SITE DIRECTED MUTAGENESIS OF β-KETOADIPATE SUCCINYL-COENZYME A TRANSFERASE II FROM ACINETOBACTER CALCOACETICUS

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

MASTER OF SCIENCE

By

Mei Sheng, B.M.
Denton, Texas
August, 1993
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The role of specific amino acid residues in \( \beta \)-ketoadipate succinyl-coenzyme A transferase II from \textit{Acinetobacter calcoaceticus} was investigated. A 1412 base pair \textit{BamHI-EcoRI} fragment carrying the \textit{catIJ} genes was amplified by polymerase chain reaction and inserted into pUC19 to generate the plasmid pCATE19. \textit{Escherichia coli} DH5\( \alpha \) (pCATE19) carrying only the \textit{catIJ} genes expressed 3-fold higher enzyme activity than the parent strain. Two mutants were constructed by site directed mutagenesis so that glutamate was replaced by a glutamine at positions Gln155 and Gln193 in the \( \beta \) subunit of the primary amino acid sequence of the CoA transferase. Both mutants produced transferase that was catalytically active suggesting that Glu155 and Glu193 do not participate directly in catalysis.
ACKNOWLEDGMENTS

I would like to acknowledge my major professor, Dr. Mark S. Shanley for accepting me as his student and giving me good guidance and encouragement to complete my graduate study. I also want to thank Dr. Gerard A. O'Donovan and Dr. G. Roland Vela for their kind guidance. I appreciate all the people who helped me in every way, especially my colleagues, Wei Chen and Michael Chen for help of many kinds. Finally, I gratefully acknowledge the assistance of my husband Xiaomin Yang and my son Chun Yang throughout this endeavor.
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CHAPTER I

INTRODUCTION

Living in a chemically dynamic state, organisms synthesize, transform, and degrade a great number of organic compounds. These activities constitute the carbon cycle. A wide range of natural compounds contain aromatic rings. Large amounts of them, such as lignin, are produced by plants. Aromatic amino acids and vitamins are constituents of every organism. All these compounds become available when organic matter is decomposed; they are converted to fossil fuels like coal and oil, or degraded for carbon and energy by living organisms in which large mass are microorganisms, bacteria and fungi. In this industrial age, a variety of artificial aromatic chemicals, such as pesticides and plastics that do not occur in nature, can enter the soil, even leach into groundwater to threaten the health of human beings. Some of these compounds are made up of chemical structures subject to bacterial biodegradation; others with complex structures or extensive ring substitutions are resistant to normal cycling of microbial action.

Nature has long evolved mechanisms for release and reuse carbon bound in chemical structures. Widespread
microorganisms, both aerobic and anaerobic, exhibit an enormous flexibility to modify, degrade, and utilize a variety of aromatic, halogenated aromatic, and polyaromatic compounds through enzymatic processes for growth. Aerobic microorganisms have made use of most of biochemical sequences and evolved various catabolic pathways. They are able to oxidize aromatic hydrocarbons using molecular oxygen, thereby facilitating cleavage reaction sequences to yield non-aromatic products which can enter the central metabolic pathways, such as the Krebs citric acid cycle.

The majority of aromatic compounds are initially converted into either catechol or protocatechuate in bacteria (Stanier and Ornston, 1973). These conversion sequences prepare the hydrocarbon benzene ring to be oxidized (dihydriodiol) for later ring fission, and tend to be specialized (Dagley, 1978 and 1985). Benzene, toluene, benzoate, and biphenyl are initially metabolized by adding oxygen to the benzene nucleus before further oxidized to catechol. Alkyl benzenes, such as m- or p-xylene and p-cymene, are first metabolized by oxidation of one methyl group of a hydrocarbon to carboxyl, which is then converted to catechol and ultimately to pyruvate. Fused polynuclear hydrocarbons, such as naphthalene and phenanthrene, can be hydroxylated and again in turn are oxidized to the catechol or protocatechuate. Some bacteria are able to form the intermediate gentisate, such as from the hydroxylation of m-
hydroxybenzoate in *Pseudomonas acidovorans*, and then cleave the ring to form fumarate and pyruvate. Phenylalanine and tyrosine are oxidized and further catabolized to fumarate and acetoacetate through the homogentisate pathway by bacteria and animals.

In mammalian liver and also in some fungi, some aromatic hydrocarbons are converted to arene oxides which can then be eliminated. These evolved eukaryotic systems may be used by the organisms for dissimilation of toxic hydrocarbons, rather than for use as growth substrates (Gibson and Subramanian, 1984).

For the ring fission substrates, catechol or protocatechuate, there are two types of cleavage reactions in microorganisms. The subsequent metabolic route of dissimilation is determined by the nature of the dioxygenase (Hayaishi, 1966) cleaving each diphenolic substrate ortho or meta to the diol. In meta cleavage, such as in some species of *Azotobacter* and *Pseudomonas*, the ring is opened adjacent to the vicinyl hydroxyl groups to form an α-hydroxymuconic semialdehyde which is subsequently dissimilated to pyruvate, fumarate, and acetaldehyde (Bayly and Dagley, 1969; Sala and Evans, 1971). In contrast, ortho-fission cleaves the aromatic ring between the two hydroxyl groups of a diphenol to produce cis, cis-muconate or β-carboxy-cis, cis-muconate, from which the metabolic channels lead into the β-ketoadipate pathway.
The \( \beta \)-ketoadipate pathway is widely distributed among bacteria for aromatic degradation. The pathway has two branches beginning with the diphenols, catechol or its analog, protocatechuate. The central reactions from the separate branches lead to the formation of a series of three common intermediates, enol-lactone of \( \beta \)-ketoadipate, free \( \beta \)-ketoadipate, \( \beta \)-ketoadipyl-coenzyme A (\( \text{CoA} \)), from which the pathway is named. \( \beta \)-ketoadipyl-CoA is cleaved to produce the central metabolic intermediates, acetyl-CoA and succinate which enter the tricarboxylic acid cycle to provide carbon and energy (Figure 1; Ornston and Yeh, 1982).

The bacterial \( \beta \)-ketoadipate pathway has been observed in bacteria of different genera. The species of the genus \textit{Pseudomonas} (in particular \textit{P. putida}) and \textit{Acinetobacter calcoaceticus} have so far provided much of the material for the study of the enzymatic properties and regulation in the pathway (Ornston, 1966a, 1966b, and 1966c; Canovas and Stanier, 1967). A striking feature among them is the parallel and chemically analogous reactions of two branches which are typical of \textit{A. calcoaceticus} (Stanier and Ornston, 1973). The two branches differ only by the presence or absence of a carboxyl substituent that may have different requirements in the active sites and that effect differential gene expression of the two homologous sets of enzymes. In the beginning, catechol is oxidized by catechol 1,2-dioxygenase to \textit{cis}, \textit{cis}-muconate that is then converted by
Figure 1. The central reactions of the β-ketoacidipate pathway in aerobic bacteria. Complex aromatic substrates are converted to one of the diphenols, catechol and protocatechuate, which is then transformed to β-ketoacidipate by the central reactions of the pathway. The β-ketoacidipate is further converted to the tricarboxylic acid cycle intermediates succinate and acetyl-CoA (Ornston and Yeh, 1982).
protocatechuate $\rightarrow$ $\rightarrow$ $\rightarrow$ succinate and acetyl-CoA
muconate lactonizing enzyme to muconolactone. Protocatechuate is oxidized by protocatechuate 3,4-dioxygenase to $\varepsilon$-carboxy-cis, cis-muconate which is then converted by $\varepsilon$-carboxymuconate lactonizing enzyme to $\gamma$-carboxy-muconolactone. Further, muconolactone is converted by muconolactone isomerase as $\gamma$-carboxy-muconolactone is converted by $\gamma$-carboxy-muconolactone decarboxylase to the first common intermediate, $\varepsilon$-ketoadipate enol-lactone. This compound is then hydrolyzed to $\varepsilon$-ketoadipate by $\varepsilon$-ketoadipate enol-lactone hydrolase. Subsequently, $\varepsilon$-ketoadipate reacts with succinyl-CoA in a reaction catalyzed by $\varepsilon$-ketoadipate succinyl-CoA transferase to yield $\varepsilon$-ketoadipyl-CoA. Finally, $\varepsilon$-ketoadipyl-CoA is thiolytically cleaved to acetyl-CoA and succinate by $\varepsilon$-ketoadipyl-CoA thiolase. In A. calcoaceticus, the overall conversions of benzoate degradation via the catechol branch and p-hydroxybenzoate dissimilation via the protocatechuate branch to acetyl-CoA are accomplished by two sets of enzymatic reactions (Figure 2). The last three common steps are catalyzed by isofunctional enzymes hydrolase I and II, transferase I and II, and thiolase I and II; whereas P. putida has only a single form of each of these enzymes.

The enzymes in the $\varepsilon$-ketoadipate pathway and its ancillary initial reactions are all inducible. However, the enzymes in the two parallel branches are physically separable and subject to the asymmetric regulation in the control of
Figure 2. The β-keto adipate pathway in *Acinetobacter calcoaceticus*. The pathway has two parallel branches that converge metabolically at the level of the β-keto adipate enol-lactone and are maintained separately in the respective metabolites at each step of the pathway. Enzymes (including three pairs of isofunctional enzymes) and structural genes are shown for the analogous metabolic transformations from catechol and protocatechuate to succinate and acetyl-CoA (Stanier and Ornston, 1973).
gene expression: analogous metabolites do not have analogous roles as inducers. In P. putida, protocatechuate 1,2-oxygenase is induced by its substrate protocatechuate, while the five subsequent enzymes are coordinately induced by a terminal intermediate, 6-ketoacidipate. In the catechol branch, cis, cis-muconate acts as a product-inducer of catA gene expression and as a substrate-inducer of the expression of the catBC genes. With respect to the nature of the metabolite inducers and enzymology, the system in A. calcoaceticus has regulatory pattern unique to the species. All enzymes for the protocatechuate branch (including the three protocatechuate branch isoenzymes) are coordinately induced by protocatechuate. The compound cis, cis-muconate induces all enzymes required (including the other three isoenzymes) for catechol as substrate. This characteristic results from the specific system of regulation in A. calcoaceticus. The structural genes pcaACBDFE encoding six enzymes for protocatechuate catabolism are clustered in a single regulatory unit and thus induced coordinately (Canovas et al., 1968; Doten et al., 1987). The genes catA and catBCEFD encoding six enzymes for the catechol branch, lie near, but separate transcriptional units and are induced both by cis, cis-muconate (Neidle and Ornston, 1986; Shanley et al., 1986). Because of this evolved specific induction and regulation system, a mutation in a gene for one of the isozymes in the last three steps of the pathway results in
complete loss of the ability to use compounds that normally feed into the corresponding branch of the $\beta$-ketoacid pathway.

In the $\beta$-ketoacid pathway of *A. calcoaceticus*, the enzyme $\beta$-ketoacid succinyl-coenzyme A transferase II encoded by the catIJ genes (formerly termed catE gene; EC 2.8.3.6.) catalyzes the transfer of the CoA moiety from the activated thioester substrate, succinyl-CoA to the $\alpha$-carboxyl group of the other substrate, $\beta$-ketoacid, to form $\beta$-ketoacidyl-CoA (Yeh and Ornston, 1981). This reaction is one of CoA thioesterification and completes the conversion of benzoate via the catechol branch to acetyl-CoA and succinate.

CoA thioesterification is a common metabolic reaction carried out by CoA transferases. In many prokaryotes, carboxylic acids are activated by the addition of CoA to form an energy-rich thioester before they are further metabolized and used as carbon and energy sources. Activation by similar homologous enzymes occurs in many mammalian tissues, such as the first step in the metabolism of ketone bodies. The CoA-transferase is a member of a family of related enzymes that catalyze the reversible transfer of the thiol group of CoA from one carboxylic acid to another. This type of acyl group activation catalyzed by CoA-transferases (Figure 3) in general is unusual in the sense that the enzymes catalyze the leaving group transfer rather than a simple displacement (Jencks, 1973). In the reaction the activating compound, the
Figure 3. The proposed scheme of the CoA transfer reaction. The reaction catalyzed by CoA transferase occurs in two steps. The CoA moiety of the CoA donor (a acyl-CoA substrate) is transferred to a γ-glutamyl residue on the enzyme to form a covalent enzyme-CoA intermediate (line 1). Then the CoA is transferred to the ketoacid acceptor (lines 2 and 3) (Jencks, 1973).
succinyl-CoA thioester that acts as a dehydrating agent, extracts oxygen from the carboxylate ion, at the same time it donates the CoA group that replaces the oxygen atom to form a part of the activated CoA product. The extraction of oxygen from the CoA acceptor carboxylate ion and the transfer of oxygen to the CoA donor carboxyl group has been demonstrated experimentally by the use of isotopically labeled compounds for the related enzyme succinyl-CoA:acetoacetate CoA-transferase (Falcone and Boyer, 1959).

Although the substrate ranges and subunit structures may differ (Table 1), different CoA transferases possess similar structures, catalytic mechanisms, and functions (Falcone and Boyer, 1959; Hersch and Jencks, 1967a and 1967b; Sramek and Frerman, 1975a and 1975b; White and Jencks, 1976; Yeh and Ornston, 1981). The CoA-transferases are all multi-subunit enzymes with similar molecular weights of approximately 100,000 Daltons. During the course of the reaction, they all undergo two conformational changes with an enzyme-CoA intermediate first formed through covalent attachment of the CoA moiety of the substrate. Though not demonstrated for the E-ketoacidate succinyl-CoA transferase, where examined, the γ-carboxyl group of a glutamyl residue on acetyl-CoA:acetoacetate CoA-transferase in E. coli bonds with the CoA moiety of substrate to form a glutamyl thioester. Also, CoA-transferases all possess a sensitive thiol group whose modification by thiol reagents results in inactivation of the
Table I. Comparison of coenzyme A transferases.

<table>
<thead>
<tr>
<th>Organism and Enzyme</th>
<th>Substrate</th>
<th>Product</th>
<th>Subunit</th>
<th>Purifying Sequence</th>
<th>Oligomer/m.w.</th>
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<td>Prokaryotes:</td>
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<tr>
<td>A. calcoaceticus</td>
<td>succinyl-CoA/β-ketoadipate</td>
<td>β-ketoadipyl-CoA/succinate</td>
<td>αββ2/β25 kDa1</td>
<td>partial</td>
<td>DNA/aa2</td>
</tr>
<tr>
<td>CoA-transferase I</td>
<td>succinyl-CoA/β-ketoadipate</td>
<td>β-ketoadipyl-CoA/succinate</td>
<td>---/---</td>
<td>partial</td>
<td>DNA/aa3</td>
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<tr>
<td>A. calcoaceticus</td>
<td>succinyl-CoA/β-ketoadipate</td>
<td>β-ketoadipyl-CoA/succinate</td>
<td>αββ2/β25 kDa1</td>
<td>partial</td>
<td>DNA/aa4</td>
</tr>
<tr>
<td>CoA-transferase II</td>
<td>succinyl-CoA/β-ketoadipate</td>
<td>β-ketoadipyl-CoA/succinate</td>
<td>αββ2/β25 kDa5</td>
<td>full</td>
<td>DNA/aa5</td>
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<tr>
<td>E. coli</td>
<td>acetyl-CoA/acetoacetate</td>
<td>acetoacetyl-CoA/acetate</td>
<td>αββ2/β25 kDa5</td>
<td>full</td>
<td>DNA/aa6</td>
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<tr>
<td>CoA-transferase</td>
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<td>Pig heart</td>
<td>succinyl-CoA/acetoacetate</td>
<td>acetoacetyl-CoA/succinate</td>
<td>dimer/β6 kDa7</td>
<td>full</td>
<td>DNA/aa8</td>
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<tr>
<td>CoA-transferase</td>
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<tr>
<td>Sheep kidney</td>
<td>acetoacetyl-CoA/succinate</td>
<td>succinyl-CoA/acetoacetate</td>
<td>dimer/β6 kDa9</td>
<td>full</td>
<td>DNA/aa9</td>
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The CoA transferases shown here are all multi-subunit proteins with similar molecular weights (m.w.) of approximately 100 kilo-Daltons (kDa). A dashed line (---) indicates that molecular weight has not been measured, or DNA sequence for deduction of its amino acid (aa) sequence is not available.

1 Yeh and Ornston, 1981.
2 Kowalchuk et al., 1991.
3 Shanley et al., 1986.
5 Sramek and FeRman, 1975a.
6 Chen and Shanley, 1993.
7 White and Jencks, 1976.
8 Lin and Bridger, 1992.
enzyme (Yeh and Ornston, 1984). The covalent enzyme-CoA intermediate, and not the free enzyme, is most susceptible to this inactivation indicating a characteristic conformational change upon formation of the enzymatically active intermediate. A cationic lysyl residue near the active site has been identified by acylation, and is presumably responsible for neutralizing the charge on the substrate CoA donor molecule during binding (Sramek and Frerman, 1975a; Frerman et al., 1977). Though the enzymes act on a variety of substrates, all of these enzymes show a high degree of specificity towards the different CoA donor while the structural requirement for the CoA acceptor substrates are less specific.

A 5.2 kilobase-pair (kbp) EcoRI fragment of DNA that carries the catBCIJFD genes has been cloned from Acinetobacter calcoaceticus chromosomal DNA. These six genes encode five enzymes of the ω-ketoadipate pathway (Figure 4; Shanley et al., 1986). This DNA fragment expressed the enzymes from the catB, catC, catIJ, catF, and catD genes in the heterologous hosts, P. putida and Escherichia coli. Wild-type E. coli DH5α does not contain genes for the ω-ketoadipate pathway. The presence of the catBCIJFD genes in E. coli should present the cell with the ability to utilize cis, cis-muconate for growth (Figure 2). However, even under conditions of high level enzyme expression in host, E. coli is unable to metabolize cis, cis-muconate further than ω-
**Figure 4.** The physical restriction and genetic map of the catBCIJFD gene region. Distances are given in base pairs from the EcoRI site. The entire EcoRI-EcoRI fragment is 5,226 base pairs in length and had been cloned into the plasmid pUC13 in previous research (Shanley et al., 1986). The position of the restriction sites and genes were confirmed by DNA sequence analysis and were drawn to scale. The direction of transcription of the catBCIJFD genes as they are depicted is from left to right.
ketoadipate. No growth on cis, cis-muconate in such strains is observed (Shanley et al., 1986).

A related CoA-transferase in E. coli uses acetyl-CoA as the CoA donor (Sramek and Preram, 1975a and 1975b), and this compound is the dominant constituent of the CoA pool in those cells (Jackowski and Rock, 1986). The CoA transferase from A. calcoaceticus has an absolute requirement for succinyl-CoA as the CoA donor (Yeh and Ornston, 1981), but the free succinyl-CoA pool may be insufficient in the intracellular CoA pools in E. coli. Thus, β-ketoadipate succinyl-CoA transferase expressed from the catIJ genes in E. coli may not be able to elevate β-ketoadipate to the level of CoA thioester for subsequent thiolytic cleavage to the TCA cycle intermediates.

The inability for E. coli to metabolize cis, cis-muconate any further than β-ketoadipate raise a caveat for the biotechnology community in their aspiration to use genetically engineered microorganisms for chemical transformations. During the reconstruction of pathways in heterologous hosts, a number of factors must be considered including transport, regulation, and availability of precursors or substrates. Before pathways for the complete mineralization of many aromatic compounds can be constructed in a recombinant strain, the structural basis for substrate specificity in the CoA-transferase must be understood and overcome.
Determination of the entire nucleotide sequence of the structural catIJ genes encoding two subunits of 8-ketoacidipate succinyl-CoA transferase II from A. calcoaceticus has allowed the deduction of the primary structure of the α and β subunits of the CoA transferase (Figure 5). The deduced 217 amino acid sequence of the β subunit contains 4 cysteinyl residues, 12 lysyl residues, and 14 glutamyl residues. The 228 amino acid sequence of α subunit contains 2, 13, and 11 such residues, respectively.

The alteration of genes and/or the proteins they encode through the substitution of specific nucleotides within a gene sequence by site-directed mutagenesis represents a fundamental tool of modern recombinant DNA technology (Rossi and Zoller, 1987). It not only allows for the analysis of the structural basis of gene and protein function but also facilitates the generation of novel gene products. The amino acid residues of the enzyme involved in binding, catalysis, assembly, and substrate specificity can be identified by site-directed mutagenesis. Substitution of a specific amino acid residue in a protein can contribute to an understanding of the relationship between protein structure and enzyme function (Caruthers, 1987).

Previous research about the detailed descriptions of the catalytic and kinetic mechanism of the 8-ketoacidipate succinyl-CoA transferase has not been performed. This enzyme from A. calcoaceticus has not been purified extensively
Figure 5. Nucleotide sequence of the catIJ genes and deduced amino acid sequence. The complete nucleotide sequence of the catIJ genes and the deduced amino acid sequence of the α and β subunits of 3-ketoacidyl-CoA transferase II from Acinetobacter calcoaceticus is shown. Several glutamyl residues surrounded by basic, positively charged residues (underlined) are likely candidates for the active site and could be involved in the formation of the covalently-linked enzyme-substrate intermediate.
**a-SUBUNIT**

Met Thr Leu Thr Leu Thr Leu Leu Ser Gly Asp Gly Ala

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| Gln Leu Leu Ile Gly Phe Gly Gly Ala Gly Glu Pro Ala Glu Leu Ile Gly Glu Leu |
|---|---|
| 70 | 80 |

| Gly Leu Ala Ile Leu Leu Leu Leu Ser Ser Gly Ser Pro Arg |
|---|---|
| 90 | 100 |

| Val Pro Gin Gly Asn Leu Ala Cys Arg Ile Gin Ala Ala Gly Met Gly Leu Leu Gly Glu |
|---|---|
| 110 | 120 |

| Thr Thr Pro Gly Thr Pro Thr Leu Ala Gly Gly Pro Thr Leu Asp Arg |
|---|---|
| 130 | 140 |

| Gln Leu Ala Leu Ala Leu Leu Leu Ser Ser Gly Ser Pro Arg |
|---|---|
| 150 | 160 |

| Val Pro Gin Gly Asn Leu Ala Cys Arg Ile Gin Ala Ala Gly Met Gly Leu Leu Gly Glu |
|---|---|
| 170 | 180 |

| Thr Thr Pro Gly Thr Pro Thr Leu Ala Gly Gly Pro Thr Leu Asp Arg |
|---|---|
| 190 | 200 |

| Gln Leu Ala Leu Ala Leu Leu Leu Ser Ser Gly Ser Pro Arg |
|---|---|
| 210 | 220 |

| Ser Thr Pro Ala Ser Ser Ala Ala Pro *** |
|---|---|
| 228 | 229 |

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**β-SUBUNIT**

Met Ser Tyr His Leu Val Thr Arg Asp Gin Ile Ala Gin Arg Val Ala Gin Arg Val |

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</table>

| Gly Leu Ile Gly Phe Gly Gly Ala Gly Glu Pro Ala Glu Leu Ile Gly Glu Leu |
|---|---|
| 70 | 80 |

| Val Pro Gin Gly Asn Leu Ala Cys Arg Ile Gin Ala Ala Gly Met Gly Leu Leu Gly Glu |
|---|---|
| 90 | 100 |

| Thr Thr Pro Gly Thr Pro Thr Leu Ala Gly Gly Pro Thr Leu Asp Arg |
|---|---|
| 110 | 120 |

| Gln Leu Ala Leu Ala Leu Leu Leu Ser Ser Gly Ser Pro Arg |
|---|---|
| 130 | 140 |

| Val Pro Gin Gly Asn Leu Ala Cys Arg Ile Gin Ala Ala Gly Met Gly Leu Leu Gly Glu |
|---|---|
| 150 | 160 |

| Thr Thr Pro Gly Thr Pro Thr Leu Ala Gly Gly Pro Thr Leu Asp Arg |
|---|---|
| 170 | 180 |

| Gln Leu Ala Leu Ala Leu Leu Leu Ser Ser Gly Ser Pro Arg |
|---|---|
| 190 | 200 |

| Ser Thr Pro Ala Ser Ser Ala Ala Pro *** |
|---|---|
| 228 | 229 |
hitherto. No crystallographic data of the protein structure from X-ray diffraction studies has been made to support specific hypotheses that can be combined with the nucleotide sequence of catIJ genes to deduce the position of active site. However, this enzyme is the only one for which a primary sequence is currently available so that the results of chemical modification studies can be correlated with the molecular structure of the protein. Other results indicate that the bacterial CoA transferases and the mammalian CoA transferases are similar in terms of catalytic pathways, general properties of protein, and amino acids at the active center.

In this research we seek to find the role that amino acid sequences play in determining enzymatic properties for later restructuring an active site to accept different substrates derived from compounds not normally handled through the pathway. As detailed in the Materials and Methods and the Results and Discussion, specific amino acid (glutamyl) residues in 8-ketoadipate succinyl-CoA transferase II have been selected and altered by site-directed mutagenesis. The specific objective of this study is to identify the critical amino acid residues at the active site in the primary sequence of 8-ketoadipate succinyl-CoA transferase by:

Subcloning the catIJ genes from the 5.2 kbp clone that carries six different 8-ketoadipate pathway genes
and constructing a plasmid carrying the catJ genes that only express β-ketoadipate succinyl-CoA transferase II activity.

Using site-directed mutagenesis to modify the amino acid residues Glu155 to Gln155 and Glu193 to Gln193 in the gene for the β subunit of the β-ketoadipate succinyl-CoA transferase II.

Verifying the sequence changes of the mutants and measuring the effects of each mutation on the enzymatic and kinetic properties of the enzyme.
CHAPTER II

MATERIALS AND METHODS

Chemicals and Enzymes

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isopropylthio-β-D-galactopyranoside (IPTG), agarose, and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Bethesda Research Laboratories. Acrylamide and cesium chloride were from International Biotechnologies, Inc. Ampicillin, ammonium persulfate, bromophenol blue, bovine serum albumin, coenzyme A, chloramphenicol, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), ethidium bromide, β-ketoadipic acid, succinic anhydride, [α-35S] dATP, Triton X-100, Tris base or Tris-HCl, urea, and xylene cyanol FF were purchased from Sigma Chemical Company. Agar and X-ray film were provided by Kodak Company. Folin phenol reagent, N,N'-methylene-bis-acrylamide, phenol, Spectrapore membrane tubing, and inorganic salts were all purchased from Fisher Scientific. The succinyl-coenzyme A used for the enzyme assay was freshly synthesized prior to use. Buffers and solutions were formulated with distilled, deionized water.
Restriction enzymes and buffers were supplied by New England BioLabs or Bethesda Research Laboratories and used as recommended by their suppliers. Polymerase chain reaction cloning kits with Vent DNA polymerase were purchased from New England BioLabs. T4 DNA ligase and buffers were from BRL and lysozyme from Sigma Chemical Company. DNA sequencing was performed according to manufacturer instructions using reagents and Sequenase T7 DNA polymerase contained in a Sequenase version 2 DNA sequencing kit purchased from United States Biochemical Co.

Bacterial Strains, Media, and Culture Conditions

The bacterial strain used in this study is *Escherichia coli* strain DH5α whose genotype is given in Table II. *E. coli* DH5α was cultured in Luria broth (LB) medium at 37°C and maintained on minimal dextrose agar plates at 4°C for four weeks (Davis et al., 1980). LB broth (Gibco Laboratories) containing 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per liter was used as a commercial preformulated preparation. When solid LB media were prepared, Bacto agar (Difco) was added at a final concentration of 1.5 per cent. After autoclaving, ampicillin was added to media at a final concentration of 100 μg per ml for selective media. When screening lacZ insertional inactivation (β-galactosidase activity), isopropylthio-β-D-galactopyranoside (IPTG) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-
Table II. List of bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F⁻, 80d λac2 ΔM15, Δ(lacZΔargF)U169, deoR, endA1, hsdR7(k-, mK+), thi-1, relA1, recA1, supE44, λ⁻, gyrA96</td>
<td>Focus (1986) 8:29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Selective marker</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt; (pBR322 PruII-EcoRI); ΔM15(HaeII), P/O&lt;sub&gt;lac&lt;/sub&gt;; multiple cloning sites(M13)</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>pPAN4</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;, multiple cloning sites, ΔM15, P/O&lt;sub&gt;lac&lt;/sub&gt; (pUC13); catECIJFD on an EcoRI fragment (A. calcoaceticus chromosome)</td>
<td>Shanley et al., 1986</td>
</tr>
</tbody>
</table>

The genotype of the strains and the selective markers of the plasmids are shown.

galactopyranoside (X-gal) were added or spread to a final concentration of 0.1 mM and 0.004 per cent (w/v). Stock solutions were stored at -20°C as 0.1 M and 2 per cent solutions respectively. Cells for transformation, plasmid purification, and enzyme assay were grown in LB selective medium as required (Sambrook et al., 1989). E. coli DH5α was made competent by calcium chloride treatment according to established procedures (Dagert and Ehrlich, 1979).
Bacterial Plasmids

The bacterial plasmids used in this study are listed in Table II. The plasmid pPAN4 carries the 5.2 kbp EcoRI-EcoRI DNA restriction fragment containing the catBCIJD genes for five β-ketoacid pathway enzymes from A. calcoaceticus inserted into the plasmid pUC13 (Figure 4; Shanley et al., 1986). The plasmid pUC19 (Yanisch-Perron et al., 1985) was used as a vector to subclone the catIJ genes on a 1.4 kbp BamHI-EcoRI fragment from the plasmid pPAN4. According to established procedures, the plasmid DNAs (25 ng in 10-20 μl) were initially transferred into DH5α after cells had been treated with 100 mM ice-cold calcium chloride to induce competency. DNA and cells were incubated together and the mixture was heat shocked at 42°C (Sambrook et al., 1989). Plasmids from transformed cells were screened by rapid mini-preparations of 1.5 ml LB cultures using TENS lysis solution (0.1 N NaOH and 0.5 per cent sodium dodecyl sulfate in Tris-EDTA buffer) (Zhou et al., 1990). Large scale amplification and purification of plasmids was accomplished from 11 cultures. When cells were grown up to early-logarithmic phase (A660 of ~0.6 - 0.8), the antibiotic chloramphenicol (0.2 g) was added into culture to prevent cell division and amplify the plasmids. Cell pellets were washed and resuspended in 5 ml of fresh lysozyme (5 mg/ml) in 50 mM Tris and 0.25 M EDTA (pH 8.0) for 10 minutes at room temperature. Then 20 ml
Triton X mixture (per 100 ml: 0.5 ml Triton X-100, 5 ml of 1 M Tris, 25 ml of 0.25 M EDTA, pH 8.0) was added. After a "clearing spin", the lysate in the supernatant was mixed with cesium chloride-ethidium bromide and spun in an ultracentrifuge at 40,000 rpm for 48 h to obtain stable gradient. Plasmids (lower band) were recovered from the gradient, extracted with 1-butanol, and dialyzed in TE buffer as previously described (Sambrook, et al., 1989).

Oligonucleotide Primers

Oligonucleotides (oligos) of 18 to 28 nucleotides (nt) in length were used for primers and were synthesized in either 0.2 or 1 µmol columns on a Miligen DNA synthesizer or on a Pharmacia Gene Assembler DNA synthesizer using β-cyanoethyl phosphoramidite method (Beaucage and Caruthers, 1981). Detritylation, activation, coupling, oxidation, capping, and deprotection were performed on the instruments according to recommended run parameters. All oligo sequences were designed using the known catI gene sequence. MicroGenie, and IBM computer program for DNA sequence analysis (Queen and Korn, 1984) was used to assure that the level of homology was sufficiently stringent to prevent nonspecific priming during DNA sequencing in polymerase chain reaction (PCR). Oligonucleotides were removed from the support columns and treated with fresh, concentrated ammonium hydroxide at 60°C for 12 to 18 hours to remove blocking
groups on the amino groups. Ammonium hydroxide was evaporated and the oligos were purified by column chromatography using a Sephadex G25 column equilibrated with distilled water to remove the free benzoyl and isobutyryl blocking groups, as well as to remove short failure sequences present in the preparation. Concentrations of oligos were measured by ultraviolet absorbance at 260 nm. Stock solutions were made by dissolving dried oligos in Tris-EDTA buffer to a final concentration of 200 pmol per µl and kept at -80°C. Working solutions at a concentration of 1 or 20 pmol per µl was made and stored at -20°C (Sambrook et al., 1989).

Subcloning and Amplification of the catIJ Genes

In order to subclone the 1.4 kbp catIJ gene fragment, the purified plasmid pPAN4 was cleaved by the restriction enzyme EcoRI (Figure 4). The fragment was extracted by agarose gel electroelution and phenol purification. Linearized DNA was used for PCR amplification since it is a more efficient target sequence than the circular form.

DNA amplification by the polymerase chain reaction (PCR) was used to introduce BamHI and EcoRI restriction sites outside of the catIJ sequence and amplify this target DNA (Figure 6). PCR is a method by which a specific DNA sequence can be amplified enzymatically in vitro using specific primers to prime DNA synthesis of the template DNA. In this
Figure 6. Schematic diagram of the subcloning of catIJ genes and generation of plasmid pCATE19. A pair of oligonucleotide primers (Table III) were used in PCR to add the BamHI and EcoRI linkers and to prime the syntheses of the catIJ fragment. The primer 'a' annealed to residues 1741-1759 and the primer 'b' hybridized to residues 3122-3140 in the sequence of the original 5.2 kbp EcoRI-EcoRI catBCIJFD fragment. The PCR product is a BamHI-EcoRI catIJ fragment with a length of 1.4 kbp that carries the catIJ genes exclusive of other cat genes. This fragment and the vector pUC19 were digested with BamHI and EcoRI and then ligated. The new plasmid pCATE19 carrying subcloned A. calcoaceticus catIJ gene fragment is shown.
5.2 kbp catBCIJFD

pUC13

EcoRI

pUC13

EcoRI cut

5.2 kbp catBCIJFD

EcoRI cut

BamHI linker

PCR amplified BamHI -EcoRI catIJ from primers 'a' and 'b'

BamHI 1.4 kbp catIJ EcoRI

EcoRI cut

BamHI cut

BamHI 1.4 kbp catIJ EcoRI

ligation

1.4 kbp catIJ

BamHI - EcoRI

pCATE19
case, two oligonucleotide primers were designed that were complementary to two small stretches of known unique sequences that flank the target DNA (one primer for each side). The primers anneal at either end of the target sequence and are oriented in opposite directions. Exponential amplification of the target sequence from primers occurs over multiple rounds of denaturation, annealing and 5' to 3' extension by a heat stable DNA polymerase (Saiki et al., 1988).

The plasmid pCATE19 was constructed by subcloning the BamHI-EcoRI catIJ gene fragment into the plasmid pUC19 from previously cloned catBCIJFD genes on the plasmid pPAN4. The catIJ genes were expressed from the lac promoter on the plasmid pUC19.

In the PCR reaction, two cloning primers (Table III), 'a' carrying a BamHI site at its 5' end and 'b' with an EcoRI site at its 5' end were hybridized to complementary regions flanking the catIJ gene sequence. The 5.2 kbp EcoRI-EcoRI catBCIJFD fragment was used as template. DNA fragments of 1.4 kbp with the BamHI-EcoRI site flanking the catIJ genes were synthesized and amplified by 3' extension from the two primers. Vent DNA polymerase, isolated from an extremely thermophilic marine archaeabacterium (New England BioLabs), was used since it is reported to have much higher fidelity of base incorporation than other thermostable DNA polymerases.
Table III. List of oligonucleotide primers.

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Sequence</th>
<th>Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>on catBCEFD:</td>
</tr>
<tr>
<td>a</td>
<td>5'E-BamHI</td>
<td>5'TAAGGATCCCTTTCCGTATGCCTAAAA3'</td>
<td>1741-1759 nt</td>
</tr>
<tr>
<td>b</td>
<td>3'E-EcoRI</td>
<td>5'TITGAATTCTGTTTCATATCCGGTTATT3'</td>
<td>3122-3140 nt</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>on catBCEFD:</td>
</tr>
<tr>
<td>c</td>
<td>5'EQ155</td>
<td>5'AAAAAAGGTCAGCGAAGATT3'</td>
<td>2920-2940 nt</td>
</tr>
<tr>
<td>d</td>
<td>3'EQ155</td>
<td>5'AAACCTTGCTGGTACCTTTTT3'</td>
<td>2920-2940 nt</td>
</tr>
<tr>
<td>e</td>
<td>5'EQ193</td>
<td>5'AAACTGATCACCGAAGCTCGAAGGC3'</td>
<td>3034-3056 nt</td>
</tr>
<tr>
<td>f</td>
<td>3'EQ193</td>
<td>5'GCTTCCGACTTTCTGATCACCCTT3'</td>
<td>3034-3056 nt</td>
</tr>
</tbody>
</table>

Primers in the list were used in the PCR to prime the synthesis and amplify the DNA sequences that were then used for subcloning and site-specific mutagenesis. The restriction enzyme recognition sequences and cut sites added in the sequence are double underlined. The base pairs substituted specifically are in bold, and therefore the corresponding codons changed are underlined.

The reaction was performed by mixing template DNA (1 ng), two primers (20 pmol/μl each), four dNTPs (10 mM each dNTP), Vent DNA polymerase (1 unit), and buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCL, pH 8.8, 2 mM MgSO₄, and 0.1 per cent Triton X-100) with BSA (100 ng/μl) in a final volume of 50 μl. Once formulated, the mixture was amplified through
28 automated cycles of temperature and time control that provided for denaturation (1 minute at 94°C); annealing (2 minutes at 58°C), and polymerization (3 minutes at 72°C) on a Techne thermal cycling module PHC-2.

The amplified products of the PCR reaction were separated on an 1.5 per cent (w/v) agarose gel. Fragments of the appropriate size were cut out and recovered by gel electroelution in Spectrapore membrane dialysis tubing. The 1.4 kbp catIJ fragment was then cut with enzymes BamHI and EcoRI, yielding a 1406 bp BamHI-EcoRI catIJ gene fragment. Later, these fragments were ligated into the plasmid pUC19 by T4 DNA ligase. Competent cells of E. coli strain DH5α were transformed by new recombinant plasmid DNA which was designated as pCATE19.

Screening and DNA Sequencing

The transformed E. coli cells were directly screened on selective LB plates containing ampicillin, and X-gal and IPTG. E. coli DH5α harboring cloned plasmid pCATE19 should be able to grow as white colonies by resistance to ampicillin and insertional inactivation of the α-peptide of β-galactosidase (Figure 7). Selected strains were further purified by streaking on the above plates. The structure of the recombinant plasmid pCATE19 was verified by the restriction analysis of plasmid mini-preparations. Enzymes BamHI and EcoRI were used again to digest pCATE19, and the
Figure 7. Circular restriction map of the plasmid pUC19. The lacZ α-peptide gene including the multiple cloning site (poly-linker site) is at right and the ampicillin resistance gene encoding a β-lactamase is at left. Restriction endonucleases that cleave the DNA once or twice are shown.
appropriate size insert (1.4 kbp) was shown after gel electrophoresis. The pCATE19 was also digested with enzyme HindIII to identify if it had the correct (and predicted) orientation of insertion.

Double-stranded DNA sequencing (Hattori and Sakaki, 1986) was used to sequence each of the BamHI-EcoRI catIJ gene fragments in order to determine there were not any other DNA sequence alterations introduced by the PCR amplification, except the substitutions expected (Higuchi, 1989). At the same time, DNA sequence analysis of the entire 1.4 kbp subclone confirmed that the BamHI and EcoRI sites had been added at the ends of the fragment and that the orientation of the insertion into the plasmid pUC19 was correct.

The plasmid pCATE19 was purified by the rapid alkaline lysis technique for DNA sequencing (Sambrook et al., 1989). Cells from 10 ml overnight cultures were lysed by lysozyme solution (0.4 per cent lysozyme in 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) at room temperature for 10 minutes, and then by 0.2 N NaOH and 1 per cent SDS for 5 minutes on ice. After adding and gently mixing with potassium acetate, the sample was centrifuged. The plasmids in the supernatant were extracted with phenol and precipitated with ethanol. The dried pellets were incubated in 20 µg RNase per ml in TE buffer to remove RNA. The plasmid DNA was denatured by alkaline treatment at 37°C. The single-stranded template was annealed to a sequencing primer
that was complementary and that flanked the region sequenced in each sequencing reaction.

Dideoxy-mediated chain-termination DNA sequencing was performed (Sanger et al., 1977). This method involves synthesizing and labeling a DNA strand from a single-stranded DNA template by bacteriophage T7 DNA polymerase in vitro. Sequencing reactions were performed according to the instructions included in Sequenase version 2.0 kit. The first synthesis step was a labeling reaction and primer extension using limited concentrations of the deoxynucleotide triphosphates (1.5 μM each of dGTP, dCTP and dTTP). The labeling mixture included radioactive [α-^35S] dATP (5 μCi) which was randomly incorporated. After priming, increased concentrations of the dNTPs (80 μM each) and one of the four dideoxy-nucleotide triphosphates (ddNTP, 8 μM each) were added for each of the four dideoxy-nucleotide termination reactions. Processive polymerizations were terminated by a ddNTP in each of four separate reactions to give complete sequence information.

Sequencing products were denatured by heating and separated by high-resolution electrophoresis on denaturing polyacrylamide buffer gradient gels. Autoradiography was done by exposing a dry gel in direct contact with Kodak XAR X-ray film for 48 h. Films were developed and sequencing results were recorded.
Site Directed Mutagenesis of the catIJ Genes

To modify the amino acid residues, Glu155 and Glu193 to Gln in the protein β subunit of β-ketoadipate succinyl-CoA transferase, the corresponding codons GAG155 and GAG193 were changed to CAG in the cloned catIJ gene sequence. These single base pair alterations were constructed by site directed mutagenesis using mismatch primers and the overlap extension technique of a two step PCR reaction (Figure 8; Ho et al., 1989; Higuchi, 1989; Jones and Howard, 1990). Two cloning primers (or 'outside' primers) 'a' and 'b' were used as above in all reactions to amplify the interest sequence. Another two pair of mutagenic primers (or overlapping primers or 'inside' primers, Table III) were used to introduce mismatched bases in the primary PCR reaction. Initially, a specific base substitution was introduced during amplification where there was a mismatch between one mutagenic primer and the target DNA. Then, two fragments with overlapping ends containing complementary mismatched bases were generated and amplified from the target gene sequence in two separate reaction tubes. Each reaction used one outside primer (or cloning primer, 'a' or 'b' in Figure 8) that hybridized at one end of the target sequence, and one inside primer (or mutagenic primer in Figure 8) that hybridized at the site for the point mutation and contains the mismatched bases. Overlapping primers annealed at the
Figure 8. Schematic diagram of site-directed mutagenesis by overlap extension. The double stranded DNA fragments and synthetic primers are represented by lines with arrows indicating the 5' to 3' orientation. A pair of mutagenic (or inside) primers annealed to the target DNA at the same segment or sequence of bases, but to the opposite strands. A single base changed (indicated by the small rectangle) in each primer is mismatched to the target sequence. The mismatches lead to the expected complementary sequence alteration in the two primary PCR products, PCR1 and PCR2. PCR1 and PCR2 fragments were purified, mixed together, denatured, and reannealed. Some fraction of the molecules annealed as shown through the overlap at their 3' ends. this short overlap of the inside primer sites allowed for extension by DNA polymerase (dotted line) to form the fusion product. All other combinations were not substrates for DNA polymerase since they had only free 5' ends annealed to template, or they were simply blunt-ended reannealed strands. By adding back the original outside primers 'a' and 'b', the mutant fusion products were further amplified by PCR.
**Step 1**

primer a

(PCR1)

site-specific mutation

target DNA

a pair of mutagenic primers

(PCR2)

PCR1 with primer mutagenesis

primer b

PCR2 with primer mutagenesis

**Step 2**

mix, remove primers, denature and renature

not a template

template

3' extension from 3' overlap ends

PCR with outside primers

mutant fusion product
same segment of the complementary, but separated strands of the catIJ gene and primed the generation of two primary DNA fragments, PCR1 and PCR2 that had overlapping ends at their 3' ends, but that were from different sections of the same template. Since the products were generated and extended from the mutagenic primers, the wild type sequence would not be amplified. In the second reaction, taking advantage of two 3' ends on two primary product that were the same as the inside primers and that overlapped, the two DNA fragments PCR1 and PCR2, were fused after combining, denaturing and annealing them. Thus, 3' extension from the overlapping ends allowed one strand synthesized from each fragment as template to produce entire molecules of the catIJ gene fragment. The other set of complementary strands that annealed are not templates for DNA synthesis since they have no free 3' hydroxyl to prime DNA synthesis. Resulting fusion products with the specific mutations were amplified further from two outside primers. Actual conditions of time and temperature cycling of the PCR were the same as described above for construction of the original clone. The mutations introduced by PCR resulted in the substitution of codons GAG155 to CAG155 and GAG193 to CAG193.

The mutated, amplified catIJ fragments were identified by size-fractionation on agarose gel, and purified by gel electroelution to remove unwanted PCR products, wild type template, and primers. The catIJ DNA fragments with
different mutations were then digested with enzymes BamHI and EcoRI and ligated into appropriately cut plasmid pUC19. Two new recombinant plasmids harboring mutations in the catIJ genes, pEQ155 and pEQ193, were transferred into competent E. coli DH5α. DNA sequencing was again performed to confirm the genetic identity of the plasmid constructs.

Measurement of Enzyme Activity

Five cell-free extracts, isolated from E. coli DH5α cells carrying plasmid pUC19, pPAN4, pCATE19, and the mutant catIJ plasmids pEQ155 and pEQ193 were assayed to determine the activities of enzymes encoded by altered catIJ genes. 500 ml bacterial cultures were grown to exponential phase (A660 of -0.4 - 0.6) in L-broth containing 100 μg ampicillin per ml and 0.1 mM inducer IPTG at 37°C, and then harvested by centrifugation. The pellets were washed and resuspended with 5 ml ice-cold enzyme extraction buffer containing 100 mM Tris-HCl, 1 mM EDTA, and 1 mM MgCl₂, pH 8.0. Cells were broken by ultrasonic oscillation using a Braun Ultrasonicator Model 2000. Cell debris was removed by centrifugation at 10,000 xg for 10 minutes.

Enzymes in cell free extracts were kept on ice when measured, and stored at -20°C. Activity of β-ketoacidic succinyl-CoA transferase II in cell-free extracts was measured spectrophotometrically at 305 nm in a quartz cuvette containing 1 ml of a mixture of 10 mM β-ketoacidic, 400 μM
succinyl-CoA, and 40 mM magnesium ion in 200 mM Tris-HCl buffer, pH 8.0, at 25°C. Succinyl-CoA was prepared fresh by adding 20 μl of 1 M succinic anhydride into 1 ml containing 4 mg coenzyme A in 100 mM sodium bicarbonate. The increase in absorbancy at A305 on a Beckman Du-40 spectrophotometer due to the increased absorptivity of the β-ketoadipyl-CoA-Mg$^{2+}$ product complex per unit time served as the measure of β-ketoadipate succinyl-CoA transferase activity (Yeh and Ornston, 1981). The Km of the enzymatic reactions were measured using IBM computer program Soft-Pac Module [KINETICS]. Protein concentrations were determined by the Lowry method using crystalline bovine serum albumin as a standard (Lowry et al., 1951). One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of substrate to product in 1 minute under the conditions of the assay (one unit equals 1 μmol minute$^{-1}$ mg protein$^{-1}$).
CHAPTER III

RESULTS AND DISCUSSION

Generation of Plasmid pCATE19

The first task performed was the subcloning of the catIJ genes from plasmid pPAN4 into the vector pUC19. The parent plasmids were purified in bulk after transformation and construction of plasmid harboring strains. The 2.7 kbp pUC19 vector and the 7.9 kbp pPAN4 carrying the 5.2 kbp EcoRI-EcoRI catBCIJFD gene fragment from the A. calcoaceticus strain ADP1 chromosome, were transformed into competent cells of E. coli strain DH5α. Strain DH5α (pUC19) grew with a ß-galactosidase-positive phenotype, and strain DH5α (pPAN4) grew as ß-galactosidase-negative cells on selective LB plates containing ampicillin and X-gal with IPTG. Restriction analysis of plasmid mini-preps showed that the transformants obtained the appropriate size plasmids. Large scale preparations of the plasmids were amplified and purified from 1 L cultures. The restriction enzymes BamHI and EcoRI were used to cut the plasmid pUC19 to get linearized plasmid pUC19 vector. EcoRI alone was used to cut the plasmid pPAN4 to obtain the catBCIJFD DNA fragment for subcloning of the catIJ genes. The 5.2 kbp fragment was used as the template for the
subsequent amplification and cloning of a sized down catIJ gene fragment.

The polymerase chain reaction was used to introduce BamHI and EcoRI sites at the margins and to amplify the catIJ gene sequence from the original 5.2 kbp EcoRI-EcoRI catBCIJPD fragment. In the PCR reaction, synthetic oligonucleotide primers introduced a linker with a 5' BamHI restriction site on one end and an EcoRI site linker onto the 3' end of the catIJ genes. A 1351 bp catIJ gene fragment with the 38 bp linker carrying a 5'BamHI site and the 29 bp linker carrying a 3'EcoRI site was generated and amplified. The amplified PCR product was visualized by agarose gel electrophoresis, purified from the gel matrix, and then digested with BamHI and EcoRI. Each amplified fragment had on its end a string of three T or A residues (Table III) that left the actual restriction endonuclease cleavage site 3 bases inside the end of the DNA fragment. This was done to increase the efficiency of the binding and cutting by the restriction enzyme since sites located immediately at the end of DNA fragments are poorly cleaved. A BamHI site was introduced on the 5' end (relative to the direction of transcription) and an EcoRI site was introduced onto the 3' end. The result was a fragment with asymmetric ends so that forced cloning could be performed with this fragment. The actual fragment was designed so that the two sites (BamHI and EcoRI) are in the proper direction of transcription relative to the lac
promoter-operator in the chosen expression vector, pUC19 (Figure 7). In addition, the asymmetric ends allowed for rapid and facile identification of the fragment in restriction endonuclease analysis. The specifically designed version of the 1.4 kbp BamHI-EcoRI catIJ fragment was then inserted into correspondingly cut plasmid pUC19, generating a 4.1 kbp plasmid designated pCATE19. Competent E. coli DH5α was transformed with the recombinant plasmid pCATE19 carrying the subcloned catIJ gene fragment. The 1.4 kbp insert was completely sequenced on both strands to confirm its identity with the original target sequence (Figure 9).

Screening and DNA Sequencing of Plasmid pCATE19

The plasmid vector pUC19 contains the α-peptide DNA sequence of the lacZ gene and an ampicillin resistance gene. The α-peptide expressed from pUC19 is capable of complementing a lacZ α-deletion in the host E. coli DH5α (AlacZAM15) to give production of a functional β-galactosidase. The α-peptide DNA sequence has been modified further by insertion of a multiple cloning site (poly-linker) that does not disrupt the translational reading frame of the peptide (Figure 7). This vector-host system allows direct screening for transformed DH5α carrying plasmid pCATE19 by plating onto LB medium containing ampicillin as well as X-gal and IPTG. Cloning a gene fragment into the multiple cloning site normally disrupts the α-peptide sequence thus resulting
Figure 9. Nucleotide sequence of the 1412 bp BamHI-EcoRI fragment carrying catI\textsuperscript{J} open reading frame (ORF) and flanking regions. A 5' BamHI linker and a 3' EcoRI linker were added by PCR with synthetic oligonucleotide primers to subclone out a new version of the catI\textsuperscript{J} gene with only 1351 bases from end to end of the coding sequence. As indicated in the figure, the catI ORF and catJ ORF are linked together by 10 bp. The catI gene encodes the 228 amino acid α subunit and catJ encodes 217 amino acid long β subunit. Each gene possesses a UAA stop signal as well. Two codons selected for site-specific alterations in the catJ DNA sequence (and hence the amino acid sequence in β subunit) made in this study are in bold type. The regions hybridized by the mutagenic primers and the cloning primers in PCR amplifications are underlined.
cat\(\text{i}\)  ATC ATA CAT AAA ACT GCA GCC ACC CTA ACC GAA GCG TCT TCC CAG ACG GAC GCT GCC
\(\alpha\)-SUBUNIT Met Ile Asp Lys Ser Ala Thr Leu Thr Glu Ala Leu Ser Gin His Asp Gly Ala
10 20
ACC ATC CTC ATT GCT GGT TTT GCA AGC GCC AGC GCC ACC GCC GAG CTC ATT GCC GCA
Thr Ile Leu Ile Gly Gly Gly Thr Ala Gly Gin Pro Ala Glu Leu Ile Asp Gly Leu
30 40
ACC ACC GCA GGT GGT TTT GGA ACA GCC GGC CAA CCC GCC GAG CTC ATT GAC GGA CTC
Ile Glu Leu Gly Arg Lys Asp Leu Thr Ile Val Ser Asn Ala Gly Asp Asp
50 60
GCA GGG GCC AGG CCG CTA AAA ACT GCC GCA TTT AAA AAG ATC AAC TTC TCC GCA GCC
Gly Ala Asp Ser Tyr Val Asp Glu Leu Tyr Ala Ala Gly Met Gln
80
CAC GCC GAC TCC CTA ATT GAC CAA CTA CAC GCC GCC CAA GCC GCC TCT GCC GCC
Glu Ala Asp Ser Tyr Val Asp Tyr Ala Ala Thr Ala Gin Ala Gin
100
GAA GCC GAC ATT CTC GCG TCT ATT GCA ACC GCC ATC ATT GCC ACC GCC GGA CTC
Glu Ala Asp Ser Tyr Val Asp Tyr Ala Ala Thr Ala Gin Ala Gin
110 120
ACC ACC GCA ACC GCT ATT TCT GCA CTA GCA GAG CTC ATT GCC CAG GCC GCC CAA
Thr Ala Asp Ser Tyr Ala Ala Thr Ala Gin Ala Gin Ala Gin
130 140
GCA GGG GCC GAC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
Ile Glu Leu Gly Asp Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala
150 160
GCA GCC GCC GGC GAA GAA GAT CCG GAA CTC ATC AAC GCA GGC AAA GAA TAC GTA
Ala Ala Gly Glu Glu Asp Pro Glu Glu Leu Met Asn Ala Gly Lys Glu Glu
170 180
ACC ACC GCA GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
Thr Ala Asp Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala
190 200
AAC GCC GCC CAC GCC ACG TAT CCA GCC ACG GGT CAG AAA TCT GTC GAC CGG ATC TAC
Asn Ala Asp Ala Ala Thr Pro Ala Thr Gly Gin Lys Cys Val Asp Arg He  Tyr Thr
210 220
ATC ATC GAT GTC GTC CCA GAA GGA CTC AAA GTC ATC
Asp He  Asp Val Val Pro Glu Gly Leu Lys Thr Leu
220

\text{cat\(\text{j}\)  ATC ACT TAT CAC AAA CTC ACC GCT TAT CAG ATC GCC CAG GCG CTT GCC CAA GAC ATT GCC
\(\beta\)-SUBUNIT Met Ser Tyr His Lys Val Thr Arg Asp Gin Ile Gin Arg Val Ala Gin Asp Ile Pro
10 20
GCA GCC TCC TAT ATT CAG ATT GCC ACC GCC ACC ACC ATT GCC ATT GCC TCT TCC
Glu Gly Ser Tyr Val Thr Thr Gin Thr Gin Thr Gin Thr Gin Thr Gin Thr Gin Thr Gin
30 40
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520
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Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala
540
in β-galactosidase-negative phenotype and a loss of β-galactosidase activity. This is observed as an inability to metabolize the chromogenic substrate X-gal and form blue colonies. Such colonies arising on the ampicillin LB plates containing X-gal are typically white due to the insertional inactivation of the α-peptide complementing activity at the multiple cloning site. However, cells carrying new plasmid pCATE19 were still β-galactosidase-positive when plated on such selective media.

The usual predilection in a β-galactosidase selection is to plate out cells transformed with the ligation mixture and pick white colonies as potential transformants. However in the case of pCATE19 ligations prepared as above, no transformants were observed that had the predicted phenotype. All transformants had the blue colony appearance indicative of a cell with no insertion at the multiple cloning site. At least no colonies arising were indicative of cells forming a functional α-peptide and effectively complementing the chromosomal LacZ deletion. Ligation and plasmid controls however gave the predicted results. Plasmid only controls gave no transformants, since the vector pUC19 was cut with two enzymes that yielded incompatible ends. These doubly digested plasmids could not reanneal and ligate to give a functional plasmid. Indeed, the BamHI and EcoRI cut pUC19 vector was missing DNA that, after ligation if possible, would result in inactivation of β-galactosidase and would not
again give blue colonies. The most obvious explanation was that some plasmid had not been cut and had carried through all of the experimental steps performed to give transformants that carried the parental pUC19 plasmid. But the plasmids isolated from these transformants were all large molecular weight plasmids with a size indicative of the sum of the sizes of pUC19 and the 1.4 kbp amplified DNA catIJ gene fragment (4.1 kbp, or 2.7 kbp + 1.4 kbp). In further plasmid analysis and confirmation, plasmids were isolated from transformants and digested by the enzymes BamHI and EcoRI. Two bands of appropriate sizes relative to the insert (1.4 kbp catIJ) and vector (2.7 kbp pUC19) were evident on gels upon staining and visualization. Also, the plasmid pCATE19 was digested separately by another enzyme HindIII, and the sizes of insert and vector on gels showed that the insert was oriented in the same direction of transcription as the lac promoter on pUC19 because only one HindIII site was located on one side (near the EcoRI site) of the catIJ genes (Figure 4).

DNA sequencing finally explained the above results where insertional inactivation of the ß-galactosidase gene was not observed. The entire BamHI-EcoRI catIJ fragment with its flanking sequences was sequenced. The sequencing results confirmed that subcloned catIJ gene sequence produced by PCR was correct; BamHI and EcoRI linkers were added as expected; and the orientation of the insertion was consistent with the
lac promoter in pUC19. In addition, no single base substitutions were made in the catIJ gene sequence that would affect interpretation of later results. Possible introduction of PCR artifacts into the sequence was feared because incorrect nucleotides might be incorporated by the thermostable DNA polymerases.

DNA sequence analysis showed that there was a ribosomal binding site (RBS) followed by a start codon ATG (AUG) within a short sequence located between the stop codon of the catJ coding sequence and the 3' EcoRI site (Figure 10). From this start codon, a new amino acid open reading frame (ORF) could be transcribed, and the translation of β-galactosidase could proceed from a message produced from the upstream lac promoter-operator. This ORF included a few bp on the 3' end of the subcloned fragment and then continued into the α-peptide DNA sequence in pUC19. Therefore, the lacZ gene was still induced by IPTG to express functional β-galactosidase in the DH5α host, even though there had been an insertion of the cloned catIJ fragment into pUC19. This in-frame gene fusion of the α-peptide and the amino terminus of the catF thiolase endowed the DH5α transformants (pCATE19) with a β-galactosidase-positive phenotype. Obviously the intergenic region between catJ and catF contained no transcriptional stop signals and this confirms previous experimental observations that the catIJ transferase and the catF thiolase are coordinately expressed.
Figure 10. Insertion of the catIJ genes into the poly-cloning site of plasmid pUC19. The upper sequence shows the pUC19 poly-cloning site without modification or insertion. The multiple cloning site part of the sequence that includes the lacZ gene (α-peptide) open reading frame (ORF) is shown and the translated amino acid sequence is numbered from the amino terminus. The lower sequence shows that the catIJ fragment made by PCR was inserted into the position between BamHI and EcoRI in the poly-cloning site of pUC19. Comparing numbers in two sequence, cloning of catIJ did interrupt the α-peptide gene initiated from the normal lacZ start codon (first bold ATG in the sequence). However, the ribosomal binding site (RBS) and the start codon (third bold ATG) of the downstream catF gene were included at the end of this cloned fragment. This ATG start with a few bp of catF gene (encoding Lys and Gln residues) and the subsequent majority of α-peptide sequence constructed a new ORF which resulted in expression of β-galactosidase activity.
The pUC19 polycloning site included in lacZ α-peptide gene:

1 2 3 4 1 2 3 4 5 6 7 8
Thr Met Ile Thr Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu Asp Pro Arg Val Pro Ser Ser Asn Ser Leu Ala
ATG ACC ATT ACC GCA ACC TGG CAT GCC TGG AGG TCG ACT CTA CAC GAT CCC CGG GTA CCG AGC TCG AAT TCA CTG GCC

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

MboII SphI PstI SalI XbaI BamHI SmaI KpnI SacI EcoRI

HindIII

Insertion of catIJ genes into the pUC19 polycloning site without lacZ gene inactivation:

**lac Promoter---------------------------**

-35

TTTACAAGCTTTTGCCGCTCGTATGGTGTACCTGAGGATGGACCGGATTACCAATTCACAGGGAACAGCTAGCATTACGCCAGCTGCACTGCGG

HindIII

9 10 11 12

ACTCTAGAGGATCCGCTTTCGTTTTTCATGCCAAAAAGCTGATGAC

**Stop(catIJ)**

**lacZ(α)** EcoRI 5 6 7 8

GTC GCT TTA GAA CCT CGT GAC TGG GAA ATC CCT GCT ACG CAA CTT AAT CGG GCT GCA GCA CAT CCC CCT TCC GCC AGC TGG CTC

Val Val Leu Glu Arg Arg Asp Trp Glu Asp Pro Gly Val Thr Gin Leu Asn Arg Leu Ala His Pro Pro Phe Ala Ser Trp Arg

**NarI**

AAT ACC GAA GAG GCC ACC GAT CGG CCT TCC CAA CAG TGG CGC AGG ATG GCA TGG CGC TCT AGC TCG TAT TTT CCT CCT

Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro Ser Gin Gin Leu Arg Ser Leu Asn Gly Glu Trp Arg Leu Met Arg Tyr Phe Leu Leu

**Stop(lacZ)**

ACG CAT CTC TCG GCT ATT TCA CAC GCG ATA TGG TGG ACT CTG AGT ATA ATC TGG TCT GAT GCC GCA TAG

Thr His Leu Cys, Gly Ile Ser His Arg Ile Trp Cys Thr Leu Ser Thr Ile Cys Ser Asp Ala Ala
DNA sequencing of the subclone showed that the DNA sequence of the BamHI-EcoRI catIJ PCR fragment was completely identical (over the whole 1412 base sequence) with the DNA sequence of same region in pPAN4. Sequences were confirmed with greater certainty by sequencing both strands to minimize errors in experimentation and interpretation. The sequence identity between the sized-down clone and the parent indicated that there was not a single base pair substitution introduced by the PCR reaction relative to the parent template. In this study, Vent DNA polymerase was used. Vent DNA polymerase has much higher fidelity of base incorporation during PCR than other thermostable DNA polymerases such as the Taq DNA polymerase. Vent DNA polymerase contains 5' to 3' polymerase activity and a 3' to 5' proofreading function as well. Reports of previous studies indicated that Taq DNA polymerase introduced many more mutations than did the Vent polymerase in PCR DNA amplifications. Taq DNA polymerase has no 3' to 5' proofreading function and can not remove misincorporated bases that even occur at very low frequency.

Construction of catIJ Mutant Strains

Among the CoA transferases known, the only known primary sequence was that of the catIJ encoded 3-ketoadipate succinyl-coenzyme A transferase II from A. calcoaceticus. All known CoA transferases have a reactive α-glutamyl side chain involved in catalysis. This glutamate residue
participates in catalysis by accepting the CoA moiety from the CoA donor and forming a covalent coenzyme A-enzyme intermediate. Identification of the actual glutamate residue involved would help to begin to define the geometry and topology of the active site of this class of enzymes. The active site glutamate residue was known to be in the β subunit. In addition, different CoA transferases have different CoA donor and acceptor specificities (Table I). Though limited structural deviation is allowed in the choice of CoA acceptors, there is a high degree of stringency and specificity of binding for the CoA donor. The structural basis of this CoA donor specificity can be understood by defining the residues that participate in binding and catalysis. Since most CoA donors are electronegative and since there is a particularly reactive lysine residue at the active site of some CoA transferases, glutamate residues adjacent to lysine residues were identified. It was assumed that the lysine residues might participate in the formation of the active site pocket. Two such residues in the primary sequence, Glu155 and Glu193 were chosen. Both have the neighboring basic lysine residues (Figure 5). These were chosen as likely candidates to test by site directed mutagenesis.

Two mutant genes encoding specifically modified enzymes were created using defined mutagenic oligonucleotide primers and PCR to introduce mismatches into the subcloned catIJ gene
fragments. As described in Chapter II, two missense transversion mutations in the nucleotide sequence, the codon GAG155 to CAG155 and the codon GAG193 to CAG193, were introduced separately. Two mutant clones were isolated that had amino acid substitutions of Glu155 to Gln155, and alternately Glu193 to Gln193 in β subunit of the enzyme. During PCR mutagenesis and amplification, primers for the BamHI 5' end and the EcoRI 3' end of the catIJ gene fragment were used each time so that the mutant catIJ fragments had a defined size and the appropriate cutting sites. The new fragments produced during the PCR amplification had the predicted length and they carried those specific ends identical to original subcloned catIJ fragments. Each mutant catIJ gene fragment also had the desired sequence changes as well. Amplified, mutated DNA was purified, cut with the restriction enzymes BamHI and EcoRI, and then inserted into correspondingly cut poly-cloning site of the plasmid vector pUC19. The new recombinant plasmids with the single missense base mutations were designated as pEQ155 and pEQ193 respectively. The mutant strains E. coli DH5α (pEQ155) and DH5α (pEQ193) were generated by transforming competent E. coli DH5α with the new recombinant plasmids.

The mutant strains grew as β-galactosidase-positive like original subclones on X-gal plates. As previously described, a fusion of the lacZ gene (encoding the α-complementing peptide) and the downstream catF gene (encoding the β-
ketoadipyl CoA thiolase) was formed. This gave an in-frame fusion peptide that used the ribosome binding site and AUG translational start of the catF gene, and that produced a functional α-peptide so that active β-galactosidase was produced. Restriction analysis showed that the plasmids from strains transformed with the mutant plasmids pEQ155 and pEQ193 all contained 1.4 kbp inserts in the same transcriptional orientation as the lac promoter of the parent vector pUC19. Double stranded DNA sequencing analysis was performed again on each of the mutant plasmids. All mutant clones that were sequenced contained the desired mutations introduced by the paired mutagenic primers in PCR. These nucleotide changes would result in the corresponding predicted amino acid alteration of a glutamate residue to a glutamine. Except for the desired changes in nucleotide sequence, results of DNA sequencing verified that no other nucleotide sequence changes were introduced during the PCR amplification. It was necessary to confirm that no other alterations were introduced since the effect of a simple, single amino acid substitution could not be interpreted if multiple substitutions were introduced. The influence of individual amino acid substitutions requires that only a single substitution be made. The sequencing was performed over the entire length of the cloned mutant gene to confirm sequence identity, as well as the presence of the desired mutation. Figure 11 shows the DNA sequencing gel from the
Figure 11. Sequence analysis of mutant pEQ155 generated by PCR and the overlap extension technique. The mutated position (codon CAG155 for amino acid Gln155) in the nucleotide sequence is denoted in bold and a line.
pCATE19     pEQ155

5'   3'     5'   3'
G   C       G   C
T   A       T   A
C   G       C   G
G   T       G   T
A   T       A   T
A   T       A   T
T   T       T   T
G   C       G   C
G   T       G   T
G   C       G   C
C   G       C   G
T   A       T   A
G   C       G   C
A   T       A   T
C   T       C   T
G   A       G   A
T   A       T   A
C   A       C   A
T   C       T   C
G   T       G   T
C   G       C   G
T   A       T   A
A   T       A   T
C   T       C   T
G   A       G   A
plasmid pEQ155 and its interpretation about the area in which the site-specific mutation was introduced. In the mutant plasmid pEQ155 a transversion mutation that substituted a GC pair with a CG base pair resulted in the modification of amino acid residue Glu155 to Gln155. This mutation is in the catJ gene encoding the B-subunit of the CoA transferase. Similarly, Figure 12 shows the DNA sequence from the plasmid pEQ193 that carries a corresponding transversion mutation that resulted in a change of a GAG glutamate codon to a CAG codon for glutamine. The change in the DNA sequence of the catJ gene on plasmid pEQ193 thus encoded an altered B-subunit of the transferase.

Enzyme Activities of Various Clones of the catIJ Genes

The enzymes in cell-free extracts from four strains (DH5α with pPAN4, pCATE19, pEQ155, and pEQ193) were assayed as described in Chapter II to observe alterations in enzyme activity. Cells were cultured in L-broth containing 100 μg ampicillin per ml and 0.1 mM IPTG to induce the synthesis of the B-ketoacyl-CoA synthetase II in E. coli. The inducer IPTG was added to induce the enzyme since it was transcribed from the endogenous lac promoter-operator on the plasmid vector pUC19. The catIJ 1.4 kbp fragment was cloned away from its own promoter and therefore required downstream insertion into an exogenous promoter to affect expression. In every case, the absorbance of product (B-ketoacyl-CoA)
Figure 12. Sequence analysis of mutant pEQ193 generated by PCR and the overlap extension technique. The mutated position (codon CAG193 for amino acid Gln193) in the nucleotide sequence is denoted in bold and a line.
increased in a linear fashion with respect to time. Linear rate data was collected to determine the reaction rate. The enzyme specific activity of β-ketoadipate succinyl-CoA transferase II encoded by the different clones of catIJ genes is presented in Table IV. Strain DH5α (pCATE19) carrying only the catIJ genes expressed approximately three times higher enzyme activity than the parent strain DH5α (pPAN4) carrying the catBCIJFD genes. Thus, removal from the original 5.2 kbp catBCIJFD gene fragment of a total 3.8 kbp of DNA lying on either side the catIJ open reading frame resulted in higher CoA transferase expression. This higher level of expression presents a benefit for purification of the native enzyme, as well as mutant forms of the enzyme, for future studies where the purified CoA transferase is desired. There are two possible explanations for this higher level of expression. First the removal of upstream DNA may result in higher levels of transcription. Abortive transcripts might be decreased, especially weak transcriptional stop signals were removed. Simple polar effects of premature transcriptional termination would be decreased since less DNA and less opportunity for RNA polymerase to terminate is present in the sized-down clone. A second possibility for the modest but significant three-fold increase in expression in the sized-down clone also relates to the removal of DNA. The parent plasmid pUC19 is a multi-copy plasmid. If the amount of DNA relative to that in the large plasmid clone
(pPAN4) is much less, the cell would have a lower metabolic load. The loss of 3.8 kbp of DNA would allow for an increase in the copy number of the plasmid. Then the higher level of gene expression could be due to high copy number and gene dosage effects.

Table IV. Specific activities of β-ketoadipate succinyl-coenzyme A transferases II encoded by different clones of catIJ genes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific Activity</th>
<th>Kinetic Data</th>
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<tr>
<td>DH5α (pUC19)</td>
<td>0</td>
<td>Km</td>
</tr>
<tr>
<td>DH5α (pPAN4)</td>
<td>57</td>
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</tr>
<tr>
<td>DH5α (pCATE19)</td>
<td>178</td>
<td>2.074</td>
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<tr>
<td>DH5α (pEQ155)</td>
<td>212</td>
<td>3.820</td>
</tr>
<tr>
<td>DH5α (pEQ193)</td>
<td>195</td>
<td>2.799</td>
</tr>
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</table>

Culture conditions, enzyme assays, and Lowry protein assays were performed as described in the Materials and Methods. The specific activity for each enzyme was determined in cell-free extracts and is given in terms of nanomoles per minute per milligram of protein. The Km is in term of micromolar β-ketoadipate (μM).
The level of expression of CoA transferase II in the sized-down clone is approximately four times that observed in fully induced cultures of A. calcoaceticus. Of course in E. coli, the enzyme expressed from the cloned gene can be purified and made free of the CoA transferase I encoded by the pcalJ genes of the protocatechuate branch of the β-ketoacidipate pathway.

Table IV also shows the results of enzyme assays on the two strains that carry the mutated catJ β-subunit genes in DH5α (pEQ155) and DH5α (pEQ193). Crude extracts from these two strains carrying the catIJ genes with different site-specific mutations still have β-ketoacidipate succinyl-CoA transferase activity. Indeed, the levels were similar to that from the subcloned catIJ genes in DH5α (pCATE19). Obviously, modification of either of the two glutamate residues did not affect catalysis in any significant way. Some other glutamate residue must be the one which is involved in the formation of the covalent enzyme substrate intermediate. The enzymes from the two mutant strains were analyzed by simple substrate velocity measurements to see if any significant alteration in the enzymes' kinetic and catalytic properties could be detected. Enzyme kinetic data were measured by varying the concentration of one substrate, β-ketoacidipate from 10 mM to 1 mM while keeping the other substrate, succinyl-CoA at a constant concentration of 400 mM. The Km of each enzyme from each strain (Table IV) was
determined using Lineweaver-Burk reciprocal plots and compared. The mutant enzymes had slightly higher Km values, indicating that the mutations had the effect of decreasing the enzymes' affinity for substrate. Of course any alteration in a protein's primary structure would result in a change in the higher order structure of the enzyme. Most such changes would be predicted to be deleterious to normal enzyme function since changes in the structure can dramatically affect function. A subtle change of a glutamate to a glutamine would result in a loss of negative charge and this might affect the enzyme. Also, all kinetic measurements were performed in crude cell extracts. Since no dramatic changes of the enzymes by these two substitutions were observed, further characterization of the mutant enzymes was not performed at this time.

DNA and Amino Acid Sequence Analyses

After this work was performed, the sequence of the pcaIJ gene encoding the single β-ketoadipate succinyl-CoA transferase from Pseudomonas putida was published (Parales and Harwood, 1992). In addition the DNA sequence of the atoDA genes encoding the homologous acetoacetate acetyl-CoA transferase from E. coli was sequenced and made available (Chen and Shanley, 1993). This enzyme functions in the dissimilation of short chain fatty acids in E. coli. Acetoacetate, butyrate and valerate are all suitable CoA
acceptor substrates. All three bacterial enzymes share an \( \alpha_2\beta_2 \) subunit structure, as well as similar essential lysine, glutamate and cysteine residues. These amino acid residues that were sensitive to inactivation by protein modifying agents were identified in studies designed to reveal the covalent modifications in the enzymes. A homology alignment of the DNA sequences of the three genes is shown in Figure 13. A great deal of sequence identity for the three genes is evident. The sequences were aligned for maximal homology and occasional gaps in the sequences have been introduced. Most of these gaps are short in-frame deletions and insertions, and maximal homology is obtained when the DNAs are aligned so that the translational frames of the three genes are preserved.

The deduced amino acid sequences of the three CoA transferases are aligned and shown in Figure 14. Inspection of the three primary protein sequences reveals that the glutamate at position 193 (Glu193) is conserved among all three enzymes. The glutamate at position 155 (Glu155) is not present in the \( E. coli \) transferase sequence and this enzyme is known to have a reactive glutamate at the active site that participates in the formation of the covalent enzyme substrate intermediate. Many of the lysines, their presence if not their actual position, are also conserved in the three bacterial CoA transferases. But since these two glutamates are no longer candidates for the active site glutamate, if
Figure 13. Comparison of the DNA sequences of three CoA transferases. The sequences for three homologous CoA transferases are shown. The sequence of the catIJ genes encoding β-ketoacidipate succinyl-CoA transferase II from A. calcoaceticus, the subject of this study is shown on the top line. Immediately below is the sequence for the pcaIJ genes encoding β-ketoacidipate succinyl-CoA transferase from P. putida. The DNA sequence of the atoDA genes from E. coli is shown on the bottom line. The atoDA genes encode a related enzyme in E. coli, acetoacetate acetyl-CoA transferase.
**Figure 14.** Comparison of the amino acid sequences of three CoA transferases. The sequences for three homologous CoA transferases are shown. The sequence of β-ketoacidic succinyl-CoA transferase II from *A. calcoaceticus*, the subject of this study is shown on the top line. Immediately below is the primary protein sequence for β-ketoacidic succinyl-CoA transferase from *P. putida*. The amino acid sequence *E. coli* acetooacetate acetyl-CoA transferase is shown on bottom line. Identity among all three enzymes at any particular residue is denoted with "=".

<table>
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<tr>
<th></th>
<th>A. calcoaceticus</th>
<th>P. putida</th>
<th>E. coli</th>
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<tbody>
<tr>
<td>Residue 1</td>
<td></td>
<td></td>
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<tr>
<td>Residue 2</td>
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<td>Residue 3</td>
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<tr>
<td>Identity:</td>
<td>=</td>
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<td>=</td>
</tr>
</tbody>
</table>


(α-SUBUNIT)

(catI) MKDSTAAAS TILIGSFQTA GQRPAELIDGL IELEGKNTLT
(pcaI) MNSATYESIA SAVGCGTDSQ TIMVGGFGTA GMPSELIDGL IATGARDLT
(atoD) MKTKMNLQG .ATGFFRGDM TIMVGGFGMG GTPSRLVEAL LEGRSDLTL

= = = = = = = = ==
VSNAGNGDY GLAKLLNTGA VRKIIICSFPR QADSYVFDELYRAGKIELLE
ISNAGNQGQ GLAAAMQSG VRKVCSPSFQR QDSYVFDELYRAGKIELE
IANGAFVQIG TLGGLVNAGR VRKVIASHGQ NPETGRQMS GEMDVWV...

= = = = = = = = ==
VPQGLACRI QAAGGGLQPI YTPTGFTGLL AEPKPLNLTE GKYVLENPI
VPQGLAERI AAGGGIGAPI FSPTQGFTGLL AEPKETREID GRMYVLENPL
VPQGTLEQI RCGGAGLQGF LTPQGVGTVV EKQQLNVTD GKTWLLERPL

== = = = = ==
KADFLAKAY KGDRQGNLVY TKASNNFQGI MAMAANVTIA QVSEAVAVGE
HADFLAKAY KGDRQGNLTY KAASNNFQGI MAMAARTIA QVQAVGAVGE
RADLALIRAH RCDQGNLNTY TQLEARNFPL TALADAVLIE EPDELVEGE

= = = = = = = = ==
LDPEWTVPG . . IFVQHWPQ VQSTPASAAP*
LDPEHILTPG . . IFVQVRVA VGSGAASSIA KAI*
LQPDHIVPGG AVIDHIIVSQ ESK*

(β-SUBUNIT)

(catJ) MSYHK.LTRD QIAQVRQQDI PEGLYVLNSL GLPTKIASYL PADKD.....
(pcaJ) MTITCHLQRT EMAQVRADAQ QEGAVYNLSL GAPTLYMNLY .GKE.....
(atoA) MRNYV.LAVG AQSLR.DGDI . . VLALG . .GLPTMVANLY PEGIH..

== = = = = ==
VFLKSENGLL AFGPFAAGE EDPELINAQK EYVTMLEGSG FFHYHDSFAM
VFLKSENLQ GMKFSAPGE EDDDLINAGK QHVLTLTQGA FFHYHDSFAM
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VPQHDHVKKG EKPPVATV YQGQKVCDR IYDLCCTIDV VPEGLKVVEK
VMDILKRTG ESLKVPECTE PTLIGACVSR IYDLCALPV TPEGLKQVEI
IAMEHCAKDQ SAKLRRCTR LTACHAVIHM LVTELAVFRF IDGKMWLTSE

VECLSFGEQL RLTCATLIDA TQG*
CADIFDPELQ KLSGVPLX*
ADGCDLAIVR AKTARPEVA ADLNTQGDL*
these lysine residues are those that function to stabilize the binding of the CoA donor, they must be brought into proximity with the active site glutamate by protein folding. A glutamate at position 50 of the £-subunit of each of two £-ketoadipate succinyl-CoA transferases and at position 44 of the £-subunit of acetoacetate acetyl-CoA transferase is highly conserved, as is the sequence of amino acids about this residue. Thus Glu50 is probably the active site glutamate sought, but confirmation awaits further testing by site-directed mutagenesis.
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