcus faecalis, an enzyme which shows only slow reaction-inactivation compared to the K. aerogenes enzyme. The isolated acyl-lyase () subunit of E. coli citrate lyase, which is not inactivated in the process of separation as extensively as the corresponding subunits from K. aerogenes, has been shown to be inhibited by p-azidobenzoyl-CoA in the dark.

Part II. Reaction-mechanism based inhibitors of citrate lyase (EC 4.1.3.6)

p-Azidobenzoate, monochloroacetate and L-malate have been shown to function as suicide substrates for bacterial citrate lyase.

p-Azidobenzoate in the dark causes a rapid and almost complete and irreversible inactivation of citrate lyases both from K. aerogenes and S. faecalis. The inactivation process follows first order kinetics; deviation being observed with the K. aerogenes enzyme with attainment of a plateau

level of activity. In presence of p-azidobenzoate (10 mM) and EDTA (10 mM), approximately 90% inactivation occurs in about 20 min, the K_{app} values being 0.09 min^{-1} for the K. aerogenes enzyme and 0.10 min^{-1} for the S. faecalis enzyme. In the presence of Mg^{2+} (3 mM), however, p-azidobenzoate (10 mM) inactivates at a markedly slower rate, the rate constants of inactivation being 0.0235 min^{-1} and 0.008 min^{-1} for the enzymes from K. aerogenes and S. faecalis, respectively. The effect is indicative of a conformational change in the enzyme complex on binding of the metal cofactor. The comparatively slower rate of inactivation of the S. faecalis enzyme in the presence of metal, which presumably reflects a correspondingly slower rate of formation of the mixed anhydride intermediate, might explain the weaker reaction-inactivation behaviour of the enzyme from this source compared to the complex from K. aerogenes. The enzyme inactivated by p-azidobenzoate is reactivated by acetic anhydride and not acetate, indicating that the mechanism of inactivation is probably through a direct deacetylation and not by the formation of a p-azibenzoyl-S-enzyme intermediate, the latter even if formed is likely to hydrolyze rapidly. Mono-chloroacetate shows similar inactivation characteristics as p-azidobenzoate, being however less effective than the latter in its inactivation.

L-Malate differs from p-azidobenzoate and monochloroacetate in the mechanism by which it inactivates citrate lyase; the enzyme inactivated by malate being partially reactivated by acetate. L-Malyl-S-enzyme intermediate

accumulation apparently occurs in this case.

Part III. Characterization of thiol groups of the acyl-carrier protein of Klebsiella aerogenes citrate lyase

The acyl-carrier protein (ACP) subunit of the K. aerogenes enzyme contains two acetylated thiol groups, one contributed by the cysteamine residue of the prosthetic group and the other by the cysteine residue in the polypeptide chain. The thiol group of the cysteamine residue in the deacetylated enzyme is acetylated by the enzyme citrate lyase ligase in presence of acetate and ATP. We have shown by the use of ^{14}C -labeled acetate that the ligase enzyme is also responsible for the modification of the cysteine residue in the ACP subunit. Post translational modifications of amino acid residues have been extensively documented but the mechanisms of such in vivo changes are not always understood.

Reaction-inactivation of K. aerogenes citrate lyase which results in the loss of the essential acetyl moiety has been shown not to deacetylate the modified cysteine residue of the ACP subunit.

The proximity of the two thiol groups generated after deacetylation of the ACP subunit with neutral hydroxylamine was studied using o-phenanthroline- Cu^{2+} complex which is known to oxidize thiol groups only to the level of disulfide. Evidence is presented for the juxtaposition of these groups which results in intrapeptide disulfide bridge formation.

PART I

PHOTOAFFINITY LABELING OF CITRATE LYASE (EC 4.1.3.6)

BY p-AZIDOBENZOYL-CoA

PHOTOAFFINITY LABELLING OF CITRATE LYASE BY p-AZIDOBENZOYL
COENZYME A

SUMMARY

p-Azidobenzoyl coenzyme A functions as a linear competitive inhibitor for (3S)-citryl-CoA in the citryl-CoA oxaloacetate-lyase reaction catalyzed by the Klebsiella aerogenes deacetyl citrate lyase complex ($K_i = 80 \mu\text{M}$; (3S)-citryl-CoA $K_m = 67 \mu\text{M}$). Inactivation is irreversible on photolysis of p-azidobenzoyl-CoA in the presence of the deacetyl citrate lyase complex. Mg^{2+} is not required for the inactivation. Inactivation is blocked by (3S)-citryl-CoA in the presence of ethylenediaminetetraacetic acid. p-Azidobenzoyl-CoA irreversibly inactivates also the acyl-lyase activity of deacetyl citrate lyase from S. faecalis, (3S)-citryl-CoA protecting the inactivation. The isolated acyl-lyase () subunit of the citrate lyase complex from E. coli which was obtained without excessive inactivation was also inhibited irreversibly on photolysis in presence of p-azidobenzoyl-CoA.

p-Azidobenzoyl-CoA has no effect on the acetyl-CoA: citrate CoA transferase activity of both the deacetyl citrate lyase complex and is isolated transferase subunit.

The stoichiometry of the CoA ester binding has been investigated by the use of p-azido- ^{14}C -benzoyl-CoA as a photoaffinity reagent with Klebsiella aerogenes citrate lyase. The labeling is exclusively on the lyase () subunit of the citrate lyase complex.

INTRODUCTION

The citrate lyase multienzyme complex (EC 4.1.3.6) has been obtained pure from a number of bacterial sources (SivaRaman, 1961; Singh and Srere, 1975; Hiremath *et al.*, 1976; Giffhorn and Gottschalk, 1978; Antranikian *et al.*, 1982; Nilekani and SivaRaman, 1983). The enzyme complex from *K. aerogenes*, which has been studied the most, has been to be assembled from three dissimilar subunits and of molecular weights of 54 000, 32 000 and 10 000, respectively. The α -subunit functions as an acyl-carrier protein (ACP) with an essential acetyl moiety in thioester linkage (Buckel *et al.*, 1971) on a covalently bound coenzyme A-like prosthetic group (Oppenheimer *et al.*, 1979). The β -subunit acts as an acyl transferase involved in the formation of (3S)-citryl-ACP in presence of citrate with the release of acetate, while the γ -subunit functions as an acyl-lyase catalyzing the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP (Dimroth and Eggerer, 1975b). Acyl-Coenzyme A (acyl-CoA) derivatives such as acetyl-CoA and propionyl-CoA have been shown to serve also as substrates for the transferase reaction, while (3S)-citryl-CoA acts as a substrate for the lyase reaction (Buckel *et al.*, 1973; Dimroth *et al.*, 1977).

The subunit structure of the citrate lyase complex from several sources has been studied in detail, but the nature and stoichiometry of the substrate binding sites have not been investigated. This chapter describes the

use of p-azidobenzoyl-CoA as a photoaffinity reagent for probing the acyl-CoA binding sites of the complex from diverse sources.

Photoaffinity labeling is usually achieved by incorporating a chemically inert but photochemically labile group in the natural ligand or an inhibitor which competes with the ligand for its specific receptor site. Reversible inactivation behaviour can be studied in the dark. By irradiation of the system, the photolabile group is converted to a highly reactive species which reacts irreversibly by chemically combining, often indiscriminately, with functional groups, specifically those located at or near the active ligand binding site because of the high local concentration of the reagent obtained there. The specificity is further enhanced by the use of scavengers in the reaction system. A number of reviews have covered the various aspects of affinity labeling (Knowles, 1972; Creed, 1974; Bayley and Knowles, 1977; Chowdhry and Westheimer, 1979).

p-Azidobenzoyl-CoA having an aryl nitrene system has been used earlier as affinity labels for enzymes using acyl-CoA, such as acyl-CoA:glycine N-acyl-transferase (Methgyl al., 1977) and transferboxylase (methylmalonyl-CoA: pyruvate carboxy-transferase) (Poto et al., 1978).

MATERIALS

Streptomycin sulfate was a product of Hindustan Antibiotics Ltd., Poona. Electrophoresis grade acrylamide N,N' methylene bisacrylamide and N,N,N',N' -tetramethylethylenediamine (TEMED) were obtained from Eastman Organic Chemicals, USA.

Peptone powder was from Bengal Immunity Co., Ltd., Calcutta; Yeast extract was a preparation of Difco Laboratories, USA.

NADH from yeast (disodium salt - $3H_2O$ grade III); Adenosine 5'-triphosphate (disodium salt) from equine muscle; citrate synthase from pig heart (type III, specific activity $155 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein); crystalline bovine serum albumin; N-hydroxysuccinimide; dithiothreitol; Trizma base and sodium dodecyl sulfate (SDS) were from Sigma Chemicals Co., USA. 5,5'-dithiobis (2-nitrobenzoate) (DTNB) was obtained from Calbiochem, USA. Dicyclohexylcarbodiimide (DCC) and 2-mercaptoethanol were from Fluka AG, Bucks SG.

p-Aminobenzoic acid was obtained from BDH Ltd., England and p-aminocarbonyl- ^{14}C -benzoic acid (21 Ci/mol) was obtained from Amersham, UK.

Analytical grade urea was obtained from BDH (India) Ltd. and was recrystallized from aqueous ethanol and stored dry over P_2O_5 in vacuum. Urea solutions were prepared fresh before use.

Solvents required for ester preparations were dried just before use. Ethyl acetate was dried by distilling over anhydrous K_2CO_3 while tetrahydrofuran was dried by

refluxing over sodium followed by distillation.

All other products were of the highest purity obtainable commercially.

Organisms:

Klebsiella aerogenes (NCTC 418) was maintained routinely on nutrient agar slants containing 2.0% agar, 0.3% beef extract, 0.5% NaCl and 0.5% peptone, pH adjusted to 7.0 by NaOH. The organism was subcultured every month. Before transferring into the citrate growth medium, the organism was inoculated into nutrient broth, and grown anaerobically to increase the cell population.

Streptococcus faecalis (strain 10 Cl) was a gift from Professor I.C. Gunsalus, University of Illinois, USA and was maintained routinely in nutrient agar stabs. Before subculture or transferring into the citrate growth medium, the organism was inoculated into an enrichment medium comprising 0.5% glucose, 0.5% lactose, 0.6% liver extract, 0.5% yeast extract, 0.6% sodium acetate and the usual trace elements, pH adjusted to 7.6 with NaOH. The organism was grown for 24 hr at 37°C and then transferred back to the stabs. The organism was subcultured every 2 - 3 weeks.

Escherichia coli (ATCC 8739 Crooke's strain) was maintained routinely on nutrient agar slants and subcultured every 2 - 3 weeks.

Buffers :

All the buffers used were prepared water, double distilled in glass. Phosphate buffers of different molarities and pH were prepared by mixing and diluting I M stock solutions

of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in the proportions given by Green and Hughes (1955). Tris-HCl buffers of desired pH were prepared by neutralizing Trizma base with 1 N HCl. All the buffers required for purification, except the 0.03 M phosphate buffer, pH 7.0 used for extraction of the enzyme from the cells, contained 1.6 mM MgSO_4 .

Alumina C gel:

Preaged alumina C gel (grade A) was obtained from Calbiochem, USA and had a solid content of 3.25%.

DEAE-cellulose:

The anion exchanger DE-52 was obtained from Whatman Ltd., England. Before use the exchanger was washed with several changes of water, the fine particles being removed in the first wash. For fast equilibration with the buffer, the exchanger was washed by decantation with three lots of 100 ml of 0.5 M potassium phosphate buffer, pH 7.5 containing 1.6 mM MgSO_4 , followed by washing three times with 200 ml lots each of the appropriate initial strength of potassium phosphate buffer, pH 7.5 containing 1.6 mM MgSO_4 used in the chromatography. The exchanger was then packed under pressure in a column of 1.5 x 30 cm. When in use, the column was run under controlled pressure to give a flow rate of 18 ml/h.

DEAE-cellulose (Cl^- -form):

The following treatment was given to the DEAE-cellulose (DE-52) used for the separation of CoA-esters:

DEAE-cellulose (20 g) was washed successively with 50 ml of 1 N HCl, 200 ml of water, 50 ml of 1 N NaOH, 200 ml of water and finally with 50 ml of 1 N HCl. All the washings were done by decantation except the acid washings which were done rapidly on a sintered glass funnel. After the final treatment with HCl, the material was washed thoroughly with water until the washings were neutral and the ion exchange resin packed under pressure in a 1 x 8 cm column.

Sepharose CL-6B:

Sepharose CL-6B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, as a wet suspension. It was thoroughly washed with water to remove the preservative used and was equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, which was also used as the elution buffer. The equilibrated Sepharose CL-6B is packed into a column (2.5 x 110 cm) under gravitational pressure. The column gave a consistent flow rate of 14 - 16 ml/h.

Ammonium sulfate fractionation :

Powdered ammonium sulfate was used in the early steps of the enzyme purification procedures. The quantities of ammonium sulfate required to get the desired saturation were read off a nomogram in which concentrations are expressed in terms of saturation at 0 - 3°C. In the later stages of purification cold, saturated solutions of ammonium sulfate neutralized with ammonia was used for the precipitation.

EXPERIMENTAL PROCEDURES

Protein determination:

Protein measurements at all stages of purification and of the isolated subunits were made by the method of Lowry et al. (1951) using bovine serum albumin as standard. The colour developed was read at 500 nm or 750 nm. Samples containing streptomycin sulfate were dialysed overnight against water before protein determination. Enzyme protein in the purified citrate lyase complex from K. aerogenes was determined from absorbance measurements at 278 nm using the absorption index $E_{1\text{cm}}^{1\%}$ of 6.2 (Singh et al., 1976).

Enzyme assay:

Two different methods were employed to determine enzyme activity. In the early stages of purification the activities were routinely measured by the procedure described by Mahadik and SivaRaman (1968). The test medium contained in a total volume of 3 ml: 150 μmol Tris-HCl, pH 8.0, 300 μmol sodium citrate and 30 μmol MgSO_4 . The reaction was initiated by adding about 10 μg of the enzyme sample. The reaction was stopped by adding 3.0 ml of 10% (w/v) trichloroacetic acid. The keto acids formed were estimated by the method of Friedman and Haugen (1943).

Citrate lyase activity of the pure enzyme complex was determined by coupled assay with malate dehydrogenase (Singh and Srere, 1971). The reaction mixture placed in 1 ml cuvette contained in a total volume of 1 ml; 50 μmol Tris-HCl buffer, pH 8.0; 5 μmol sodium citrate; 3 μmol MgSO_4 ; 10 - 15 μg malate dehydrogenase and 0.2 μmol NADH.

The reaction was initiated by adding 0.5 ug of citrate lyase contained in a volume of 10 ul. The temperature was maintained at 30°C. The progress of the reaction was monitored by the decrease in optical density at 340 nm. A decrease of 6.22 in the optical density correspond to 1 umol of oxaloacetate formed. The amount of enzyme required for catalysing the cleavage of 1 umol of citrate per min represents 1 U of the enzyme.

Enzymatic assay of individual subunits:

Acyl transferase. Acyl transferase activity of the deacetyl citrate lyase complex and of the isolated - subunit was determined from the initial velocity of the CoA transfer from acetyl-CoA to citrate in presence of EDTA by estimation of citryl-CoA produced with citrate synthase in presence of DTNB (Dimroth et al., 1977; Srere et al., 1963). The reaction mixture contained in a total volume of 1 ml: 50 umol Tris-HCl buffer, pH 8.0; 5 umol citrate; 2 umol DTNB; 0.5 umol acetyl-CoA, 1.1 U of citrate synthase and 10 umol EDTA. The reaction was initiated by the addition of 25 ug of deacetyl citrate lyase or 10 ug of isolated -subunit.

Acyl lyase. Acyl lyase activity of the deacetyl enzyme complex and of the isolated -subunit was determined from the initial rate of oxaloacetate formation on cleavage of (3S)-citryl-CoA in the presence of Mg^{2+} , oxaloacetate being assayed with malate dehydrogenase (Buckel et al., 1973). 1 ml of reaction mixture contained 50 umol

Tris-HCl buffer, pH 8.0; 3 μmol MgSO_4 ; 10 μg malate dehydrogenase; 0.2 μmol NADH and 0.5 μmole citryl-CoA. The reaction was initiated by adding 25 μg of deacetyl citrate lyase or isolated α -subunit.

Optical density:

Optical density measurements were carried out on a Beckman DU, Model-26 (Spectrophotometer) or Shimadzu Model UV-210 A spectrophotometer. Enzyme kinetics were done mainly on Shimadzu instrument equipped with a strip chart recorder.

pH measurements :

A Phillips precision pH meter, Model PR-9405 and a Global digital pH meter Model DPH 500 with a combination glass electrode were used for all pH measurements.

Conductivity measurements:

Conductivity measurements were carried out on a Systronics Model 303 direct reading conductimeter.

Ultracentrifugation:

Ultracentrifugation runs were carried out in an analytical Spinco ultracentrifuge Model E equipped with phase plate Schlieren optics and a Rotor Temperature indicator and Control device for maintaining constant temperature. All runs were carried out at $2 - 5^\circ\text{C}$ and 59 780 rpm and were used to check the homogeneity of the enzyme preparations.

Ultrafiltration:

Concentration of the enzyme solutions obtained in the

final step of isolation of the holoenzyme and of the isolated component subunits were done by ultrafiltration in an Amicon ultrafiltration cell of 10 ml capacity using a PM-10 membrane.

Polyacrylamide gel electrophoresis:

Disc gel electrophoresis was carried out by the method described by Davis (1964). 5% acrylamide gels were prepared by polymerization with 0.07% ammonium persulfate. The separating gel contained Tris-HCl buffer, pH 8.9, the sample and stacking gels were omitted. Excess ammonium persulfate was removed by a preliminary electrophoresis for 1 h. Samples containing 50 + 100 ug of protein, 30% sucrose and Bromophenol blue as tracking dye in a total volume of 50 - 100 ul were loaded on top of the gel. Electrophoresis was carried out at 4°C and a constant current of 4 mA/tube using Tris-glycine, pH 8.3. Protein bands were visualized by staining the gels for 2 - 10 h with 0.20% Coomassie Brilliant Blue R250 prepared in water containing 45.4% methanol and 9.2% glacial acetic acid. Destaining was carried out by diffusion in a solution containing 7.5% glacial acetic acid and 5% methanol in water.

Growth of organisms:

Klebsiella aerogenes (NCTC 418) was grown without aeration for 18 - 20 h at 30°C in 10 L flasks filled to the neck with the citrate medium described by Dagley and Daves (1953a). The medium consisted of trisodium citrate $2H_2O$, 9g; KH_2PO_4 , 2g; $(NH_4)_2SO_4$, 10 g and $MgSO_4 \cdot 7H_2O$

0.4g per litre of the solution. The pH was adjusted to 7.0 with 2 N NaOH. $(\text{NH}_4)_2\text{SO}_4$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were autoclaved separately in 1.0 litre of water, to avoid precipitation of Mg^{2+} as magnesium phosphate, and mixed with the rest of the medium just before inoculation. The organism was adapted to the citrate growth medium by two successive subcultures, the final inoculum was 10% of the final medium. Cells were grown for about 20 h and harvested by centrifugation at 12 000 g in a Model A-12 Sharples Super Centrifuge. The yield was about 20 - 22 g of wet cells for each 10 L of the medium. The cells were frozen at -20°C for 24 h before extraction of the enzyme. The citrate lyase activity was found to be stable for more than a week when the cells were kept frozen at -20°C .

Streptococcus faecalis, strain 10 C1 was grown in an enrichment medium before inoculating in citrate growth medium. The ingredients per litre of the citrate growth medium are, trisodium citrate, $2\text{H}_2\text{O}$, 10 g; KH_2PO_4 , 2 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; yeast extract 5 g; and peptone powder, 5 g. The pH was adjusted to 7.2 with 2 N NaOH. The cells were harvested after growing for a period of 24 - 30 h at 37°C , in a Sharples supercentrifuge and were washed once with 2 L of 0.03 M potassium phosphate buffer pH 7.0. 10 L of the medium yielded about 10 - 15 g of wet cells. The cells were found to be unstable on storing and thus processed further within a week of freezing at -20°C .

Escherichia coli (Crooke's strain) was grown without aeration at 30°C for 18 - 22 h in a medium described by Dagley (1954). The composition of the medium was as follows: KH_2PO_4 , 90 g; trisodium citrate, 50 g; peptone, 56 g; glucose, 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g and water to make upto 10 L after adjusting the pH to 7.2 with 5 N NaOH. After a growth of 18 - 22 h the medium was supplemented with glucose (1 g/litre) and after 1 h the cells were harvested and frozen at -20°C.

Extraction of cells:

The frozen cells of K. aerogenes, S. faecalis and E. coli were thawed and suspended in 0.03 M potassium phosphate buffer, pH 7.0 in the proportion of 1 g wet cells to 3.5 - 4.0 ml of buffer and disrupted in a Biosonik III sonicator (Brownmill Scientific Co., USA). Sonication was done twice for three minutes each at 20 kc (300 W). A batch of 100 - 220 g of cells were worked upon at a time. Cell debris was removed by centrifugation at 50 000 g for 60 min in a Spinco Model-L ultracentrifuge, and the clear supernatant was adjusted to a protein concentration of 0.5% with the same buffer used for sonication.

PURIFICATION OF K. AEROGENES CITRATE LYASE

Citrate lyase complex from K. aerogenes was purified by a modification of the procedure described earlier (Mahadik and SivaRaman, 1968), sonic extracts being treated with ATP and acetate for reactivating any deacetyl citrate lyase formed on storing (Tikare, 1979). All the steps of

purification were carried out at 0 - 4°C and without storage at any stage. All the buffers used for purification contained 1.6 mM MgSO_4 for stabilization of the enzyme. The outline of the purification procedure is described below.

Treatment with ATP and acetate:

The sonic extract in which the protein was adjusted to a concentration of 0.5% was treated with ATP and acetate at a final concentration of 0.5 mM and 1 mM, respectively. The solution was kept stirred for 45 min to reactivate any deacetyl citrate lyase formed during storage, by the action of deacetyl citrate lyase ligase.

Streptomycin sulfate treatment:

Nucleic acids were precipitated by stirring the above solution with 1.4% streptomycin sulfate for about 30 min. After this the solution was allowed to stand for another 30 min and then the precipitate was removed by spinning at 50 000 g for 40 min.

Fractionation by Alumina C gel:

First gel treatment. The supernatant obtained in the previous step was treated with 10 ml of alumina C gel under constant stirring. 5% of the total activity was adsorbed by the gel at this step. The gel was separated by centrifugation at 3 000 g for 15 min and discarded.

Second gel treatment. To the supernatant obtained after the first gel treatment step more of the same gel was added (200 ml/600 ml of the extract) under gently stirring. After stirring for 15 min and standing for 15 min, the gel

was separated by centrifugation at 3 000 g for 20 min and the supernatant was discarded. About 90 - 95% of the enzyme activity was adsorbed on the gel.

Elution of activity from alumina C gel. The enzyme adsorbed on alumina C gel was eluted in 0.05 M phosphate buffer, pH 7.0 (200 ml/600 ml extract) by homogenizing in a glass tube with a close fitting ground glass pestle. The gel was discarded after removal by centrifugation and the supernatant containing the enzyme activity was treated further.

Fractionation by Ammonium sulfate:

Powdered ammonium sulfate was added to the eluate from alumina C gel to a saturation of 0.2 (20 g/200 ml) and the mixture stirred for 15 min. The precipitate formed was removed by centrifugation for 30 min at 50 000 g and discarded. The supernatant containing the enzyme activity was then treated with ammonium sulfate to 0.5 saturation (38 g/200 ml). The solution was stirred for 15 min and then allowed to stand for 15 min longer. The precipitate containing the enzyme activity was collected by centrifugation at 50 000 g and dissolved in the minimum volume of 0.05 M potassium phosphate buffer, pH 7.5 (5 - 6 ml). The solution was then dialysed overnight against 500 ml of the same buffer to remove ammonium sulfate.

DEAE-cellulose chromatography:

The dialysed solution (8 - 9 ml) was centrifuged at 10 000 g for 15 min to remove turbidity and the clear solution was loaded on a DEAE-cellulose column (1.5 x 30 cm)

which was equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. Protein was eluted by a linear concentration gradient of phosphate buffer, pH 7.5, from 0.01 M to 0.3 M. Fractions of 4 ml were collected and an aliquot from each fraction was checked for enzyme activity. Fractions containing maximum activity were pooled (about 25 - 30 ml) and the protein precipitated by adding dropwise an equal volume of cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 50 000 g for 30 min and dissolved in 2 ml of 0.05 M potassium phosphate buffer, pH 7.5.

Gel filtration:

The enzyme solution obtained from the previous step was loaded on a Sepharose CL-6B column (2.5 x 110 cm) preequilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Elution was carried out with the same buffer and fractions of 3 ml were collected on an automatic fraction collector. Each fraction was checked for enzyme activity and the fractions with high enzyme activity were pooled (about 20 ml). The protein was either collected by precipitation with cold neutral saturated ammonium sulfate solution or concentrated by ultrafiltration through a PM-10 membrane.

PURIFICATION OF *S. FAECALIS* CITRATE LYASE

Purification of citrate lyase from *S. faecalis* was carried out according to the procedure described by Hiremath et al. (1976). A brief outline of the procedure is

described here.

Treatment with ATP and acetate:

The cell free extract was adjusted to 0.5% protein and then treated with acetate and ATP (1 mM and 0.5 mM final concentration respectively). The mixture was kept stirred for 45 min.

Streptomycin sulfate treatment:

Streptomycin sulfate (1% final concentration) was added under constant stirring. The precipitate formed was removed after 1 h of stirring by centrifugation at 50 000 g for 45 min.

Alumina C gel fractionation :

First gel treatment. The supernatant from the above step was treated with alumina C gel (3 ml/200 ml extract) and stirred for 10 min. The gel was removed by spinning and discarded.

Second gel treatment. The supernatant obtained in the above step was treated with more of alumina C gel (33 ml/200 ml of extract) and stirred for 15 min. The gel was then separated by centrifugation at 2 000 g and the supernatant discarded. About 95% of the activity present was adsorbed on the gel.

Washing of the gel. The gel containing the enzymic activity was homogenized in 0.01 M potassium phosphate buffer, pH 7.0 (100 ml/200 ml extract). After 10 min the gel was removed by centrifugation and the washings discarded.

Elution of activity from the gel. The gel was then homogenized in 0.05 M potassium phosphate buffer, pH 7.0 (100 ml/200 ml extract) for 15 min, removed by centrifugation and discarded. The supernatant containing the enzyme activity was treated further.

Ammonium sulfate fractionation:

The enzyme solution from the above step was treated with powdered ammonium sulfate to 0.5 saturation (29 g/100 ml). After stirring for 10 min the precipitate formed was removed by centrifugation at 50 000 g for 30 min and discarded. Powdered ammonium sulfate was added to the supernatant obtained from the previous step to get 0.8 saturation (23 g more) and the solution was stirred for 30 min. The precipitated enzyme was collected by centrifugation at 50 000 g for 30 min and dissolved in 0.05 M potassium phosphate buffer (4 - 5 ml) and dialysed against 500 ml of the same buffer for 16 h.

DEAE-cellulose chromatography:

The turbidity from the dialysed protein solution was removed by centrifugation at 10 000 g for 10 min and loaded on a DEAE-cellulose column (1 x 30 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The column was eluted by a linear concentration gradient of phosphate buffer from 0.05 M to 0.75 M. Fractions with maximum enzyme activity were pooled and the protein precipitated by adding powdered ammonium sulfate to 0.8 saturation. The precipitate was collected by centrifugation

and dissolved in a minimum volume of 0.1 M potassium phosphate buffer, pH 7.5 (about 2.0 ml).

Gel filtration:

The enzyme solution was then loaded on a Sepharose CL-6B column (1.5 x 120 cm) which was equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. Elution was done with the same buffer and fractions of 3 ml size were collected. Enzyme activity was checked in the usual manner and the fractions with highest enzyme activity were pooled. Concentration was done either by precipitation with ammonium sulfate (0.8 saturation) or by ultrafiltration using a PM 10 membrane).

PURIFICATION OF E. COLI CITRATE LYASE

Citrate lyase from E. coli was purified according to the procedure described by Nilekani and SivaRaman (1983). A brief outline of the procedure is described here.

Streptomycin sulfate treatment:

The cell-free extract was treated with streptomycin sulfate at final concentration of 1.4%. After stirring for 45 min the precipitated nucleic acid was removed by centrifugation at 50 000 g for 45 min.

Salmine sulfate treatment:

The enzyme activity was then precipitated by treating the above supernatant with freshly prepared 0.5% salmine sulfate in 0.05 M phosphate buffer, pH 7.4 at a proportion of 10 ml/100 ml of supernatant having a protein concentration of 3 mg/ml. After stirring for 30 min the

precipitated protein was collected by centrifugation at 50 000 g for 40 min.

Elution of citrate lyase activity from salmine sulfate precipitate:

The protein pellet from the above step was suspended and homogenized for 20 min in 0.05 M phosphate buffer, pH 7.4 containing 0.5 M NaCl (15 ml/100 ml of extract). The undissolved material was removed by centrifugation and to the clear supernatant 0.05 M phosphate buffer, pH 7.4 was added to dilute the sodium chloride concentration to 0.15 M, where upon the enzyme was reprecipitated.

Treatment with CM-cellulose:

The diluted solution was then treated with CM-52 cellulose equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 0.15 M NaCl and stirred for 10 - 15 min. On removal of CM-cellulose a clear solution was obtained.

Alumina C gel treatment:

Alumina C gel was added to the above supernatant (30 ml/100 ml of CM-cellulose treated extract) and after 15 min of stirring the gel was removed by spinning at 3 000 g for 20 min and discarded.

Ammonium sulfate fractionation:

Finely powdered ammonium sulfate was added to the above solution till 0.6 saturation and stirred for 20 min. The precipitated protein was then collected by centrifugation at 50 000 g for 30 min and dissolved in 0.05 M phosphate

buffer, pH 7.4 (4 - 5 ml) and dialysed against 0.01 M phosphate buffer, pH 7.4 for 12 h.

DEAE-cellulose chromatography:

The dialysed protein solution (8 - 9 ml) was spun at 10 000 g to remove turbidity and the clear solution was loaded on the DEAE-cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.4. Protein was eluted from the column by a linear concentration gradient (0.01 to 0.12 M) of phosphate buffer, pH 7.4. Citrate lyase activity in each fraction was monitored; the fractions containing maximum activity were pooled (about 25 ml) and the protein precipitated by slow addition of an equal volume of cold, neutralized saturated ammonium sulfate solution. The precipitate was separated by centrifugation at 50 000 g for 30 min and dissolved in 0.05 M phosphate buffer, pH 7.4 (about 2 ml).

Gel filtration:

The above protein solution was then subjected to gel-filtration through a Sepharose CL-6B column (2.5 x 110 cm) equilibrated with 0.05 M phosphate buffer, pH 7.4. Elution was done with the same buffer and fractions of 3 ml were collected. Enzyme activity was checked as usual and the fractions containing high enzyme activity were pooled and protein precipitated by addition of an equal volume of cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 50 000 g for 20 min and dissolved in 0.05 M phosphate buffer, pH 7.4 (about 1 ml).

PREPARATION OF COENZYME A DERIVATIVES

Preparation of p-azidobenzoyl-CoA:

p-Azidobenzoyl-CoA was prepared by acylation of coenzyme-A with N-hydroxysuccinimide ester of p-azidobenzoic acid by the procedures described earlier (Lapidot *et al.*, 1967; Al-Arif and Blecher, 1969; Lau *et al.*, 1978). All the steps were carried out in the dark.

(a) Preparation of p-azidobenzoic acid. p-Azidobenzoic acid was prepared from p-aminobenzoic acid by diazotization and treatment with sodium azide. p-Aminobenzoic acid (10 mmol) was suspended in 50 ml of 4 N sulfuric acid (precooled to 0°C) and dissolved by dropwise addition of a solution of sodium nitrite (12 mmol in 12 ml of water). After p-aminobenzoic acid is completely dissolved (about 15 - 20 min) a 12 ml solution of sodium azide (12 mmol) was added. After 60 min of stirring at 0 - 4°C, the precipitate formed was collected by filtration and washed thoroughly with ice cold water till the washings were neutral. The precipitate of p-azidobenzoic acid was then air dried.

(b) Preparation of N-hydroxysuccinimide ester of p-azidobenzoic acid. p-Azidobenzoic acid (10 mmol) was dissolved in a solution of N-hydroxysuccinimide (10 mmol) in dry ethyl acetate (50 ml). To this a solution of dicyclohexylcarbodiimide (10 mmol) in dry ethyl acetate (about 5 ml) was added and the reaction mixture was kept at room temperature for 20 h. Dicyclohexylurea which precipitates out was removed by filtration and the filtrate

was concentrated under vacuum in a rotary evaporator. The white crystals of the ester were recrystallized from ethanol and was used without further purification. This ester was stable when stored in dark.

(c) Preparation of p-azidobenzoyle-CoA. 20 mg of CoA (about 2.5 μmol) was dissolved in 2 ml of water and the pH was adjusted to 8.0 by adding KHCO_3 (about 2 mmol). To this was added a solution of N-hydroxysuccinimide ester of p-azidobenzoic acid (20 molar excess to CoA) in 3 ml of tetrahydrofuran that had been freshly distilled over sodium. The reaction mixture, a single phase, was kept stirred. The reaction was complete within 4 h as indicated by the disappearance of free -SH groups on titration of aliquots with DTNB. After an interval of 2 h, 1 ml more of tetrahydrofuran was added to maintain a homogeneous solution. After the reaction was complete the mixture was diluted to 100 ml with cold water and loaded on a DEAE-cellulose (Cl^- -form) column for the separation of the CoA ester.

Preparation of acetyl-CoA:

Acetyl-CoA was prepared by a method similar to the preparation of succinyl-CoA as described by Simon and Shemin (1953). 10 mg of coenzyme A was dissolved in 1 ml of water and the pH was adjusted to 8.0 by adding KHCO_3 . To this was added acetic anhydride to a final concentration of 4.7 mM. The Mixture was kept in an ice-bath and stirred. Formation of acetyl-CoA was monitored by checking

an aliquot of 10 μ l for the disappearance of thiol groups by titration with DTNB. The reaction was complete within 10 min as monitored by the disappearance of sulfhydryl groups. To prevent spontaneous hydrolysis of acetyl-CoA at the alkaline pH, the mixture was acidified to pH 4 - 5 by 1 N HCl.

Preparation of (3S)-citryl-CoA:

(3S)-citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in presence of *K. aerogenes* deacetyl-citrate lyase and EDTA. The procedure was similar to that described by Dimroth *et al.* (1977). The incubation mixture contained in a total volume of 2 ml: 100 μ mol Tris-HCl buffer, pH 8.0; 200 μ mol citrate; 20 μ mol acetyl-CoA; and 20 μ mol EDTA. The reaction was started by adding 100 μ g of deacetyl-citrate lyase from *K. aerogenes*. After about 60 min the mixture was acidified with HCl to pH 3.0, diluted to 100 ml with water and applied to a column of DEAE-cellulose (Cl⁻-form) for purification.

Purification of the coenzyme A esters:

CoA-esters were purified by DEAE-cellulose (Cl⁻-form) chromatography as described by Moffat and Khorana (1961). The solution containing the CoA-derivatives were diluted to 100 ml with distilled water and loaded on a DEAE-cellulose column (1 x 8 cm). The column was washed with water till the eluate showed no absorbance at 260 nm. The column was then developed with a 500 ml volume of a linear gradient of 0 to 0.2 M LiCl in 0.003 M HCl. Fractions of

6 - 7 ml were collected at a flow rate of 60 ml/h. The presence of CoA derivatives were checked by the absorbance at 260 nm. Appropriate fractions were pooled and concentrated on a rotary evaporator under reduced pressure at 30°C. Removal of LiCl was achieved by passing the concentrate (2 - 3 ml) through a Sephadex G-15 column (1 x 50 cm) equilibrated with water.

Estimation of CoA derivatives:

(a) p-Azidobenzoyl-CoA. p-Azidobenzoyl-CoA was estimated from its extinction coefficient value of $22.2 \pm 0.4 \text{ mM}^{-1}$ at 265 nm in 0.1 M sodium phosphate buffer, pH 7.0 (Lau *et al.*, 1977).

(b) (3S)-citryl-CoA. Citryl-CoA was estimated enzymatically using citrate synthase (Buckel *et al.*, 1973). An aliquot of citryl-CoA solution was taken into 1 ml of assay mixture containing 50 μmol Tris-HCl, pH 8.0 ; 2 μmol DTNB and about 1.1 U of citrate synthase. The increase of absorbance at 412 nm was used for determination of citryl-CoA.

(c) Acetyl-CoA. Acetyl-CoA was determined using citrate synthase and oxaloacetate (Srere *et al.*, 1963). 1 ml of test medium contained 0.05 M Tris-HCl, pH 8.0; 2 μmol DTNB; 1 μmol oxaloacetate and 1.1 U of citrate synthase. 10 μl of acetyl-CoA solution was added and the absorbance at 412 nm was noted after the completion of the reaction.

Preparation of deacetyl citrate lyase:

Deacetyl citrate lyase was obtained by treatment of the purified native enzyme complex from K. aerogenes, S. faecalis or E. coli with neutral hydroxylamine according to the procedure of Buckel et al. (1973). Citrate lyase enzyme complex (about 10 mg protein) in 1 ml of 0.05 M potassium phosphate buffer, pH 7.5 was treated with neutralized hydroxylamine (0.2 M final concentration). The solution was incubated at 25°C for 30 min. After this period excess hydroxylamine was removed by passing the solution through a column of Sephadex G-25 (1 x 40 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Protein was monitored by the absorbance at 280 nm. The fractions containing protein were pooled. Aliquots checked for citrate cleavage showed complete loss of activity.

Isolation of citrate lyase subunits:

Subunits of K. aerogenes citrate lyase. The subunits of citrate lyase complex from K. aerogenes were isolated in electrophoretically pure and catalytically active state after dissociation with urea by the procedure described by Dimroth and Eggerer (1975b). 25 mg of pure citrate lyase from K. aerogenes in a total volume of 3 ml of 0.01 M potassium phosphate buffer, pH 7.0 was treated with 5 M urea and incubated for 2 h at 0 - 4°C to dissociate the subunits. The solution was loaded on a DEAE-cellulose column (0.8 x 6 cm) which was previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.0 containing 4 M urea.

Elution of protein from the column by the same buffer yielded the first fraction, which appeared within first 15 ml. After collecting about 25 ml of eluate, urea from the column was washed out by passing 25 ml of 0.01 M potassium phosphate buffer, pH 7.0. The column was then further eluted with the same buffer but containing 0.4 M NaCl in addition, which yielded the second fraction. The final elution was done with 0.01 M potassium phosphate buffer, pH 7.0 containing 0.4 M NaCl and 4 M urea. All the fractions were immediately dialyzed against buffer systems described in detail by Dimroth and Eggerer (1975b). The subunits in the three fractions were characterized by their individual reactions and by their molecular size in SDS-gel electrophoresis.

Subunits of *E. coli* citrate lyase. Biologically active subunits of citrate lyase complex from *E. coli* were isolated after dissociation with 6 M urea in 50 mM potassium phosphate buffer, pH 7.4 (Nilekani and SivaRaman, 1983). *E. coli* citrate lyase (15 mg) in 1.0 ml of 0.05 M phosphate buffer, pH 7.4, containing 10 mM dithiothreitol was treated with recrystallized urea to a final concentration of 8 M and was incubated at 15°C for 16 - 18 h. At the end of this period the protein solution was loaded on a Sepharose CL-6B column (1.5 x 110 cm) which was previously equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 10 mM 2-mercaptoethanol and 6 M urea. Elution was done with the same buffer and fractions of

1 ml size were collected and checked for protein at 280 nm. Pooled fractions comprising protein peaks were dialyzed immediately against buffers without urea as described by Dimroth and Eggerer (1975a). Homogeneity was checked by SDS-polyacrylamide gel electrophoresis.

Kinetics:

K_m for citryl-CoA and K_i for p-azidobenzoyl-CoA were calculated from the double reciprocal plots by least squares analysis. Assay mixtures at 25°C contained 50 mM Tris-HCl buffer pH 8.0; 3 mM $MgSO_4$; 12.5 ug deacetyl citrate lyase; varying concentrations of (3S)-citryl-CoA (upto 50 uM) and p-azidobenzoyl-CoA (0 - 80 uM). Initial rates of reaction were calculated by measuring the oxaloacetate formed by coupling with malate dehydrogenase.

Photoactivation of p-azidobenzoyl-CoA:

Photoactivation was carried out under a Philips Blacklight lamp 125 W (wavelength of maximum intensity 365 nm). The sample solutions were kept in a glass well and positioned 2 cm from the lamp. Solutions were precooled and temperature maintained at 0 - 4°C in an ice bath. Mixing was achieved using a magnetic stirrer. In control experiments it was found that the photolysis conditions do not inactivate the enzymes.

Covalent labeling:

Incorporation of radioactivity into protein on photolysis in presence of the ^{14}C -labeled p-azidobenzoyl-

CoA was measured by the filter paper disc method described by Mans and Novelli (1961). The irradiation mixture contained in a total volume of 300 μ l: 75 μ g of K. aerogenes deacetyl citrate lyase; 50 mM Tris-HCl, pH 8.0; 3 mM MgSO_4 and varying amounts of p-azidobenzoyl-CoA (0 - 100 μ M). Protein samples were irradiated for five min as described above and a 50 μ l aliquot was withdrawn for the determination of residual lyase activity and a 200 μ l sample was withdrawn and transferred on to a disc of Whatman No. 3 MM chromatographic paper (2.3 cm dia) and dried in a current of hot air for 30 s. The protein was then fixed in the matrix of the paper by dipping into an ice-cold solution of 10% TCA for 60 min. The discs were then washed successively by suspending in 5% TCA solution for 15 min, twice in ether-ethanol mixture (v/v) for 30 min each, and finally in diethyl ether for 15 min to remove alcohol and water. The discs were then air dried and placed in a standard 20 ml counting vial. 10 ml a dioxane based scintillation cocktail (5 g 2,5-diphenyloxazole and 100 g naphthalene per litre of dioxane) was added and radioactivity counts were taken on a Beckman Model LS-100 scintillation system after aging for 48 h in the dark. Known amounts of the ^{14}C -labeled ester preparation were dried on similar discs and counted directly for determination of its specific radioactivity.

Distribution of radioactivity in K. aerogenes citrate lyase subunits :

SDS-polyacrylamide gel electrophoresis of the enzyme photolyzed in the presence of the radioactive reagent was carried out according to the method of Weber and Osborn (1969). Samples were prepared by heating aliquots (50 - 100 μ l) containing about 200 μ g protein with 1% SDS in a boiling water bath for 3 min and loaded on 10% polyacrylamide gels containing 1% SDS. Electrophoresis was carried out at a constant current of 8 mA/tube using 0.01 M sodium phosphate buffer, pH 7.2. Gels were stained with Coomassie Brilliant blue R-250 and destained by diffusion in a 7.5% acetic acid solution containing 5% methanol. Detection of radioactivity in the protein bands was carried out by the method described by Saitoh et al. (1980). The gel regions containing the individual subunits were cut, dried in ethanol and suspended in 10 ml of a scintillation fluid of the following composition: toluene, 900 ml; 2,5-diphenyloxazole, 5 g; Bio Solv BBS-3 tissue solubilizer, 10 ml and 4 M NH_4OH , 20 ml. Samples were incubated for 2 days at room temperature in the dark before counting.

RESULTS

Purification of citrate lyases from *K. aerogenes*, *S. faecalis* and *E. coli* :

The results obtained in a typical experiments are summarized in Tables I.1, I.2 and I.3, respectively. It can be seen from these tables that treatment of the sonic extracts with ATP and acetate result in an increase in the total activity. This can be attributed to the acetylation of the deacetyl citrate lyases formed on storing of cells, by the action of citrate lyase ligase present in the extracts.

The final yields and specific activities were comparable with earlier reports by Mahadik and SivaRaman (1968) for *K. aerogenes*, Hiremath et al. (1976) for *S. faecalis* and Nilekani and SivaRaman (1983) for *E. coli* citrate lyase.

The purification steps resulted in ultracentrifugally and electrophoretically pure enzymes both from *K. aerogenes* and *S. faecalis*. The electrophoretic profile of pure citrate lyase from the sources are shown in Figure I.1 (a and b). Purity of the enzymes were also judged from the subunit profiles of enzyme complexes. Figure I.2 (a and b) shows the SDS-polyacrylamide gel electrophoresis and as reported earlier (Singh et al., 1974; Hiremath et al., 1976) the enzyme complexes dissociated into three non-identical subunits, -subunit (M_r 54 000); -subunit (M_r 32 000) and -subunit (M_r 10 000 for *K. aerogenes* and 14 000 for *S. faecalis*). The enzyme from *E. coli* was

TABLE I.1 : Purification of citrate lyase from
Klebsiella aerogenes

Wet weight of cells : 140 g

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	550	4.5	4703	1.9	-
Cell-free extract + acetate + ATP	550	4.5	6188	2.5	100
Streptomycin sulfate treatment	545	3.1	5406	3.2	87
Euate from Alumina C gel	210	3.3	4158	6.0	667
Ammonium sulfate fraction (0.2 - 0.5 Sat.) dissolved in 0.05 M buffer, pH 7.5	5.5	34.3	3773	20.0	61
DEAE-cellulose chromatography/ ammonium sulfate precipitation	1.8	29.5	3398	64.0	55
Sepharose CL-6B fractions after concentration over PM-10 membrane by ultrafiltration	1.5	12.0	1548	86.0	25

TABLE I.2 : Purification of citrate lyase from
Streptococcus faecalis

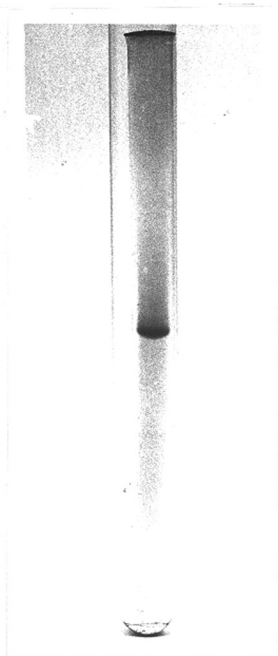
Wet weight of cells : 100 g

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	350	4.5	3937	2.5	-
Cell-free extract + acetate + ATP	350	4.5	7245	4.6	100
Streptomycin sulfate fraction	345	3.5	6520	5.4	90
Elution from alumina C gel	150	1.94	4347	15.0	60
Ammonium sulfate fraction (0.5 - 0.8 saturation)	3.6	21.5	3477	45.0	48
Ammonium sulfate fraction after dialysis	6 6.0	11.1	2608	39.0	36
DEAE-cellulose chromatography/ ammonium sulfate precipitation	2.0	19.7	2173	55.0	30
Sephacrose CL-6B fractions after concentration	1.5	9.0	1215	90.0	16

TABLE 1.3 : Purification of citrate lyase from*Escherichia coli*

Wet weight of cells : 120 g

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	400	15.0	8400	1.4	100
Streptomycin sulfate treatment	395	8.1	7980	2.5	95
Salmine sulfate treatment/elution in 0.05 M phosphate buffer, pH 7.5 containing 0.5 M NaCl	60	15.0	5460	6.0	65
CM-cellulose treatment	215	2.0	5208	12.0	62
Alumina C gel treatment	240	1.35	4872	15.0	58
Ammonium sulfate fraction	6.0	20.8	4368	35.0	52
Ammonium sulfate fraction after dialysis	9.0	13.4	3612	30.0	43
DEAE-cellulose chromatography/ ammonium sulfate precipitation	2.0	19.25	2772	72.0	33
Sephacrose CL-6B/ Ammonium sulfate precipitation	1.4	10.0	1680	120.0	20



(a)



(b)

FIG. I.1 POLYACRYLAMIDE GEL ELECTROPHORESIS OF
NATIVE CITRATE LYASE

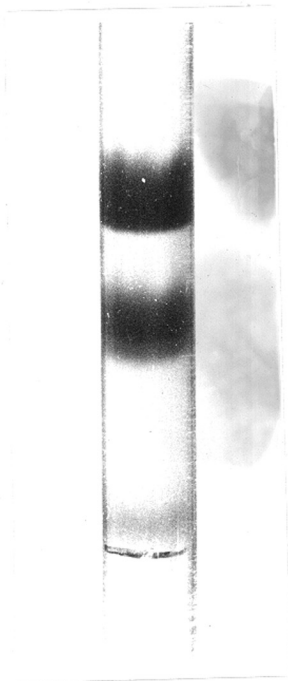
(a) K. aerogenes citrate lyase

(b) S. faecalis citrate lyase

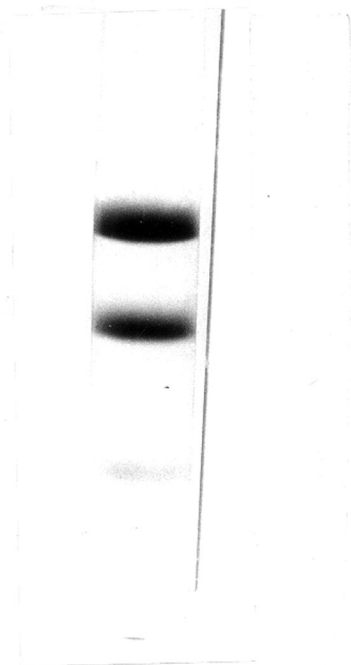
5% gel; Tris-glycine buffer, pH 8.3;

4 mA/tube, 5 h

Protein loaded 50 ug.



(a)



(b)

FIG. I.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
OF CITRATE LYASE

- (a) K.aerogenes citrate lyase
- (b) S. faecalis citrate lyase

10% gel; Sodium phosphate buffer, pH 7.3
containing 0.1% SDS; 8 mA/tube; 4.5 h
Protein loaded 100 ug.

polydisperse both in ultracentrifuge and in polyacrylamide gel electrophoresis. The SDS-polyacrylamide gel electrophoresis profile, however, showed the presence of only the three corresponding subunits of 85 000, 54 000 and 32 000 daltons (Figure I.3) as reported earlier (Nilekani and SivaRaman, 1983).

Isolation of subunits :

Component subunits of K. aerogenes citrate lyase were isolated in electrophoretically pure and catalytically active state after dissociation with urea by the procedure described by Dimroth and Eggerer (1975b).

The component subunits of E. coli citrate lyase were isolated by the procedure described earlier by Nilekani and SivaRaman (1981). The isolated subunits were catalytically active and homogenous in SDS-polyacrylamide gel electrophoresis (Figure I.3).

Preparation and Purification of CoA-esters :

p-Azidobenzoyl-CoA was prepared by acylation of coenzyme A with a 20% molar excess of N-hydroxysuccinimide ester of p-azidobenzoic acid. p-Azido- ^{14}C -benzoyl-CoA was prepared by the same procedure using p-amino- ^{14}C -benzoic acid as the precursor. Less materials were used to get the final preparation with a high specific radioactivity. The ester was purified by DEAE-cellulose (Cl^- form) chromatography using a linear gradient of LiCl (0 to 0.2 M) containing 0.003 N HCl . Figure I.4 shows the elution profile of the ester from the DEAE-cellulose column. The

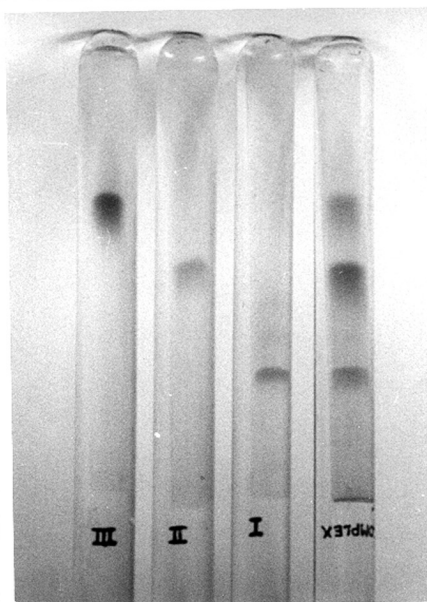


FIG. 1.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
E. coli CITRATE LYASE COMPLEX AND OF ITS
ISOLATED SUBUNITS

100µg of citrate lyase complex (or 50 µg of each subunit) electrophoresed on 7.5% gel in 50 mM sodium phosphate buffer, pH 7.3 containing 0.1% SDS at a constant current of 8 mA/tube for 4 h.

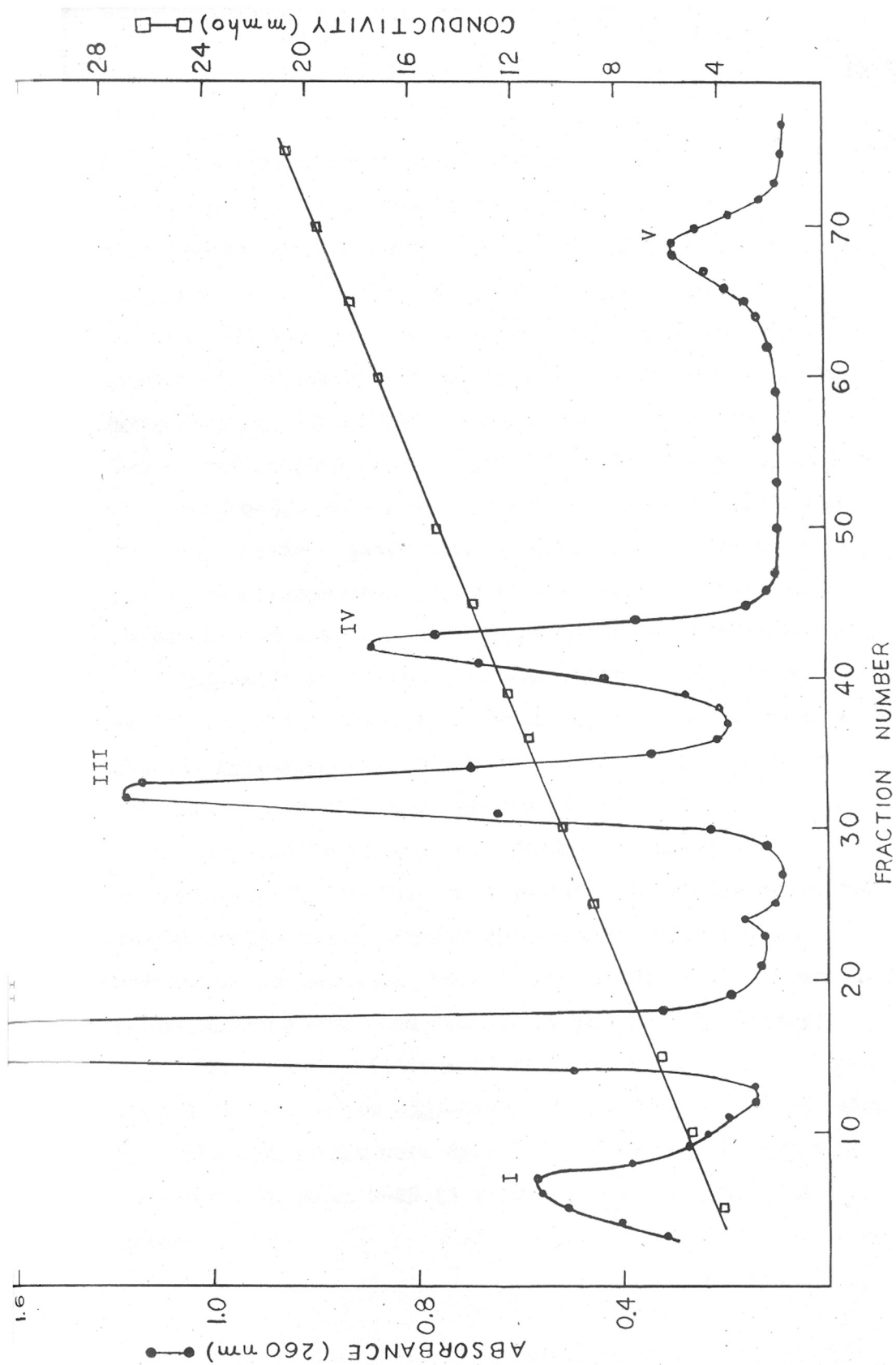


FIG. 4. PURIFICATION OF $\bar{P}$ -AZIDOBENZOYL-CoA BY DEAE-CELLULOSE (Cl^- -FORM) CHROMATOGRAPHY

compounds comprising a peak were characterized by their absorption spectra. p-Azidobenzoyl-CoA shows a characteristic peak around 300 nm in addition to an absorption maxima at 265 nm (Figure I.5). Peak IV was found to contain p-azidobenzoyl-CoA which elutes out in buffer of a conductivity of around 13 - 14 mmho under the conditions of separation. Peak II was characterized as N-hydrosuccinimide ester of p-azidobenzoic acid and Peak III as coenzyme-A. Peak I and V were not characterized, but from the earlier observation by Moffatt and Khorana (1961) peak V could represent oxidized CoA (CoA-S.S.-CoA) which is associated with commercial preparations of coenzyme A.

(3S)-citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in presence of deacetyl citrate lyase from *K. aerogenes* and EDTA by a method similar to that described by Dimroth and Eggerer (1977). Figure I.6 shows the purification of citryl-CoA by DEAE-cellulose chromatography. In this case the fractions were characterized enzymatically using citrate synthase as described in Materials and Methods. Peak I was unidentified. Peak II contains acetyl-CoA and Peak III comprises (3S)-citryl-CoA. Under the conditions of separation acetyl-CoA eluted around 10 mmho while (3S)-citryl-CoA elutes out at 15 mmho.

The CoA esters were desalted by passing through a column of Sephadex G-25 (1 x 50 cm) equilibrated with water.

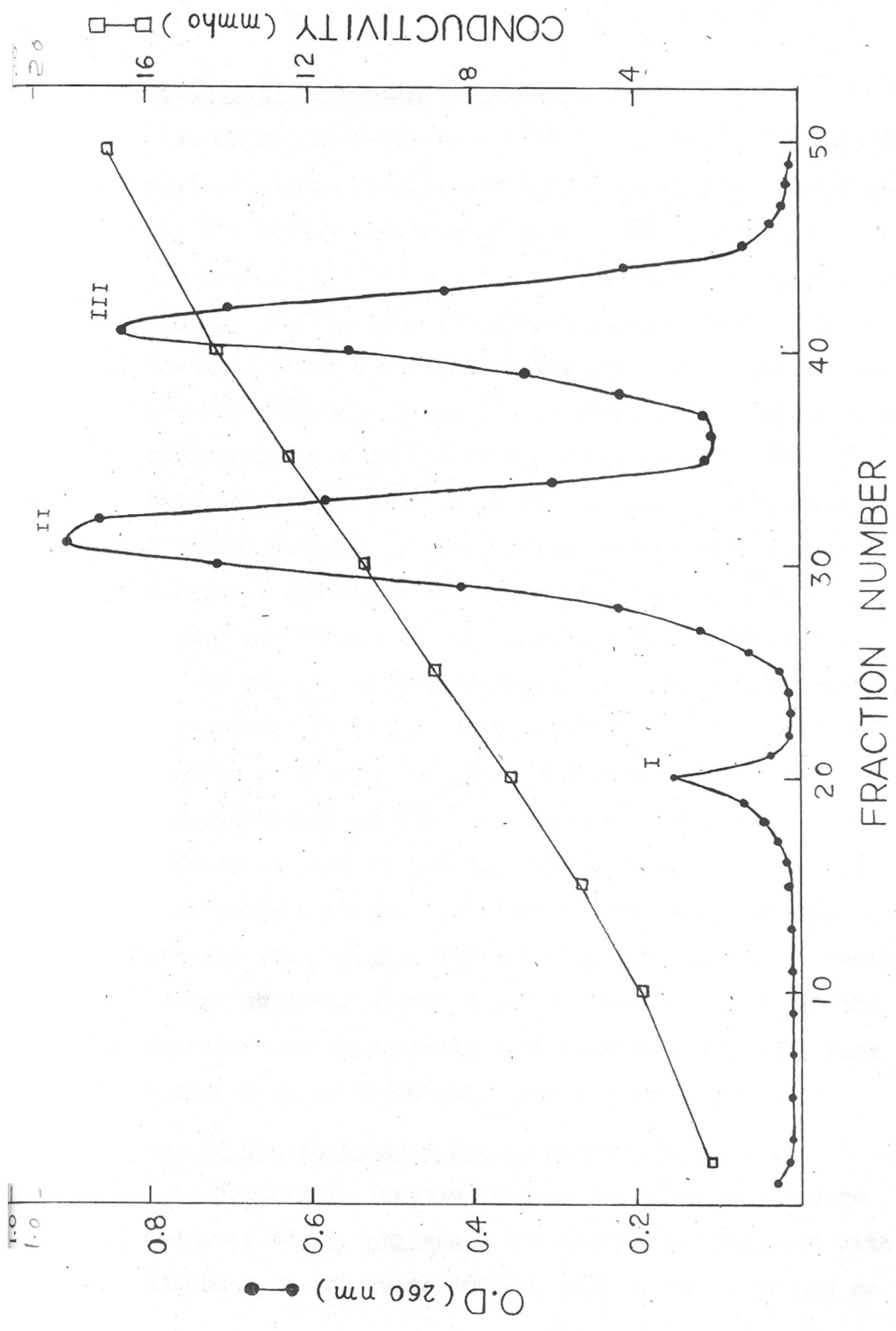


FIG.I. 5. PURIFICATION OF (3S)-CITRYL-CAA BY DEAE-CELLULOSE (Cl⁻-FORM) CHROMATOGRAPHY

Photolability of p-azidobenzoyl-CoA :

As described by Knowles (1972) irradiation of p-azidobenzoyl-CoA leads to photodecomposition of the azido group to yield a highly reactive nitrene. This property of p-azidobenzoyl-CoA was checked by the method described by Lau *et al.* (1977). The absorption spectra of p-azidobenzoyl-CoA shows a characteristic peak around 300 nm due to the azido group (Figure I.). This peak disappears on photodecomposition of the azido group. A solution containing p-azidobenzoyl-CoA was taken in a stoppered 1 ml quartz cuvette and irradiated through the transparent side under a Philips black light lamp (125 W, maximum intensity at 365 nm) at 2 cm distance for fixed periods of time. At the end of each exposure the absorption spectra was recorded. It can be seen from Figure I. that the absorption at 300 nm decreases and simultaneously the absorption maxima at 265 nm decreases with a shift in absorption maximum to 260 nm. These results are in agreement with the reported behaviour of the azidoaryl-CoA derivative (Lau *et al.*, 1977). Complete photodecomposition was found to occur within 5 min of irradiation. All the photoactivation experiments with p-azidobenzoyl-CoA were therefore done by irradiation for a period of 5 min.

Effect of p-azidobenzoyl-CoA on citrate lyase :

No detectable inactivation of the citrate cleavage activity of the *K. aerogenes* holoenzyme was observed with p-azidobenzoyl-CoA (upto 160 μ M) both in the dark and on

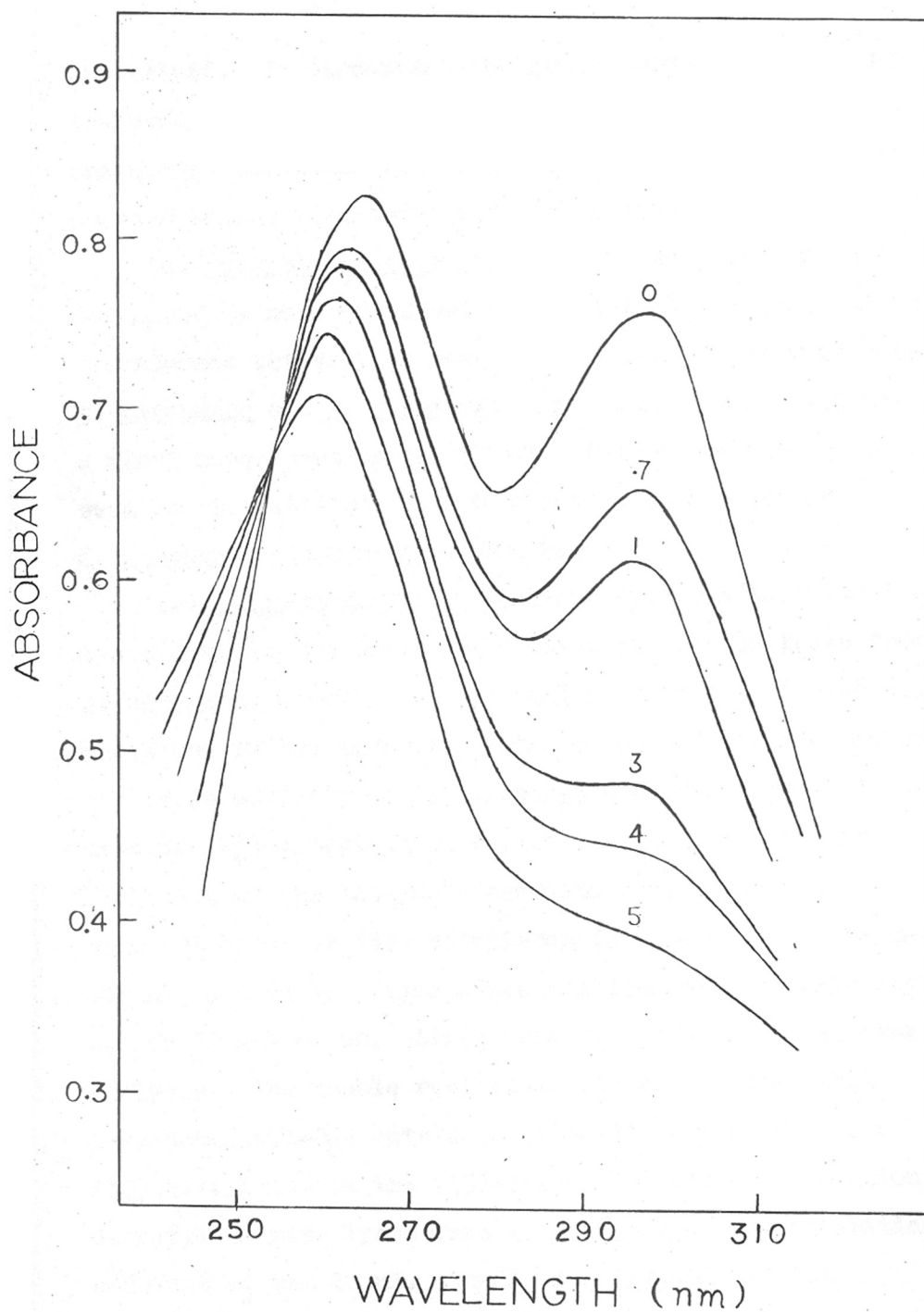


FIG. I-6. ABSORPTION SPECTRA OF *p*-AZIDOBENZOYL-CoA AFTER IRRADIATION FOR FIXED PERIODS OF TIME

photolysis. To circumvent the protection offered by the acylated prosthetic group, effects on the individual enzymatic activities of the deacetyl enzyme complex were studied in the acetyl-CoA mediated reactions.

(a) Acyl transferase activity. p-Azidobenzoyl-CoA was found to have no effect on the acetyl-CoA mediated acyl transferase activity of deacetyl citrate lyases from both K. aerogenes and S. faecalis. The reagent was tried upto a final concentration of 160 μM . The reagent had no effect even on the isolated -transferase subunit of the K. aerogenes citrate lyase complex.

(b) Lyase activity. p-Azidobenzoyl-CoA inhibited the lyase activity in the dark of deacetyl citrate lyase from K. aerogenes as well as S. faecalis. Kinetic studies were performed in the dark to see the effect of the reagent on the lyase activity of K. aerogenes deacetyl citrate lyase towards (3S)-citryl-CoA. Figure I. shows the Lineweaver-Burk plot of the data obtained with varying initial concentrations of (3S)-citryl-CoA (0 - 50 μM) both in the absence and at two fixed concentrations of p-azidobenzoyl-CoA of 30 and 60 μM . Lines were fitted by least squares analysis. The double reciprocal plots indicated that p-azidobenzoyl-CoA acts as a competitive inhibitor for (3S)-citryl-CoA in the (3S)-citryl-CoA cleavage reaction by deacetyl citrate lyase from K. aerogenes. The inhibition constant K_i was 80 μM . The K_m for (3S)-citryl-CoA determined from the data was 67 μM .

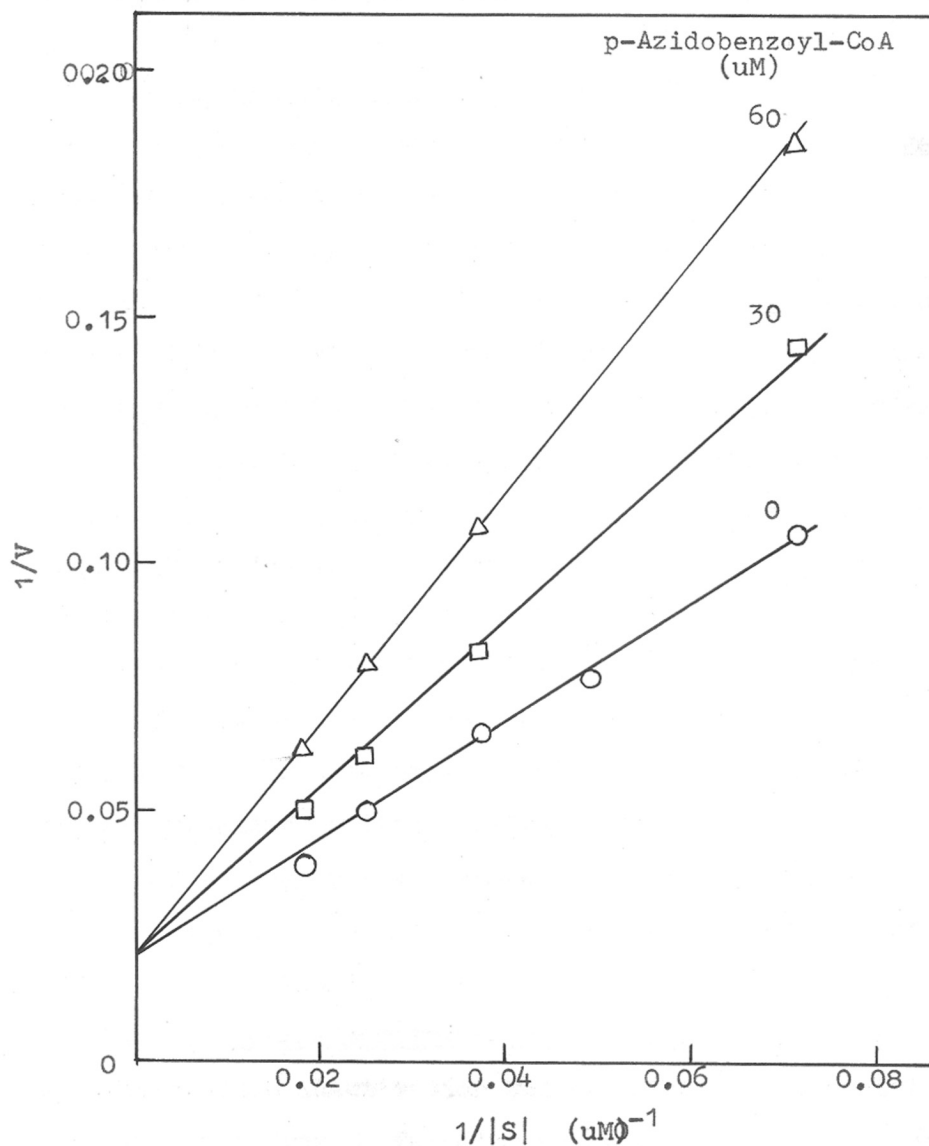


FIG. I.7 The competitive inhibition of the lyase activity of *K. aerogenes* deacetyl citrate lyase by p-azidobenzoyl-CoA with (3S)-citryl-CoA as the varied substrate. Initial velocity is expressed as $\mu\text{mol. of OAA formed per min.}$

Photoaffinity labelling of citrate lyase with p-azidobenzoyl-CoA :

(a) K. aerogenes citrate lyase. The data on the effect of photoactivated p-azidobenzoyl-CoA on K. aerogenes deacetyl citrate lyase are summarized in Table I.4.

Deacetyl citrate lyase treated with p-azidobenzoyl-CoA (final concentration upto 168 μ M) and photolysed for 5 min as described in Materials and Methods showed no loss in acyl transferase activity. The photoactivated reagent was also without effect on the activity of the isolated transferase

subunit of K. aerogenes citrate lyase. However, from Table I.5 it can be seen that under these conditions the lyase activity of deacetyl enzyme was irreversibly inactivated to an extent of 25%, 50% and 58% in the presence of 80 μ M, 160 μ M and 168 μ M. concentrations of p-azidobenzoyl-CoA, respectively. As seen from the Table I.4 the inactivation of lyase activity of the deacetyl enzyme was similar both in the presence of EDTA (10 mM final concentration) as well as in the presence of Mg^{2+} (3 mM final concentration).

(b) S. faecalis citrate lyase. A similar pattern of irreversible inactivation was observed with deacetyl citrate lyase from S. faecalis on irradiation in presence of p-azidobenzoyl-CoA. The data is summarized in Table I.5. Like in K. aerogenes, the transferase activity of the deacetyl citrate lyase of S. faecalis is unaffected on irradiation for 5 min in presence of p-azidobenzoyl-CoA

TABLE 1.4 : Effect of photoactivated p-azidobenzoyl-CoA on transferase and lyase activities of Klebsiella aerogenes citrate lyase. ^a

Enzyme	Buffer	p-Azido- benzoyl- CoA (uM)	% inhibition	
			Trans- ferase Activity	Lyase Activity
Deacetyl citrate lyase	50 mM Tris-HCl	0	0	0
	pH 8.0 +	80	0	25
	3 mM Mg ²⁺	112	0	42
		160	0	50
		168	0	58
	50 mM Tris-HCl	0	0	0
	pH 8.0 + EDTA	80	0	25
		160	0	48
Acyl- trans- ferase subunit	50 mM Tris-HCl	0	0	
	pH 8.0	80	0	
		160	0	

^aSamples contained in a total volume of 0.3 ml: 150 ug deacetyl enzyme or about 25 ug isolated subunit. Aliquots were withdrawn after 5 min irradiation and activities assayed as described under Experimental Procedure. Controls in which p-azidobenzoyl-CoA was absent showed no loss in the enzymatic activities.

(final concentration upto 160 μ M). While under the same conditions the lyase activity was inactivated to a maximum extent of 57% by 160 μ M of the reagent.

TABLE I.5 : Effect of photoactivated p-azidobenzoyl-CoA on transferase and lyase activities of Streptococcus faecalis citrate lyase^a

Enzyme	p-Azidobenzoyl-CoA (μ M)	% Inhibition	
		Trans-ferase activity	Lyase activity
Deacetyl citrate lyase	0	0	0
	40	0	21
	80	0	38
	120	0	55
	160	0	57

^aSamples contained in a total volume of 200 μ l: 120 μ g deacetyl citrate lyase from S. faecalis; 50 mM Tris-HCl, pH 8.0; 3 mM MgSO_4 and the indicated amounts of p-azidobenzoyl-CoA. After irradiation for 5 min, 50 μ l aliquots were assayed for residual lyase activity.

(c) Isolated lyase subunit of E. coli. The isolated -subunit of K. aerogenes citrate lyase showed very low activity, probably being extensively and irreversibly

denatured during the isolation procedure as reported by Dimroth and Eggerer (1975b). The effect of p-azidobenzoyl-CoA on the isolated subunit was therefore not studied. The E. coli -subunit when isolated after denaturation of the holoenzyme with urea and gel filtration through Sepharose CL-6B column in the presence of urea (Nilekani and SivaRaman, 1983), retained relatively higher activity on removal of the denaturant. The effect of the reagent was therefore studied on the isolated -subunit of E. coli citrate lyase. The data on the effect of p-azidobenzoyl-CoA of the E. coli lyase subunit is summarized in Table I.6. The inactivation pattern of the isolated -subunit of E. coli citrate lyase was similar to that of the lyase activity of deacetyl citrate lyase from K. aerogenes (Table I.4).

TABLE I.6 : Inactivation of isolated -subunit of E. coli citrate lyase by p-azidobenzoyl-CoA^a

Enzyme	p-azidobenzoyl-CoA (μ M)	% Inhibition
Acyl-lyase	0	0
subunit of	70	28
<u>E. coli</u>	105	51
citrate lyase	140	58

^aThe irradiation mixture in a total volume of 0.3 ml contained 100 μ g of lyase subunit; 50 mM Tris-HCl,

pH 8.0; 3 mM MgSO_4 and the indicated amounts of p-azidobenzoyl-CoA

Protection studies with (3S)-citryl-CoA :

A further evidence that p-azidobenzoyl-CoA is an active site directed photoaffinity label is obtained from the ability of (3S)-citryl-CoA to protect the lyase activity from irreversible inactivation on irradiation in presence of the reagent. Table I.3 summarizes the effect of (3S)-citryl-CoA on inactivation of the lyase activity of both K. aerogenes and S. faecalis citrate lyase by photoactivated p-azidobenzoyl-CoA.

In case of K. aerogenes citrate lyase the inactivation in presence of 112 μM p-azidobenzoyl-CoA was completely blocked up 200 μM (3S)-citryl-CoA while inactivation of S. faecalis enzyme by 160 μM reagent was completely blocked by 212 μM (3S)-citryl-CoA.

Stoichiometry of binding :

Quantitative binding studies were carried out with p-azido- ^{14}C -benzoyl-CoA and deacetyl citrate lyase from K. aerogenes. Aliquots of enzyme solutions, irradiated in presence of various concentrations of p-azidobenzoyl-CoA were separately taken for measuring residual enzyme activity and to estimate covalent incorporation of the radioactive reagent as described in Materials and Methods. Enzyme controls photolysed without reagent showed no loss of activity and were taken as 100% activity. A plot of the inactivation of the lyase activity towards (3S)-

TABLE I.7 : Protection of lyase activity of deacetyl citrate lyase from K. aerogenes and S. faecalis from inactivation by photoactivated p-azidobenzoyl-CoA^a.

Enzyme	p-Azidobenzoyl-CoA (uM)	Citryl-CoA (uM)	% Inhibition %
<u>Klebsiella</u> <u>aerogenes</u> deacetyl citrate lyase	0	0	0
	112	0	42
	112	44	31.5
	112	74	25
	112	200	0
<u>Streptococcus</u> <u>faecalis</u> deacetyl citrate lyase	0	0	0
	160	0	57
	160	42.5	42
	160	85	20
	160	212	0

^aThe reaction mixture contained in a total volume of 0.3 ml: 150 ug of deacetyl enzyme; 50 mM Tris-HCl buffer, pH 8.0; 10 mM EDTA; and the indicated amounts of the inhibitor and (3S)-citryl-CoA. The mixture was irradiated for 5 min and 0.05 ml aliquots assayed for residual lyase activity.

citryl-CoA as a function of the covalent incorporation of p-azido- ^{14}C -benzoyl-CoA after photolysis is shown in Figure I.7.. The stoichiometry of the binding was calculated using a value of 575 000 for the molecular weight of K. aerogenes citrate lyase complex (Mahadik and SivaRaman, 1968).

It will be seen from Figure I.7 that the decrease in enzyme activity is linear with the irreversible incorporation of ^{14}C -labeled reagent till about 30% inactivation. To check whether the incorporation of ^{14}C -labeled reagent under limiting conditions was selectively at the active site or not, the photolysis with p-azido- ^{14}C -benzoyl-CoA (50 and 100 μM) were carried out in the presence of (3 β)-citryl-CoA (200 μM) and EDTA (10 mM). The results are summarized in Table I.8. The reactions were carried out in the absence of Mg^{2+} to prevent action on the substrate, the absence of the metal having been shown to have no effect on inactivation with photoactivated p-azidobenzoyl-CoA (Table I.4). Under these conditions the enzyme retained its full lyase activity and the incorporation of radioactivity was completely blocked. In the presence of 50 μM of the radioactive aryl-CoA derivative the enzyme was inactivated 25% in the absence of the substrate. This would suggest that the linearity upto atleast 25% inactivation represents specific labeling of the active site by p-azidobenzoyl-CoA.

On the basis of this assumption an estimate of the total active sites per mol of the enzyme was found by

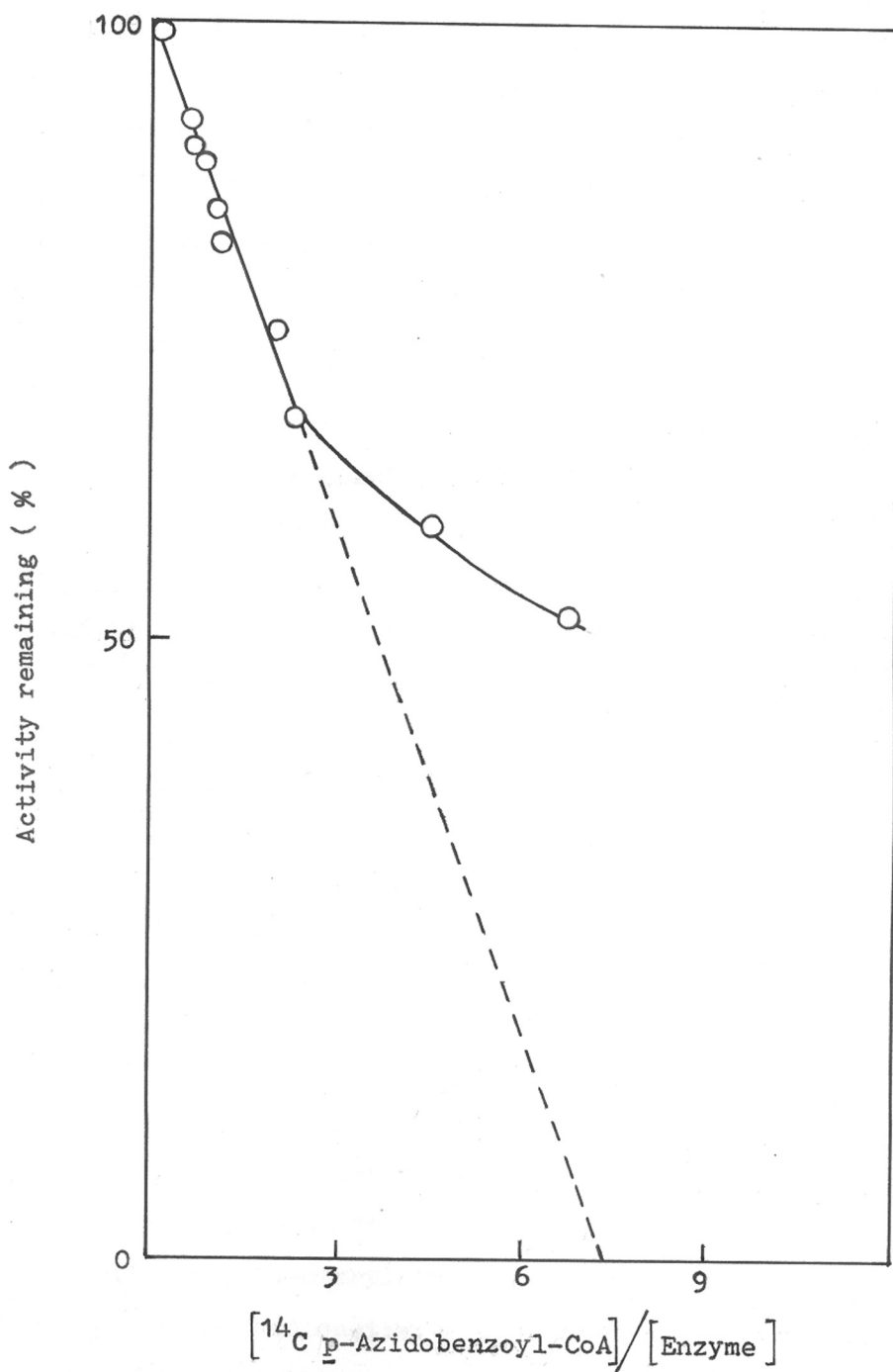


FIG. I.8. Determination of the number of active sites of lyase β -subunit per mol of deacetyl citrate lyase complex accessible to p -azidobenzoyl-CoA.

extrapolation of the linear part of the plot by fitting of least squares line for the data upto 25% inactivation. This extension was observed to include also the value for 30% inactivation. The stoichiometry of the number of active sites per mol of enzyme obtained in this manner was 7.4. The extended extrapolation would make this only an approximate estimate.

TABLE I.8 : Non-specific labeling of *K. aerogenes* citrate lyase complex on irradiation in presence of p-azido- ^{14}C -benzoyl-CoA^a

p-Azidobenzoyl CoA (μM)	(3S)-citryl- CoA (μM)	Inhibition %	^{14}C -reagent/ Enz	
			Total	Non-specific ^b
50	0	25	2.0	0
50	200	0	0	0
100	0	38	6.4	3.4
100	200	2	1.0	0

^a*K. aerogenes* deacetyl citrate lyase (125 μg) was irradiated for 5 min in 0.3 ml of 50 mM tTris-HCl, pH 8.0; containing 3 mM MgSO_4 and the indicated amounts of ^{14}C -labeled reagent and (3S)-citryl-CoA. Residual activity and protein bound radioactivity was measured as described in Materials and Methods.

^bDifference between value calculated from extrapolated linear region of the plot in Figure I.7.

At concentrations of the reagent required for a higher degree of inactivation, photolysis apparently results in significant nonspecific incorporation even in the presence of Tris which has been shown to function as a scavenger for the highly reactive nitrene groups which are away from the binding site (Bayley and Knowles, 1977). Nonspecific binding of p-azidobenzoyl-CoA has also been reported by Lau et al. (1977) for acyl glycine N-acyl transferase of beef liver at values above about 30% inactivation.

Distribution of covalent labeled radioactivity :

An enzyme sample photolysed in presence of the [^{14}C]-labeled p-azidobenzoyl-CoA was dissociated and the constituent subunits separated by SDS-polyacrylamide gel electrophoresis. The electrophoretic profile of the separated subunits and the radioactivity distribution in the gel are represented in Figure I. 8. The presence of radioactivity exclusively in the gel region containing the lyase subunit would confirm the evidence obtained from the kinetic data (Figure I.), Figure I.7 and Table I.4 that the active site of lyase subunit is specifically labeled on photolysis in presence of the azidoaryl-CoA.

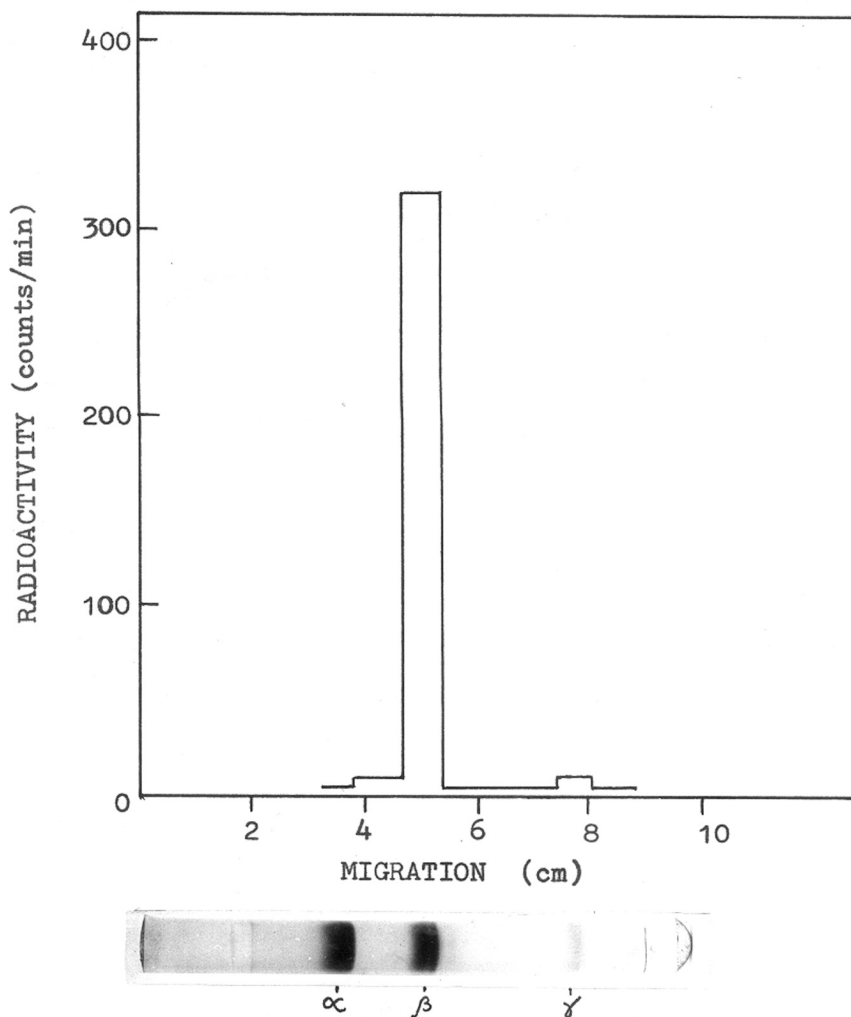


FIG. I.9 SDS-Polyacrylamide gel electrophoresis of *K. aerogenes* deacetyl citrate lyase labeled by photolysis in the presence of p-azido- ^{14}C -benzoyl-CoA.

The histogram shows the profile of radioactivity in the separated subunits

DISCUSSION

Lau et al. (1977) have described the synthesis and characterization of p-azidobenzoyl-CoA and have effectively used it as a reagent for the photoaffinity labeling of the active sites of acyl-CoA : glycine N-acyltransferase. Wood and his coworkers have used the reagent as a photoaffinity label for methylmalonyl-CoA transcarboxylase (Poto et al., 197). p-Azidobenzoyl-CoA has thus been shown to be useful for investigating the reactive sites of enzymes which utilize CoA esterified with an aliphatic acid. In our studies, p-azidobenzoyl-CoA has been shown to have no inhibitory effect on the acetyl-CoA dependent acyltransferase activity of both K. aerogenes and S. faecalis deacetyl citrate lyases. That the enzyme complex might have hindered the photoaffinity reagent from reaching the active site on the transferase subunit has been ruled out, since the isolated acyl-transferase -subunit of the K. aerogenes enzyme is also not inhibited by p-azidobenzoyl-CoA on photoactivation. The active site of the transferase subunit itself apparently does not accept p-azidobenzoyl-CoA, probably on account of the bulky aryl group. Buckel et al. (1973) have shown that among several acyl-CoA derivatives checked for their ability to substitute for acetyl-CoA, only propionyl-CoA was effective while malonyl-CoA, malylyl-CoA, butyryl-CoA and valeryl-CoA were inactive.

The substrate binding sites of the lyase subunits of

K. aerogenes and S. faecalis enzyme complex, however, accept the aryl-CoA ester. The enzymes from the two sources represent distinct types on the basis of their reaction-inactivation behaviour, the former showing rapid inactivation while the later is only weakly inactivated. However, both show comparable extents of inactivation by p-azidobenzoyl-CoA under similar conditions of treatment. The kinetic data obtained with K. aerogenes deacetyl citrate lyase establish that p-azidobenzoyl-CoA acts as a potent competitive inhibitor of the acyl lyase activity, having an inhibitor constant $K_i = 80 \text{ uM}$. The reagent presumably binds specifically to the CoA-ester site of the lyase subunits. Further evidences were (a) the observation that ^{14}C -labeled reagent was found exclusively with the lyase subunit on photoirradiation; (b) during photolysis with different concentrations of the ^{14}C -labeled reagent a linear relationship is observed between incorporation of the ^{14}C -label into the protein and loss in acyl lyase activity under limiting conditions of the reagent and (c) the incorporation of the ^{14}C -labeled reagent is blocked in the presence of the substrates (3S)-citryl-CoA, the activity being completely retained. The reagent would thus be useful for selectively labeling the lyase active sites of the deacetyl citrate lyase enzyme complex, as the reagent has been shown to have no action on the acetyl-CoA binding sites of the acyl-transferase -subunits.

The lyase -subunit has an absolute requirement for

divalent metal ions such as Mg^{2+} for its activity (Dimroth and Eggerer, 1975b); Buckel et al., 1973). SivaRaman and SivaRaman (1979) have shown more recently by metal binding studies using $^{54}MnCl_2$ that a conformational change is brought about in the enzyme complex from K. aerogenes on binding with divalent metal ions. In the present studies, the binding of p-azidobenzoyl-CoA to the active site of the lyase subunit and the subsequent irreversible inactivation on photolysis have been shown to occur both in the presence of Mg^{2+} and in the presence of excess of EDTA. These results might suggest that (3S)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of Mg^{2+} .

Although the K. aerogenes citrate lyase enzyme complex has been shown to have a hexameric (, ,)₆ structure (Carpenter et al., 1975; Dimroth and Eggerer, 1975a), careful estimation of the prosthetic group components by chemical and microbiological assay methods have indicated that only four acyl-carrier proteins carry pantetheine moieties (Singh et al., 1976). This has posed the problem of the function of subunit combinations which are devoid of prosthetic groups. Buckel et al. (1973) have earlier observed that certain acyl-CoA such as acetyl-CoA, propionyl-CoA and (3S)-citryl-CoA can replace the prosthetic group and mediate citrate cleavage catalyzed by deacetyl citrate lyase. Robinson et al. (1976) have suggested that

the subunit combinations which are devoid of prosthetic group might function in citrate cleavage mediated by acetyl-CoA. The approximate estimate obtained in the present investigation of about seven acyl-CoA binding sites which are available on the lyase subunits in a mol of the citrate lyase enzyme complex from K. aerogenes is suggestive of the fact that all the lyase structure subunits in the complex are accessible to p-azidobenzoyl-CoA or (3S)-citryl-CoA. Thus in the intact organism the cleavage of citrate can proceed possibly both via the built-in prosthetic group and through the acyl-CoA mediated reaction.

PART II

REACTION - MECHANISM BASED INHIBITORS OF
CITRATE LYASE (EC 4.1.3.6)

REACTION MECHANISM BASED INHIBITORS OF
CITRATE LYASE (EC 4.1.3.6)

SUMMARY

p-Azidobenzoate, monochloroacetate and L-malate have been shown to be suicide substrates of the purified native citrate lyase enzyme complexes from Klebsiella aerogenes and Streptococcus faecalis.

Incubation of Klebsiella aerogenes citrate lyase with p-Azidobenzoate followed by photolysis resulted in inactivation without formation of any cross-linking between subunits. p-Azidobenzoate rapidly and irreversibly inactivates purified native citrate lyases from both the sources in the dark. Inactivation in presence of EDTA was markedly higher than in presence of Mg^{2+} . In presence of EDTA (10 mM) and p-azidobenzoate (10 mM), the first-order kinetics of inactivation were observed upto about 90% inactivation, with apparent rate constants of inactivation (K_{app}) of 0.09 min^{-1} for the Klebsiella aerogenes complex and 0.10 min^{-1} for the Streptococcus faecalis enzyme. In presence of $MgSO_4$ (3 mM) and p-azidobenzoate (10 mM) the rates of inactivation were markedly lowered; the Klebsiella aerogenes enzyme showed a marked deviation from the first order kinetics. The Streptococcus faecalis enzyme showed a markedly lower inactivation rate ($K_{app} = 0.008 \text{ min}^{-1}$) than the Klebsiella aerogenes enzyme ($K_{app} = 0.0235 \text{ min}^{-1}$) in presence of metal. The lower rates of inactivation, presumably through a lower rate of formation of the mixed anhydride

intermediate with the Streptococcus faecalis enzyme under conditions required for substrate cleavage possibly accounts for its slow reaction-inactivation behaviour compared to the Klebsiella aerogenes enzyme. The effect of Mg^{2+} in lowering inactivation rates is indicative of a conformational change in the complex on binding of the metal cofactor. The mechanism of inactivation was analysed by reactivation studies with acetic anhydride and acetate. After removal of excess p-azidobenzoate, reactivation was observed only with acetic anhydride and not with acetate indicating that the inactivation is a result of deacetylation. A probable mechanism of deacetylation is through the formation of the mixed acetic acid - p-azidobenzoic acid anhydride intermediate which diffuses away from the active site leaving the enzyme in an inactive form. Similar results were observed with monochloroacetate only the rate of inactivation was less rapid than in the case of p-azidobenzoate.

L-Malate, however, showed a difference from p-azidobenzoate and monochloroacetate in its mechanism of inactivation. In this case partial reactivation was obtained with acetate, suggesting that the L-malyl-S-enzyme intermediate is also formed subsequent to the formation of acetic acid - malic acid mixed anhydride.

INTRODUCTION

Bacterial citrate lyase (EC 4.1.3.6) has been obtained pure from diverse sources and has been shown to contain three non-identical subunits (Carpenter et al., 1975). The enzyme from K. aerogenes, which has been studied the most, contains a 10 000 dalton acyl-carrier protein () subunit (Dimroth et al., 1973) with a covalently bound CoA-like prosthetic group (Robinson et al., 1976; Oppenheimer et al., 1979) carrying an essential acetyl moiety (Buckel et al., 1971). The cleavage of citrate by the enzyme complex is catalyzed in a sequence of two steps (Buckel et al., 1973). The first step involving an acyl-transferase reaction mediated by the -subunit (54 000 dalton) to yield the citryl-ACP intermediate which is subsequently cleaved in presence of divalent metals by a reaction catalyzed by the acyl-lyase (-subunit of 32 000 dalton) (Dimroth and Eggerer, 1975b). Citrate lyases undergo characteristic reaction-inactivation (Singh and Srere, 1971) through the loss of the essential acetyl group to yield deacetyl enzyme (Buckel et al., 1971a). Although details of the mechanism of this deacetylation are not understood, the acyl-transferase has been reported to catalyze the hydrolysis of the citryl-ACP intermediate through acyl transfer to water to yield deacetyl-ACP (Dimroth and Eggerer, 1975b). Some citrate lyases such as the complexes from K. aerogenes and R. gelatinosa (Singh and Srere, 1971; Kummel et al., 1975) undergo rapid reaction-inactivation in vitro, while

others such as the complexes from S. diacetilactis and S. faecalis (Singh and Srere, 1975; Hiremath et al., 1976) are inactivated at markedly slower rates.

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 (Jencks, 1973), in that a single displacement mechanism operates unlike the double displacement of the classical CoA transferases (Dimroth et al., 1977b). Kinetic evidences indicate that in citrate lyase an anhydride intermediate between the substrate and the acetyl moiety of the prosthetic group is generated without the formation of an enzyme-CoA intermediate in acetyl-CoA mediated reaction or an acyltransferase-carbonyl-S-ACP intermediate in the acetyl-S-ACP mediated step.

With substrate analogues such as 3-fluoro-3-deoxy citrate (3-fluoro citrate), two different types of reaction-mechanism based inhibitions have been observed (Rokita et al., 1982). From the known reaction mechanism of citrate lyase acyl-transferase, the formation of an anhydride intermediate of the substrate analogue and acetic acid is expected. On the basis of this mechanism, Rokita et al. (1982) have indicated that a suicide substrate might act through two possible routes. The anhydride could either diffuse away from the active site leaving the enzyme in the deacetylated and inactive form or it could subsequently yield the substrate

analogue-carbonyl-S-ACP intermediate as a dead-end product. In the case of 3-fluorocitrate equal extents of both types of inactivation were observed, approximately half of active sites being deacetylated and the other half being converted to the 3-fluorocitryl-S-enzyme which cannot structurally undergo the retroaldol cleavage.

In the previous chapter the use of p-azidobenzoyl-CoA as a photoaffinity probe for the active sites of the lyase subunits has been described. As an extension of this study it was thought of interest to determine whether p-azidobenzoate would act as reaction-mechanism based photoreactive cross-linking reagent in the event of p-azidobenzoyl-S-ACP being formed through the initial acyl-transferase reaction. The present chapter describes the suicidal inactivation of citrate lyases from *K. aerogenes* and *S. faecalis* through deacetylation in presence of p-azidobenzoate. The mechanisms of inhibition of the enzyme by two other suicide substrates, monochloroacetate and L-malate, are also described.

MATERIALS AND METHODS

Materials:

L-Malic acid, malate dehydrogenase from pig heart (specific activity of $945 \text{ } \mu\text{mol. min}^{-1} \text{ mg}^{-1}$), NADH from yeast (disodium salt - $3\text{H}_2\text{O}$, grade III) and primary standard grade Tris were obtained from Sigma Chemical Co. USA. Monochloroacetic acid was obtained from E. Merck, Darmstadt. p-Aminobenzoic acid was from BDH, England. Acetic anhydride was from HPC Ltd., India.

p-A p-Azidobenzoic acid was prepared from p-aminobenzoic acid via a diazonium salt intermediate as described by Lau et al. (1977). The procedure is described briefly in Part I of this thesis.

The reagents and buffers required for purification of citrate lyase from K. aerogenes and S. faecalis were similar to those described in Part I of this thesis.

All other chemicals and reagents were of the highest quality available commercially.

Methods :

Protein determinations :

Protein was routinely determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. Enzyme protein in the purified complex from K. aerogenes was determined from absorbance measurement at 278 nm using absorption index $E_{1\%}^{1\text{cm}}$ of 6.2 (Singh et al., 1976).

Enzymes :

Citrate lyase from K. aerogenes was prepared by the method described by Mahadik and SivaRaman (1968) and modified by Tikare (1979). A brief outline of the procedure has been described in Part I of this thesis. The preparations were homogeneous both on analytical ultracentrifugation and polyacrylamide gel electrophoresis. The preparation had a specific activity of about $90 \text{ } \mu\text{mol. min}^{-1} \text{ mg}^{-1}$.

Citrate lyase from S. faecalis was purified according to the procedure of Hiremath et al. (1976). The procedure has already been described in Part I of this thesis.

Analytical ultracentrifugation and polyacrylamide gel electrophoresis showed that the preparations were homogeneous. The enzyme from the final purification step had a specific activity of about $90 \text{ } \mu\text{mol. min}^{-1} \text{ mg}^{-1}$.

Enzyme assay :

Enzyme activity of pure citrate lyase and of samples from the inactivation studies was determined by coupled assay with malate dehydrogenase (Singh and Srere, 1971). The assay medium contained in a total volume of 1.0 ml: 50 mM Tris-HCl, pH 8.0; 5 mM MgSO_4 ; 5 mM sodium citrate; 0.2 mM NADH and 1.1 U of malate dehydrogenase. The reaction was initiated by the addition of 10 μl of citrate lyase solution containing about 0.5 - 1.5 μg of the pure enzyme. The assay was carried out at 25°C and the optical density at 340 nm was monitored in a Shimadzu UV 210 spectrophotometer. The coupling enzyme was not inhibited by the test compounds under the assay conditions.

Inhibitors :

Stock solutions of 100 mM concentration of the three acids, p-azidobenzoic acid, monochloroacetic acid and L-malic acid were prepared in distilled water and neutralized by the addition of potassium bicarbonate.

Photoactivation in presence of p-azidobenzoate :

Native citrate lyase from *K. aerogenes* (150 μg) in a total volume of 0.3 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM p-azidobenzoic and 10 mM EDTA was incubated

at 30°C for 10 min in the dark. The solution was then cooled in an ice bath and irradiated under a Philips Black light lamp (365 nm) for a period of 5 min. An aliquot was checked for residual activity and the remaining solution was subjected to SDS-polyacrylamide gel electrophoresis as described in Part I of this thesis except that the mercaptan was omitted from all buffers.

Effect of p-azidobenzoate, monochloroacetate and L-malate on citrate lyase activity :

Time-dependent inactivation of citrate lyase from *K. aerogenes* and from *S. faecalis* was studied by incubating a solution of the test compound with the enzyme at 25°C in the dark. The incubation mixture contained in a total volume of 0.2 ml: 30 ug of pure citrate lyase from either *K. aerogenes* or *S. faecalis*; 50 mM Tris-HCl buffer, pH 8.0; 3 mM $MgSO_4$ or 10 mM EDTA and required amounts of one of the neutralized stock solution containing the test compound (2.5 or 10 mM final concentration). The mixture was incubated at 25°C and at regular intervals of time, aliquots (10 ul) of the incubation mixture were withdrawn and assayed for residual citrate lyase activity. The enzyme activity at zero time was arbitrarily taken as 100%. Control experiments were run in which the enzyme was incubated at 25°C in a system of the same composition as the incubation system except that the test solution was omitted. Enzyme activity was determined by the malate dehydrogenase coupled assay as described earlier. The decrease in absorbance

at 340 nm was monitored and the initial rate of oxaloacetate formation was calculated from the extinction coefficient value of NADH at 340 nm.

Protection by acetate :

The effect of acetate in protecting the inactivation of *K. aerogenes* citrate lyase by p-azidobenzoate was investigated as follows: The pure enzyme (30 ug) was incubated at 25°C in a total volume of 0.2 ml solution containing 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; and sodium acetate (1 - 50 mM). After an incubation period of 5 min, p-azidobenzoate solution was added to give a final concentration of 10 mM of the inhibitor. The mixture was then incubated for 30 min and an aliquot of 10 ul was checked for citrate lyase activity by the malate dehydrogenase coupled assay procedure.

Reactivation studies :

K. aerogenes citrate lyase (150 - 200 ug) was incubated at 25°C in a total volume of 1 ml containing 50 mM Tris-HCl, pH 8.0; 10 mM EDTA and the acid inhibitor final concentrations 10 or 20 mM. After an incubation period of 30 or 60 min the system was subjected to reactivation by treatment with acetate or acetic anhydride.

(a) Reactivation by acetate. Test for reactivation with acetate was done by treating the inhibited enzyme with sodium acetate (0.1 M) as described by Rokita et al., (1982). 20 ul of the inactivation mixture was treated with 20 ul of 0.2 M sodium acetate and incubated for 5 min at 35°C. After

the incubation the reactivation mixture was transferred into a 1 ml cuvette containing the malate dehydrogenase coupled assay system and the decrease in absorbance at 340 nm was monitored.

(b) Reactivation with acetic anhydride. The inactivated enzyme solution was cooled rapidly in an ice-bath and acetic anhydride was added to a final concentration of 4.7 mM and stirred frequently. After a period of 5 min, an aliquot was withdrawn and checked for reactivation by the coupled malate dehydrogenase assay procedure.

Test for reactivation with acetic anhydride was also carried out after removal of excess inhibitor. The enzyme solution after inactivation by treatment with the inhibitor was passed down a column of Sephadex G-25 (1 x 20 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5 containing 1.6 mM MgSO_4 and eluted with buffer of the same composition. Protein was monitored by adsorption at 280 nm and protein fractions were pooled and aliquots separately tested for residual activity and for reaction with acetic anhydride as described above.

RESULTS

Photolysis of citrate lyase in presence of p-azidobenzoate :

Citrate lyase from K. aerogenes was progressively inactivated on incubation in the dark with p-azidobenzoate (10 mM) and EDTA (10 mM) over a period extending upto 20 min at 25°C. SDS-polyacrylamide gel electrophoresis carried

out after photolysis for 5 min showed the presence of the 54 000 (), 32 000 () and 10 000 () daltons subunits only. No cross-linked subunit species was detected indicating that p-azidobenzoyl-S-ACP is probably not formed in the inactivation process.

Inactivation of citrate lyase by p-azidobenzoate :

Interaction of p-azidobenzoate with citrate lyase. The effect of p-azidobenzoate in the dark on citrate lyases from K. aerogenes and S. faecalis is shown in Figure II.1 and the kinetic constants are summarized in Table II.1. Controls in which the enzyme was incubated with EDTA (10 mM) or MgSO_4 (3 mM) in the absence of the reagent did not show any significant inactivation under the conditions of incubation. From Figure II.1 it can be seen that the reagent is an effective time-dependent inactivator of citrate lyase in presence of EDTA. The inactivation shows first-order kinetics upto about 90% inactivation. The first order inactivation rate constants K_{app} were 0.09 min^{-1} and 0.1 min^{-1} at a concentration of 10 mM p-azidobenzoate with K. aerogenes and S. faecalis citrate lyase, respectively (Table II.1). In presence of EDTA, the S. faecalis enzyme is inactivated slightly more rapidly than the K. aerogenes enzyme. The enzyme from K. aerogenes showed deviation from first order kinetics and the attainment of a plateau level of residual activity.

In presence of Mg^{2+} (3 mM) inactivation by 10 mM p-azidobenzoate was greatly reduced ($K_{app} = 0.0235$) for K. aerogenes citrate lyase and 0.008 for S. faecalis).

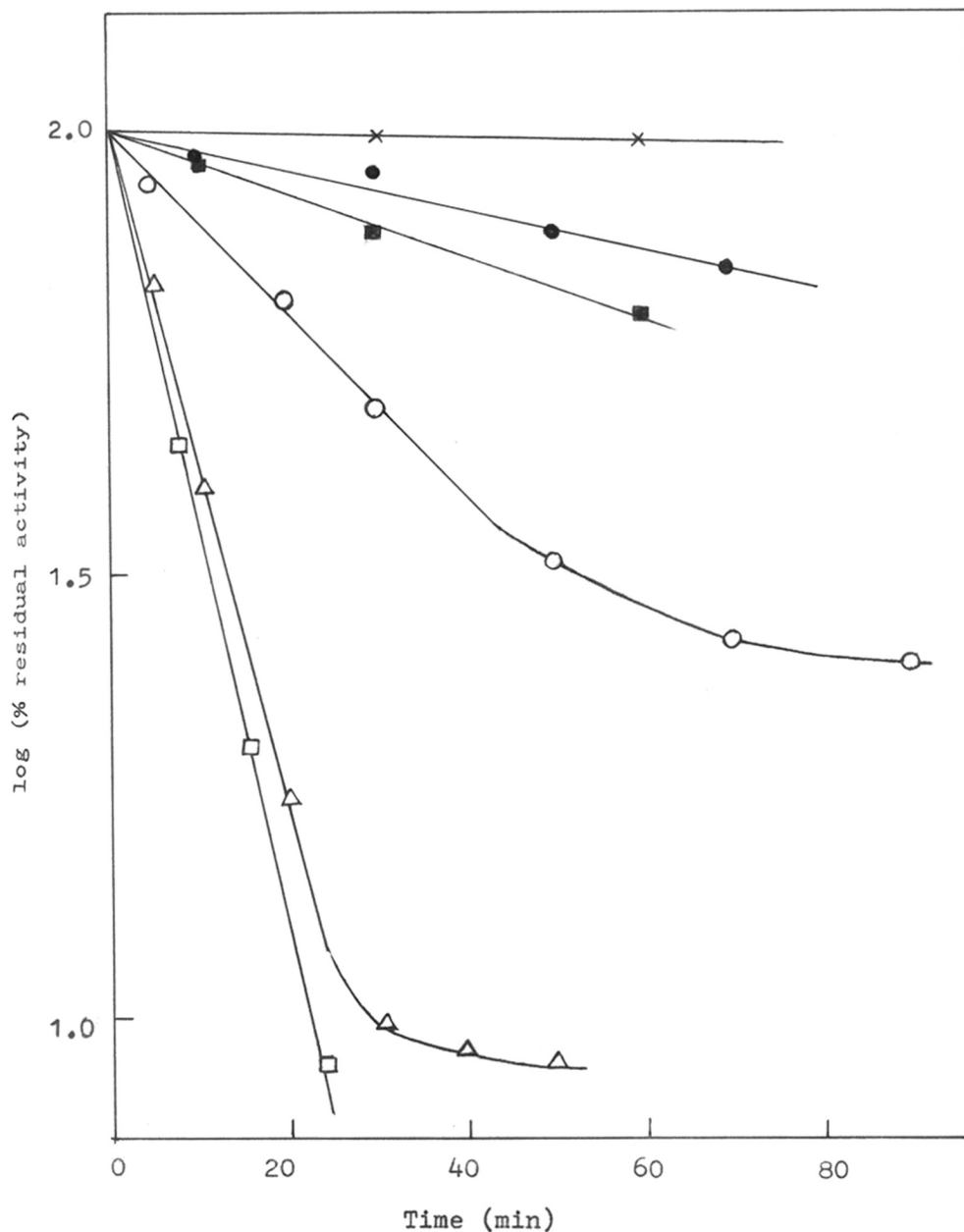


FIG. II.1 INACTIVATION OF CITRATE LYASE BY p-AZIDOBENZOATE
 Control, ().
K. aerogenes enzyme: 2.5 mM p-azidobenzoate + 3 mM MgSO_4 ();
 10 mM p-azidobenzoate + 3 mM MgSO_4 (); 10 mM p-azidobenzoate
 + 10 mM EDTA ().
S. faecalis enzyme: 10 mM p-azidobenzoate + 3 mM MgSO_4 ();
 10 mM p-azidobenzoate + 10 mM EDTA ().

TABLE II.1. Effect of p-azidobenzoate, monochloroacetate and L-malate on citrate lyase enzyme complex from K. aerogenes and S. faecalis.

Enzyme	Preincubation	$10^3 \times K_{app}^* (\text{min}^{-1})$
<u>K. aerogenes</u> citrate lyase	p-Azidobenzoate (10 mM)	23.5
	‡ Mg^{2+} (3 mM)	
	p-Azidobenzoate (10 mM) + EDTA (10 mM)	90
<u>S. faecalis</u> citrate lyase	p-Azidobenzoate (10 mM)	8.1
	+ Mg^{2+} (3 mM)	
	p-Azidobenzoate (10 mM) + EDTA (10 mM)	100
<u>K. aerogenes</u> citrate lyase	Monochloroacetate (10 mM)	10.5
	+ EDTA (10 mM)	
	Monochloroacetate (20 mM)	20
	+ EDTA (10 mM)	
	Monochloroacetate (20 mM)	6.5
	+ Mg^{2+} (3 mM)	
<u>K. aerogenes</u> citrate lyase	L-Malate (10 mM)	29.5
	+ EDTA (10 mM)	
<u>S. faecalis</u> citrate lyase	L-Malate (10 mM)	6.2
	+ EDTA (10 mM)	

* Values of K_{app} were obtained from the linear regions of plots of $\log (\% \text{residual activity})$ versus time of incubation with reagent, $K_{app} = 2.303 \times \text{slope}$.

In contrast to the relative inactivation of the enzymes from the two sources in presence of EDTA, the enzyme from K. aerogenes is inactivated at a markedly higher rate than the S. faecalis enzyme in presence of Mg^{2+} . The K. aerogenes enzyme, unlike the S. faecalis enzyme, again exhibited deviation from first-order inactivation kinetics. The rate of inactivation was dependent on the concentration of p-azidobenzoate, the rate constants in presence of Mg^{2+} being 0.005 min^{-1} with 2.5 mM reagent and 0.0235 min^{-1} with 10 mM reagent.

The inactivation of bht enzymes was irreversible since the removal of excess reagent by gel filtration did not reverse the inactivation.

Reactivation of p-azidobenzoate inhibited citrate lyase. To study the mechanism of inactivation by p-azidobenzoate, reactivation characteristics were monitored by taking advantage of the differential reactivation abilities of acetic anhydride and acetate (Rokita et al., 1982). Table II.2 summarizes the reactivation characteristics of p-azidobenzoate inactivated citrate lyase from K. aerogenes. Acetate (0.1 M) incubation did not result in any reactivation both before and after removal of excess inhibitor. Acetic anhydride at a final concentration of 4.7 mM also failed to reactivate the enzyme in the presence of excess inhibitor. However, on removal of excess reagent, the inactive citrate lyase was reactivated to an extent of 70% by 4.7 mM acetic anhydride. Acetate under these conditions had no reactivation effect.

TABLE II.2. Mode of inactivation of *K. aerogenes* citrate lyase by p-azidobenzoate.

Pretreatment	System for Reactivation	Residual activity (%)	Reactivation (%)
p-Azidobenzoate (10 mM) + EDTA (10 mM)	-	17	-
	Acetate (0.1 M) before removal of excess reagent	16	0
	Acetic anhydride	15	0
p-Azidobenzoate (10 mM) + EDTA (10 mM) followed by removal of excess reagent by gel filtration	Acetate (0.1 M)	12	0
	Acetate (0.1 M)	12	0
	Acetic anhydride	82	70

The enzyme was initially inactivated by incubating with p-azidobenzoate for 20 min. Reactivation characteristics were monitored with acetate or acetic anhydride as described under Materials and Methods.

Protection from inactivation by p-azidobenzoate :

The presence of nucleophilic acetate ion in the incubation mixture prevented the inactivation of K. aerogenes citrate lyase by p-azidobenzoate. Table II.3 shows that the inactivation of citrate lyase decreases markedly with increase in the concentration of added acetate in the incubation mixture. Acetate has been shown to participate in the acetyl-exchange reaction catalyzed by the acyl-transferase subunit of citrate lyase, evidently through the generation of acetic anhydride as an intermediate (Dimroth et al., 1977b). The protection offered by added acetate against inhibition by p-azidobenzoate supports the hypothesis that the inactivation of citrate lyase by p-azidobenzoate is through the formation of mixed p-azidobenzoate-acetic acid - acetic acid anhydride intermediate, the formation of which is blocked in presence of the added acetate.

TABLE II.3. Protection of citrate lyase from K. aerogenes from inhibition by p-azidobenzoate

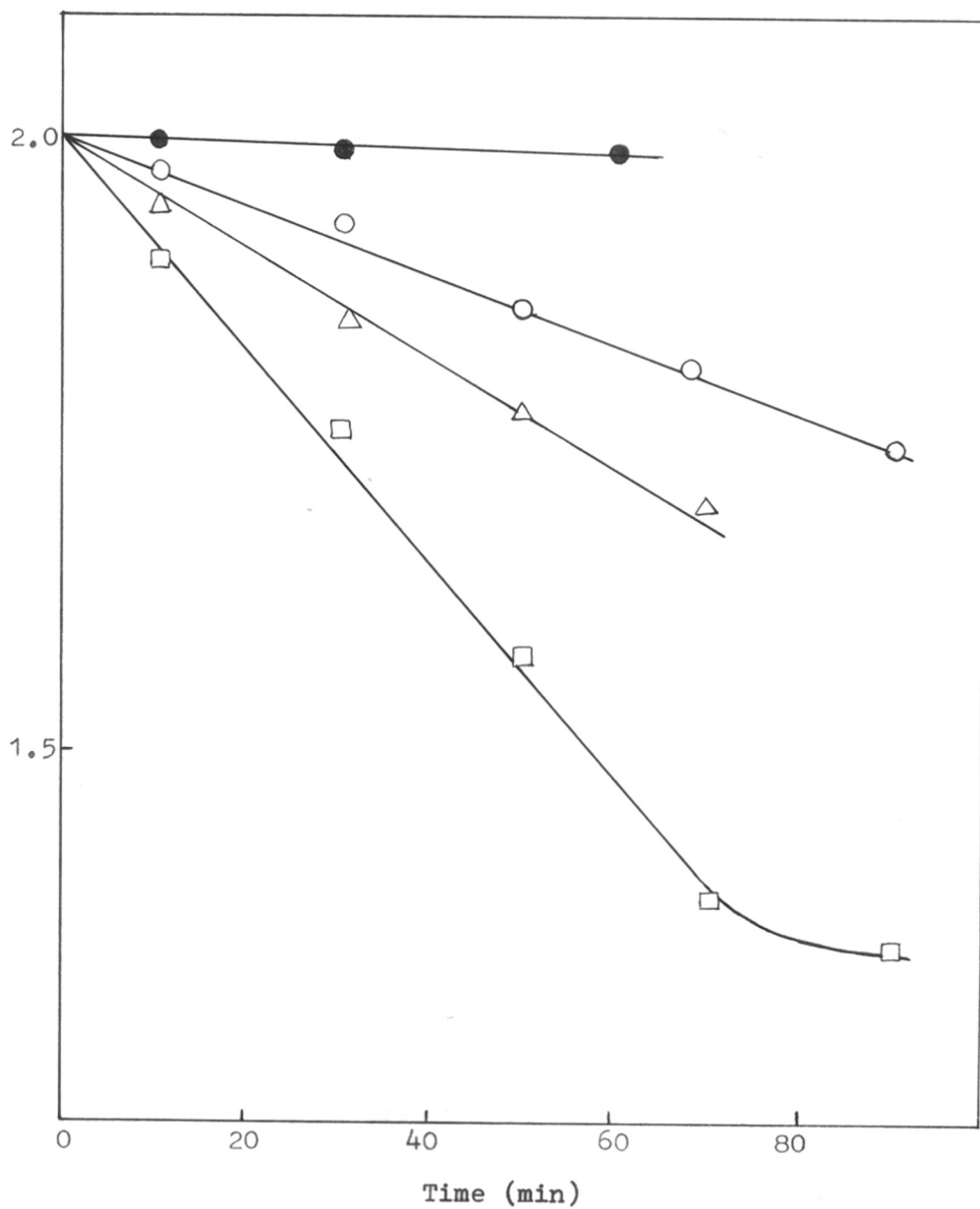
p-Azidobenzoate (mM)	Acetate (mM)	Residual activity (%)
0	0	100
10	0	8.5
10	1	12.5
10	5	35
10	50	88

The reaction mixture contained in a total volume of

0.2 ml : 30 ug pure enzyme; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 10 mM p-azidobenzoate and the indicated amounts of acetate. The mixture was incubation for a total period of 30 min and 10 ul aliquots were assayed for residual activity.

Citrate lyase inactivation by monochloroacetate :

Citrate lyase was inactivated by monochloroacetate and the kinetics of inactivation followed the same pattern as that observed with p-azidobenzoate. From Table II.1 and Figure II.2 it can be seen that the rate of inactivation by monochloroacetate is much slower compared to that observed with p-azidobenzoate. The apparent first-order rate constants of inactivation with 20 mM monochloroacetate in presence of EDTA (10 mM) and Mg^{2+} (3 mM) are 0.02 min^{-1} and 0.0065 min^{-1} , respectively. The first order inactivation constant in presence of 10 mM monochloroacetate and 10 mM EDTA was about 9-fold slower (0.01 min^{-1} vs 0.090 min^{-1}) than that of p-azidobenzoate under similar conditions. The reactivation characteristics of the monochloroacetate-inactivated citrate lyase from K. aerogenes is shown in Table II.4. As in the case of the p-azidobenzoate-inactivated enzyme, there is no reactivation by acetate while 41% reactivation resulted on incubation with acetic anhydride even without removal of excess inhibitor.



Control (●); 20 mM monochloroacetate + 3 mM MgSO₄ (○);
FIG. II.2 INACTIVATION OF *K. aerogenes* CITRATE LYASE BY
 MONOCHLOROACETATE

Control (●); 20 mM MgSO₄ +

TABLE II.4. Mode of inactivation of K. aerogenes citrate lyase by monochloroacetate.

Pretreatment	System for reactivation	Residual activity (%)	Reactivation (%)
Monochloro-acetate (20 mM) + EDTA (10 mM)	-	32	0
	Acetate (0.1 M)	25	0
	Acetic anhydride (4.7 mM)	73	41

Citrate lyase from K. aerogenes was treated with 20 mM Monochloroacetate in presence of 10 mM EDTA and incubated for 60 min prior to the reactivation studies with acetate or acetic anhydride.

Inactivation of citrate lyase by L-malate :

The effect of L-malate on citrate lyase from K. aerogenes and S. faecalis is shown in Figure II.3. The rate of inactivation by L-malate was more rapid with K. aerogenes citrate lyase compared with the S. faecalis enzyme. L-Malate (10 mM) in presence of EDTA (10 mM) inactivates K. aerogenes citrate lyase to an extent of 58% within 30 min, while the S. faecalis enzyme is inactivated only to an extent of 16%, the apparent first-order rate constants being 0.03 min^{-1} and 0.06 min^{-1} , respectively. From

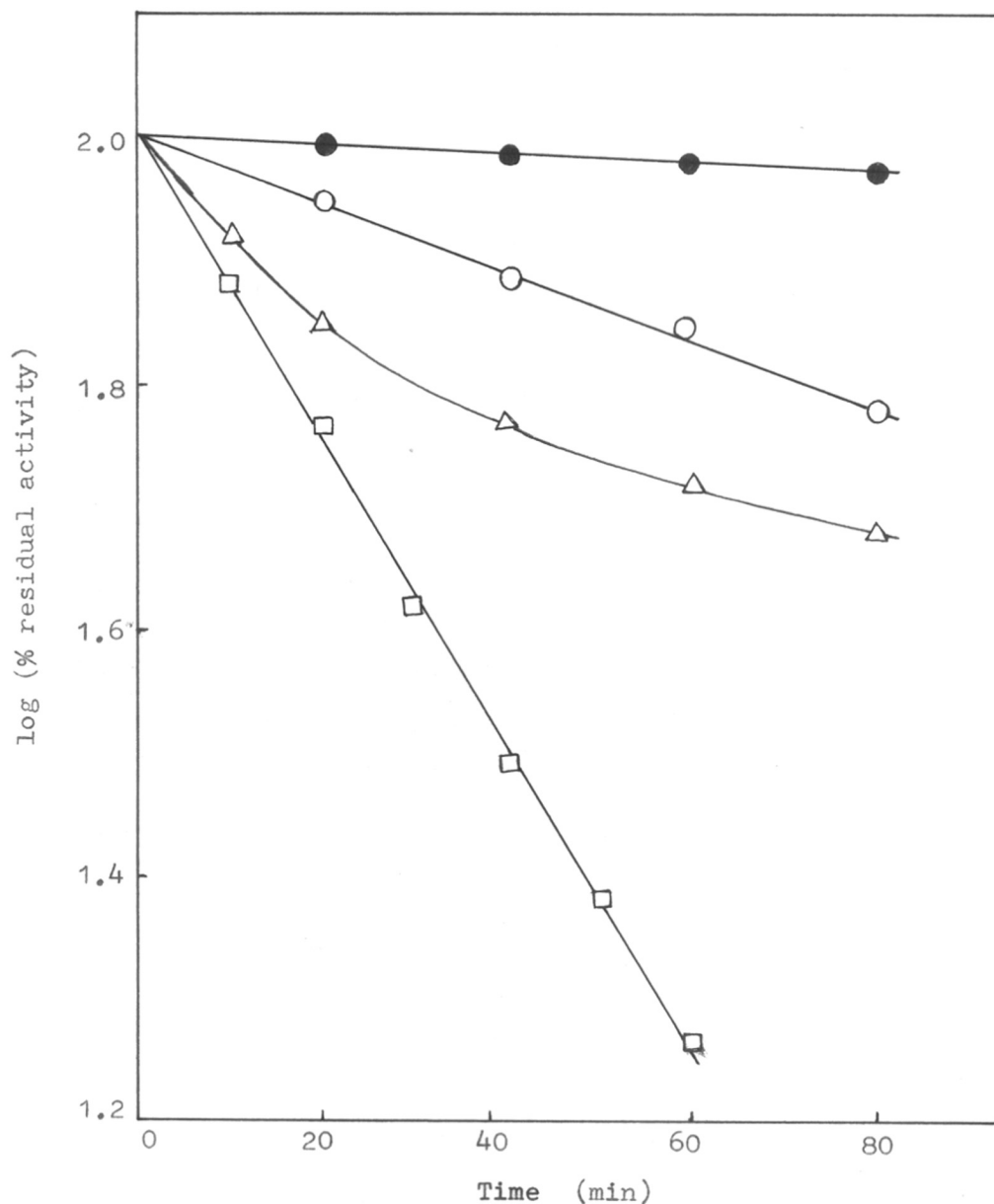


FIG. II.3 INACTIVATION OF CITRATE LYASE BY L-MALATE

Control (●).

K. aerogenes enzyme: 10 mM malate + 3 mM MgSO₄ (○);
10 mM malate + 10 mM EDTA (△).

S. faecalis enzyme: 10 mM malate + 10 mM EDTA (□).

Figure II.3 it can be seen that time dependent inactivation of K. aerogenes citrate lyase by L-malate (10 mM) in presence of Mg^{2+} (3 mM) shows deviation from first-order kinetics of inactivation and attains a plateau around 45 - 50% residual activity.

The reactivation characteristics of L-malate inactivated citrate lyase from K. aerogenes are shown in Table II.5. It is evident that the mode of inactivation by L-malate differs from that of p-azidobenzoate and monochloroacetate. About 20% reactivation results on incubation with acetate (0.1 M) and about 30% reactivation on incubation with acetic anhydride (4.7 mM), even without removal of excess inhibitor. The data would indicate that both formation of the maly-S-enzyme intermediate as well as deacetylation occur. The latter inactive state could also result from the hydrolysis of the maly-enzyme intermediate.

TABLE II.5. Mode of inactivation of K. aerogenes citrate lyase by L-malate.

Pretreatment	System for reactivation	Residual activity (%)	Reactivation (%)
L-Malate (10 mM) + EDTA (10 mM)	-	35	-
	Acetate (0.1 M)	55	20
	Acetic anhydride (4.7 mM)	65	30

Subsequent to the reactivation studies, the enzyme from K. aerogenes was inactivated for 30 min. in presence of L-malate (10 mM) and EDTA (10 mM)

DISCUSSION

Suicide substrates inactivate enzymes irreversibly through reaction-mechanism based formation of toxic or futile intermediates. Rokita *et al.* (1982) have observed a reaction-mechanism based inhibition of bacterial citrate lyase by the citrate analogue, β -fluorocitrate. Inactivation of citrate lyase can be expected at two steps during the turnover of the enzyme. In the event of the substrate analogue participating in the first step of acyl-exchange, a mixed anhydride of the citrate analogue and acetic acid derived from the enzyme acetyl moiety would result. The mixed anhydride could diffuse away from the active site leaving the enzyme stranded in a deacetylated and inactive form. Alternatively the acyl-S-enzyme derivative of the substrate analogue is formed which cannot undergo retroaldol cleavage and thus the enzyme accumulates in this inactive form. Hydrolysis of the latter intermediate could also occur and leave the enzyme in the deacetylated inactive form (Eq. 1).

The mode of inactivation by 3-fluorocitrate has been shown to involve both routes, an immediate deacetylation as well as an accumulation of 3-fluorocitryl-S-enzyme and its subsequent slow hydrolysis. The mode of inactivation of citrate lyase by p-azidobenzoate has been characterized in the present studies from an approach similar to that suggested by Rokita et al. (1982). The distinction between deacetylated enzyme and an inactive acyl-S-enzyme intermediate is based on the differential diagnostic test suggested by Rokita et al. (1982) dependent on reactivation either with acetic anhydride or with acetate. Acetic anhydride specifically reactivates deacetyl citrate lyase (Buckel et al., 1971; Srere et al., 1972) while acetate can reactivate only the inactive acyl-S-enzyme intermediate by an acyl exchange reaction (Rokita et al., 1982). The enzyme inactivated by p-azidobenzoate could only be reactivated with acetic anhydride and not by acetate, suggesting that deacetylation of the enzyme is the cause for inactivation. Deacetylation results probably through the formation of a mixed anhydride of enzyme acetate and p-azidobenzoate, which could diffuse away from the active site leaving the enzyme in the deacetyl inactive form. p-Azidobenzoyl-S-enzyme intermediate even if formed would be highly prone to hydrolysis because of the presence of an aromatic ring with an azido group adjacent to the carbonyl-carbon making it electron deficient and thus prone to nucleophilic attack.

An interesting observation is the markedly lower rate of inactivation of citrate lyase by p-azidobenzoate in the presence of Mg^{2+} . The effect is more pronounced in the case of the S. faecalis enzyme. While in the absence of metal, the rates of inactivation of the enzyme from both sources are comparable, the rate of inactivation of the S. faecalis is about 3 times slower than that of the K. aerogenes enzyme in presence of Mg^{2+} (3 mM). The removal of metal has been shown not to cause any dissociation-association effect in citrate lyase from K. aerogenes in presence of 50 mM Tris-HCl buffer, pH 8.0 (SivaRaman and SivaRaman, 1979), while the S. faecalis enzyme is stable even in the absence of metal and in low ionic strength buffer systems (Hiremath et al., 1976). The metal effect could therefore be attributed to a conformational change in the complex. Evidence for such a change in the complex K. aerogenes citrate lyase complex has been shown earlier from the Mn^{2+} binding characteristics of the purified enzyme (SivaRaman and SivaRaman, 1979). The conformational change brought about by divalent metal apparently effects the reactivity of the acyl transferase subunit, although the acyl exchange reaction does not require metal. The markedly greater lowering in acyl exchange rates observed with the S. faecalis enzyme as compared to the K. aerogenes enzyme in presence of metal might also explain the slower rates of inactivation of the former. Accumulation of citryl-S-enzyme, the product of the acyl transferase step

in the citrate cleavage reaction could result in enhanced deacetylation as citryl-S-ACP is known to undergo hydrolysis in presence of the acyltransferase () subunit. The lower rate of anhydride intermediate formation which precedes citryl-S-ACP production would ensure a lower steady state concentration of the labile intermediate.

Another distinguishing feature in the behaviour of the K. aerogenes enzyme towards p-azidobenzoate is the deviation from first-order inactivation kinetics and the attainment of a plateau level of residual activity. Such behaviour has been reported earlier by Rokita et al. (1982) in studies on the inhibition of K. aerogenes citrate lyase by 3-fluorocitrate. As suggested by these authors, the deviation may be a result of the negative cooperativity of the inactive subunits in the acyl-transferase reaction.

Chloroacetate, which has an electron withdrawing Cl atom substituting for a methylene hydrogen, also inactivates the enzyme in a manner similar to inhibition by p-azidobenzoate but at a slower rate.

L-Malate inactivates citrate lyases from K. aerogenes and from S. faecalis apparently through the formation of a malyl-S-enzyme intermediate, reactivation being observed on incubation of the inactivated enzyme with acetate (0.1 M). The reactivation is partial (20%) under these conditions tried. Treatment with acetic anhydride (4.7 mM) also results in partial (30%) reactivation). The deacetyl enzyme could have resulted either from the diffusion of

the anhydride intermediate away from the active site or from hydrolysis of the malyl-S-enzyme intermediate. The inactivation with L-malate was also more rapid with the K. aerogenes than with the S. faecalis enzyme. Irreversible inactivation of K. aerogenes citrate lyase had been observed earlier by Bowen and Rogers (1963a), although the mechanism of the enzyme action was not understood at the time. The evidence obtained in the present study indicates that malyl-S-enzyme formation may be one of the modes of inactivation of the enzyme by malate. R,S-malyl-CoA has been shown by Buckel et al. (1973) to be inactive in mediating citrate cleavage by deacetylated citrate lyase. On the basis of this finding, malyl-S-ACP can also be expected not to participate in an acyl exchange reaction with citrate. Accumulation of malyl-S-ACP would thus result in the conversion of the active acetyl-enzyme to an inactive malyl-derivative.

PART IIII

CHARACTERIZATION OF THIOL GROUPS OF THE ACYL-CARRIER
PROTEIN OF Klebsiella aerogenes CITRATE LYASE

S ACYLATED RESIDUES OF THE ACYL CARRIER PROTEIN SUBUNIT
OF *Klebsiella aerogenes* CITRATE LYASE

SUMMARY

Oxidation of the isolated deacetyl acyl-carrier protein subunit with Cu^{2+} - α -phenanthroline complex, a reagent which oxidizes -SH groups only to the level of disulfide, results in a decrease of -SH group content from about 2 - 0.3 SH/mol of ACP. An estimate of the molecular size of the oxidized subunit by SDS-polyacrylamide gel electrophoresis showed that absence of interpeptide cross-linking. These results indicate that oxidation leads exclusively to the formation of intrapeptide disulfide bridge formation. The thiol groups of the cysteamine and the cysteine residues of the Acyl-carrier protein subunit are apparently located in close proximity to permit bridging through a disulfide linkage.

$|^{14}\text{C}|$ -acetyl labeled citrate lyase from *K. aerogenes* was obtained by acetylation of deacetyl citrate lyase with a partially purified preparation of citrate lyase ligase of *K. aerogenes* in presence of $|^{14}\text{C}|$ -acetate and ATP. Radioactivity was found exclusively on the ACP () subunit of the $|^{14}\text{C}|$ -acetyl labeled citrate lyase. The presence of protein bound radioactivity after complete reaction-inactivation of the $|^{14}\text{C}|$ -labeled enzyme which results in the complete deacetylation of the cysteamine residue of the prosthetic group, suggests that the S-acetylation of the

cysteine residue in deacetyl acyl-carrier protein subunit is catalyzed by citrate lyase ligase.

INTRODUCTION

Citrate lyase (EC 4.2.3.6) which catalyzes the cleavage of citrate in a two step sequence of reaction (Singh et al., 1974; Dimroth and Eggerer, 1975b) is a multienzyme complex containing two enzymes and an acyl-carrier protein (ACP) (Carpenter et al., 1975). The most extensively studied of these complexes is the one from K. aerogenes, the ACP subunit of which has been shown to contain an S-acylated cysteine residue in addition to the essential S-acetyl moiety on the cysteamine residue (Dimroth et al., 1973) of the (phosphoribosyl)-dephospho-CoA prosthetic group (Robinson et al., 1976; Oppenheimer, et al., 1979). During the course of citrate cleavage the acetyl group is exchanged first with citrate to yield a citryl-ACP intermediate which is then cleaved with liberation of oxaloacetate and regeneration of acetyl-ACP. It has also been found that during the turn over of citrate, the enzyme undergoes a phenomenon of reaction-inactivation which has been attributed to the loss of essential acyl moiety presumably through the hydrolysis of the unstable citryl-ACP intermediate (Singh and Srere, 1971; Dimroth and Eggerer, 1975b). The HS-enzyme that is formed is inactive towards citrate as substrate. The HS-citrate lyase can be reactivated either chemically

by acetic anhydride (Singh et al., 1972) or enzymatically by citrate lyase ligase in presence of ATP and acetate (Schmellenkamp and Eggerer, 1974). Treatment of the deacetyl enzyme with $|^{14}\text{C}|$ -iodoacetate in presence of excess of DTE (10 mM) results in labeling of only the cysteamine residue (Dimroth and Eggerer, 1975a) and on the basis of this affinity labeling it was postulated that only the cysteamine residue carries the essential acetyl moiety.

Though the thioester groups of the acyl-carrier protein have been shown to play an important role in the mechanism of the action of citrate lyase, little is known about the nature and function of the cysteine acetyl group of the subunit. This part of the thesis describes some of the properties of the acylated thiol residues of the ACP subunit of K. aerogenes citrate lyase enzyme complex and the probable role of citrate lyase ligase in modification of the cysteine residue of the subunit to acetyl-cysteine.

MATERIALS AND METHODS

Materials :

All the chemicals used were of the highest purity available commercially.

1,10-Phenanthroline, monohydrate (o-phenanthroline), Trizma base and DTT were obtained from Sigma. Acrylamide and N,N' methylene bisacrylamide were of electrophoresis grade and obtained from Eastman Organic Chemicals,

Rochester, USA. Sodium dodecyl sulfate (SDS) was a preparation of Hico Products, Pvt. Ltd., Bombay. 5-5'-Dithiobis (2-Nitrobenzoate) (DTNB) was from Calbiochem, USA.

Sodium $|1 - {}^{14}\text{C}|$ acetate (47.6 Ci/mol) was obtained from Bhabha Atomic Research Centre, Bombay and was diluted with cold acetate to about 3 Ci/mol before use.

Analytical grade hydroxylamine hydrochloride ($\text{NH}_2\text{OH} \cdot \text{HCl}$) was from BDH Ltd. and a neutralized hydroxylamine solution, pH 7.0 was prepared by mixing equal volumes of 14% NaOH and 28% $\text{NH}_2\text{OH} \cdot \text{HCl}$ (Stadtman, 1957). Fresh solutions were prepared before use.

Citrate lyase :

Citrate lyase enzyme complex from K. aerogenes was prepared according to the procedure of Mahadik and SivaRaman (1968) and an outline of the procedure has been discussed in Part I of this thesis. The enzyme had a specific activity of about $70 - 90 \text{ } \mu\text{mol. min}^{-1} \text{ mg}^{-1}$ and were homogeneous in analytical ultracentrifuge and polyacrylamide gel electrophoresis.

Experimental Procedures:

Protein estimations :

Protein estimations were generally done by the method of Lowry et al. (1951). Protein of pure citrate lyase was estimated by the method of Singh et al. (1976) using an absorbance index of $E_{1\text{cm}}^{1\%} = 6.2$ at 278 nm.

Isolation of acyl carrier protein subunit :

The purified citrate lyase complex from *K. aerogenes* was deacetylated and dissociated with DTT-urea and the individual subunits separated essentially by the procedure described by Carpenter *et al.* (1975). Pure citrate lyase (25 mg protein in a total volume of 2.0 ml of 0.05 M potassium phosphate buffer, pH 7.0 was treated with 6 M urea and 10 mM DTT. The solution was incubated for 12 - 16 h at 4°C and then loaded on a Sepharose CL-6B column (1 x 100 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0 containing 6 M urea and 10 mM 2-mercaptoethanol. Elution was carried out by the same buffer and fractions of 1.1 ml were collected at a flow rate of 8 - 9 ml/h. Protein was monitored by measuring absorbance at 280 nm and 260 nm. The purity of the subunits are judged by SDS-polyacrylamide gel electrophoresis. The fractions containing the ACP subunits were pooled and dialyzed against 0.01 M potassium phosphate buffer, pH 7.0 containing 10 mM mercaptoethanol (2 x 1 litre) and stored frozen at -20°C. For thiol group estimation an aliquot was dialysed against 0.01 M potassium phosphate buffer, pH 7.4 for 4 h at 0 - 4°C and assayed immediately.

Oxidation with o-phenanthroline - Cu^{2+} complex :

Catalytic oxidation of thiol groups with o-phenanthroline- Cu^{2+} complex of deacetyl acyl carrier protein was carried out according to the method of Kobashi (1968). The isolated deacetyl ACP subunit

(0.8 mg) in 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4 was treated with a mixture of α -phenanthroline and copper at a final concentration of 100 μ M and 20 μ M, respectively. The reaction mixture was then incubated for 30 min at 25°C. Quenching of the reaction was achieved by adding 0.5 ml of 20% SDS solution. Aliquots of the above solution were separately taken for SDS-polyacrylamide gel electrophoresis and for thiol group estimation. Sulfhydryl groups of K. aerogenes ACP subunit were estimated with DTNB before and after oxidation of the subunit.

Estimation of thiol groups :

The estimation of sulfhydryl groups in the isolated deacetyl acyl-carrier protein of K. aerogenes citrate lyase was carried out after dialysis using DTNB according to the method described by Ellman (1959), with and without added DTT. DTNB titration of unoxidized ACP was also performed in the presence of DTT and arsenite by the method of Zahler and Cleland (1968) to check for autooxidation if any, during the dialysis step. Deacetyl acyl-carrier protein (200 - 300 μ g) was treated with 0.1 ml of 0.05 M Tris-HCl buffer, pH 9.0 and 0.1 ml of 3 mM DTT and incubated for 30 min at 30°C. 0.2 ml of 100 mM Tris-HCl, pH 8.1 and 1.5 ml of sodium arsenite (5 mM in 40 mM EDTA) were then added and the total volume was made upto 2.9 ml with distilled water. The solution was then treated with 0.1 ml of 3 mM DTNB in 50 mM acetate buffer, pH 5.0 and increase in optical density at 412 nm was

monitored.

Molecular weight of the ACP subunit was taken as 9378 (Beyreuther, 1978) for the calculation of the |SH| content/|ACP|.

SDS-polyacrylamide gel electrophoresis :

SDS-polyacrylamide gel electrophoresis of the native citrate lyase and of the isolated subunits were carried out under denaturing conditions according to the procedure of Weber and Osborn (1969). 10% gel tubes were prepared in 50 mM sodium phosphate buffer, pH 7.3 using 0.07% ammonium persulfate for polymerization. Protein samples were prepared by heating at 100°C for 3 min in presence of 1% SDS and 10 mM 2-mercaptoethanol. The solutions were made dense with 30% sucrose and loaded on the tubes. Bromophenol blue was used as the tracking dye.

Electrophoresis was carried out at a constant current of 8 ma/tube. The gels were stained with 0.25% Coomassie Brilliant Blue for 6 - 12 h and destained by diffusion in methanol-acetic acid-water mixture.

The effect of oxidation by o-phenanthroline-Cu²⁺ complex on the molecular size of ACP subunit was checked by subjecting the protein to SDS-gel electrophoresis in buffer systems without 2-mercaptoethanol. The molecular weight of the ACP subunit before and after oxidation was estimated from a plot of log molecular weight versus relative mobility of the protein. The three non-identical subunits (54 000 dalton), (32 000 dalton)

and (10 000 dalton) (Carpenter et al., 1975) of the native citrate lyase complex from K. aerogenes were taken as standard marker proteins for determination of molecular weights.

Preparation of citrate lyase ligase :

The acetate: HS-[acyl-carrier protein] enzyme ligase (AMP) (citrate lyase ligase) was partially purified from K. aerogenes by the method described earlier by Hiremath et al. (1976). Cell extracts were fractionated upto the alumina C gel treatment step as described in Part I of the thesis. The enzyme solution was then treated with excess of alumina C gel (45 ml gel/200 ml extract). At this step citrate lyase activity is completely adsorbed on the gel, while the ligase activity remains in the supernatant. Alumina C was removed by centrifugation at 3 000 g for 15 min and the clear supernatant containing the ligase activity was concentrated by ultrafiltration using a PM-30 membrane. The ligase preparation was free from citrate lyase activity. The ligase activity was checked by incubating with deacetyl citrate lyase prepared from K. aerogenes as described by Kummel et al. (1975). The preparation had a specific activity of 8 U/mg protein.

Preparation of ^{14}C -acetyl citrate lyase from K. aerogenes :

The ^{14}C -acetyl labeled enzyme was prepared by acetylation of HS-citrate lyase with citrate lyase ligase in presence of ^{14}C -acetate and ATP. Citrate lyase from

K. aerogenes was deacetylated with neutralized hydroxylamine (Buckel et al., 1973) before the DEAE-cellulose step in the purification procedure described in Part I of the thesis. Excess reagent was separated by filtration through a Sephadex G-25 column (1.5 x 40 cm) using 50 mM potassium phosphate buffer, pH 7.5, containing 1.6 mM MgSO_4 . The protein containing fractions were pooled and an aliquot withdrawn from the fraction showed complete loss of citrate lyase activity as judged by malate dehydrogenase coupled assay. The deacetyl enzyme (200 mg) in 5 to 6 ml of the phosphate buffer was treated with citrate lyase ligase (10 mg of the partially purified enzyme) in presence of ATP and acetate (8 μCi) at a final concentrations of 6 mM and 0.5 mM, respectively. The mixture was incubated at 25°C for 30 min. Citrate lyase ligase was adequate to reactivate to about 50% of the original citrate lyase activity. The protein was then precipitated by addition of an equal volume of cold saturated ammonium sulfate solution and collected by centrifugation for 30 min at 50 000 g and 4°C. The protein was dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.6 and subjected to DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B essentially as described in Part I of the thesis.

Detection of ^{14}C -acetyl labeled subunit :

The radioactivity distribution in the subunits was determined by dissociating ^{14}C -acetyl citrate lyase by

SDS followed by SDS-gel electrophoresis. [^{14}C]-acetyl-citrate lyase (200 ug) obtained as described above, was treated with 1% SDS in the absence of 2-mercaptoethanol and heated in a boiling water bath for 2 min and then subjected to SDS-polyacrylamide gel electrophoresis as described earlier. The gel was then stained, destained and the gel region corresponding to subunits , and were cut and checked for radioactivity by the method of Saitoh *et al.* (1980) as described briefly in Part I of the thesis.

Reaction-inactivation :

[^{14}C]-acetyl-citrate lyase (0.5 mg) was reaction-inactivated in a total volume of 0.5 ml of Tris-HCl buffer, pH 8.0, containing 0.5 mmol citrate and 1.5 umol MgSO_4 , by incubation for 15 min at 30°C . An aliquot of 10 ul was checked for citrate lyase activity by coupled assay with malate dehydrogenase to confirm complete inactivation. After complete inactivation the enzyme solution was dialyzed exhaustively against 0.05 M potassium phosphate buffer, pH 7.5 and the remaining protein bound radioactivity was measured by transferring the solution into a 10 ml of dioxane based scintillant cocktail containing 100 g naphthalene and 5 g PPO per litre of dioxane. Radioactive counts were taken in a Beckman Model LS-100 scintillation system.

RESULTS

Isolation of deacetyl acyl-carrier protein :

Acyl-carrier protein in deacetylated form was prepared by dissociation of the native citrate lyase from *K. aerogenes* by 6 M urea in the presence of 10 mM DTT followed by separation of subunits by gel filtration through Sepharose CL-6B column. A typical elution profile is shown in Figure III.1. It can be seen from Figure III.1 that the larger subunits and separate with overlap in the intermediate fractions. The fractions on the leading edge of the α -subunit peak and the trailing end of the β -subunit component were therefore pooled separately for isolation of individual subunits. SDS-polyacrylamide gel electrophoresis revealed that fractions 48 - 54 contained only α -subunit, fractions 54 - 60 both α - and β -subunits, while fractions 61 - 65 contained only β -subunit. The pooled fractions containing single protein components were taken for isolation of the individual subunits. The α -subunit emerged distinctly from the other two subunits and fractions 68 - 76, both inclusive, were pooled for the isolation of the β -subunit. The presence of α -subunit is easily detectable since it shows a higher 260 nm absorbance because of the presence of the CoA-like prosthetic group and low content of aromatic amino acids. Figure III.2 shows the analytical SDS-polyacrylamide gel electrophoresis patterns of the native enzyme and of the isolated subunits. This figure shows that electrophoretically homogeneous components of *K. aerogenes* citrate

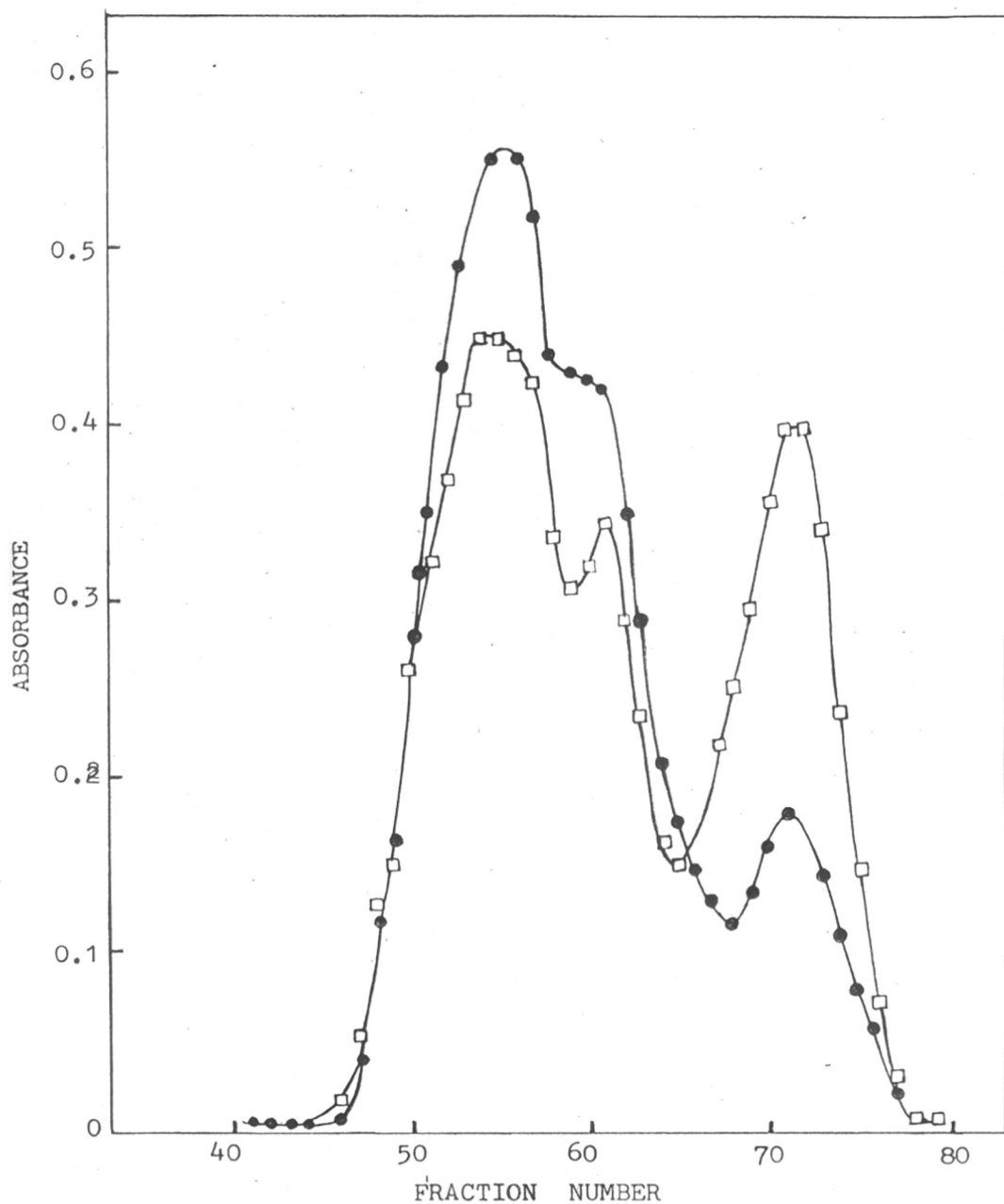


FIG. III.1. SEPARATION OF *K. aerogenes* CITRATE LYASE SUBUNITS BY FILTRATION THROUGH SEPHAROSE CL 6B (1 x 100 cm) IN PRESENCE OF 6 M UREA.

●—●, 280 nm ; □—□, 260 nm.

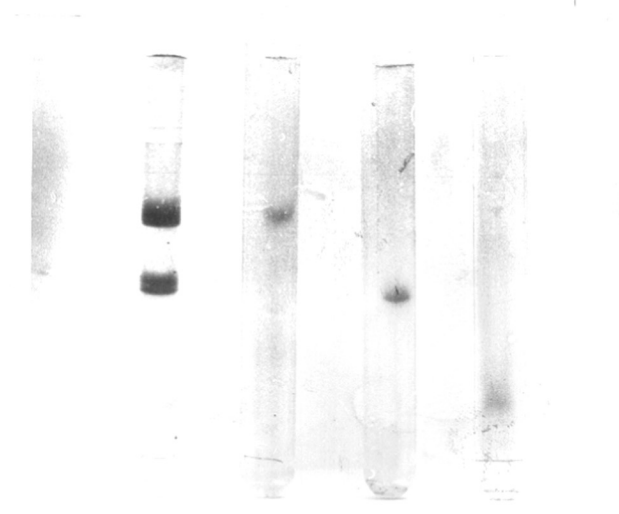


FIG. III.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
K. aerogenes CITRATE LYASE COMPLEX
AND OF ITS ISOLATED SUBUNITS

100 ug of citrate lyase complex (or 50 ug of each subunit) electrophoresed on 10% gel in 50 mM sodium phosphate buffer, pH 7.3 containing 0.1% SDS at a constant current of 8 mA/tube for 4 h.

lyase can be obtained by the separation procedure described above.

Oxidation of deacetyl-ACP:

The effect of oxidation by o-phenanthroline - Cu^{2+} complex on the content of sulfhydryl groups of the isolated deacetyl ACP subunit is shown in Figure III.3 and Table III.1.

The content of sulfhydryl groups in the deacetyl acyl-carrier protein was approximately two, both in the absence as well as in the presence of added DTT. Reoxidation apparently does not occur during dialysis. The content of two sulfhydryl groups per mol of deacetyl acyl-carrier protein is in agreement with the presence of a cysteine and a cysteamine residue in the ACP molecule (Dimroth, 1975). From Table III.1 it can be seen that oxidation with o-phenanthroline- Cu^{2+} complex results in the decrease of thiol group content from 2 to 0.3 -SH/mol ACP. SDS-polyacrylamide gel electrophoresis of aliquots from the reaction mixture showed that the molecular weight of oxidized subunit was unchanged. This was established by electrophoresis along with the unoxidized ACP subunit (shown in Figure III.4).

These results indicate that no interpeptide cross-linking occurs and that the oxidation results exclusively in intrapeptide disulfide bridge formation between the thiol groups of the cysteamine residue on the prosthetic group and of the cysteine in the polypeptide chain of

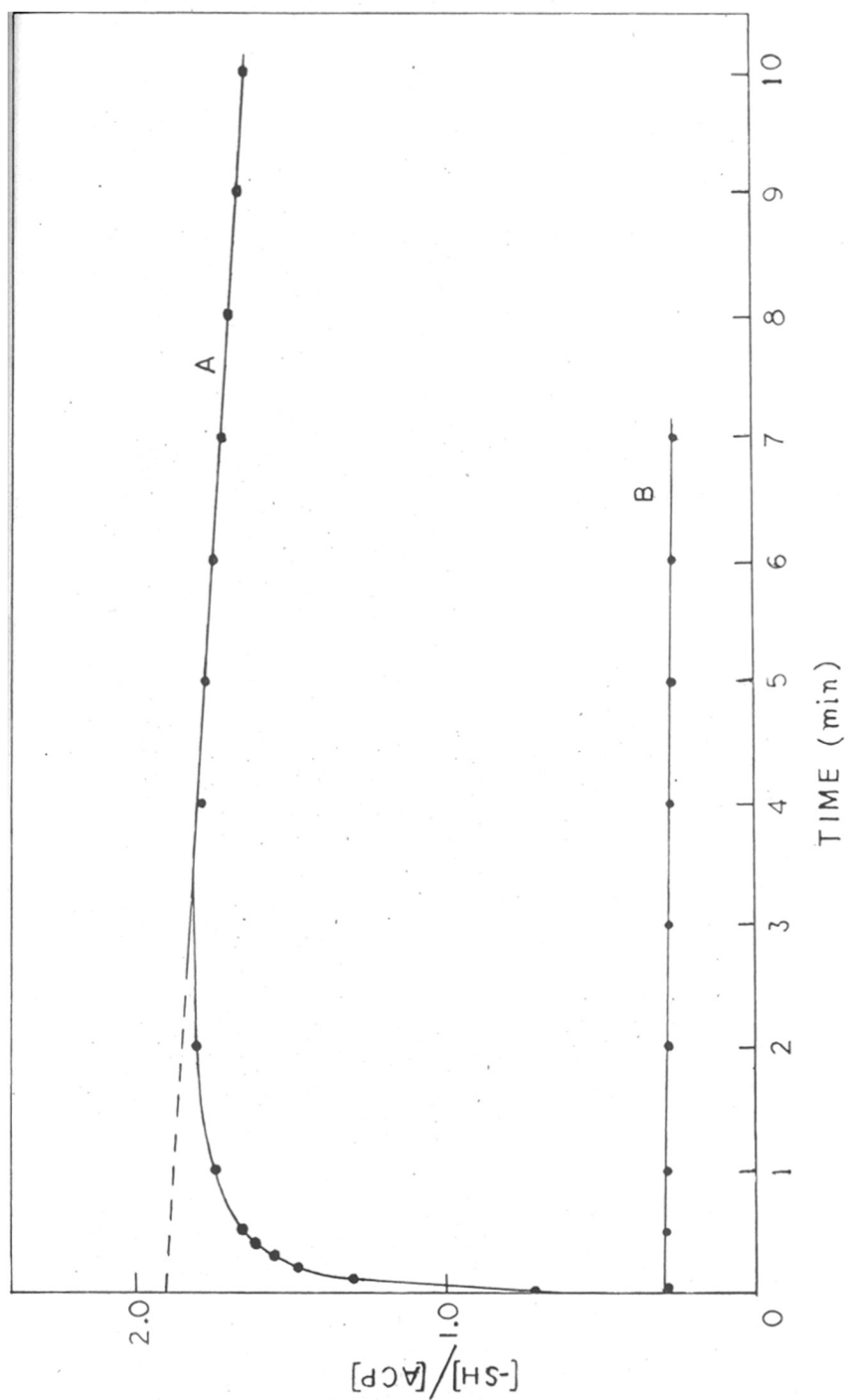


FIG.III.3. ESTIMATION OF THIOL GROUPS OF DEACETYL ACYL-CARRIER PROTEIN
SUBUNIT OF K. aerogenes CITRATE LYASE BY DTNB TITRATION
A — Before oxidation with o-phenanthroline- Cu^{2+} complex
B — After oxidation with o-phenanthroline- Cu^{2+} complex

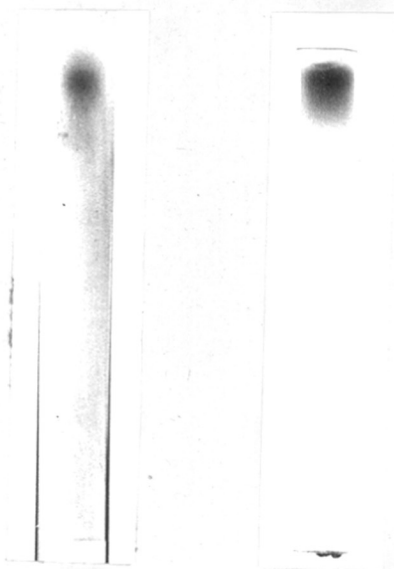


FIG. III.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
ACYL-CARRIER PROTEIN SUBUNIT OF
K. aerogenes CITRATE LYASE

- (a) Before oxidation with o-phenanthroline- Cu^{2+} complex.
- (b) After oxidation with o-phenanthroline- Cu^{2+} complex.

10% gel; Sodium phosphate buffer, pH 7.3
containing 0.1% SDS; 8 mA/tube; 4 h.

the ACP subunit.

TABLE III.3 : Oxidation of sulfhydryl groups of deacetyl ACP by o-phenanthroline- Cu^{2+} complex

System	-SH/mol of ACP ^a
Deacetyl ACP	1.9 ^b 2.0 ^c
Deacetyl ACP + o-phenanthroline- Cu^{2+} complex	0.3

The isolated deacetyl ACP was oxidized with o-phenanthroline- Cu^{2+} complex (as described under Materials and Methods) and the reduction in thiol groups estimated. The values given are an average of three separate estimations.

^a M_r = 9378 (Beyreuther *et al.*, 1978)

^bEstimated without DTT

^cEstimated in presence of DTT and Arsenite.

Acetylation of HS-citrate lyase by citrate lyase ligase :

Preparation of $|^{14}\text{C}|$ -acetyl citrate lyase from HS-citrate lyase by partially purified citrate lyase ligase in presence of $|1-^{14}\text{C}|$ -acetate and ATP, by the procedure described in the Experimental Procedures, results in electrophoretically pure $|^{14}\text{C}|$ -labeled citrate lyase.

The final preparation had a specific activity of about $30 \text{ } \mu\text{mol. min}^{-1} \text{ mg}^{-1}$ and a specific radioactivity of 13070 cpm/mg.

Figure III.5 shows the subunit profile and the distribution of radioactivity in the subunits of $|^{14}\text{C}|$ -acetyl citrate lyase obtained on acetylation of deacetyl citrate lyase with citrate lyase ligase in the presence of $|^{14}\text{C}|$ -acetate and ATP. The radioactivity is associated exclusively with the region in the gel containing the 10 000 dalton ACP () subunit, the 54 000 daltons acyl-transferase () and the 32 000 dalton acyl-lyase () subunits carrying no radioactive label. Citrate lyase ligase thus specifically acetylates the ACP () subunit of citrate lyase from K. aerogenes.

Effect of reaction-inactivation on $|^{14}\text{C}|$ -acetyl citrate lyase :

Earlier work carried out in this laboratory (Tikare, 1979) had established that total reaction-inactivation of K. aerogenes citrate lyase results in the complete loss of acetyl groups on the cysteamine moieties of the prosthetic group, while the acetylated-cysteines are intact. The characterization of the acetylated residues present on the reaction-inactivated enzyme complex had been done by a modification of the method described for fatty acid ACP (Simoni et al., 1967). Citrate lyase was reaction-inactivated by incubating with $0.2 \text{ mmol citrate (pH 8.0)}$ and $0.16 \text{ } \mu\text{mol MgSO}_4$ for 15 min at 30°C. The free

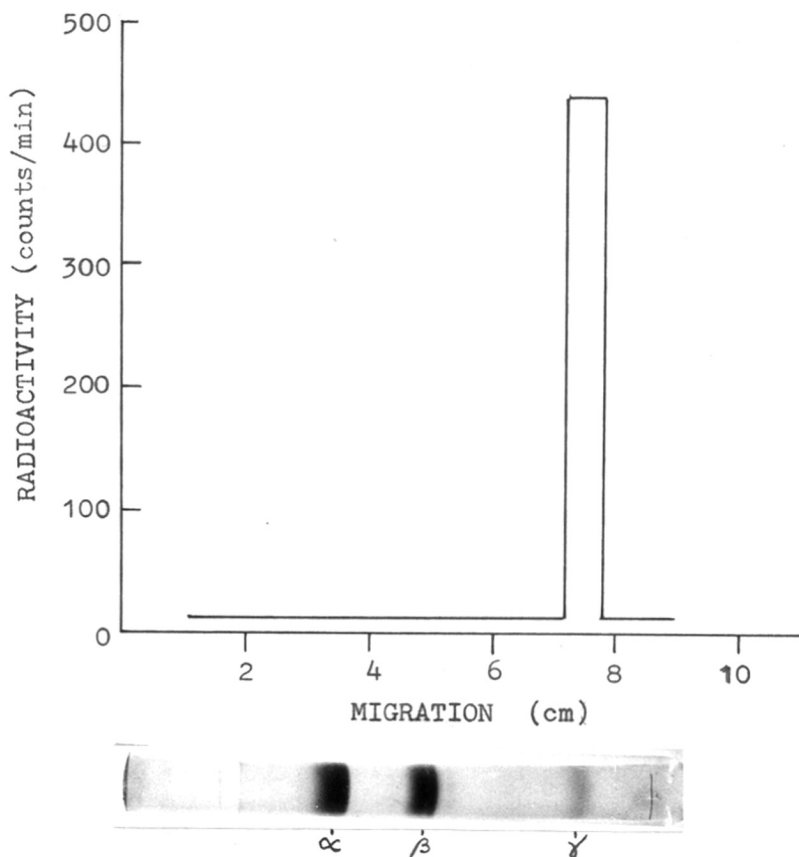


FIG. III.5 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
OF ^{14}C -ACETYL CITRATE LYASE

The histogram shows the profile of radioactivity in the separated subunits of K. aerogenes citrate lyase.

sulfhydryl groups of the reaction-inactivated enzyme were blocked with cold iodoacetate. The thioester linkages were then cleaved with neutralized hydroxylamine and dithiothreitol and the liberated thiol groups reacted with iodo- ^{14}C -acetate. The S- ^{14}C -carboxymethylated residue in the protein was characterized by paper electrophoresis of the acid hydrolysate. The experimental conditions used for the characterization of the ^{14}C -carboxymethylated residue were similar to those described by Dimroth *et al.* (1973). These experiments had shown that the cysteamine residues of the reaction-inactivated enzyme were completely deacetylated, while the cysteines which were originally acylated remained blocked even after the inactivation. Reaction-inactivation was thus shown to result in the selective deacetylation of the acetyl-S-cysteamine residues of the prosthetic group.

The effect of reaction-inactivation on the specific radioactivity of the ^{14}C -acetyl citrate lyase was determined in the present study and the results are shown in Table III.2. ^{14}C -Acetyl citrate lyase was subjected to reaction-inactivation as described in Experimental Procedures. After complete inactivation, as judged by malate dehydrogenase coupled assay, the protein bound radioactivity was measured after removal of liberated acetate by exhaustive dialysis. The presence of about 30% of the initial radioactivity in protein bound form, after complete reaction inactivation would indicate that

the $|^{14}\text{C}|$ -acetyl moieties retained by the enzyme are on the cysteine residue of the ACP subunits. The results are also suggestive of the role of citrate lyase ligase in the modification of the cysteine residues of the ACP subunits to acetyl-S-cysteines in addition to the acetylation of the prosthetic group in presence of ATP and acetate. The acetylation of the cysteine residue occurs even under conditions where HS-citrate lyase is only partially reactivated. The results also provides an indirect evidence that the acetyl moiety of cysteine residue does not take part in the reaction mechanism of the enzyme as it is not lost during the reaction inactivation process.

TABLE III.2 : Effect of reaction-inactivation on protein-bound radioactivity of $|^{14}\text{C}|$ -acetyl citrate lyase

Enzyme	cpm/mg
$ ^{14}\text{C} $ -acetyl citrate lyase	13070
$ ^{14}\text{C} $ -acetyl citrate lyase after reaction-inactivation	44120

$|^{14}\text{C}|$ -Acetyl citrate lyase (430 ug) was incubated in a total volume of 1 ml of the reaction mixture containing 1 mol citrate; 3 umol, MgSO_4 and 50 umol, Tris-HCl buffer, pH 8.0. Aliquots tested after 15 min incubation at 30°C showed complete loss of citrate lyase activity. Radio-activity was measured as described in Experimental Procedures.

Reactivation

Reaction-inactivation studies :

To check a possible role for the acetyl-cysteine residue in the reaction-inactivation process, progress curves for the cleavage of citrate by native citrate lyase from K. aerogenes and that of the deacetyl citrate lyase reactivated with acetic anhydride by affinity labeling in presence of excess DTT were compared. Such affinity labeling with acetic anhydride in the presence of excess mercaptans results in the ipreferential acetylation of the cysteamine residue of the prosthetic group (Dimroth et al., 1977b). Native citrate lyase (150 ug) was treated in a volume of 1 ml of 0.05 M Tris-HCL, pH 8.0 with 10 mM DTT and incubated for 30 min at 25°C. An aliquot tested showed complete loss of activity. The solution was cooled to 0 - 4°C in an ice-bath and acetic anhydride was then added to a final concentration of 4.7 mM. After 5 min of incubation the mixture was passed down a Sephadex G-25 column (1 x 20 cm) to remove excess reagents. Elution was done with 0.05 M potassium phosphate buffer, pH 7.5 and fractions containing enzyme were then used for obtaining progress curve for citrate cleavage. Progress curves were obtained by malate dehydrogenase coupled assay procedure as described in Experimental Procedure of Part I. Half life of citrate lyase activity and the rate constants (K) for inactivation were calculated from first order plots as described and Srere, (1971). Figure III.6 shows the plot of log of enzyme activity

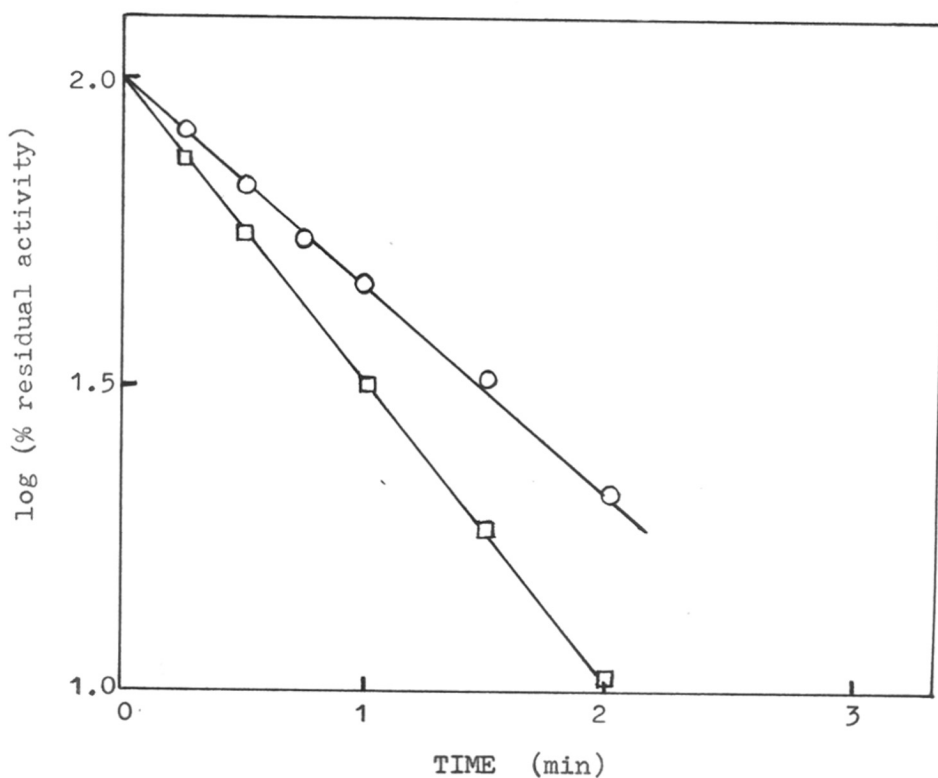


FIG. III.6. FIRST ORDER PLOT FOR THE REACTION-
INACTIVATION OF CITRATE LYASE.

Native citrate lyase (□) ; deacetyl citrate lyase
reactivated with acetic anhydride in presence of DTT (○).

remaining at any time, expressed as % of the original activity versus time. The activity at zero time was arbitrarily taken as 100%.

Table III.3 summarizes the half lives and rate constant for reaction-inactivation of the native enzyme and of the affinity labeled preparation obtained by treatment of the deacetyl enzyme in presence of excess DTT where only the prosthetic group is reacetylated. It can be seen from the table that the half-life of the enzyme reactivated with acetic anhydride in presence of excess DTT is inactivated at a slightly lower rate than the native enzyme.

TABLE III.3 : Reaction-inactivation of native citrate lyase and acetic anhydride reactivated citrate lyase

Enzyme	K (min ⁻¹)	t ^{1/2} * (min)
Native citrate lyase	1.15	0.6
Acetic anhydride reactivated citrate lyase	0.79	0.9

Reaction systems were contained in 0.05 M Tris-HCl buffer, pH 8.0; 5 mM citrate, and 3 mM MgSO₄.
Temperature 25°C.

DISCUSSION

DINB titration of the ACP () subunit of *K. aerogenes* citrate lyase shows the presence of two -SH groups/mol, which is in agreement with the presence of a single cysteine and a single cysteamine residue in the ACP subunit (Dimroth et al., 1975). Dimroth et al. (1973) have earlier shown that both of the thiol groups are acylated in the native citrate lyase enzyme complex and this has posed the problem of the nature of the biologically active acyl-carrier in the enzyme complex. Indirect evidence for the cysteamine as the biologically active acyl-carrier was provided by affinity labeling of deacetyl citrate lyase with iodoacetate in presence of excess DTE, which results exclusively in the carboxymethylation of the cysteamine residue to yield a carboxymethylated complex inaccessible to reactivation either chemically by acetic anhydride or enzymatically by citrate lyase ligase (Dimroth and Eggerer, 1975). Tikare (1979) has provided direct evidence for cysteamine being the biologically active acyl-carrier by a differential labeling method similar in principle to the procedure described for the fatty acid synthetase system (Simoni et al., 1967). Using this technique it has been shown that only the cysteamine residue is deacetylated during reaction-inactivation, while in the completely inactivated enzyme the cysteine group is still acylated (Basu et al., 1983). In the present studies, the effect of acetylation of deacetyl citrate lyase with ^{14}C -acetate in the

presence of ATP by citrate lyase ligase has been studied. Under these conditions only the ACP () subunit has been found to be labeled specifically, while the acyl transferase () and the acyl lyase () subunits carry no covalently bound $|^{14}\text{C}|$ -acetyl moieties. Complete reaction-inactivation of the $|^{14}\text{C}|$ -acetyl citrate lyase, which results in the deacetylation of all the prosthetic group moieties results in a product that retains partially $|^{14}\text{C}|$ -acetyl groups apparently on the cysteine residue of the ACP subunit. The results would also suggest that the acetylation of the cysteine residue in deacetyl ACP subunit is catalyzed by citrate lyase ligase. The ligase in presence of acetate and ATP acetylates both the cysteamine moiety of the prosthetic group and the cysteine of the polypeptide chain.

Numerous instances of the presence of amino acid residues with modified side chains as constituents of proteins have been reported in the literature (Wold, 1981). The in vivo mechanisms of such modifications are not often understood. The results obtained with reaction-inactivated $|^{14}\text{C}|$ -acetyl labeled citrate lyase in the present study implicate citrate lyase ligase in the post translational modification of cysteine residues in the ACP subunits of the K. aerogenes citrate lyase complex.

Cu^{2+} -o-phenanthroline complex has been shown by Kobashi (1968) to oxidize -SH groups only to the level of disulfide in presence of molecular oxygen. In proteins

the thiol groups which are proximal to each other are cross-linked by disulfide bridge formation. Oxidation of the isolated deacetyl acyl-carrier protein subunit of citrate lyase from *K. aerogenes* with Cu^{2+} -9-phenanthroline complex leads to the oxidation of free -SH groups as estimated by DTNB titration without the appearance of any higher molecular weight species detectable on SDS-polyacrylamide gel electrophoresis. These observations would indicate the exclusive formation of intrapeptide disulfide bridge on -SH oxidation. The cysteamine moiety of the prosthetic group and the cysteine residue of the polypeptide chain in the deacetyl acyl-carrier protein subunit are apparently located in close proximity permitting bridging by -S-S- bond formation. The bond distance of the -S-S- bridge has been shown to be about 2Å (Murphy, 1976).

The cysteine acetyl group does not reduce the reaction-inactivation rate. The enzyme which is reactivated with acetic anhydride in presence of excess DTT on the contrary shows a slower rate of reaction-inactivation.

A possible role of the in vivo modification of the cysteine residue by citrate lyase ligase may be to protect against intrapeptide disulfide bridge formation with the juxtaposed sulfhydryl residue on the prosthetic group of the enzyme in the deacetylated form.

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Photoaffinity Labeling of *Klebsiella aerogenes* Citrate Lyase by *p*-Azidobenzoyl Coenzyme A[†]

Amaresh Basu, Subhalakshmi Subramanian, and Churya SivaRaman*

ABSTRACT: *p*-Azidobenzoyl coenzyme A functions as a linear competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA oxaloacetate-lyase reaction catalyzed by the *Klebsiella aerogenes* deacetylcytrate lyase complex ($K_i = 80 \mu\text{M}$; (3*S*)-citryl-CoA $K_m = 67 \mu\text{M}$). Inactivation is irreversible on photolysis of *p*-azidobenzoyl-CoA in the presence of the deacetylcytrate lyase complex. Mg^{2+} is not required for the inactivation. Inactivation is blocked by (3*S*)-citryl-CoA in

the presence of ethylenediaminetetraacetic acid. *p*-Azidobenzoyl-CoA has no effect on the acetyl-CoA: citrate CoA transferase activity of both the deacetylcytrate lyase complex and its isolated transferase subunit. The stoichiometry of the CoA ester binding has been investigated by the use of *p*-azido[¹⁴C]benzoyl-CoA as a photoaffinity reagent. The labeling is exclusively on the lyase β subunit of the citrate lyase complex.

The citrate lyase multienzyme complex (EC 4.1.3.6) from *Klebsiella aerogenes* is assembled from three dissimilar subunits α , β , and γ of molecular weights 54 000, 32 000, and 10 000, respectively (Carpenter et al., 1975; Dimroth & Eggerer, 1975a). The γ subunit functions as an acyl-carrier protein (ACP)¹ with an essential acetyl function in thioester linkage (Buckel et al., 1971) on a covalently bound CoA-like prosthetic group (Oppenheimer et al., 1979). The α subunit acts as an acyl transferase involved in the formation of (3*S*)-citryl-ACP¹ in the presence of citrate with the release of acetate, while the β subunit functions as an acyl lyase catalyzing the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP¹ (Dimroth & Eggerer, 1975b). Acyl coenzyme A (acyl-CoA) derivatives such as acetyl-CoA and propionyl-CoA have been shown to serve also as substrates for the transferase reaction, while (3*S*)-citryl-CoA acts as a substrate for the lyase reaction (Buckel et al., 1973; Dimroth et al., 1977). The mechanism of action of the citrate lyase complex requires the oscillation of the prosthetic group (or of acyl-CoA in the acetyl-CoA-mediated reaction) between the active sites on the α and β subunits (Srere & Singh, 1974).

The paper reports the use of *p*-azidobenzoyl-CoA as a photoaffinity reagent for probing the acyl-CoA binding sites of the complex.

Experimental Procedures

Products. NADH, *N*-hydroxysuccinimide, sodium dodecyl sulfate (NaDodSO_4),¹ citrate synthase, and malate dehydrogenase were from Sigma. DTNB¹ was from Calbiochem.

Sephacel CL-6B, Sephadex G-10, and Sephadex G-25 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was from Whatman. *p*-Amino[carboxyl-¹⁴C]benzoic acid (21 Ci/mol) was obtained from Radiochemicals Centre. All other products were of the highest purity obtainable commercially.

Preparation of CoA Esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Simon & Shemin, 1953). (3*S*)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in the presence of *K. aerogenes* deacetylcytrate lyase and EDTA¹ (Dimroth et al., 1977) and assayed with citrate synthase in the presence of DTNB.

p-Azidobenzoyl-CoA was synthesized as described by Lau et al. (1977). *p*-Azido[¹⁴C]benzoyl-CoA was synthesized from *p*-amino[carboxyl-¹⁴C]benzoic acid without excessive dilution of the radioactivity. The concentration of the reagent was determined by using $\epsilon_{265\text{nm}} = 22.2 \text{ mM}^{-1}$ (Lau et al., 1977).

All acyl-CoA preparations were purified as described by Moffat & Khorana (1961) by chromatography on a DEAE-cellulose (Cl⁻ form) column using a linear gradient from 0 to 0.2 M LiCl containing 0.003 N HCl. Samples of the CoA esters were concentrated at 25 °C in a rotary evaporator and desalted by filtration through a Sephadex G-10 column (1.0 × 50 cm).

Purification of Citrate Lyase. Citrate lyase complex from *Klebsiella aerogenes* (NCTC 418) was purified by a modification of the procedure described earlier (Mahadik & SivaRaman, 1968), sonic extracts being treated with ATP and acetate at final concentrations of 0.3 and 1 mM, respectively, for reactivating any deacetylcytrate lyase (Leena, 1979).

[†] From the Biochemistry Division, National Chemical Laboratory, Poona 411 008, India. Received December 4, 1981; revised manuscript received March 29, 1982. NCL Communication No. 2770. A.B. and S.S. had the support of Research Fellowship Awards from the Council of Scientific and Industrial Research, New Delhi.

¹ Abbreviations: ACP, acyl-carrier protein; (3*S*)-citryl-ACP, (3*S*)-citrylacyl-carrier protein; acetyl-ACP, acetylacyl-carrier protein; NaDodSO_4 , sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Effect of Photoactivated *p*-Azidobenzoyl-CoA on Transferase and Lyase Activities of *Klebsiella aerogenes* Citrate Lyase^a

enzyme	buffer	<i>p</i> -azido- benzoyl- CoA (μ M)	% inhibition	
			trans- ferase act.	lyase act.
deacetyl citrate lyase	50 mM Tris-HCl (pH 8.0) + 3 mM Mg ²⁺	0	0	0
		80	0	25
		112	0	42
		160	0	50
		168	0	58
	50 mM Tris-HCl (pH 8.0) + EDTA	0	0	0
acyltransferase subunit	50 mM Tris-HCl (pH 8.0)	80	0	25
		160	0	48
		0	0	0
		80	0	0
		160	0	0

^a Samples contained, in a total volume of 0.3 mL, 150 μ g of deacetyl enzyme or about 25 μ g of isolated α subunit. Aliquots were withdrawn after a 5-min irradiation and activities assayed as described under Experimental Procedures. Controls in which *p*-azidobenzoyl-CoA was absent showed no loss in the enzymatic activities.

Preparations were homogeneous in the ultracentrifuge and in polyacrylamide gel electrophoresis and had a specific activity varying between 70 U/mg of protein and 90 U/mg of protein.

Enzyme protein in the purified complex was determined from absorbance measurements at 278 nm by using the absorption index $E_{1\text{cm}}^{1\%}$ of 6.2 (Singh et al., 1976).

Preparation of Deacetyl citrate Lyase. Deacetyl citrate lyase was obtained by treatment of the purified native enzyme with neutral hydroxylamine according to the procedure of Buckel et al. (1973).

Isolation of Citrate Lyase Subunits. Component subunits were isolated in the electrophoretically pure and catalytically active state after dissociation with urea by the procedure described by Dimroth & Eggerer (1975b). Protein measurements of the isolated subunit preparations were made by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Assay of Enzymatic Activities. Citrate cleavage activity of the native enzyme complex was determined by coupled assay with malate dehydrogenase (Singh & Srere, 1971).

Acyl transferase activity of the deacetyl citrate lyase complex and of the isolated α subunit was determined from the initial velocity of the CoA transfer from acetyl-CoA to citrate in the presence of EDTA by estimation of the citryl-CoA produced with citrate synthase in the presence of DTNB (Dimroth et al., 1977; Srere et al., 1963).

Acyl lyase activity of the deacetyl enzyme complex and of the isolated β subunit was determined from the initial rate of oxaloacetate formation on cleavage of (3S)-citryl-CoA in the presence of Mg²⁺, oxaloacetate being assayed with malate dehydrogenase (Buckel et al., 1973).

Kinetics. The K_m for citryl-CoA and K_i for *p*-azidobenzoyl-CoA were calculated from the double-reciprocal plots by least-squares analysis. *p*-Azidobenzoyl-CoA was tested at two different concentrations of 30 and 60 μ M with varied initial concentrations of (3S)-citryl-CoA.

Photoactivation of *p*-Azidobenzoyl-CoA. Photoactivation was carried out under a Philips black light lamp (125 W) with the sample placed in a glass well kept at a distance of 2 cm. Solutions were precooled and maintained at 0–4 °C in an ice bath. Mixing was achieved by using a magnetic stirrer.

Incorporation of radioactivity into protein on photolysis in the presence of the ¹⁴C-labeled reagent was measured by the filter paper disk method (Mans & Novelli, 1961). Dried disks were counted in 10 mL of dioxane-based scintillant on a Beckman Model LS-100 scintillation system.

NaDodSO₄-polyacrylamide gel electrophoresis of the enzyme photolyzed in the presence of the radioactive reagent was carried out according to the method of Weber & Osborn (1969). Samples were prepared by heating aliquots (50–100 μ L) containing about 200 μ g of protein with 1% NaDodSO₄ in a boiling water bath for 3 min. Gels were stained with Coomassie brilliant blue R-250 and destained by diffusion. Detection of radioactivity in the protein bands was carried out by the method described by Saitoh et al. (1980).

Results

Effect of *p*-Azidobenzoyl-CoA. (1) Acyl Transferase Activity. *p*-Azidobenzoyl-CoA had no effect on the acetyl-CoA-mediated acyl transferase activity, both of deacetyl citrate lyase from *K. aerogenes* and of the isolated α subunit. The reagent was tried up to 160 μ M final concentration.

(2) Lyase Activity. Kinetic studies were performed in the dark to see the effect of *p*-azidobenzoyl-CoA on the lyase activity of *K. aerogenes* deacetyl citrate lyase toward (3S)-citryl-CoA. Assay mixtures at 25 °C contained 12.5 μ g of deacetyl citrate lyase, varying concentrations of (3S)-citryl-CoA (up to 50 μ M), 0–60 μ M *p*-azidobenzoyl-CoA, 50 mM Tris-HCl buffer, pH 8.0, and 3 mM MgSO₄ in a final volume of 1.0 mL. Double-reciprocal plots indicated that *p*-azidobenzoyl-CoA was a competitive inhibitor with a K_i of 80 μ M. The K_m for (3S)-citryl-CoA determined in this experiment was 67 μ M.

Photoaffinity Labeling of Citrate Lyase with *p*-Azidobenzoyl-CoA. The data on the effect of photoactivated *p*-azidobenzoyl-CoA on *K. aerogenes* deacetyl citrate lyase are summarized in Table I. Deacetyl citrate lyase treated with *p*-azidobenzoyl-CoA (final concentrations 80–168 μ M) and photolyzed for 5 min showed no loss in acyl transferase activity. The photoactivated reagent was also without effect on the activity of the isolated transferase α subunit of *K. aerogenes* citrate lyase. Under these conditions the lyase activity of the deacetyl enzyme was inhibited 25%, 50%, and 58% in the presence of 80, 160, and 168 μ M concentrations of *p*-azidobenzoyl-CoA, respectively. The inactivation of lyase activity was similar both in the presence of EDTA (10 mM final concentration) and in the presence of Mg²⁺ (3 mM final concentration).

Another piece of evidence that *p*-azidobenzoyl-CoA is an active-site directed photoaffinity label is the protection from irreversible inactivation of the lyase activity by (3S)-citryl-CoA (Table II). Inactivation in the presence of 112 μ M reagent is completely blocked by 200 μ M (3S)-citryl-CoA.

Table II: Protection of Deacetyltransferase by (3S)-Citryl-CoA from Inhibition by Photoactivated *p*-Azidobenzoyl-CoA^a

<i>p</i> -azidobenzoyl-CoA (μM)	citryl-CoA (μM)	% inhibition
0	0	0
112	0	42
112	44	31.5
112	74	25
112	200	0

^a The reaction mixture contained, in a total volume of 0.3 mL, 150 μg of deacetyl enzyme; 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and the indicated amounts of the inhibitor and (3S)-citryl-CoA. The mixture was irradiated for 5 min, and 0.05-mL aliquots were assayed for residual lyase activity.

The isolated β subunit of *K. aerogenes* citrate lyase showed very low activity; thus it was probably being extensively and irreversibly denatured during the isolation procedure as reported by Dimroth & Eggerer (1975b). The effect of *p*-azidobenzoyl-CoA on the isolated subunit was therefore not studied.

Quantitative binding studies were carried out with *p*-azido[¹⁴C]benzoyl-CoA and deacetyltransferase from *K. aerogenes*. A plot of the inactivation of the lyase activity toward (3S)-citryl-CoA as a function of the covalent incorporation of *p*-azido[¹⁴C]benzoyl-CoA after photolysis is shown in Figure 1. The activity of the enzyme which had been photolyzed in the presence of various concentrations of *p*-azidobenzoyl-CoA (0–100 μM) was determined by assaying aliquots from the irradiated mixture. Controls photolyzed without reagent showed no loss of activity and were taken as 100% activity. The stoichiometry of binding was calculated by using a value of 575 000 for the molecular weight of *K. aerogenes* citrate lyase complex (Mahadik & SivaRaman, 1968).

It will be seen from Figure 1 that the decrease in enzyme activity is linear with the irreversible incorporation of ¹⁴C-labeled reagent until about 30% inactivation. To check whether the incorporation of ¹⁴C-labeled reagent under limiting conditions was selectively at the active site or not, the photolysis with *p*-azido[¹⁴C]benzoyl-CoA (50 μM) was carried out in the presence of (3S)-citryl-CoA (200 μM) and EDTA (10 mM). This reaction was carried out in the absence of Mg²⁺ to prevent action of the enzyme on the substrate, the absence of the metal having been shown to have no effect on inactivation with photoactivated *p*-azidobenzoyl-CoA (Table I). Under these conditions the enzyme retained its full lyase activity, and the incorporation of radioactivity was completely blocked. In the presence of 50 μM of the radioactive aryl-CoA derivative, the enzyme was inactivated 25% in the absence of the substrate. This would suggest that the linearity up to at least 25% inactivation represents specific labeling of the active site by *p*-azidobenzoyl-CoA.

On the basis of this assumption an estimate of the total active sites per mole of enzyme was found by extrapolation of the linear part of the plot by fitting of the least-squares line for the data up to 25% inactivation. This extension was observed to include also the value for 30% inactivation. The stoichiometry of the number of active sites per mole of enzyme obtained in this manner was 7.4. The extended extrapolation would make this only an approximate estimate.

At concentrations of the reagent required for a higher degree of inactivation, photolysis apparently results in significant nonspecific incorporation even in the presence of Tris which functions as scavenger (Bayley & Knowles, 1977). Nonspecific binding of *p*-azidobenzoyl-CoA has also been reported for acylglycine *N*-acyltransferase of beef liver at values above

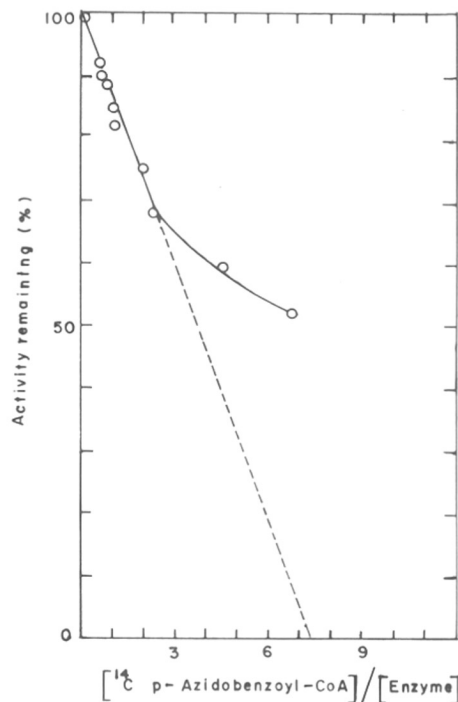


FIGURE 1: Determination of the number of active sites of lyase β subunits per mole of deacetyltransferase complex accessible to *p*-azidobenzoyl-CoA. The irradiated mixture in 300 μL contained 75 μg of deacetyltransferase, 50 mM Tris-HCl, pH 8.0, 3 mM MgSO₄, and varying amounts of *p*-azido[¹⁴C]benzoyl-CoA (0–100 μM). After 5-min photolysis, a 50-μL aliquot was withdrawn for the determination of lyase activity and a 200-μL sample withdrawn for estimation of ¹⁴C incorporation.

about 30% inhibition (Lau et al., 1977).

An enzyme sample photolyzed in the presence of the ¹⁴C-labeled *p*-azidobenzoyl-CoA was dissociated, and the constituent subunits were separated by NaDodSO₄-polyacrylamide gel electrophoresis. The electrophoretic profile of the separated subunits and the radioactivity distribution in the gel are represented in Figure 2. The presence of activity exclusively in the gel region containing the lyase subunit would confirm the evidence obtained from the kinetic data, Figure 1, and Table II that the active site of this subunit is specifically labeled on photolysis in the presence of the azidoaryl-CoA.

Discussion

p-Azidobenzoyl-CoA has been shown to have no inhibitory effect on the acyltransferase activity of *K. aerogenes* deacetyltransferase. That the enzyme complex might have hindered the photoaffinity reagent from reaching the active site on the transferase α subunit has been ruled out, since the isolated α subunit is also not inhibited by *p*-azidobenzoyl-CoA on photoactivation. The active site of the transferase subunit itself apparently does not accept *p*-azidobenzoyl-CoA, possibly on account of the bulky aryl group.

The substrate binding site of the lyase subunit, however, accepts the aryl-CoA ester. The kinetic data establish that *p*-azidobenzoyl-CoA acts as a potent competitive inhibitor of the acyl lyase activity. The reagent presumably binds specifically to the CoA ester sites of the lyase subunits. Again during photolysis a linear relationship is observed between incorporation of the ¹⁴C-labeled reagent and loss in activity

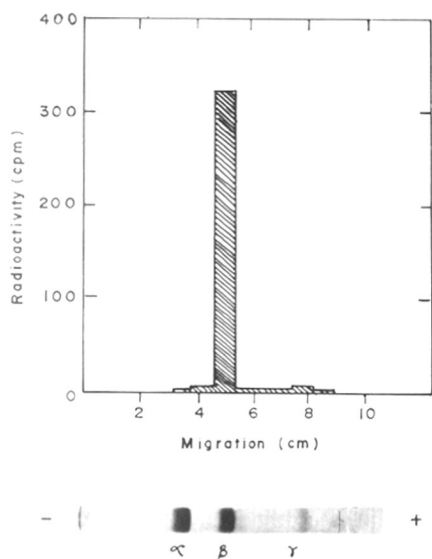


FIGURE 2: NaDodSO₄-polyacrylamide gel electrophoresis of deacetyl citrate lyase labeled by photolysis in the presence of *p*-azido-[¹⁴C]benzoyl-CoA. The irradiated mixture contained, in 0.5 mL, 400 μg of deacetyl citrate lyase, 50 mM Tris-HCl, pH 8.0, 3 mM MgSO₄, and 300 μM *p*-azido-[¹⁴C]benzoyl-CoA. An aliquot of about 0.2 mL was taken for electrophoresis. Gels were stained and sliced for determination of covalently bound radioactivity as described under Experimental Procedures. The histogram shows the profile of radioactivity in the separated subunits.

under limiting conditions of the reagent, the label being exclusively on the lyase β subunit. The reagent would thus be useful for selectively labeling the lyase active sites, having no action on the acetyl-CoA binding sites of the acyltransferase α subunits.

The lyase activity requires the presence of divalent metal ions such as Mg²⁺ (Dimroth & Eggerer, 1975b; Buckel et al., 1973). Divalent metal binding has been shown more recently to bring about a conformational change in the enzyme complex from *K. aerogenes* (SivaRaman & SivaRaman, 1979). In the present studies, the binding of *p*-azidobenzoyl-CoA to the lyase subunit and the subsequent irreversible inactivation on photolysis have been shown to occur both in the presence of Mg²⁺ and in the presence of an excess of EDTA. These results might suggest that (3S)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of Mg²⁺.

Although *K. aerogenes* citrate lyase has been shown to have a hexameric $\alpha_6\beta_6\gamma_6$ structure (Carpenter et al., 1975; Dimroth & Eggerer, 1975a), careful estimation of the prosthetic group components have indicated that only four acyl-carrier proteins carry pantetheine moieties (Singh et al., 1976). This has posed the problem of the function of subunit combinations which are devoid of prosthetic groups. An earlier suggestion has been made that these subunits might function in citrate cleavage

mediated by acetyl-CoA (Robinson et al., 1976). The approximate estimate of about seven sites on the lyase subunits complex obtained in the present investigation is suggestive of the fact that all the lyase subunits are accessible to citryl-CoA. Thus in the intact organism the cleavage of citrate can proceed both via the built-in prosthetic group and through the acyl-CoA-mediated reaction.

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

Subhalakshmi Subramanian, Amaresh Dasu and Churya Sivarman

Biochemistry Division, National Chemical Laboratory
Poona - 411 008; India

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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 (α), 32 000 (β) and 10 000 (γ) daltons respectively (1). The smallest subunit (γ) 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 α subunit functions as an acyl-transferase catalysing the formation of (3S)-citryl-ACP with liberation of acetate. The β 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-hydroxy-ethyl)-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 (α) and lyase (β) 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 μ l) 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.

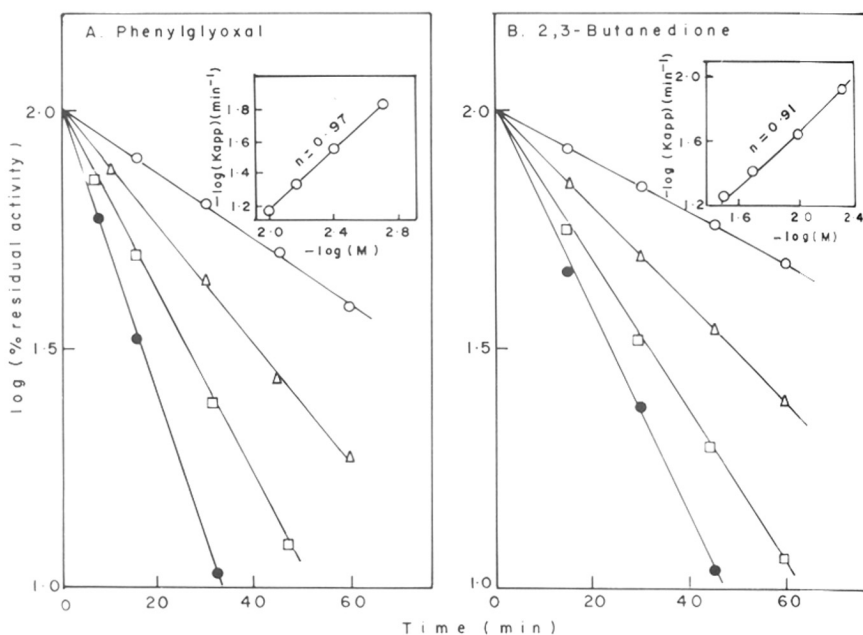


Figure 1: Inactivation of the acyl-transferase activity of deacetyl citrate lyase by α -carbonyl reagents. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA and various concentrations of the reagents. (A) Phenylglyoxal 2 mM (○), 4 mM (△), 7 mM (□), 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).

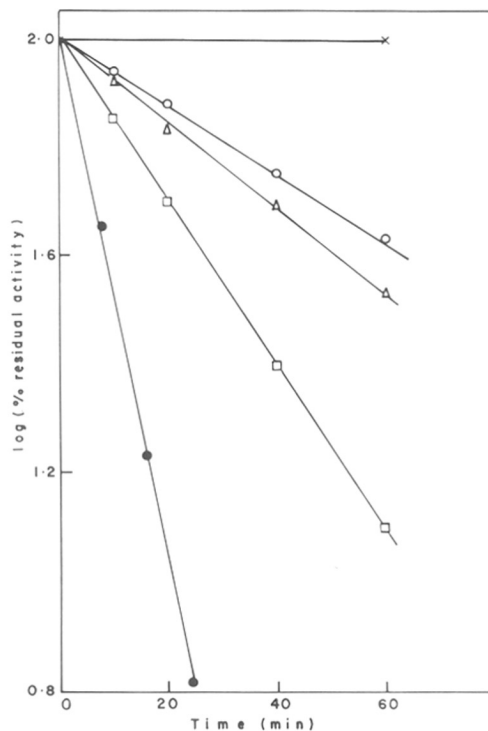


Figure 2 : Effect of ligands on the rate of inactivation of the acyl-transferase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with the ligands for 5 min at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA followed by incubation with 5 mM phenylglyoxal. Control without phenylglyoxal (x), 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 α -subunit where a value of $n = 0.93$ was obtained with phenylglyoxal.

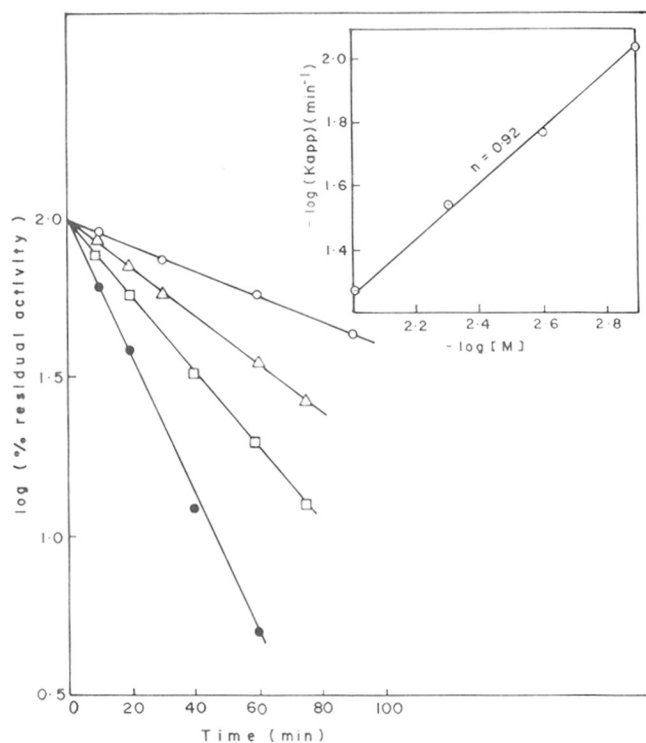


Figure 3 : Inactivation of the acyl-lyase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 3 mM $MgSO_4$ and phenylglyoxal 1.25 mM (○), 2.5 mM (△), 5 mM (□) or 10 mM (●). Aliquots were assayed for lyase activity 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.

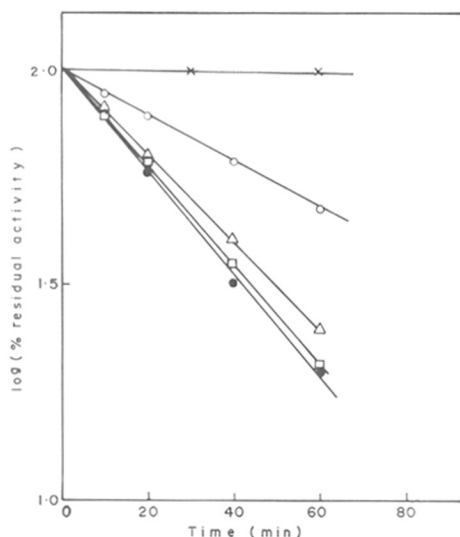


Figure 4 : Protection of lyase activity of deacetyl citrate lyase from inactivation by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with various ligands in presence of EDTA. Conditions were the same as described in Figure 2. Control (x), no added ligand (●), 10 mM citrate (□), 0.5 mM citryl-CoA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

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 (α) 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 α -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.

ACKNOWLEDGEMENTS

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S-ACYLATED RESIDUES OF THE ACYL-CARRIER PROTEIN SUBUNIT OF
Klebsiella aerogenes CITRATE LYASE

Amaresh Basu, Subhalakshmi Subramanian, Leena S. Hiremath*
and Churya SivaRaman

Biochemistry Division, National Chemical Laboratory,
Poona 411008 India

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SUMMARY Oxidation of the isolated deacetyl acyl-carrier protein subunit of citrate lyase from Klebsiella aerogenes with Cu^{2+} -o-phenanthroline complex leads exclusively to intrapeptide disulfide bridge formation indicating that the cysteamine and the cysteine residues are located in close proximity. The S-acetylation of the cysteine residue in deacetyl acyl-carrier protein subunit is catalysed by a citrate lyase ligase preparation in presence of acetate and ATP. Reaction-inactivation of citrate lyase results in deacetylation of the S-acetyl cysteamine residue of the prosthetic group but not of the S-acylated cysteine residue in the acyl-carrier protein.

Citrate lyase (EC 4.1.3.6) which catalyses the cleavage of citrate in a two step sequence of reactions (1) is a multienzyme complex containing two enzymes and an acyl-carrier protein (ACP)[†] (2). The most extensively studied of these complexes is the one from Klebsiella aerogenes, the ACP subunit of which has been shown to contain an S-acylated cysteine residue in addition to the essential S-acetyl moiety on the cysteamine residue of the CoA-like prosthetic group (3). Citrate lyase from K. aerogenes undergoes rapid inactivation in the course of the reaction it catalyses with loss of the essential acetyl groups to give inactive deacetyl (HS-) citrate lyase (4). The HS-citrate lyase is re-

* Present Address: Department of Biochemistry, University of Kentucky, Medical Center, Lexington Ky 40536.

† Abbreviations: ACP, acyl-carrier protein; SDS, sodium dodecyl sulfate; DTNB, 5,5'-(2-nitrobenzoic acid); DTE, dithioerythritol.

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activated either chemically by acetic anhydride (4) or enzymatically by citrate lyase ligase in presence of acetate and ATP(5).

Some of the properties of the acylated thiol residues of the ACP subunit of *K. aerogenes* citrate lyase and the probable role of citrate lyase ligase in modification of the cysteine residue of the subunit are reported here.

MATERIALS AND METHODS

Iodo [$2-^{14}\text{C}$] acetic acid (57 Ci/mol) was obtained from Radiochemical Centre, Amersham and diluted with cold iodoacetate to a sp. act. of 20 Ci/mol. Sodium [$1-^{14}\text{C}$] acetate (47.6 Ci/mol) from the Bhabha Atomic Research Centre, Bombay, was diluted with cold acetate to about 3 Ci/mol before use.

S-Carboxymethyl derivatives of cysteine and cysteamine were prepared by standard procedures (6,7).

Citrate lyase The pure enzyme (sp. act. $\sim 70 \mu\text{mol} \cdot \text{min}^{-1} \text{mg}^{-1}$) was obtained from *K. aerogenes* and assayed as described earlier (8).

Deacetylated ACP subunit Citrate lyase was dissociated with 6 M urea in the presence of 10 mM dithiothreitol and the deacetylated ACP subunit isolated in electrophoretically pure state by the procedure described by Carpenter *et al.* (2), a Sepharose CL-6B column (1 x 100 cm) being used for the separation. Protein in the isolated subunit preparation was determined by the method of Lowry *et al.* (9) using bovine serum albumin as standard.

Oxidation with o-phenanthroline- Cu^{2+} complex The separated deacetyl ACP subunit (0.8 mg) in 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4 was treated with o-phenanthroline and copper at concentrations of 100 μM and 20 μM respectively (10). The mixture was incubated for 30 min at 25°C and the reaction quenched by adding 0.5 ml of 20% SDS solution. Aliquots of the reaction system were separately taken for SDS-polyacrylamide gel electrophoresis and thiol group estimation. Sulfhydryl groups were estimated with DTNB⁺ (11) before and after the oxidation of the subunit.

SDS-polyacrylamide gel electrophoresis SDS-gel electrophoresis was carried out by the method of Weber and Osborn (12) in the absence of 2-mercaptoethanol.

Characterization of the S-acylated residues The S-acetyl residue involved in the reaction mechanism was characterized by a modification of the method described for fatty acid ACP (13). Citrate lyase (1 mg) was reaction inactivated in a total volume of 0.2 ml containing 0.2 mmol, citrate (pH 8.0); 0.16 μmol , MgSO_4 by incubation for 15 min at 30°C. An aliquot was checked for citrate lyase activity by coupled assay with malate dehydrogenase (14) to confirm complete inactivation. The free sulfhydryl groups of the reaction-inactivated enzyme were blocked with cold iodoacetate. The thioester linkages were then cleaved with neutralized hydroxylamine and dithiothreitol and the liberated thiol groups reacted with iodo [^{14}C] acetate. The S- [^{14}C] carboxymethylated residue in the protein was characterized by paper electrophoresis of the acid hydrolysate. The experimental conditions used for the differential labeling and characterization of the

[^{14}C]carboxymethyl residue were similar to those described by Dimmoth et al. (3). A mixture of reference compounds, S-carboxymethyl cysteine and S-carboxymethyl cysteamine (2 μmol each), was also separated under identical conditions on a separate strip of paper and their positions after migration visualised by spraying with 0.1% ninhydrin in acetone. The strip with the hydrolysed S-carboxymethylated enzyme was cut into pieces at the end of the run and radioactivity located using a dioxane based cocktail and a Beckman LS-100 C counter.

Citrate lyase ligase The acetate: HS- acyl-carrier protein enzyme ligase (AMP) was partially purified from *K. aerogenes* as described earlier (15). The ligase preparation was free from citrate lyase activity.

[^{14}C]-acetyl citrate lyase The [^{14}C]-acetyl labeled enzyme was prepared by acetylation of HS-citrate lyase with citrate lyase ligase in presence of [^{14}C]-acetate and ATP. Citrate lyase from *K. aerogenes* was deacetylated with neutralized hydroxylamine (16) before the DEAE-cellulose step in the purification procedure (17) and excess reagent separated by filtration through Sephadex G-25 (1.5 x 40 cm) using 0.05 M potassium phosphate buffer, pH 7.6 containing 1.6 mM MgSO_4 . An aliquot withdrawn from the protein fraction showed complete loss of activity. The deacetyl-enzyme (250 mg) in 5 to 6 ml of the phosphate buffer was treated with citrate lyase ligase in presence of ATP and acetate (8 μCi), final concentrations of 6 mM and 0.5 mM, respectively; and incubated at 25°C for 30 min. Adequate citrate lyase ligase was used to reactivate to about 50% of the initial activity. The protein was then precipitated by addition of an equal volume of cold saturated ammonium sulfate solution and collected by centrifugation for 30 min at 50 000 g and 4°C, dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.6 and subjected to DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B essentially as described earlier (17).

The radioactivity distribution in the subunits was determined after dissociation of [^{14}C]-acetyl citrate lyase (200 μg) with 1% SDS in the absence of 2-mercaptoethanol followed by SDS-gel electrophoresis. The gel was stained, destained and the regions corresponding to the subunits were cut and checked for radioactivity as described by Saitoh et al. (18).

The extent of deacetylation of [^{14}C]-acetyl citrate lyase on reaction-inactivation was estimated by exhaustive dialysis of the completely inactivated enzyme against 0.05 M potassium phosphate buffer, pH 7.5 and determination of the remaining protein-bound radioactivity.

RESULTS

Oxidation of deacetyl ACP The effect of o-phenanthroline- Cu^{2+} on the content of -SH groups of the isolated deacetyl ACP subunit is shown in Table 1. The content of 2-SH/mol in deacetyl ACP is in agreement with the presence of a cysteine and a cysteamine residue in the ACP molecule (19). Oxidation with the reagent

Table 1. Oxidation of sulfhydryl groups of deacetyl ACP by o-phenanthroline-Cu²⁺ complex.

System	-SH/mol of ACP ^a
Deacetyl ACP	1.9
Deacetyl ACP + o-phenanthroline-Cu ²⁺ complex	0.3

The isolated deacetyl ACP was oxidized with o-phenanthroline-Cu²⁺ complex (as described under Materials and Methods) and the reduction in thiol groups estimated. The values given are an average of three separate estimations.

^aM_r = 9379 (20).

results in a decrease to about 0.3 -SH/mol. SDS-gel electrophoresis of aliquots from the reaction mixture showed that the molecular weight of the oxidized subunit was unchanged. This was established by electrophoresis along with the unoxidized ACP. Interpeptide cross-linking is apparently excluded and oxidation results exclusively in intrapeptide disulfide bridge formation between the thiol groups of the cysteine residue on the prosthetic group and of the cysteine residue in the polypeptide chain of the ACP subunit.

Acetylation of HS-citrate lyase by citrate lyase ligase The distribution of the radioactive label in the subunits of [¹⁴C]-acetyl citrate lyase obtained on acetylation of HS-citrate lyase by citrate lyase ligase in presence of [¹⁴C]-acetate and ATP is shown in Figure 1. Acetylation by the ligase is specifically of the ACP subunit, radioactivity being associated exclusively with the region in the gel containing the 10 000 daltons ACP (γ) subunit, the 54 000 daltons acyl transferase (α) and the 32 000 daltons acyl lyase (β) subunits carrying no radioactive label.

S-Acylated residues of reaction-inactivated citrate lyase The characterization of the S-acylated residues present in reaction-inactivated citrate lyase is shown in Figure 2. It can be seen from Figure 2 that total reaction-inactivation of citrate lyase

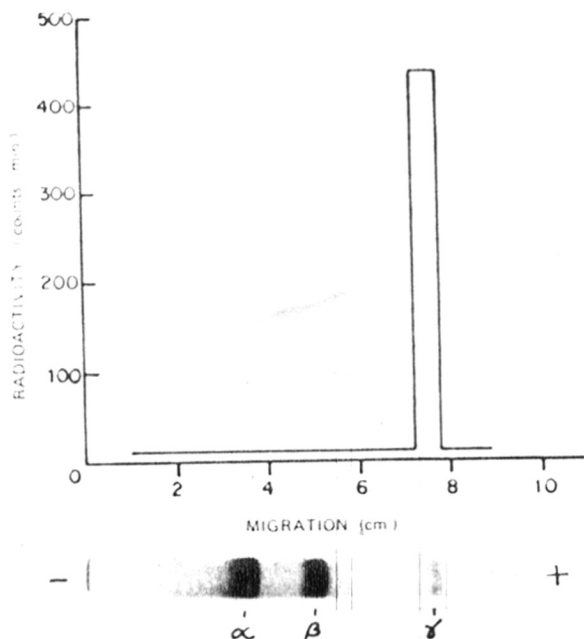


Figure 1. 2D-polyacrylamide gel electrophoresis of [^{14}C]-acetyl citrate lyase prepared as described under Materials and Methods. The gel was stained and sliced for determination of protein bound radioactivity. The histogram shows the profile of radioactivity in the separated acyl transferase (α), acyl lyase (β) and ACP (γ) subunits.

results in the complete loss of acetyl groups on the cysteamine moiety of the prosthetic group, while S-acetylated cysteine residues are still present in the inactivated enzyme. Reaction-inactivation results in the selective deacetylation of the acetyl-S-cysteamine residues of the prosthetic groups.

Partial deacetylation was also observed on reaction-inactivation of [^{14}C]-acetyl citrate lyase. The specific radioactivity of the active enzyme and of the reaction-inactivated complex are shown in Table 2. The presence of about 30% of the initial radioactivity in protein bound form after total reaction-inactivation and the finding that the cysteamine residues of the prosthetic

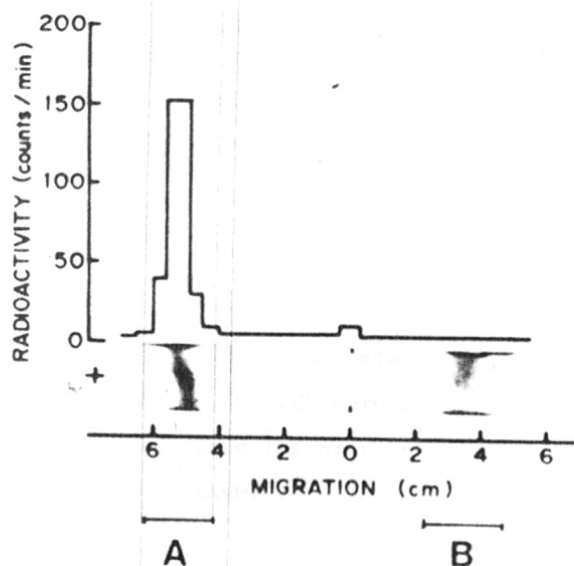


Figure 2. The histogram shows the distribution of radioactivity between the S-carboxymethyl derivatives formed after differential labeling of S-acetylated residues of reaction-inactivated citrate lyase with iodo[^{14}C]-acetate. See text for details. A, S-carboxymethyl cysteine; B, S-carboxymethyl cysteamine.

groups in the inactivated complex are fully deacylated would indicate that the [^{14}C]-acetyl moieties are retained only on the cysteine residues of the ACP subunits. The results are suggestive of the role of citrate lyase ligase in the modification of the

Table 2. Effect of reaction-inactivation on protein-bound radioactivity of [^{14}C]-acetyl citrate lyase.

Enzyme	cpm/mg
[^{14}C]-acetyl citrate lyase	13070
[^{14}C]-acetyl citrate lyase after reaction-inactivation	4120

[^{14}C]-Acetyl citrate lyase (430 μg) was incubated in a total volume of 1 ml of the reaction mixture containing 1 mmol citrate; 3 μmol , MgSO_4 and 50 μmol , Tris-HCl buffer, pH 8.0. Aliquots tested after 15 min incubation at 30°C showed complete loss of citrate lyase activity. Radioactivity was measured as described in the text.

cysteine residues of the ACP subunits to acetyl-S-cysteines in addition to the acetylation of the prosthetic groups in presence of acetate and ATP. The acetylation of the cysteine residue occurs even under conditions where HS-citrate lyase is only partially reactivated.

DISCUSSION

The presence of an S-acylated cysteamine and an S-acylated cysteine residue in the ACP subunit of citrate lyase from K. aerogenes has posed the problem of the nature of the biologically active acyl-carrier in the enzyme complex (3). Affinity labeling of HS-citrate lyase with iodoacetate in presence of excess DTE was shown by Dimroth and Eggerer (21) to result exclusively in the carboxymethylation of the cysteamine residue to yield a carboxymethylated complex inaccessible to reactivation either chemically with acetic anhydride or enzymatically by citrate lyase ligase. The present studies provide direct evidence that the cysteamine residue functions as the biologically active acyl-carrier, the reaction-inactivated citrate lyase being devoid of acetyl-S-cysteamine while still retaining S-acetylated cysteine residues.

Numerous instances of the presence of amino acid residues with modified side chains as constituents of proteins have been reported in the literature (22). The in vivo mechanisms of such modifications are not often understood. The data presented here suggest the involvement of citrate lyase ligase in the acetylation of cysteine residues in the ACP subunits of the K. aerogenes citrate lyase complex. The possible function of this modification might be to protect against intrapeptide disulfide bridge formation with the juxtaposed sulfhydryl residue on the prosthetic group of the enzyme in the deacetylated form.

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