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A TRIGGER FOR RADICAL GENARATION?
INVESTIGATING THE TERNARY PFL-AE/ADOMET/PFL
COMPLEX

presented by

Ziyang Su

has been accepted towards fulfillment
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Master of Science degree in Department of Chemistry

Major Professor's Signature

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Date

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A TRIGGER FOR RADICAL GENERATION?
INVESTIGATING THE TERNARY PFL-AE/ADOMET/PFL COMPLEX

By

Ziyang Su

A THESIS

Submitted to
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Abstract

A TRIGGER FOR RADICAL GENERATION?

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The activation of pyruvate formate-lyase (PFL) by its activating enzyme PFL-AE employs a novel radical mechanism, which utilizes a Fe-S cluster and S-Adenosylmethionine (SAM or AdoMet) to facilitate the generation of a putative adenosyl radical. PFL-AE is one representative member of the so-called "Radical –SAM" superfamily, which is thought to contain more than 600 members, all working under a common radical mechanism. Previous studies on PFL-AE have indicated close association of SAM to the Fe-S cluster of PFL-AE, suggesting a pathway from one sulfide of the cluster to the sulfonium of SAM for the inner sphere electron transfer. This thesis work focuses on how the substrate PFL interacts with PFL-AE and the cosubstrate SAM in the ternary PFL-AE/SAM/PFL complex, and how the pro-*S* hydrogen atom on glycine 734 of PFL is abstracted by PFL-AE and SAM. Multiple routes were undertaken to prepare a mutant of PFL, G734A. The activity assays of PFL(G734A) were performed to study how the mutation would affect the activation of PFL. The alternative cofactor Te-SAM was also employed to investigate the ternary complex PFL-AE/Te-SAM/PFL by EPR spectroscopy.

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Also, I would like to thank the great professors in my committee and great friends I have met in MSU. They have made my life easier and happier.

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

CHAPTER II

EXPRESSION AND CHARACTERIZATION OF PYRUVATE FORMATE-LYASE (G734A)

CHAPTER III

ATTEMPTS TO IMPROVE PREPARATION OF PYRUVATE FORMATE-LYASE (G734A)

CHAPTER IV

USEAGE OF ALTERNATIVE SUBSTRATE Te-SAM

CHAPTER V

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF ABBREVIATIONS

AdoMet/SAM: *S*-Adenosyl-L-methionine

EPR: Electron paramagnetic resonance

ENDOR: Electron-nuclear double resonance

LB: Luria-Bertani

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

PMSF: Phenylmethanesulfonyl fluoride

DTT: Dithiothreitol

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Tris: Tris-(hydroxymethyl) aminomethane

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Ni-NTA: Nickel-nitrilotriacetic acid agarose

UV-vis: Ultraviolet-visible

OD: Optical density

kDa: kiloDalton

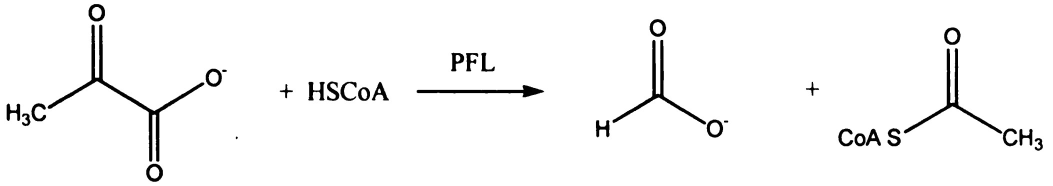
Kbp: Kilo base pairs

Chapter I

INTRODUCTION

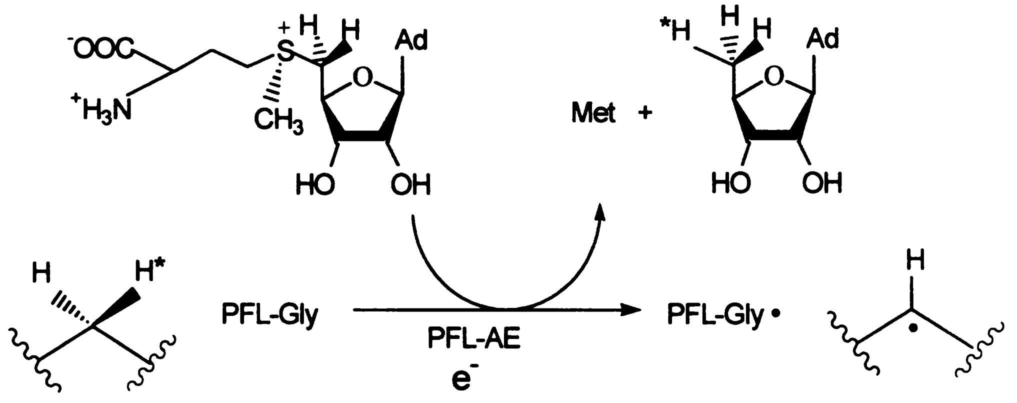
I.1 Pyruvate formate-lyase

The enzyme pyruvate formate-lyase (PFL) catalyzes the reversible conversion of pyruvate and coenzyme A (CoA) to formate and acetyl-CoA, which is the first committed step in anaerobic glucose metabolism in *E.coli* (Scheme I.1).[1, 2] PFL has the distinction of being the first enzyme identified to contain a stable and catalytically essential glycyl radical.[3, 4] This remarkable discovery was made using electron paramagnetic resonance (EPR) spectroscopy in combination with isotopic labeling, together with analysis of the products of oxygenolytic cleavage of the radical-containing protein. This discovery brought up two key questions: first, what is the mechanism by which a glycyl radical mediates the C-C bond cleavage of pyruvate; second, how is the glycyl radical generated? The first question has been the subject of active investigation for nearly 30 years. It is known that the glycyl radical does not directly participate in catalysis, but rather serves as a source of an unpaired electron that can be relayed to the active site in the form of a cysteinyl-thiyl radical, which is directly involved in homolytic cleavage of the pyruvate C-C bond.[5, 6] Uncertainty



Scheme I.1 PFL catalyzed interconversion of pyruvate and CoA to acetyl-CoA and formate remains regarding the precise role of the glycyl radical in catalysis. This thesis is a part of the integrated effort to answer the second question, how the glycyl radical is generated in pyruvate formate-lyase.

PFL from *E.coli* is a homodimeric protein of 170 kDa. Early work demonstrated that PFL was activated under anaerobic conditions by an activating enzyme, in the presence of *S*-adenosylmethionine, Fe(II), and reduced flavodoxin as a reductant.[7, 8] Subsequent work identified the glycyl radical was located on residue G734 of PFL.[1, 3] Concomitant with generation of the glycyl radical, *S*-adenosylmethionine was cleaved to produce methionine and 5'-deoxyadenosine (Scheme I.2). Label transfer studies demonstrated that a 5' hydrogen atom of the 5'-deoxyadenosine product originated from the G734 residue of PFL.[9] The H atom abstraction from G734 was shown to be stereospecific for the pro-*S* hydrogen.[9]



Scheme I.2 AdoMet-dependent PFL glycyl radical generation by PFL-AE

I.2 Pyruvate formate-lyase activating enzyme

Pyruvate formate-lyase activating enzyme (PFL-AE) generates the glycyl radical at G734 on PFL in the presence of a cosubstrate, AdoMet. PFL-AE was initially isolated aerobically from *E.coli* and found to be a 28 kDa monomer with a broad absorbance from 310 to 550 nm, suggesting the presence of a covalently bound cofactor.[1] The first isolation of PFL-AE in its native state under strictly anaerobic conditions and identification of the presence of an iron-sulfur cluster in PFL-AE were achieved by Broderick and coworkers.[10, 11] It was also demonstrated that the Fe-S cluster of PFL-AE is required for enzymatic activity. A complete description of the states of the cluster present in the enzyme was provided by Mössbauer spectroscopy.[12] The major component was the cuboidal $[3Fe\text{-}4S]^{+}$ cluster, accounting for 66% of the total iron, followed by $[2Fe\text{-}2S]^{2+}$ (12% of the total Fe), $[4Fe\text{-}4S]^{2+}$ (8% of the total Fe) and linear $[3Fe\text{-}4S]^{+}$ (~10% of the total Fe). When PFL-AE was anaerobically reduced with dithionite, complete conversion of all cluster types to $[4Fe\text{-}4S]^{2+/+}$ clusters was observed by Mössbauer spectroscopy. The $[4Fe\text{-}4S]^{1+}$ was identified to be the catalytically active cluster by a 'single turnover' experiment.[13] Deazariboflavin-mediated photoreduction afforded quantitative reduction of PFL-AE to the $[4Fe\text{-}4S]^{1+}$ state. Spin quantitation of the EPR spectra showed a 1:1 correspondence between the amount of PFL glycyl radical generated and the amount of $[4Fe\text{-}4S]^{1+}$ cluster present in PFL-AE prior to addition of PFL and suggested that the final cluster state is $[4Fe\text{-}4S]^{2+}$. This is the first direct quantitative spectroscopic evidence that the $[4Fe\text{-}4S]^{1+}$ of PFL-AE is the catalytically relevant cluster, and this cluster provides the electron necessary for the reductive cleavage of AdoMet.

Site-directed mutagenesis studies have identified Cys29, Cys33, Cys36 as cluster ligands in PFL-AE.[14] In general, a similar cluster-binding motif comprised of only three cysteines (CXXXCXXC) is a common feature to all of the Fe-S/SAM-dependent enzymes for which the gene sequence is known.[15-19]

I.3 Adenosylmethionine-dependent iron-sulfur enzymes

Iron-sulfur clusters are among the most ubiquitous and versatile metal centers found in biological systems. Electron transfer was the first function recognized; beyond that, there have been other roles for iron-sulfur clusters identified, including mediating redox and non-redox catalysis, and gene regulation. Aconitase is an enzyme that utilizes a site-differentiated [4Fe-4S] cluster as a Lewis acid to interconvert citrate and isocitrate.[20] Iron-responsive protein (IRP),[21] fumarate-nitrate reduction protein (FNR),[22] SoxR[23] utilize iron-sulfur clusters to respond to changes in levels of iron, oxygen and superoxide respectively.

More recently, a new role for Fe-S cluster has emerged as a number of enzymes has been identified that utilize Fe-S clusters and AdoMet to initiate radical catalysis. This Fe-S cluster-mediated radical catalysis includes the generation of catalytically essential glycyl radicals,[3, 10, 24, 25] the generation of substrate radical intermediates,[26] cofactor biosynthesis,[27-29] and repair of DNA damage.[30] This novel protein family was first identified in 2001 and is thought to contain more than 600 members, and was named the "Radical-SAM Superfamily".[31] Some of the reactions catalyzed by members of this superfamily are shown in Figure I.1. Despite the remarkable diversity of the reactions catalyzed by these enzymes, it is currently

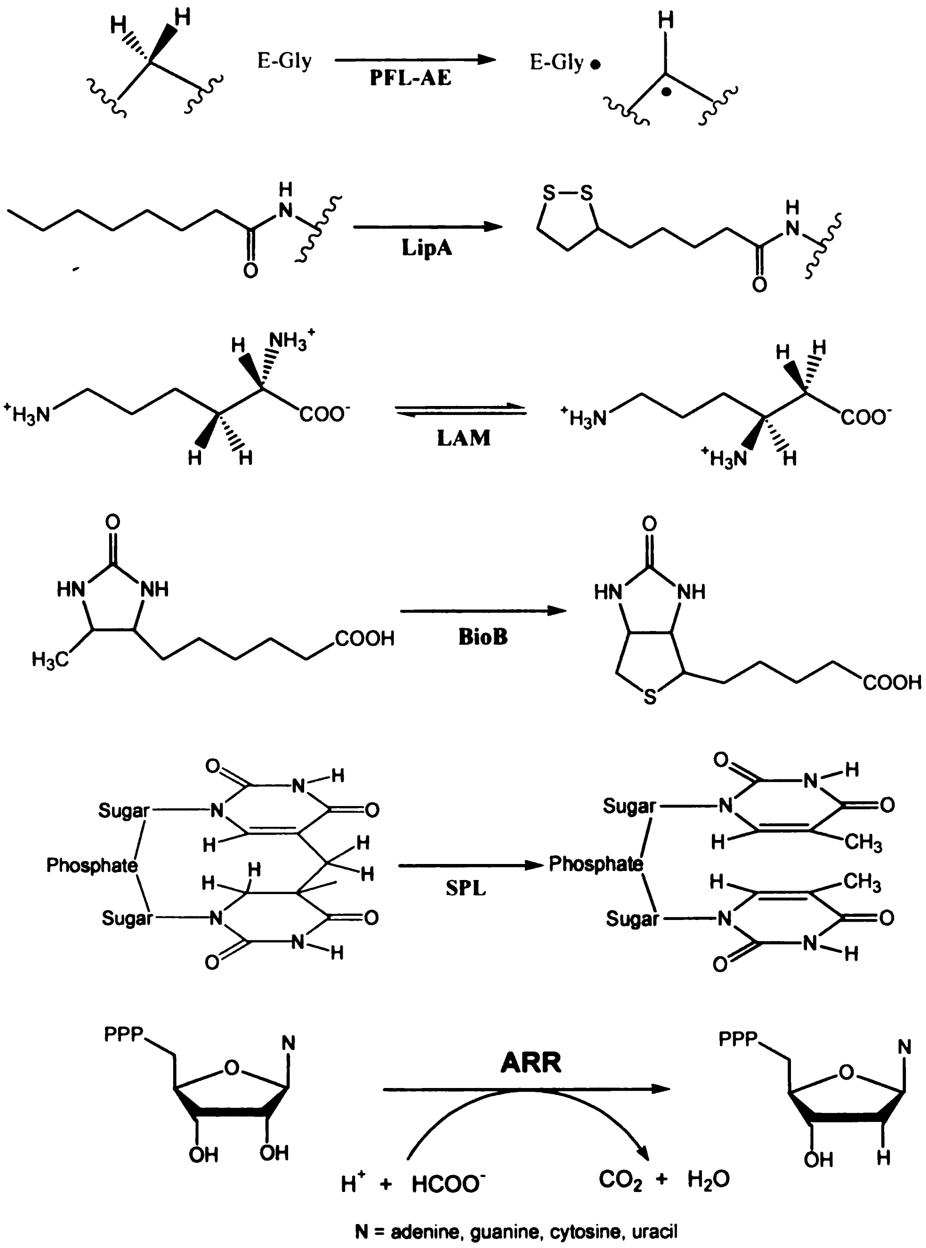


Figure I.1 Reactions catalyzed by representative members of the radical-SAM superfamily. PFL-AE, pyruvate formate-lyase activating enzyme; LipA, lipoate synthase; LAM, lysine 2,3-aminomutase; BioB, biotin synthase; SPL, spore photoproduct lyase; ARR, anaerobic ribonucleotide reductase.

thought that all of these reactions, as well as those radical-SAM reactions yet undiscovered, involve a common mechanism. A three cysteine CXXXCXXC cluster binding motif is conserved in all members of the superfamily (Figure I.2); this suggests a unique Fe site in the [4Fe-4S] cluster, which has been indicated to interact with AdoMet mediating reductive cleavage of S-C bond and radical generation.[32-35] The work described here focuses on the glycyl radical generation of PFL by PFL-AE in the presence of AdoMet.

SP Lyase	86	IPFATG**C**MGH**C**HY**C**YLQTT
PFL-AE	24	ITFFQG**C**LMR**C**LY**C**HNRDT
aRNR-AE	20	VLFVTG**C**LHK**C**EG**C**YNRST
Biotin Synthase	47	SIKTGA**C**PQD**C**KY**C**PQTSR
Lipoate Synthase	48	MILGAI**C**TRR**C**PF**C**DVAHG
LAM	132	LLITDM**C**SMY**C**RH**C**TRRRF

Figure I.2 Sequence alignment of the Fe-S cluster-binding motif in the radical SAM enzymes

I.4 Interaction of the [4Fe-4S] cluster of PFL-AE with AdoMet

Electron-nuclear double resonance (ENDOR), which probes coupling between electronic and nuclear spins,[36] has been used to define the interaction of AdoMet with the [4Fe-4S] cluster of PFL-AE. Isotopically labeled *S*-adenosylmethionines were prepared with ^{17}O at the carboxylate oxygen, with ^{13}C at the carboxylate carbon, and with ^{15}N at the amine nitrogen.[35] The observation of ENDOR signals for all three nuclei demonstrated for the first time that AdoMet forms a classical five-membered chelate ring with the unique iron of the [4Fe-4S] cluster of PFL-AE (Figure I.5).[35] Field-dependence of the ^{13}C, ^{17}O, and ^{15}N ENDOR data further suggests that the unique iron serves as an anchor to hold the cosubstrate AdoMet in place for reaction.[37] Furthermore, ENDOR of methyl-^{2}H-AdoMet and methyl-^{13}C-AdoMet reveals local orbital overlap of AdoMet with the [4Fe-4S] cluster.[34] It was proposed that this orbital overlap occurred via interaction of one of the μ_3-bridging sulfides of the $[4Fe\text{-}4S]^+$

cluster with the sulfonium of SAM (Figure I.3); this close contact provides a pathway for the potential inner-sphere electron transfer from the cluster to AdoMet, which is expected to facilitate homolytic bond cleavage and creation of the adenosyl radical.[34,]

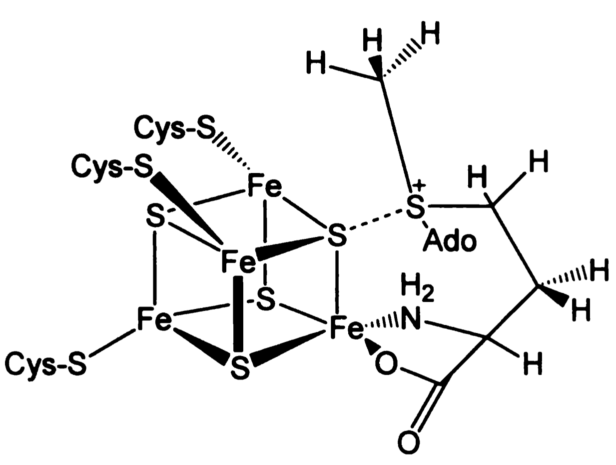


Figure I.3 Interaction of SAM to the [4Fe-4S] cluster of PFL-AE

[35] On the basis of the above observations and discussion, a mechanism for the Fe-S/AdoMet-driven radical chemistry catalyzed by PFL-AE was proposed (Figure I.4) by our group.[38]

EPR and ENDOR results provide significant insight into the mechanism by which PFL is activated by PFL-AE in the presence of AdoMet, yet many questions remain unresolved here: How does PFL-AE recognize the gly-734 residue in pyruvate formate-lyase? How do PFL, AdoMet and the iron-sulfur cluster of PFL-AE interact so as to trigger inner sphere electron transfer and S-C bond cleavage? How does the 5'-deoxyladenosyl radical abstract the *pro*-S hydrogen from the glycine residue of PFL? Concerns about the thermodynamic aspects of the radical catalysis remain as well, such as the dissociation constant of the interaction between PFL and PFL-AE, and between AdoMet and PFL-AE.

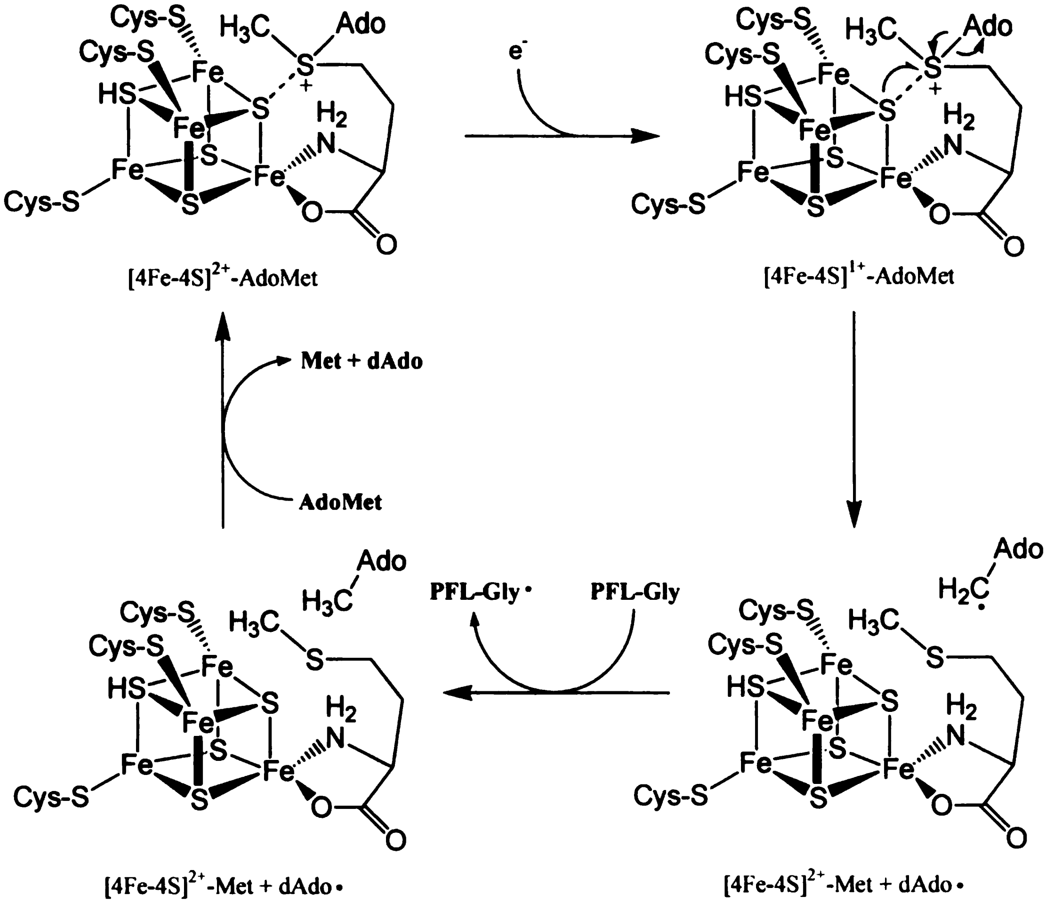


Figure I.4 proposed mechanism for the activation of PFL by PFL-AE and AdoMet. Inner sphere electron transfer from a bridging sulfide of the [4Fe-4S]$^{1+}$ cluster to the sulfonium of AdoMet causes C-S bond homolysis, which produces a 5'-deoxyadenosyl radical and methionine. The adenosyl radical abstracts a hydrogen from Gly734 of PFL and 5'-deoxyadenosine and methionine are replaced with another AdoMet. The source of the electron is proposed to be a reduced flavodoxin

To address the question how PFL-AE recognizes the gly-734 residue in PFL, a series of peptides homologous to the gly-734 site of PFL were synthesized and tested.[9] Among them, a heptamer peptide with gly-734 replaced by L-alanine totally abolished the H atom abstraction by PFL-AE. This suggested that it is very likely a mutant protein of PFL G734A would be inert as well in terms of radical activation. One goal of this work is to address the substrate recognition properties of PFL-AE. A mutant PFL(G734A) was cloned and purified to test its activity as a substrate for

PFL-AE.

Another goal of this project is to investigate the ternary complex PFL-AE/AdoMet/PFL by ENDOR spectroscopy. The spectroscopic results obtained so far are restricted to the binary complex PFL-AE/AdoMet, in the absence of the substrate PFL. Since the binary complex is not active and does not generate a radical, some important structural/electronic changes must occur upon adding PFL. Either AdoMet or PFL will be isotopically labeled for ENDOR purpose. In case of mutant PFL(G734A), if no turnover occurs, ENDOR of sample PFL-AE/labeled AdoMet/PFL(G734A) could detect the interaction between the $[4Fe\text{-}4S]^{1+}$ cluster of PFL-AE and AdoMet in the presence of PFL; on the other hand, sample PFL-AE/AdoMet/labeled PFL(G734A) can provide information about the interaction between the cluster and the substrate PFL. Furthermore, sample with wild type PFL can be probed by ENDOR via labeled PFL. In this case, the ternary complex PFL-AE/labeled AdoMet/PFL would generate a glycyl radical and the $[4Fe\text{-}4S]^{1+}$ cluster would be oxidized to $[4Fe\text{-}4S]^{2+}$ (EPR silent). Subsequently ENDOR could detect the interaction between AdoMet and the glycyl radical. Taken together, study of the ternary complex would allow a full analysis of the radical generation process.

CHAPTER II

EXPRESSION AND CHARACTERIZATION OF PYRUVATE FORMATE-LYASE (G734A)

II.1 Introduction

One key question of the radical generation mechanism is how PFL-AE recognized the glycine 734 as the active site in pyruvate fomate-lyase. In attempts to investigate the substrate recognition properties of PFL-AE, a series of peptides homologous to the active site of PFL were synthesized and tested for substrate efficiency.[9] Among them, a heptamer peptide with glycine-734 replaced by L-alanine totally abolished the H atom abstraction by PFL-AE. It would be interesting to study the mutant of PFL, replacing glycine at 734 by L-alanine, to determine if it can act as a substrate for PFL-AE.

If PFL (G734A) cannot act as a substrate as expected, ENDOR spectroscopy can be performed on various PFL-AE/AdoMet/ PFL (G734A) ternary complexes. Sample with labeled AdoMet can help to detect the interaction between $[4Fe-4S]^+$ cluster and AdoMet in presence of PFL (G734A); sample with labeled PFL (G734A) can facilitate detection of the interaction between $[4Fe-4S]^+$ and PFL (G734A), which can not be obtained from binary complex PFL-AE/AdoMet. At the same time, EPR spectroscopy will be employed to help monitor the oxidation state and cluster type of PFL-AE.

II.2 Experimental methods

II.2.1 Materials

The plasmids pKK-PFL and pMG-AE were obtained as a generous gift from John Kozarich (Merck). 5-Deazariboflavin was synthesized in our laboratory according to published procedures,[39, 40] and characterized using NMR and Mass Spectrometry. All other chemicals were obtained commercially and used as received.

II.2.2 Growth and expression of PFL (G734A)

The mutated PFL (G734A) gene was subcloned into the plasmid pKK-PFL. PKK-PFL (G734A) was then used to transform *E. coli* strain BL21(DE3)pLysS (from Novagene). A single colony of transformed cells was used to inoculate 50 mL LB media containing 50μg/mL ampicillin (LB/Amp). This culture was grown for 16h to saturation and then used to inoculate LB/Amp in 6 flasks containing 800 mL media each. The culture was then allowed to grow for 24 h at 37°C in shaker at 250 rpm before being harvested by centrifugation at 8,000 rpm for 15 min (Sorvall GS3 rotor). The supernatant was decanted and the cells freezed at -80°C.

II.2.3 Purification of PFL (G734A)

PFL (G734A) was purified from the BL21(DE3) pLysS/pKK-PFL(G734A) cells. Cell paste (typically 10-15 g) was suspended in enzymatic lysis buffer (5 mL per gram of cell paste) containing 20 mM Hepes (pH 7.2), 1% (w/v) Triton X-100, 5% (w/v) glycerol, 10 mM $MgCl_2$, 8 mg lysozyme, 1 mM PMSF, and trace amounts (approximately 0.1mg each) RNase A and DNase I. The suspension was agitated and then incubated at ambient temperature for 1 h. The lysed cells were centrifuged at 15,000 rpm for 20 min at 4°C.

The crude extract was purified by fast protein liquid chromatography (FPLC) in three steps: first, the crude extract (approximately 50 mL) was loaded onto an Accell Plus QMA Anion Exchange column equilibrated with buffer A (20 mM Hepes, 1mM DTT, pH 7.2). The column was washed with 300 mL of buffer A prior to running a gradient from buffer A to buffer B (20 mM Hepes, 500 mM NaCl, 1 mM DTT, pH 7.2) over 900 mL. PFL eluted at approximately 250 mM NaCl. Fractions containing > 70% pure PFL (as judged by SDS-PAGE on a 12% Tris-HCl gel) were combined, flash frozen and stored at -80°C. The second step was to load fractions from the ion exchange column onto a gel filtration column (Superose 12 10/300 GL, Amersham Bioscience) with buffer A, PFL eluted approximately after 40 min (1 mL/min). Fractions containing > 75% pure PFL were combined and dialyzed against buffer C (40 mM Hepes, pH 7.2, 1 M ammonium sulfate, 1 mM DTT), and centrifuged to remove precipitated protein. Thirdly, the supernatant was loaded onto a hydrophobic interaction column (Phenyl-Sepharose, Pharmacia 16/10) equilibrated with buffer C. The column was washed with 50 mL of buffer C prior to running a gradient from buffer C to buffer A over 50 mL, followed by a wash with 50 mL of buffer A. PFL was eluted through the last half of the gradient. Fractions containing >80% pure PFL were combined, concentrated, flash-frozen and stored at -80°C.

II.2.4 Preparation of PFL-AE

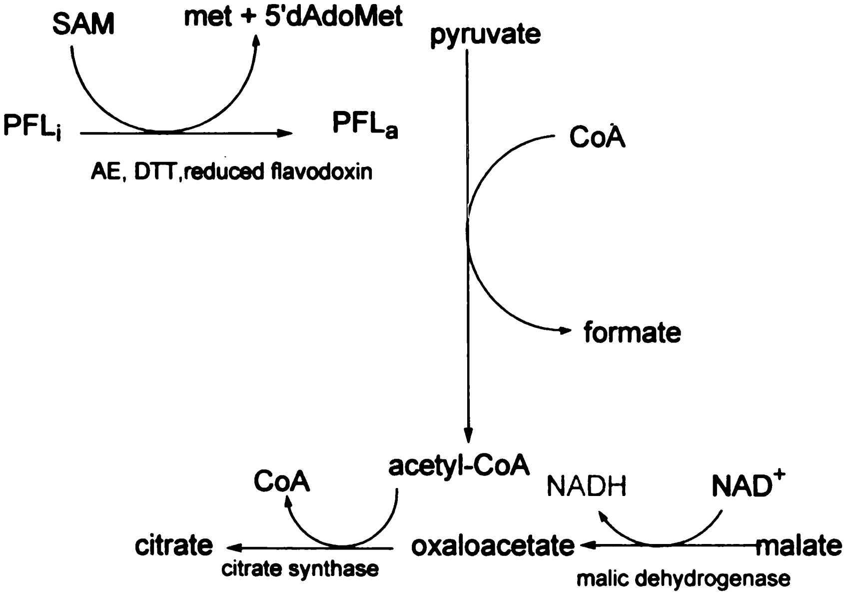
The plasmid pCAL-n-AE3 (from Stratagene) was transformed into *E.coli* BL21(DE3)pLysS strain. PFL-AE was overexpressed and purified as described in the published methods.[11, 34]

II.2.5 Protein assays

Routine determinations of proteins concentrations were done by the method of Bradford,[41] using a kit purchased from Bio-Rad, and bovine serum albumin as a standard.

II.2.6 Activity assay of PFL (G734A)

Scheme 1 illustrates how the activity assay was performed. Under strict anaerobic conditions (O_2 level < 1ppm), the PFL-AE reaction mix was made to a final volume of 450 µL: 0.10 M Tris chloride, pH 8.1, 0.15 M NaCl, 10 mM oxamate, 8 mM DTT, 0.6 µM PFL-AE, 50 µM PFL_i, 0.2 mM AdoMet, and 0.2 mM 5'-Deazariboflavin (stock solution dissolved in DMSO). This mix was prepared in the anaerobic chamber by combining reagents from stock solutions in the order listed to the final concentrations indicated. In all cases, 5'-deazariboflavin acted as a photoreductant and was added last in all cases. The samples were then allowed to sit in ambient light in the chamber for at least 30min, before an aliquot was removed for assay of active PFL through the coupling assay.



Scheme II.1 Activity assay of PFL

The coupling assay mix contained 0.1 M Tris chloride, pH 8.1, 3 mM NAD^+, 55μM CoA, 0.1 mg BSA, 10 mM pyruvate, 10 mM malate, 20 units citrate synthase, 300 units malic dehydrogenase, and 10 mM DTT. All reagents except DTT were combined and made anaerobic by freeze-pump-thaw cycles. The DTT was added after bringing the anaerobic mix into the anaerobic chamber. To assay PFL activity, 690μL of this mix and 10μL of the activated PFL described above were combined in a quartz cuvette, which was then sealed with a septum and brought out of the anaerobic chamber to monitor the production of NADH by the increase in absorbance at 340 nm. If PFL-AE and AdoMet activate PFL, then PFL_a will catalyze the conversion of pyruvate and CoA to formate and acetyl-CoA. The remaining reactions illustrated in Scheme 1 couple acetyl-CoA production to NAD reduction, which is monitored by UV-vis. Therefore, the production of NADH is related to PFL activation. PFL's activity was calculated as follows:

$$U\ PFL_a\ (\text{in cuvette}) = \text{Rate}\ (AU/sec) \times 1/\varepsilon\ (mol/L{*}Au) \times \text{Vol}\ (L) \times 60\ (sec/min) \times 10^6\ (\mu mol/mol) \qquad \text{Equation II.1}$$

In the above equation, Rate is the production of NADH, ε is the extinction coefficient of NADH, Vol is the volume of the solution under test in the cuvette. The assay was repeated every other 20 min until the reading reached saturation.

II. 3 Results and Discussion

II.3.1 Expression and purification of PFL (G734A)

PFL (G734A) was overexpressed from BL21(DE3)pLysS cells harboring the pKK-PFL plasmid (SDS-PAGE gel see Figure II. 1). The cells were lysed by an enzymatic procedure. Crude extract was loaded onto columns of Biological Systems in the order of ion exchange, gel filtration and hydrophobic interaction (chromatograms see Figure II.2, II.3, II.4, respectively). Fractions off the three columns yield > 90% pure PFL (SDS-PAGE gel see Figure II.5).

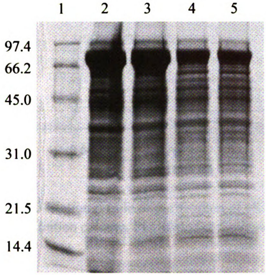


Figure II.1 SDS-PAGE analysis of pKK-PFL (G734A) cells. Lane 1, molecular marker (kDa); lane 2-3, overnight culture; lane 4-5, cells before harvesting

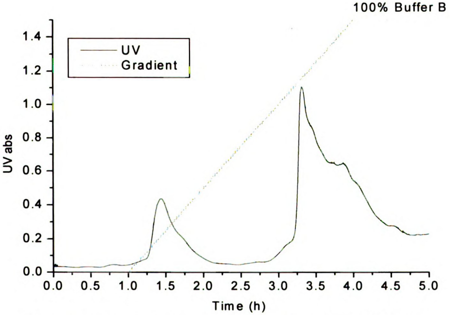


Figure II.2 Elution profile of purification of pKK-PFL (G734A) for the Anion Exchange column, PFL eluted at approximately 3.3 hours.

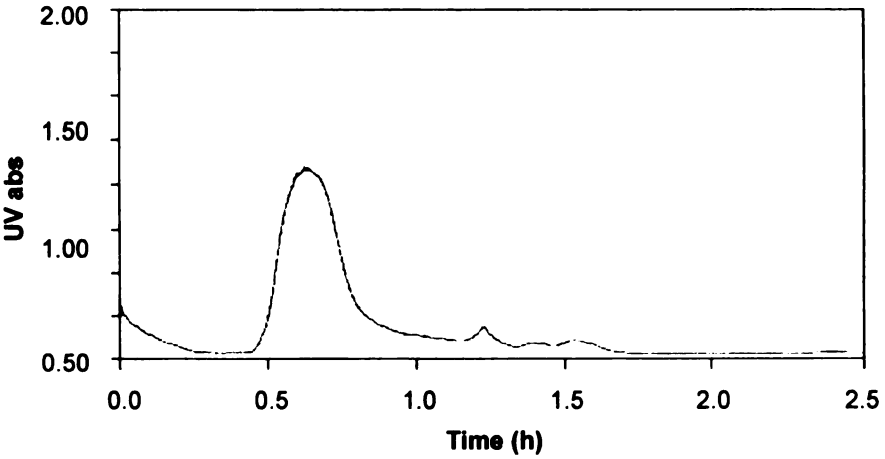


Figure II.3 Elution profile of purification of pKK-PFL (G734A) for the Gel Filtration column, PFL eluted at approximately 0.6 hours.

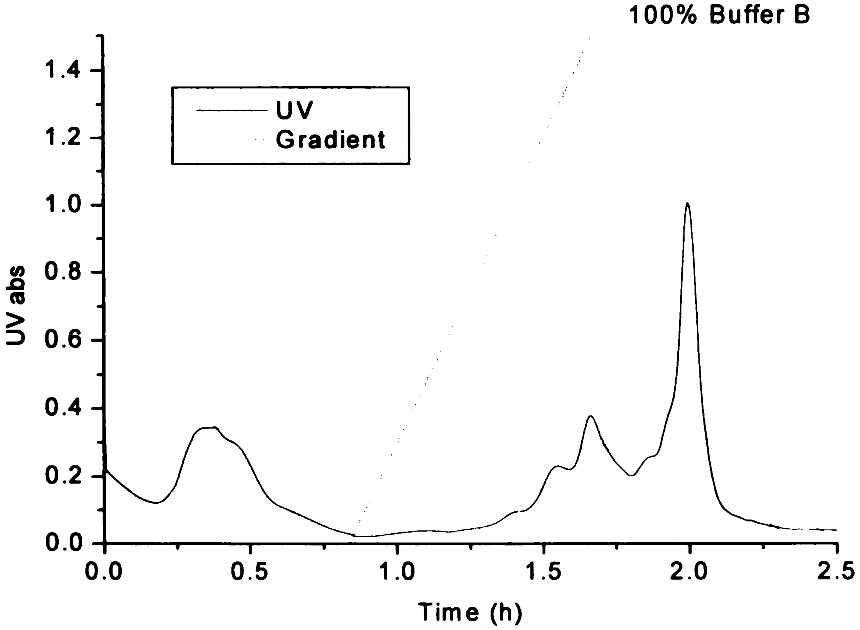


Figure II.4 Elution profile of purification of pKK-PFL (G734A) for the Phenyl Sepharose column, PFL was eluted through last half of the gradient.

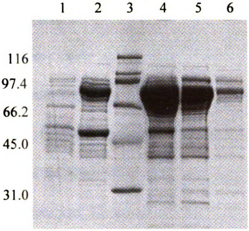


Figure II.5 SDS-PAGE analysis of purified pKK-PFL (G734A) off the three chromatographic columns. Lane 1, fraction 21; lane 2, fraction 23; lane 3, molecular marker (kDa); lane 4-6, fraction 24-26. Fractions 23-26 were pooled.

II.3.2 Expression and purification of PFL-AE

PFL-AE was purified from BL21(DE3)pLysS cell harboring the pCAL-n-AE3 expression vector. Proteins off the second run of the gel filtration column showed high purity (Figure II.5). The purest fractions were combined, concentrated and stored under nitrogen in small aliquots at -80°C.

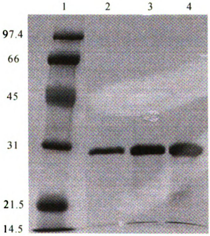


Figure II.6 SDS-PAGE analysis of PFL-AE. Lane 1, molecular marker (kDa); lane 2-4, purified PFL-AE.

II.3.3 Activity assay of PFL (G734A)

Activity assays were carried out for the purified PFL(G734A) and isolated PFL-AE and AdoMet, according to the procedures described previously. As a result, the activated mutant PFL (G734A) showed specific activity of 0.685 unit/mg, which is approximately 0.5% of the value obtained for wild type PFL (200 units/mg).[9] As discussed in the introduction part, this mutant G734A was not expected to be an active substrate for PFL-AE. It is most likely that the activity comes from a small amount of wild-type PFL, which was coexpressed from the *E.coli* strain simultaneously with the mutant. The wild-type PFL expressed has almost the same size, ion affinity, hydrophobic affinity as the mutant G734A, thus making it difficult to be removed via normal purification methods. In order to obtain more pure PFL (G734), alternative methods need to be explored, which will be discussed in the following Chapter.

CHAPTER III

ATTEMPTS TO IMPROVE THE PREPARATION OF PYRUVATE FORMATE-LYASE (G734A)

III.1 Introduction

The small fraction of activity observed from the activity assay of mutant PFL(G734A) was assumed to be the result of trace amount of wild-type PFL in the sample, which is difficult to be removed via normal purification methods. In order to obtain more pure mutant, two methods have been undertaken: one is to clone PFL (G734A) into plasmids with an N-terminal Histidine tag to generate PFL (G734A)-His_6, and purify the protein through metal affinity chromatography. Since the wild-type PFL does not contain a His-tag, affinity chromatography would allow separation of the wild type PFL and the mutant. Two plasmids were employed, pET-28a and pET-30 Ek/LIC. The former requires digestion with the restriction enzymes; the latter is ligation independent, does not require restriction digestion.

The second method that was chosen to obtain pure G734A PFL mutant was 'gene gorging'. The goal of this method was to make precise mutations in the *E.coli* genome at high frequencies that resulted in efficient replacement of the wild type allele with the modified sequence.[42] In this method, a special knockout strain of *E.coli* was employed, in which the wild-type PFL gene was deleted. The cloning of PFL (G734A) into the knockout strain will then allow the production of only mutated PFL protein, without any wild-type present. The activity assay of the pure PFL mutant should be able to provide information about how the mutation from glycine to alanine at residue 734 affects the function of PFL.

III.2 Experimental methods

III.2.1 Materials

Pfu Turbo polymerase was purchased from Stratagene. Restriction endonucleases and T4 ligase were purchased from New England Biolabs. Oligonucleotide primers were synthesized by Integrated DNA Technologies and are listed in Table III.1. Plasmids pET-28a, pET-30 Ek/LIC were obtained from Novagene, as were competent *E. Coli* Novablue and BL21(DE3)pLysS cells. Nickel-nitrilotriacetic acid agarose (Ni-NTA) column and Ni-HisTrap column (1 mL) were purchased from Amersham Biosciences. *E.coli* knockout strains KO_pflB were obtained from Nara Institute of Science and Technology (Nara, Japan). Bradford dye-binding reagent for protein quantification was obtained from Pierce. All buffers and chemicals used were of the highest quality available and used as received.

Table III.1 Sequence of primers used in cloning.

Primer	Sequence	Purpose
ZS1	5'-CCGAATTCTCCGAGCTTAATGAAAAG-3'	Forward primer for cloning PFL(G734A) into pET28a[a]
ZS2	5'-CGCCAAGCTTTTACATAGATTGAGTGAA-3'	Reverse primer for cloning PFL(G734A) into pET28a[b]
ZS3	5'-GCCGCCGCTAGCTCCGAGCTTAATGAAAAG-3'	Forward primer for cloning PFL(G734A) into pET28a[c]
ZS4	5'-GACGACGACAAGATGTCCGAGC-3'	Forward primer for cloning PFL (G734A) into pET30 Ek/LIC[d]
ZS5	5'-GAGGAGAAGCCCGGTTTACATAGATTG-3'	Reverse primer for cloning PFL (G734A) into pET30 Ek/LIC[e]

[a] Underlined bases represent *Eco*RI restriction site

[b] Underlined bases represent *Hin*dIII restriction site

[c] Underlined bases represent *Nhe*I restriction site

[d,e] Underlined bases represent required overhangs for sense and antisense primers

III.2.2 General procedures

The polymerase chain reaction (PCR) was carried out using Dr. Geiger's thermocycler, gene amp PCR system 2400 (from Perkin Elmer). Each amplification reaction contained 20 pmol of each primer, 20 nmol of each deoxynucleoside tripohsphate, 1 U of Pfu Turbo polymerase, 5 μL of 10x Pfu Turbo polymerase buffer and 1-3 μg of DNA [pKK-PFL (G734A)] in a final volume of 50 μL. Polymerase was always added last. After a 2 min denaturation step at 95 °C, 30 cycles of the following program were performed: 15 seconds at 95 °C, 30 seconds at 55 °C, and 90 seconds at 72 °C. The mix was finally cooled down to 4 °C. DNA sequencing was carried out at the Michigan State University Genomics Technology Support Facility.

III.2.3 Cloning of PFL (G734A) into plasmid pET-28a

The PFL (G734A) gene was amplified from pKK-PFL plasmid by PCR using primers ZS1/ZS2 and ZS3/ZS2 (Table 1). Primer ZS1 contained an *Eco*RI restriction site flanked by a 2 base GC clamp and the first 18 bases of the PFL (G734A) gene. Primer ZS2 contained a *Hin*dIII restriction site flanked by a 4 base GC clamp and the last 18 bases of the PFL (G734A) gene, which included the stop codon. Primer ZS3 contained a *Nhe*I restriction site flanked by a 6 base GC clamp and the first 18 bases of the PFL (G734A) gene. Two pairs of primers (ZS1/ZS2 and ZS3/ZS2) were used to amplify PFL gene by PCR independently. Subsequent to amplification by PCR, the 2283 bp fragment was purified from agarose gel, and digested with *Eco*RI/*Hin*dIII, and *Nhe*I/*Hin*dIII, respectively. The digested fragments were ligated into plasmid pET28a that had been similarly digested. All procedures were carried out by standard methods.[43]

III.2.4 Cloning of PFL (G734A) into plasmid pET-30 Ek/LIC

Plasmid pET-30 Ek/LIC is a member of Ligation Independent Cloning (LIC) vectors that do not require digestion with restriction enzymes. pET-30 Ek/LIC kit was purchased from Novagen, and contained the following components: pET-30 Ek/LIC vector, T4 DNA polymerase (LIC-qualified), T4 DNA polymerase buffer, dATP, EDTA, Novablue GigaSingles competent cells, BL21(DE3)pLysS competent cells. Two primers ZS4 and ZS5 (Table 1) were synthesized for the purpose of PCR. ZS4 contains required overhangs and the first 7 bases of PFL (G734A) gene. ZS5 contains required overhangs and the last 12 bases of PFL (G734A) gene. PCR was performed according to the general procedures with Pfu Turbo DNA polymerase. After amplification, the 2283 bp target insert was purified from agarose gel and treated with T4 DNA polymerase (from the kit). The treated target was subsequently annealed to the vector pET-30 Ek/LIC and transformed into competent *E.coli* cells. All procedures after the PCR were carried out according to pET Ek/LIC User Protocols.[44] The identity of the PFL (G734A) gene for the plasmid pET-30 Ek/LIC was confirmed by DNA sequencing, and the resulting plasmid construct was designated pET-30 Ek/LIC-pfl (G734A).

III.2.5 Expression and purification of the pET-30 Ek/LIC-pfl (G734A) gene product

A single colony of *E. coli* BL21(DE3)pLysS harboring the pET-30 Ek/LIC-pfl

(G734A) plasmid was selected from a Luria–Bertani (LB) agar plate supplemented with 10 μgmL^{-1} of kanamycin, used to inoculate 50 mL of LB media containing 10 μgmL^{-1} of kanamycin, and grown for 12 h at 37 °C with shaking (250 rpm). This culture was used to inoculate LB/Kan in 6 flasks containing 800 mL media each. When an OD_{600} of ~0.6 was reached, expression of the gene PFL(G734A) was induced by addition of IPTG to a final concentration of 1 mM and was allowed to grow for an additional 3 h. Cells were harvested by centrifugation at 18,000 rpm at 4 °C for 30 min, frozen with liquid nitrogen and stored at -80°C.

Cell paste (typically 10-15 g) was suspended in enzymatic lysis buffer (5 mL per gram of cell paste) containing 50 mM Hepes, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1% (w/v) Triton X-100, 5% (w/v) glycerol, 8 mg lysozyme, 1 mM PMSF and trace amounts (approximately 0.1mg each) RNase A and DNase I. The suspension was agitated and incubated at ambient temperature for 1 h. The lysed cells were centrifuged at 15,000 rpm for 20min at 4°C. The crude extract (approximately 25mL) was loaded onto a Ni-NTA superflow column (Pharmacia, now Amersham Bioscience) equilibrated with 100mL loading buffer (50mM Hepes, 300mM NaCl, 5mM β-ME, 5% (w/v) glycerol, 10mM imidazole). The column was washed with a step gradient from loading buffer to elution buffer of high concentration of imidazole (500mM, other components are the same as loading buffer) in 4 steps: 10% of elution buffer followed by 20%, 50% and 75%, with two column volumes (40 mL) in each step. PFL (G734A) eluted during the 50% step. Fractions containing >80% proteins were combined and frozen. A different Nickel column (HisTrap HP, 1mL, from Amersham Bioscience) was also used to purify pET-30 Ek/LIC-pfl (G734A), less amount of crude extract (approximately 7mL) was loaded onto this column. Loading buffer contains 20 mM Hepes, pH 7.2, 10mM $MgCl_2$, 5% glycerol and 10mM Imidazole; elution buffer contains all the same except 500 mM Imidazole. Similar program was run for this column as the Ni-NTA column.

III.2.6 Usage of knockout strain of *E.coli* to prepare PFL (G734A)

Kanamycin-resistant *E.coli* knockout strain with the deletion of wild-type PFL gene was ordered from Japan, denoted KO_pflB. It was obtained as a solid material and was stored at 4°C. In order to transform PFL (G734A) gene into KO_pflB, chemically competent cells had to be prepared. Calcium chloride was used to make competent cells.[43, 45] Five solutions were prepared, as listed in Table III.2. All of the solutions were sterilized by autoclaving. On Day One, 5 mL of LB was inoculated with a single colony of the strain KO_pflB, kanamycin was added to a concentration of 10 μg/ml. The media was placed in a shaker at 250 rpm at 37°C overnight. Day Two in the morning, 100 mL of LB/kan medium was inoculated with 1.0 mL of the overnight culture. While the cells were grown at 37°C, solutions A, B and C were chilled in an ice bath. Also, a 500 mL centrifuge bottle was filled with ~25% bleach and allowed to stand on the benchtop for approximately 2 h. As soon as the cells reached OD_{600} of 0.4-0.6, the centrifuge bottle was rinsed several times with the sterile H_2O prepared earlier. All the following steps were performed in a sterile hood and cells were kept cold at all times. The cells were transferred to the centrifuge bottle, harvested in prechilled rotor at 8,000 rpm for 5 min at 4 °C. The medium was decanted and gently resuspended the cells in solution A, harvested by centrifugation at 8,000 rpm for 5 min at 4 °C. After decanting the salt solution, cells were gently resuspended in solution B on ice. Cells were allowed to stand on ice for a minimum of 30 min before being harvested by centrifugation. After removal of solution B, cells were gently resuspended in 4.0 mL of 100 mM $CaCl_2$ / 15% glycerol (solution C). Aliquots (0.1-0.25 mL) of the competent cells were transferred to sterile, labeled, chilled microcentrifuge tubes. Each tube was quick-frozen in liquid N_2 and then stored at -80 °C.

The gene pKK-PFL(G734A) was transformed into the competent cells prepared above. Cells were grown at the same conditions as described in section III.2.5 to express PFL(G734A).

Table III.2 Solutions for making competent cells

A	0.9% NaCl, 100 mL
B	100 mM $CaCl_2$, 50mL
C	100 mM $CaCl_2$, 15% glycerol (w/v), 100 mL
D	1.2 L distilled H_2O
E	100 mL LB medium (500 mL Erlenmeyer flask)

III.3 Results and Discussion

III.3.1 Cloning of PFL(G734A) into plasmid pET-28a

The PFL(G734A) gene was amplified from pKK-PFL plasmid by PCR using primers ZS1/ZS2 and ZS3/ZS2 (Table III.1), as judged by agarose gel (Figure III.1). Purified gene was digested with *Eco*RI/*Hin*dIII or *Nhe*I/*Hin*dIII, respectively. Difficulties were encountered while trying to perform a double digest of the plasmid pET-28a with two pairs of enzymes shown above. Multiple attempts were undertaken to digest the plasmid yet all were unsuccessful. At this point, a ligation independent vector pET-30 Ek/LIC was chosen for cloning.

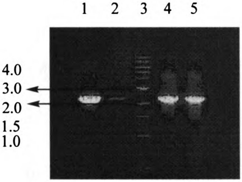


Figure III.1 Agarose gel of PCR products from pKK-pfl(G734A) with primers ZS1, ZS2, ZS3. Lane 1-2, gene amplified by primers ZS1/ZS2; lane 3, molecular marker (Kbp); lane 4-5, gene amplified by primers ZS2/ZS3.

III.3.2 Cloning of PFL(G734A) into pET-30 Ek/LIC and expression of the gene product

The PFL(G734A) gene (2.3 Kbp) was amplified from PCR as judged by agarose gel (Figure III.2). The amplified target was then treated with T4 DNA polymerase and annealed to the vector pET-30 Ek/LIC, leading to a plasmid construct pET-30 Ek/LIC-pfl(G734A). The presence of the mutated PFL gene was confirmed by

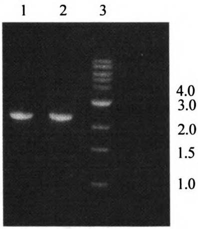


Figure III.2 Agarose gel of PCR products from pKK-pfl(G734A) with primers ZS4 and ZS5. Lane 1-2,PCR products; lane 3, molecular marker (Kbp) sequencing. Proteins overexpressed from BL21(DE3)pLysS cells harboring the pET-30 Ek/LIC-pfl(G734A) plasmid (SDS-PAGE gel see Figure III.3).

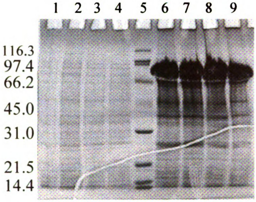


Figure III.3 SDS-PAGE analysis of pET-30 Ek/LIC-pfl (G734A)cells lane 1-4, cell pastes before induction; lane 5, molecular marker (KDa); lane 6-9, cell pastes 3 hr after induction with IPTG

III.3.3 Purification of pET-30 Ek/LIC-pfl(G734A)

Cells were lysed by an enzymatic procedure; crude extract was loaded onto Ni-NTA affinity column (Figure III.4). Protein eluted from the column was analyzed

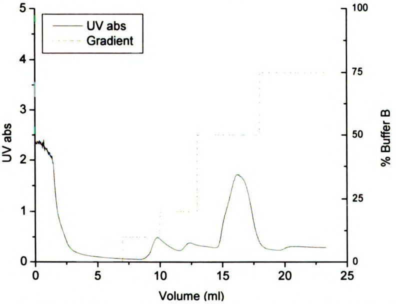


Figure III.4 Elution profile of purification of pET-30 Ek/LIC-pfl (G734A) by the Ni-HisTrap column. PFL eluted at approximately 16 min.

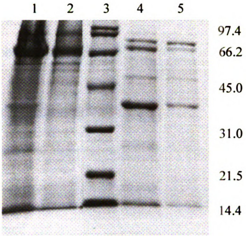


Figure III.5 SDS-PAGE analysis of pET-30 Ek/LIC-pfl (G734A) fractions off the Ni His Trap _1mL column. Lane 1-2, 4-5, fractions from the column; lane 3, molecular maker (kDa).

by SDS-PAGE. The band corresponding to the eluted protein appeared at lower position (approximately 70 kDa) (Figure III.5) than expected for a normal size PFL protein (85 kDa). SDS-PAGE was repeated to make sure that the protein gel was running properly. In order to investigate this problem, whole cell samples were examined. It was found that a strong band of expressed PFL at 85 kDa was present in these samples. However, after the enzymatic lysis was performed a protein of smaller molecular mass (70 kDa) was again obtained. This indicated that the enzyme PFL(G734A) was cleaved during lysis. To prevent protease from cleaving the enzyme, more cells were grown and lysed in a lysis buffer containing Protease Inhibitor Cocktail set III (from CALBIOCHEM) in addition to PMSF. However, this modification did not stop the cleavage of PFL(G734A). N-terminal amino acid sequence of the cleaved sample showed that the first five amino acids of the obtained protein were different from the ones expected for PFL (MSELN). The sequencing confirmed the cleavage of the PFL protein during lysis and indicated the need for a different method of obtaining mutated PFL protein.

III.3.4 Knockout strain of *E.coli* KO_pflB

Chemically competent cells of KO_pflB were prepared as described in the experimental part. Transformation of the gene pKK-pfl(G734A) into these cells was performed but it turned out to be inefficient. For majority of the experiments, no bacterial colonies were obtained on media plates. In a single experiment, a few colonies were observed from one transformation; and a well isolated colony was used to inoculate a cell culture. However, no overexpression of the PFL protein was observed (Figure III.6). No band present at 85 kDa on the SDS-PAGE gel. Another protein with mass of 55 kDa was expressed to a certain level. In order to investigate the best conditions to increase the efficiency of transformation, different amounts of DNA were used (from 1 to 20 μg), and different heatshock times (between 30 s and 90 s) were applied. But none of these efforts showed any obvious improvement. These indicated that it is very likely the cells made were not competent enough. Recently, electroporation has been widely used for transformation procedures. In this method, an intense electric field is applied to make the cell membranes transiently permeable, allowing DNA to enter the cell. It is suggested that the electroporation method be used to make the KO_pflB cells competent, subsequently transform, and express the mutant PFL(G734A).

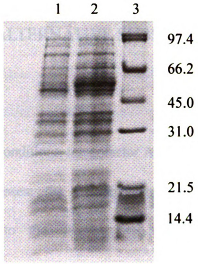


Figure III.6 SDS-PAGE analysis of pKK_pfl (G734A) in knock out strain KO_pflB lane 1,pre-induced cells; lane 2, post-induced cells; lane 3, molecular marker (KDa).

CHAPTER IV

USAGE OF ALTERNATIVE SUBSTRATE Te-AdoMet

IV.1 Introduction

Under physiological conditions, the cofactor AdoMet is not stable. It degrades by three independent processes: the pH-dependent intramolecular attack of the α-carboxylate group onto the γ-carbon to afford homoserine lactone and 5'-methylthioadenosine (MTA); the pH-dependent deprotonation at C-5' with ensuing chemistry that releases adenine and *S*-ribosylmethionine; and the pH-independent racemization about the sulfomium to give the (*R*,*S*)-diastereomer (Figure IV.1).[46] Decay studies of AdoMet and its selenium and tellurium analogues, Se-AdoMet and Te-AdoMet, were carried out at 37 °C and constant ionic strength. The result showed that Se-AdoMet degraded significantly in both pH-dependent processes, while Te-AdoMet was inert to both pH-dependent processes at 37 °C and pH values ranging from 0.5 to 12.[46]

Based on the inertness of Te-AdoMet demonstrated in the decay studies, it was assumed that Te-AdoMet cannot function as a cosubstarte for the activation of PFL by PFL-AE as AdoMet does. If no turnover occurs, in the presence of Te-AdoMet, various interactions can be probed in the ternary complex PFL-AE [4Fe-4S]$^{1+}$/ PFL/ Te-AdoMet by ENDOR spectroscopy. Sample with labeled Te-AdoMet could be used to detect the interaction between [4Fe-4S]$^{1+}$ and Te-AdoMet in presence of the substrate PFL. Sample with labeled PFL could probe the interaction between

$[4Fe\text{-}4S]^{1+}$ and the substrate PFL, which cannot be done with regular AdoMet due to the turnover. Features of the ternary complex PFL-AE/PFL/Te-AdoMet, together with information from complex PFL-AE/PFL(G734A)/AdoMet would provide comprehensive insight into the activation process of PFL.

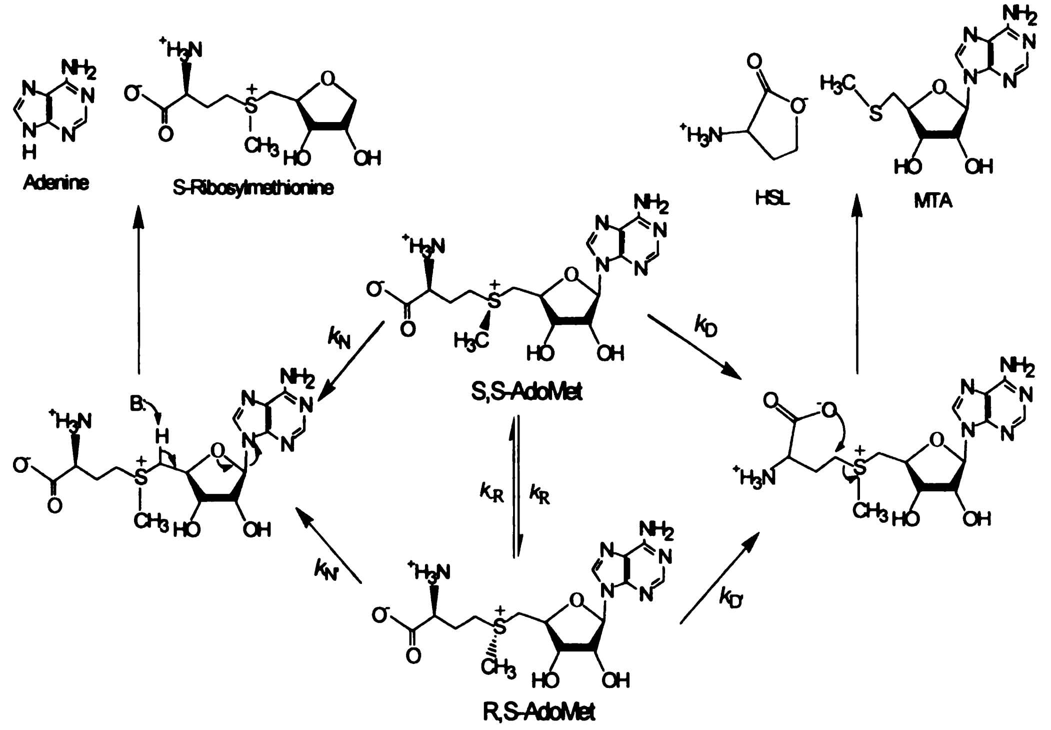


Figure IV.1 Three degradation pathways of AdoMet

IV.2 Experimental methods

IV.2.1 Materials

Te-AdoMet was obtained as a gift from Dr. Squire Booker. It came as sulfate salt, dissolved in water and stored at -80 °C. 5-Deazariboflavin was synthesized in our laboratory. All other chemicals were obtained commercially and used as received.

IV.2.2 Activity assay of wild-type PFL with Te-AdoMet

Activity assay was performed as described in Chapter II, section II.2.6. Two samples were prepared and test simultaneously: one with Te-AdoMet, the other with AdoMet for control. To assay PFL's activity, 695 μL of coupling mix and 5 μL of the activated PFL_i were combined in a quartz cuvette, the production of NADH was monitor by UV-vis at 340 nm.

IV.2.3 EPR Spectroscopy of ternary complex PFL/PFL-AE/Te-AdoMet

EPR first-derivative spectra were obtained at X-band on a Bruker ESP300E spectrometer equipped with a liquid He cryostat and a temperature controller from Oxford Instruments. Spectra were recorded at 12 K for $[3Fe\text{-}4S]^{1+}$ and $[4Fe\text{-}4S]^{1+}$, and at 60 K to detect glycyl radical. Spin concentrations in the protein samples were determined by calibrating double integrals of the EPR spectra recorded under nonsaturating conditions (i) with a standard sample of 0.1 mM Cu(II) and 1 mM EDTA solution for the cluster signals, or (ii) with a 1.04 mM $K_2(SO_3)_2NO$ solution for the glycyl radical signals.

To prepare an EPR sample of the ternary complex PFL/PFL-AE/Te-AdoMet, all procedures were carried out anaerobically in an inert atmosphere glove box (Mbraun) with O_2 level ≤ 3 ppm. All buffers, solvents and bath water were deoxygenated using a vacuum/inert gas manifold before being taken into the glove box. Solid chemicals were pumped in as solids. Ice was pre-chilled with liquid nitrogen before pumping into the glove box.

A PFL-AE photoreduction mix was prepared first in an eppendorf to a volume of 200μL with the following: Tris (50 mM, pH 8.1), 5'-deazariboflavin (100 μM), isolated PFL-AE (200 μM), DTT (1 mM), glycerol (20%, w/v). The sample was then transferred to an EPR tube. The EPR tube was capped and inserted in a beaker tightly packed with ice-water. The sample was illuminated on ice using a 500 W halogen lamp for 30 min with the lamp situated about 5 cm from the beaker. After illumination, two equivalents of Te-AdoMet (21.4 mM in sulfate solution) were added. Lastly, 200 μL of inactive PFL_i mix was added to the EPR tube with the following components: PFL (200 μM), Tris (1M, pH 8.1), 5'-deazariboflavin (100 μM), oxamate (20 mM), DTT (1 mM), glycerol (20%, w/v). The tube was then brought out of the glove box, and stored in liquid nitrogen for EPR.

IV.3 Results and Discussion

IV.3.1 Activity assay of wild-type PFL with Te-AdoMet

Activity assay of wild type PFL with Te-AdoMet was performed in an analogous manner as done for AdoMet. Unexpectedly, sample of Te-AdoMet showed PFL activity as well as AdoMet. 45 min after PFL's activation, units of PFL_a in the cuvette: for AdoMet, 0.36 units; for Te-AdoMet, 0.04 units. These values were calculated according to Equation (1) in Chapter II, section II.2.6. Since the purpose of this experiment is to test whether Te-AdoMet can act as a cosubstrate, not to quantify the specific activity of PFL, readings were not continued until saturation. This results indicates though Te-AdoMet was inert in the three major degradation ways, it is readily reductively cleaved by PFL and PFL-AE. The following EPR spectra further confirmed that Te-AdoMet can act as cosubstrate for PFL/PFL-AE.

IV.3.3 EPR of ternary complex PFL/PFL-AE/Te-AdoMet

EPR spectra of PFL/PFL-AE/Te-AdoMet were recorded at 12 K (Figure IV.2) and 60 K (Figure IV.3). If PFL-AE was fully photoreduced to $[4Fe\text{-}4S]^{1+}$ and all being consumed to activate PFL in the presence of Te-SAM, no EPR signal should be detected at 12 K (since only EPR-silent $[4Fe\text{-}4S]^{2+}$ would be present at that time). Therefore, the observed signal ($g = 2.01, 1.92, 1.88$) in the EPR spectrum at 12 K is probably due to incomplete reduction of PFL-AE. Compared to the nearly axial signal ($g = 2.01, 1.88, 1.87$) for photoreduced PFL-AE in the presence of SAM,[34] the deviation of signal in Figure IV.2 may come from different binding interaction of PFL-AE with Te-AdoMet. Or it may also be a result of PFL's presence, since PFL is expected to have close association with PFL-AE and AdoMet as well.

Spectrum at 60 K (Figure IV.3) exhibited a signal typical of glycyl radical ($g = 2.00$, a doublet splitting as seen in Figure IV.4). Due to high noise and low concentration, the signal was not sharp as usual, yet it confirmed the activation of PFL in the presence of Te-AdoMet.

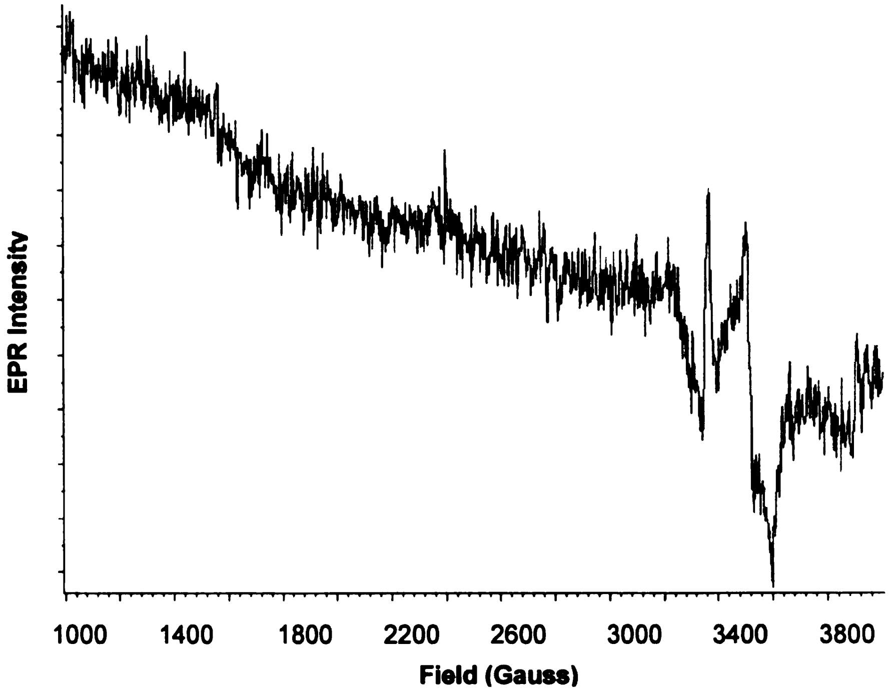


Figure IV.2 X-band EPR spectrum of ternary complex PFL/PFL-AE/Te-AdoMet. g values: 2.01, 1.92, 1.88. Conditions of measurement, T=12K; microwave frequency, 9.47 GHz; modulation amplitude, 10.084 G; modulation frequency, 100 KHz, 3 scans.

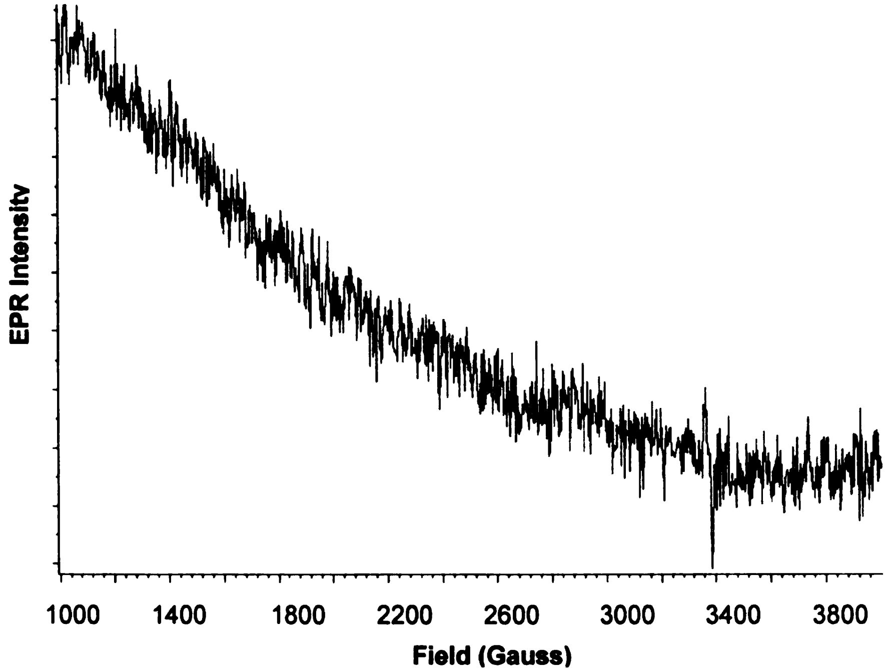


Figure IV.3 X-band EPR spectrum of ternary complex PFL/PFL-AE/Te-AdoMet. g values:2.00. Conditions of measurement, T=60K; microwave frequency, 9.47 GHz; modulation amplitude, 10.084 G; modulation frequency, 100 KHz, 3 scans.

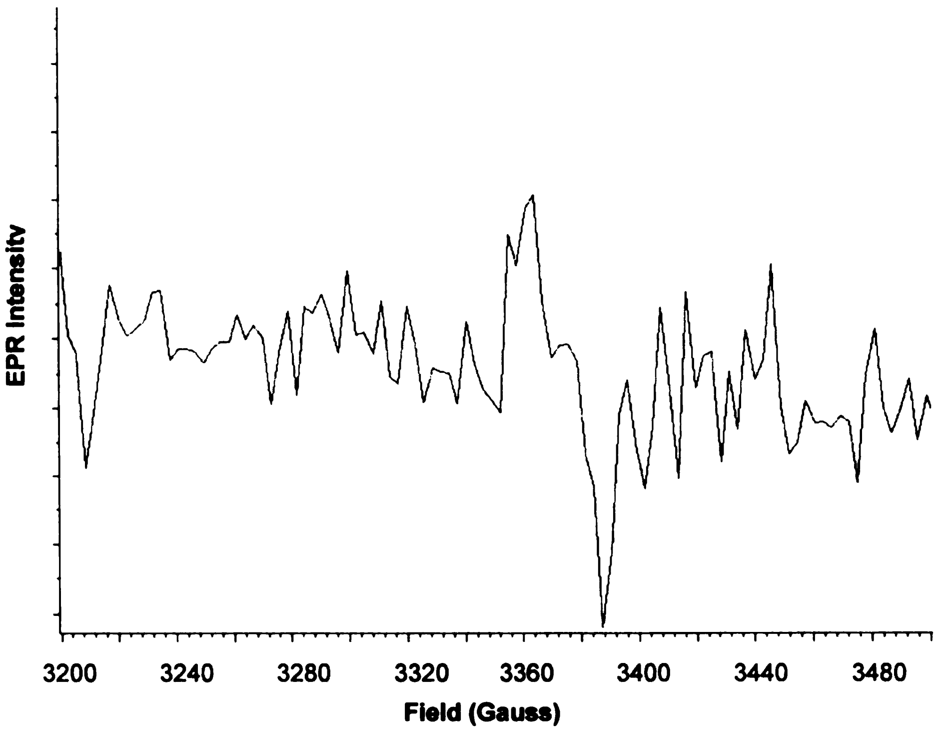


Figure IV.4 Blow-up of Figure IV.3 at the signal site. Details of the doublet splitting can be seen from field 3200 Gauss to 3500 Gauss.

CHAPTER V

CONCLUSIONS

To probe one central question in the activation of pyruvate formate-lyase--how PFL-AE recognizes glycine 734, the active site of PFL, one mutant PFL(G734A) in plasmid pKK has been successfully overexpressed from *E. coli.* The enzyme was purified by three chromatography methods in the order of ion exchange, gel filtration and hydrophobic interaction. Purified mutant was used to perform the activity assay of PFL. Unexpectedly, activity of 0.685 unit/mg was observed, which is approximately 0.5% of the wild-type value. Since mutant PFL(G734A) is presumably inactive, the weak activity observed is very likely due to trace amount of wild-type PFL remained, which is difficult to be removed via normal purification methods.

In order to obtain more pure PFL(G734A), several different attempts have been made. One is to clone PFL(G734A) gene into plasmids with an N-terminal His-tag, subsequently the mutant can be separated from wild-type via affinity chromatography. Another method is the usage of knockout strain of *E. coli,* in which wild-type PFL gene had already been deleted, thus transformation of mutant gene PFL(G734A) into knockout strain will express only the mutant PFL(G734A). Difficulties were encountered in the first method. PFL(G734A) with His-tag attached cannot be purified successfully. In the second method, further effort needs to be taken to make the cells chemically competent.

One analogue to the cosubstrate AdoMet, Te-AdoMet, was presumably inert for the activation of PFL. However, both activity assays of Te-AdoMet and EPR spectra of ternary complex PFL-AE/Te-AdoMet/PFL demonstrated that Te-AdoMet can function as a cosubstrate for the activation of PFL by PFL-AE.

Future work is directed to the preparation of PFL(G734A) from the knockout strain of *E. coli*. The purified mutant will be used to perform activity assay, EPR, ENDOR of the ternary complex to probe the interaction among PFL-AE, AdoMet and PFL, ultimately elucidate the radical generation process.

Reference

1. Conradt, H., Hohmann-Berger, M., Hohmann, H.-P, Blasch-kowski, H. P., Kanppe, J., *Arch. Biochem. Biophys.* **1984,** 228, 133-142.

2. Knappe, J., Neugebauer, F. A., Blaschkowski, H. P., and Ganzler, M., *Proc. Natl. Acad. Sci.* **1984,** 81, 1332-1335.

3. Wagner, A. F., Frey, M., Neugebauer, F. A., Schafer, W., Knappe, J., *Proc. Natl. Acad. Sci.* **1992,** 89, 996-1000.

4. Unkrig, V., Neugebauer, F. A., Knappe, J., *Eur. J. Biochem.* **1989,** 184, 723-728.

5. Reddy, S. G., Wong, K. K., Parast, C. V., Peisach, J., Magliozzo, R. S., and Kozarich, J. W., *Biochemistry* **1998,** 37, 558-563.

6. Parast, C. V., Wong, K. K., Lewisch, S. A., and Kozarich, J. W., *Biochemistry* **1995,** 34, 2393-2399.

7. Knappe, J., Blaschkowski, H. P., *Methods Enzymol.* **1975,** 41.

8. Knappe, J., Schmitt, T., *Biochem. Biophys. Res. Commun.* **1976,** 71, 1110-1117.

9. Frey, M., Rothe, M., Wagner, A. F., Knappe, J., *J. Biol. Chem.* **1994,** 269, 12432-12437

10. Broderick, J. B., Duderstadt, R. E., Fernandez, D. C., Wojtuszewski, K., Henshaw, T. F., and Johnson, M. K., *J. Am. Chem. Soc.* **1997,** 119, 7396-7397.

11. Broderick, J. B., Henshaw, T.F., Cheek,J., Wojtuszewski, K., Trojan,M. R., McGhan, R., Smith, S. R., Kopf, A., Kibbey, M., Broderick,W. E., *Biochem. Biophys. Res. Commun.* **2000,** 269, 451-456.

12. Krebs, C., Henshaw, T. F., Cheek, J., Huynh, B. H., Broderick, J. B., *J. Am. Chem. Soc.* **2000,** 122, 12497-12506.

13. Henshaw, T. F., Cheek, J., and Broderick, J. B., *J. Am. Chem. Soc.* **2000,** 122, 8331-8332.

14. Kulzer, R., Pils, T., Kappl, R., Huttermann, J., Knappe, J., *J. Biol. Chem.* **1998,** 273, 4897-4903.

15. Rodel, W., Plaga, W., Frank, R., and Knappe, J., *Eur. J. Biochem.* **1988,** 177, 153-158.

16. Sun, X., Eliasson, R., Pontis, E., Andersson, J., Buist, G., Sjoeberg, R.-M., Reichard, P., *J. Biol. Chem.* **1995,** 270, 2443-2446.

17. Hayden, M. A., Huang, I., Bussiere, D. E., and Ashley, G. W., *J. Biol. Chem.* **1992,** 267, 9512-9515.

18. Fajardo-Cavazos, P., Salazar, C., and Nicholson, W. L., *J. Bacteriology* **1993,** 175, 1735-1744.

19. Ruzicka, F. J., Lieder, K. W., and Frey, P. A., *J. Bacteriology* **2000,** 184, 469-476.

20. Beinert, H., Kennedy, M. C., and Stout, C. D., *Chem. Rev.* **1996,** 96, 2335-2374.

21. Harrison, P. M., and Arosio, P., *Biochem. Biophys. Acta* **1996,** 1275, 161-203.

22. Khoroshilova, N., Popescu, C., Munck, E., Beinert, H., and Kiley, P. J., *Proc. Natl. Acad. Sci.* **1997,** 94, 6087-6092.

23. Hidalgo, E., Ding, H. G., and Demple, B., *Trends Biochem. Sci.* **1997,** 22, 207-210.

24. Leuthner, B., Leutwein, C., Schulz, H., Horth, P., Haehnel, W., Schlitz, E., Schagger, H., and Heider, J., *Molecular Microbiology* **1998,** 28, 615-628.

25. Ollagnier, S., Mulliez, E., Schmidt, P. P., Eliasson, R., Gaillard, J., Deronzier, C., Bergman, T., Graslund, A., Reichard, P., and Fontecave, M., *J. Biol. Chem.* **1997,** 272, 24216-24223.

26. Lieder, K. W., Booker, S., Ruzicka, F. J., Beinert, H., Reed, G. H., and Frey, P. A., *Biochemistry* **1998,** 37, 2578-2585.

27. Duin, E. C., Lafferty, M. E., Crouse, B. R., Allen, R. M., Sanyal, I., Flint, D. H., and Johnson, M. K., *Biochemistry* **1997,** 36, 11811.

28. Busby, R. W., Schelvis, J. P. M., Yu, D. S., Babcock, G. T., and Marletta, M. A., *J. Am. Chem. Soc.* **1999,** 121, 4706.

29. Ollagnier-de Choudens, S., and Fontecave, M., *FEBS Letters* **1999,** 453, 25.

30. Rebeil, R., Sun, Y., Chooback, L., Pedraza-Reyes, M., C., K., Begley, T. P., Nicholson, W. L., *J. Bacteriology* **1998,** 180, 4879-4885.

31. Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F., and Miller, N. E.,

Nucleic Acids Research **2001,** 29, 1097-1106.

32. Cheek, J. B., J. B., *J. Biol. Inorg. Chem.* **2001,** 6, 209-226.

33. Krebs, C., Broderick, W.E., Henshaw, T. F., Broderick, J. B., Huynh, B. H., *J. Am. Chem. Soc.* **2002,** 124, (6), 912-913.

34. Walsby, C. J., Hong, W., Broderick, W. E., Cheek, J., Ortillo, D.,Broderick, J. B., Hoffman, B. M., *J. Am. Chem. Soc.* **2002,** 124, 3143-3151.

35. Walsby, C. J., Ortillo, D., Broderick, W.E., Broderick, J.B., Hoffman, B.M., *J. Am. Chem. Soc.* **2002,** 124, 11270-11271.

36. Hoffman, B. M., *Acc. Chem. Res.* **2003,** 36, 522-529.

37. Walsby, C. J., Ortillo, D., Yang, J., Nnyepi, M. R., Broderick, W. E., Hoffman, B. M., Broderick, J. B., *Inorg. Chem.* **2005,** 44, 727-741.

38. Buis, J. M., Broderick, J. B., *Arch. Biochem. Biophys.* **2005,** 433, 288-296.

39. Beinert, H., Holm, R. H., Munck, E., *Science* **1997,** 277, 653-659.

40. Ruzicka, F. J., Lieder, K. W., Frey, P.A., *Journal of Bacterioloy* **2000,** 184, 469-476.

41. Bradford, M., *Analytical Biochemistry* **1976,** 72, 248.

42. Herring, C. D., Glasner, J. D., Blattner, F. R., *Gene* **2003,** 331, 153-163.

43. Sambrook, J., Frtitsch, E. F., Maniatis, T., Molecular Cloning: A Laboratory Manual. **1989.**

44. Novagen, pET vector manuals.

45. Cohen, S. N., *Proc. Natl. Acad. Sci.* **1972,** 69, 2110.

46. Iwig, D. F., Booker, S. J., *Biochemistry* **2004,** 43, 13496-13509.