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Identification and characterization of dihydrolipoamide dehydrogenase deficiency

Liu, Te-Chung, Ph.D.

Case Western Reserve University (Health Sciences), 1994
IDENTIFICATION AND CHARACTERIZATION OF DIHYDROLIPOAMIDE DEHYDROGENASE DEFICIENCY

by

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Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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*We also certify that written approval has been obtained for any proprietary material contained therein.
IDENTIFICATION AND CHARACTERIZATION OF DIHYDROLIPOAMIDE DEHYDOGENASE DEFICIENCY

Abstract

by

TE-CHUNG LIU

This study involved identification of two missense mutations in an E3-deficient patient, characterization of the kinetic and physical properties of these two missense E3 proteins and two other active base missense mutant proteins which were created by site-directed mutagenesis.

Fibroblasts cultured from the patient contained about 6% of the E3 activity of cells from a normal subject. Western and Northern blot analyses indicated that the patient's cells had a reduced amount of protein but normal amounts of E3 mRNA when compared to those of control cells. Direct sequencing of E3 cDNA derived from the patient's RNA as well as each subclone of the cDNA revealed that the patient had two substitution mutations in the E3 coding region. One identified mutation changed a single nucleotide from A to G, resulting in the substitution of E (GAA) for K-37 (AAA) (E3 K-37 to E). The other point mutation was a nucleotide change from C to T, resulting in the substitution of L (CTG) for P-453 (CCG) (E3 P-453 to L).

The specific activity of purified recombinant E3 K-37 to E was decreased to 50% and recombinant E3 P-453 to L was decreased to 0.04% when compared to the wild type E3. Kinetic studies of the E3 K-37 to E showed that the reaction mechanism
of this mutant protein followed the 'Ping Pong' mechanism. The $K_m$ for
dihydrolipoamide and NAD$^+$ remain the same as E3, however the $K_{cat}$ decreased to
50% when compared to E3. The chemical and physical properties of E3 K-37 to E and
E3 P-453 to L together with E3 E-452 to Q and E3 H-457 to Q were analyzed.
Molecular sieving showed that the dimerization of E3 K-37 to E, E3 E-457 to Q, and
E3 H-452 to Q was not affected by the mutation, while only 50% of E3 P-453 to L was
in the dimer form. Quantitation studies of FAD showed that the amount of FAD of the
E3 E-457 to Q, and E3 H-452 to Q decreased by 10%, E3 K-37 to E decreased by
30% while E3 P-453 to L decreased by 70%. UV-visible, Fluorescence and CD
spectral represented the lower amount of FAD of E3 K-37 to E, and E3 P-453 to L.
The E3 H-452 to Q showed slow reduction of FAD without formation of NADH. The
E3 E-457 to Q showed that the smaller peak at 510 nm and the formation of NADH at
peak 460 nm was maintained with a slow decreasing rate. The CD spectra also showed
that the four mutations decreased the $\alpha$-helix and $\beta$-sheet content and caused the
conformation change of the E3 mutant protein.
Dedication

This work is dedicated to my father, Pin-Hsiung Liu, for his endless love of science, and to my wife and daughter for their continuous support.

"Science consists of concise descriptions, called general laws, of the recurrences of phenomena. In kinship study the phenomena are kinterms like father in English or bapa in the Murngin language in northwestern Australia, and the recurrences take the following form. In English the kinterm uncle for father's brother recurs for mother's brother, but in Murngin bapa for father's brother recurs for father and not for mother's brother (gawel). In English the term son applied to a male child by his father recurs as the term applied to the same child by his mother, but in Murngin gatu (father's child) recurs for brother's child (English nephew), and not for mother's child (waku). Then the general laws take the form of 'equivalence rules', by which the recurrences are concisely described."

Pin-Hsiung Liu

Foundations of Kinship Mathematics
Acknowledgment

To all friends whom I met in the United States.
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List of Abbreviations

AD: Asparaginate dehydrogenase
ApyAD: Acetylpyridine adenine dinucleotide
BCKADC: Branched-chain α-keto acid dehydrogenase complex
CD: Circular dichroism
CoA: Coenzyme A
DHL: Dihydrolipoamide
DMSO: Dimethylsulfoxide
DTT: Dithiothreitol
E₁: α-keto acid dehydrogenase
E₂: Dihydrolipoyl acyltransferase
E₃: Dihydrolipoamide dehydrogenase
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethyleneglycol-bis-(β-amino-ethylether) N,N'-tetraacetic acid
FAD: Flavin adenine dinucleotide
FCS: Fetal calf serum
GCS: Glycine cleavage system
GR: Glutathione reductase
GrR: Glutaredoxin
GSH:DT: GSH:disulfide transhydrogenase
GSR: Bis-γ-glutamylcystine reductase
H-protein: Hydrogen carrier protein
Hepes: 4-(2-hydroxyethyl)-1-Piperazine ethanesulphonic acid
KGDC: α-ketoglutarate dehydrogenase complex
L-protein: Lipoamide dehydrogenase
PDC: Pyruvate dehydrogenase complex
P-protein: Glycine dehydrogenase
PBS: Phosphate buffered saline
PMSF: Phenylmethyl sulfonylfluoride
SDS: Sodium dodecylsulfate
TCA: tricarboxylic acid
TFA: Trifluoroacetic acid
TEMED: N,N,N',N'-Tetramethylenediamine
TPP: Thiamine pyrophosphate
T-protein: Aminomethyltransferase
Tris: Tris(hydroxymethyl)aminomethane
TR: Thioredoxin reductase
TrR: Thioredoxin
TTR: Typanothione reductase
RR: Ribonucleotide reductase
U: International unit (μmol/min)
Chapter I
Introduction

A. General Introduction of dihydrolipoamide dehydrogenase (E3)

1. The family of pyridine nucleotide-disulfide oxidoreductases

E3 belongs to the enzyme family of pyridine nucleotide-disulfide oxidoreductases. The enzymes in this family have an active disulfide, an active base, and FAD to transfer electrons between the electron acceptor (NAD$^+$ or NADP$^+$) and a dithiol substrate in the active site. Williams et al. (1) have characterized the electron transfer from the active disulfide to pyridine nucleotides (FAD vs NAD$^+$ or NADP$^+$) of E3, glutathione reductase (GR) and thioredoxin reductase (TR) by pyridine nucleotide-disulfide oxidoreductase. Williams et al. (1) purified E3, GR and TrR from *Escherichia coli* B and studied their physical and chemical properties. The other members of the pyridine nucleotide-disulfide oxidoreductases family include mercuric ion reductase (MR), trypanothione reductase (TTR), asparagusate dehydrogenase (AD) and bis-$\gamma$-glutamylcystine reductase (GSR). The background information of the enzymes mentioned above are described below.

2. Dihydrolipoamide dehydrogenase (E3)

Straub (2) purified a flavoprotein from pig heart muscle based on the following physical properties of the protein: it precipitated at the pH 4.6, and between 50 to 65 % ammonium sulfate saturation. It was insoluble in 3 % alcohol, was heat stable at 43 °C for 10-15 minutes, and absorbed with aluminum hydroxide. This protein has FAD as its prosthetic group and oxidizes NADH to NAD$^+$. Therefore, the protein is named diaphorase or coenzyme factor. The first absorption spectrum of the oxidized diaphorase was published by Straub in 1939 (2). In 1958, Massey (3) found that the diaphorase oxidized NADH by transferring an electron to lipoic acid to form dihydrolipoic acid. Therefore, diaphorase was renamed as lipoyl dehydrogenase (4-5).
Massy (6) later found that lipoyl dehydrogenase also is a component of α-ketoglutarate dehydrogenase complex (KGDC). The KGDC oxidizes α-ketoglutarate; resulting in the release of CO2; the transfer of a succinyl group to CoASH; and the concomitant reduction of NAD+. Therefore, lipoyl dehydrogenase catalyzes a reversible oxidoreduction reaction of lipoamide-NADH couple. Under physiological conditions, the lipoyl dehydrogenase oxidizes dihydrolipoamide by the formation of lipoamide and reduction of NAD+. Today, we call this flavoprotein dihydrolipoamide dehydrogenase or E3.

E3 contains both an active disulfide and FAD that catalyzes the following reaction:

\[ \text{Dihydrolipoamide} + \text{NAD}^+ \leftrightarrow \text{Lipoamide} + \text{NADH} + \text{H}^+ \]

The study of E3 kinetics (7) showed that the two substrates of E3 follow the 'Ping Pong' mechanism. In this model the substrate dihydrolipoamide comes in, the product lipoamide goes out, the substrate NAD⁺ comes in and the product NADH goes out. In the entire reaction, FAD is reduced to FADH and the FADH is reoxidized to FAD again.

3. Glutathione reductase (GR)

GR activity was discovered in the pig liver extract. GR catalyzes the oxidation of NADPH and the formation of NADP⁺ with the reduction of glutathione (GSSG) and the formation of reduced glutathione (GSH) (8). GR was accidentally purified by Asnis et al. from the Warburg-Christian system (9). These investigators were attempting to purify a NADP-linked reductase which reduced Furacin (5-nitro-2-furaldehyde semicarbazone). Since that time, GR has been purified from several other sources, including Escherichia coli (1 and 9), yeast (10), germinated peas (11), rat liver (12) and human erythrocyte (13). Like E3, the kinetics of GR is a 'Ping Pong'
mechanism (11 and 14) and the GR also contains an active disulfide and FAD which catalyze the reaction shown below:

\[ \text{NADPH} + \text{GSSG} \rightleftharpoons \text{NADP}^+ + 2 \text{GSH} \]

The \( K_m \) for NADPH is 4.7 uM, the \( K_m \) for GSSG is 17 uM, and the \( V_{\text{max}} \) is \( 1.6 \times 10^4 \) moles of NADPH oxidized/min/mole of flavin (or \( K_{\text{cat}} = 267 \) S\(^{-1}\)) (15). Under physiological conditions, GSH maintains the reduced form of other thiol groups, i.e., glutaredoxin (GrR), GSH:disulfide transhydrogenase (GSH: DT), and ribonucleotide reductase (RR) (Figure 1). The glutathione reductase system includes three enzymes (GR, GSH:DT, and RR) and 4 substrates (NADPH, GSH, GrR, and ribonucleotide diphosphate [rNDP]). GR reduces GSSG forming GSH; GSH maintains the reduced state of GrR by GSH:DT; and RR catalyzes the synthesis of deoxyribonucleotide diphosphate (dNDP) from rNDP (9). The dNDP is further phosphorylated to the deoxyribonucleotide triphosphate (dNTP) by nucleotide diphosphate kinase. The reactions catalyzed by the glutathione reductase system as described below (15-16).

\[ \text{NADPH} + H^+ + \text{GSSG} \rightleftharpoons \text{NADP}^+ + 2 \text{GSH} \]
\[ 2\text{GSH} + \text{GrS}_2 \rightleftharpoons \text{GSSG} + \text{Gr(SH)}_2 \]
\[ \text{Gr(SH)}_2 + \text{rNDP} \rightleftharpoons \text{dNDP} + \text{GrS}_2 + \text{H}_2\text{O} \]
\[ \text{dNDP} + \text{ATP} \rightleftharpoons \text{dNTP} + \text{ADP} \]

4. Thioredoxin reductase (TR)

Laurent et al. (17) isolated the thioredoxin system which includes thioredoxin reductase (TR) and thioredoxin (TrR). Furthermore, Laurent et al. studied the synthesis of deoxyribonucleotides triphosphate (dNTP's) by the thioredoxin system. TR has an active disulfide and FAD that catalyzes the first step of the reaction, which is described below.

\[ \text{TrRS}_2 + \text{NADPH} + H^+ \rightleftharpoons \text{TrR(SH)}_2 + \text{NADP}^+ \]
The biological function of TrR is dependent on the presence of a disulfide bond. In *Escherichia coli*, TrR has a -C-G-P-C- disulfide as its active center (18). The thioredoxin-dependent reduction of rNDP uses two enzymes, TR and RR, and 3 substrates, TrS₂, NADPH and NDP to catalyze the synthesis of dNDP from rNDP. The reactions catalyzed by TR and RR are described below (16-18).

\[
\text{TrS}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Tr(SH)}_2 + \text{NADP}^+
\]

\[
\text{NDP} + \text{Tr(SH)}_2 \rightleftharpoons \text{dNDP} + \text{TS}_2
\]

5. **Mercuric ion reductase (MR)**

Mercuric ion reductase (MR)[EC. 1. 16. 1. 1.] (19) catalyzes the reduction of mercuric ion (Hg(II)) in the following detoxification reaction.

\[
\text{Hg(SR)}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Hg}^0 + \text{NADP}^+ + 2\text{RSH}
\]

The mercury detoxification system is encoded by the *mer* operon in Tn 501. The *mer* operon includes a specific Hg(II) uptake system that is encoded by *merP* and *merT* genes and reductase that is encoded by *merA* gene (20). The mercuric reductase (MR) has an active disulfide and FAD cofactor, and the enzyme belongs to the pyridine nucleotide disulfide oxidoreductase family. Besides the active disulfide, the MR has another C-terminal disulfide pair. The active disulfide, the C-terminal disulfide (from the other polypeptide chain), and FAD participate in reduction of Hg(II) to the less toxic form Hg⁰(21).

6. **Trypanothione reductase (TTR)**

Trypanothione reductase [EC. 1. 6. 4. 8.] (22-23), found in trypanosomatid parasites, reduces trypanothione (marcyclic disulfide N¹,N⁸-bis [gluthionyl] spermidine). The trypanosomatids have no glutathione reductase enzyme system. Therefore, the trypanothione/trypanothione reductase system provides protection against oxidative stress. The reaction is described below.

\[
\text{TS}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{T(SH)}_2 + \text{NADP}^+
\]
7. Asparaguate dehydrogenase (AD)
Asparaguate dehydrogenase (24) is found in asparagus. It catalyzes the reduction of asparagusic acid (AsgS₂). The reaction is listed below.

\[ \text{AsgS}_2 + \text{NADH} + H^+ \leftrightarrow \text{Asg(SH)₂} + \text{NAD}^+ \]

Asparagusic acid resembles that of lipoic acid (see Table 1). Asparaguate dehydrogenase's physiological function is considered to be the oxidation of protein-bound dihydrolipoic acid with \( \text{NAD}^+ \) as electron acceptor and may serve as one of the components of \( \alpha \)-ketoacid dehydrogenase complexes.

8. Bis-\( \gamma \)-glutamylcystine reductase (GSR)

Bis-\( \gamma \)-glutamylcystine reductase (25) is found in halophilic archaebacterium *Halobacterium halobium*. Halobacteria do not contain glutathione but have \( \gamma \)-glutamylcysteine (\( \gamma \)-Glu-Cys) instead. The bis-\( \gamma \)-glutamylcystine reductase maintains the chemically reduced state of *Halobacterium halobium*. The substrates of the pyridine nucleotide-disulfide oxidoreductases are listed in Table 1.

B. The characteristics of E₃

Mammalian dihydrolipoamide dehydrogenase [EC 1.8.1.4] catalyzes the transfer of electrons from dihydrolipoamide to \( \text{NAD}^+ \). The catalytic mechanism involves an active disulfide and an active base in E₃. E₃ is a common component of pyruvate dehydrogenase (PDC), \( \alpha \)-ketoglutarate dehydrogenase (KGDC), and branched-chain \( \alpha \)-keto acid dehydrogenase complexes (BCKADC) (26-28). The sequential reactions catalyzed by the \( \alpha \)-ketoacid dehydrogenase complex are shown in Figure 2. E₃ is the same in the above enzyme complexes in mammals as supported by the following evidence: E₃ is interchangeable between PDC and KGDC (29); the antibody against pig heart E₃ cross reacts with PDC, KGDC and BCKADC (30); E₃ is a single copy gene with one isolated cDNA clone (31, 33). Recently, pea leaf mitochondrial E₃ showed that it is also a common component of the L-protein of the
glycine cleavage system (Figure 3). The evidence demonstrates that the polyclonal antibodies raised against the L-protein of the glycine cleavage system also cross reacts with E3 of the α-ketoacid dehydrogenase. Using L-protein cDNA as a probe, the Northern analysis of pea leaf, stem root and seed RNA shows that the E3 mRNA is expressed at high levels in all tissues, whereas, the H-protein is expressed primarily in leaves. Southern analysis indicates that the L-protein gene is a single copy in the genome (34).

The cDNA of human E3 has been cloned by Otulakowski et al. (32) and Pons et al. (33). The cDNA contains a 1527 bp open reading frame and codes for 509 amino acid residues. The first 35 amino acid residues are the mitochondrial leader sequence. The mature E3 has 474 amino acid residues with molecular mass of 50919 daltons. The 3' untranslated region of the E3 cDNA has two polyadenylation sites, 234 bp apart. The E3 mRNA reflects the alternative splicing, resulting in two mRNA species of 2.2 and 2.4 kb in length. The human E3 is a single copy gene located on chromosome #7 (31).

The cDNA sequence of the E3 has been identified in eucaryotes, eubacteria and archaeabacteria. In eucaryotes, the E3 sequence has been determined in human liver (33), human small cell carcinoma (32), pig adrenal medulla (32), pea leaf (34), Trypanosoma brucei (35) and yeast (36). In eubacteria, E3 cDNA sequence has been identified in Escherichia coli (37), Azotobacter vinelandii (38), Bacillus stearothermophilus (39), Bacillus subtilis (40), Pseudomonas fluorescens (41), Pseudomonas putida (LPD-Val, LPD-glc, and LPD-3) (42-44), and Staphylococcus aureus (45). In archaeabacteria, the E3 cDNA sequence has been reported for Haloferax volcanii (46).

Archaeabacteria, Trypanosoma brucei, cyanobacteria and glycine-utilizing bacteria have no α-ketoacid dehydrogenase complexes. The pyruvate and α-
Ketoglutarate are converted to acyl-CoA by pyruvate (or α-ketoglutarate):ferredoxin oxidoreductase (47) in all of those organisms except for *Trypanosoma brucei*. *Trypanosoma brucei* can not utilize pyruvate, but uses the glycolytic pathway.

Pyruvate is excreted into the host's blood stream, after glucose is utilized in the glycolytic pathway (48). Pyruvate (or α-ketoglutarate):ferredoxin oxidoreductase catalyzes the thiamin diphosphate-dependent decarboxylation reaction of the α-ketoacids. One electron per molecule of substrate is transferred to the enzyme bound iron-sulfur cluster and the ion-sulfur cluster then reduces ferredoxin. Therefore, the existence of E3 may imply another function beside α-ketoacid dehydrogenase complexes activity.

In aerobic eubacteria and eukaryotes, E3 re-oxidize dihydrolipoamide residues reduced in the formation of acyl-CoA. In archaebacteria (49), *Escherichia coli* (50), *Trypanosoma brucei* (51) and 3T3-L1 adipocytes (52), a protein with a similar function to E3 has been identified but it is bound to the plasma membrane or cell membrane. The enzyme may be involved in transporting solutes into and out of the cell, as supported by *Escherichia coli* deficient in lipoic acid synthesis. This strain can grow in minimal medium supplemented with acetate and succinate. Lactose permease and phosphoenol pyruvate-glucose phosphotransferase are unaffected by this lipoic acid deprivation, but the binding-protein-dependent transport of ribose, galactose and maltose is severely reduced. Furthermore, when lipoic acid is supplied in the growth media, these lipoic acid dependent transporters are completely inhibited by the dithiol-specific arsenite (50). A new E3 activity was detected in a strain of *Escherichia coli*, containing a deletion for the *lpd* gene which codes for the E3 common to the PDC, KGDC and GCS (53). In 3T3-L1 adipocytes, the E3 activity is based on the phenylarsine oxide inhibition which is similar to E3 and the inhibition block of insulin-activated hexose transport by 3T3-L1 adipocytes.
From the evolutionary point of view, E3 diverges at the level of kingdoms (eubacteria, archaebacteria and eucaryote). The archaebacteria contains a ferredoxin oxidoreductase analog which uses F420 as an electron acceptor. The eucaryote has undergone symbiosis with a respiratory bacterium (the ancestor of mitochondria) resulting in machinery for oxidative phosphorylation and α-ketoacid dehydrogenase complexes (54).

In *Clostridium litoralis* and *Eubacterium acidaminophilum*, the glycine cleavage system involves a thioreductase-like E3 to catalyze the following reactions (55).

\[
\text{Dihydrolipoamide } + \text{ TrS}_2 \rightleftharpoons \text{ Lipoamide } + \text{ Tr(SH)}_2
\]

\[
\text{Tr(SH)}_2 + \text{ NADP}^+ \rightleftharpoons \text{ TrS}_2 + \text{ NADPH } + \text{ H}^+
\]

The overall reaction is:

\[
\text{Dihydrolipoamide } + \text{ NADP}^+ \rightleftharpoons \text{ Lipoamide } + \text{ NADPH } + \text{ H}^+
\]

In addition, through the use of seleno protein PA, the glycine reductase and glycine cleavage can integrate together for glycine catabolism. The reaction equations are described as below.

\[
\text{Glycine } + \text{ THF } + \text{ R(S)}_2 \rightleftharpoons \text{ CH}_2=\text{THF } + \text{ CO}_2 + \text{ NH}_3 + \text{ R(SH)}_2
\]

\[
\text{CH}_2=\text{THF} + \text{ 2 NAD(P) + ADP + Pi } \rightleftharpoons \text{ THF } + \text{ CO}_2 + \text{ 2 NAD(P)H } + \text{ ATP}
\]

\[
\text{Glycine } + \text{ R(SH)}_2 + \text{ ADP } + \text{ Pi } \rightleftharpoons \text{ Acetate } + \text{ NH}_3 + \text{ ATP } + \text{ R(S)}_2
\]

The overall reaction is:

\[
\text{2 Glycine } + \text{ 2 NAD(P) } + \text{ 2 ADP } + \text{ 2 Pi } \rightleftharpoons \text{ Acetate } + \text{ 2 NH}_3 + \text{ 2 ATP } + \text{ 2 CO}_2
\]

*Pelobacter carbinolicus*, a strict anaerobe, uses only 2,3-butanediol, methylacetoin, acetoin, and ethylene glycol serve as substrates for growth (56). *Pelobacter carbinolicus* can grow on those substrates because the following enzymes
are used to transfer acetoin, 2,3-butanediol, and methylacetoin to acetyl CoA, acetaldehyde, and acetone. These enzymes include acetoin:2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR), E3, dihydrolipoamide acetyltransferase (E2). The hypothetical cleavage of acetoin in Pelobacter carbinolicus is shown in Figure 4. The ability of bacteria to degrade acetoin is identified in many aerobic bacteria, Micrococcus ureae (57), Bacillus subtilis (58), Alcaligenes eutrophus (59).

In summary, E3 has evolved from archaeabacteria where it might have originally served as a membrane transport protein. During evolution in bacteria, E3 was first recruited into the glycine reductase system and glycine decarboxylase system. These systems were the predecessors of the glycine cleavage system in higher eucaryotic cells. E2 and E3 were recruited into an enzyme system that included acetoin:2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR), E3, and dihydrolipoamide acetyltransferase (E2). Finally, E3 has been recruited into the α-keto acid dehydrogenase complexes in the higher eucaryote with or without retaining its original functions.

C. Three α-keto acid dehydrogenase complexes

The α-keto acid dehydrogenase complexes include pyruvate dehydrogenase (PDC), α-ketoglutarate dehydrogenase (KGDC) and branched-chain α-keto acid dehydrogenase complexes (BCKADC). The α-keto acid dehydrogenase complexes have α-ketoacid dehydrogenase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) that catalyze the overall reaction as shown below.

\[
\alpha\text{-keto acid} + \text{CoA} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{acyl CoA} + \text{NADH} + \text{H}^+ 
\]

The partial reactions are shown in Figure 3. E1 catalyzes the irreversible oxidative decarboxylation of the α-keto acid forming CO2 and acyl-lipoamide. E2
catalyzes the transfer of the acyl group to CoA forming acyl CoA and dihydrolipoamide. E3 catalyzes the reoxidation of the dihydrolipoamide and the transfer of the electron to NAD⁺. E₁ and E₂ show substrate specificity while E₃ is the common component for all α-keto acid dehydrogenase complexes. The substrates and products of α-keto acid dehydrogenase complexes are listed in Table 2.

The overall PDC reaction is a 'Ping Pong' mechanism (60). The order of the substrates entering and products leaving the reaction are shown below:

![Diagram](Image)

The E₁-catalyzed reaction of PDC follows the 'Ping Pong' mechanism (60). The order of the substrates entering and leaving the reaction are shown below:

![Diagram](Image)

E₂ follows a sequential mechanism (61). The order of the substrates entering and leaving the reaction are shown below:

![Diagram](Image)
E3 follows the 'Ping Pong' mechanism (7). The order of the substrates entering and leaving the reaction are shown below:

```
  DHL  L  NAD^+  NADH  
   ↓    ↓    ↓     ↓    
   E    F    E    E    
```

Product inhibition experiments showed competitive inhibition of acetyl CoA vs. CoA and NADH vs. NAD^+. These results imply that both products change the binding of the substrate. The inhibition is caused by an increase of the K_m, but did not affect the V_max.

Noncompetitive inhibition is found between NAD^+ vs. acetyl-CoA and CoA vs. NADH. This implies that inhibition of E2 by acetyl CoA does not affect the K_m of NAD binding to E3, but affects the V_max of E3. Similarly, the inhibition of E3 by NADH did not affect the K_m of CoA binding to E2, but affected the V_max of E2.

Pyruvate vs. acetyl-CoA and pyruvate vs. NADH exhibit uncompetitive inhibition. This implies that the inhibition of E2 by product acetyl-CoA or inhibition of E3 by product of NADH affects the V_max for E1 and the K_m for pyruvate. No other kinetic data for other α-keto acid dehydrogenase complexes have been reported, but it is likely to have the same kinetic patterns of PDC.

D. E3 deficiency

1. The features of E3 deficiency

E3 is the common component of the three α-keto acid dehydrogenase complexes. A deficiency in E3 leads to the deficiency of all these three α-keto acid dehydrogenase complexes. The features of E3 deficiency include lactic acidosis, neurological dysfunction, increased serum amino acids and increased urinary organic acids.
Lactic acidosis forms as a result of pyruvate accumulation along with the accumulation of the reducing equivalent, NADH in the cytosol. This excess pyruvate is reduced to lactate by lactate dehydrogenase driven by an elevated cytoplasmic NADH/NAD⁺ ratio. The brain appears to be exquisitely sensitive to E₃ deficiency since it predominantly utilizes glucose as a fuel source. This observation is supported by the fact that areas of high metabolic activity such as the brain stem and basal ganglia manifest with the most severe lesions as a result of E₃ deficiency.

The PDC, KGDC and BCKADC also play a major role in amino acid metabolism as well as in energy metabolism. In case of deficiencies of the three α-keto acid dehydrogenase complexes, the metabolism of branched-chain amino acids is impeded, resulting in the accumulation of serum amino acids and urinary organic acids.

In BCKADC deficient patients, the color and odor of urine are similar to maple syrup. The disease, which is caused by the deficiency of BCKADC, is called maple syrup urine disease. This can result from a deficiency of either E₁, E₂, or E₃ (62).

E₃ is also a component of the L-protein in glycine cleavage system (GCS). GCS deficiency leads to hyperglycinemia. Treatment with branched-chain fatty acids, such as the antiepileptic drug dipropylacetate, inhibits P-protein and causes ketotic hyperglycinemia (63, 64). Therefore, the patient with nonketotic hyperglycinemia could be diagnosed as deficient in P-, H-, or T-protein activity of the GCS. However, the patient with ketotic hyperglycinemia may have a deficiency of BCKADC, L-protein (E₃), and the enzymes in group one of lactic acidosis.

In summary, the features of E₃ deficiency involves lactic acidosis, maple syrup urine, and hyperglycinemia. However, in the clinical symptoms of lactic acidosis, maple syrup urine, and hyperglycinemia may also result from many of the other enzyme deficiencies as described above. Therefore a definitive diagnosis of the E₃ deficiency can only be made by the enzymatic assay of E₃.
2. Case report

The first report of E3 deficiency was published by Haworth et al. (65). Three patients (C. M., G. M. and D. M) were studied in this report. Both G. M. and D. M. had increased levels of glutamate, proline and alanine in their plasma and cerebrospinal fluid (CSF). G. M. and D. M. also had increased urinary pyruvate and α-ketoglutarate levels. D. M. had lower enzyme activity in both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes assayed in vitro. Blass et al. (66) also published a case report of E3 deficiency. In this report, one patient showed the enzymatic activities from fibroblasts of both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes which were lower than the control values. Since then, several other cases have been studied (67-76). Taylor et al. (68) used an E3 assay to diagnose the E3 deficiency. Otulakowski et al. (76) characterized the E3 deficiency by Western blot analysis of the patient's fibroblasts. Furthermore, Liu et al. (77) identified two point mutations in E3 cDNA synthesized from an E3 deficient patient.

E. X-Ray Crystal structure

1. General structure of NAD± binding site

Rossmann et al. (78) and Branden (79), when studying the structure of the NADH dehydrogenases (s-malate dehydrogenase, lactate dehydrogenase, liver alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase), found that the structure of the NAD± binding region was similar in all of the above mentioned dehydrogenases. This domain is composed of six strands of parallel β-sheets (βA, βB, βC, βD, βE and βF) and 4 α-helices (αB, αC, αE and α1F; see Figure 5). The order of these β-sheet and α-helix, from amino acids sequence N-terminal to C-terminal, is βA, αB, βB, αC, βC, twist, βD, αE, βE, α1F to βF. Both helices αB and αC are on one side of the sheet while αE and α1F are on the other side. The NAD binds to the C-terminal amino
acids of the β-sheet and to the N-terminal amino acids of the α-helix. The amino acids of strands βA, βB, βC, αB and αC are the adenine nucleotide monophosphate (AMP) binding site of this domain, while the strands of βD, βE, βF, αE and α1F consist of the nicotiamide mononucleotide binding site. The crevice (the space between βA and βE) is the pyrophosphate moiety of the NAD⁺ binding site.

2. The FAD binding site of E3

The understanding of the E3 FAD binding site is based on the E3 X-ray crystallographic and structural studies from Azotobacter vinelandii (80), Pseudomonas putida (81) and Pseudomonas fluorescens (82). Furthermore, the E3 FAD binding site was studied by homologous comparison of the structure of glutathione reductase to the amino acid sequence of the E3 (83). In addition, a three dimensional structural model for the human E3 was constructed based on the three dimensional structure of glutathione reductase (84). The E3 has 4 different domains: the FAD (Figure 6), the NAD⁺, the central, and the interface domain (Figure 7). These domains of the molecule form the homodimer with the FAD, NAD⁺, and lipoamide binding sites, and the active center. A cartoon of the E3 dimer is shown in Figure 8.

The FAD binding site is composed of the FAD, the central and the interface domains. The proposed amino acids that participate in the binding of FAD are listed in Table 3. Instead of the common FED-ABC sheet in NAD⁺ dehydrogenase, the FAD binding domain in the E3 has a D-ABC β-sheet structure. The carboxyl ends of these parallel strands, which face the active site, are the contact areas for the FAD. In the FAD binding domain, αC helix is expanded to the αII and the αIII helices. The active disulfide is in front of the αII helix. The amino acids which interact with the FAD are located in the carboxyl end of the β-strand. The peptide backbone of amino acids G-17, K-37, T-44, I-121, and G-149 interact with FAD (main chain contact). The side chains of amino acids E-36, T-44, and K-54 contact to FAD (side chain contact). The
twist between βC and βD contains 3 β strands (βM-1, βM-2 and βM-3), which form the β-meander of the FAD domain. In the NAD domain, the side chains of V-188, E-192, and R-280 contact the FAD. In the center domain, the peptide backbone of amino acids N-268, I-314, D-316 and A-328 and a side chain of D-316 interact with FAD. In the interface domain, H'-452 has the main chain contact to the FAD.

3. The NAD binding site

The NAD binding domain of E3 has the D-ABC β-sheet structure and is similar to the FAD binding domain. The amino acids involved in the NAD binding site are located in the NAD and the interface domains. The proposed NAD⁺ contacts the NAD domain on the carboxyl end of the β-strand, at the peptide backbone of residues G-187, V-188, E-208, K-232, V-243, R-280, and M-327. In addition, NAD⁺ contacts the peptide backbone of residue Q-373 of the interface domain and the side chains of E-208 and R-280 of the NAD domain. Table 4 summarizes all the amino acids comprising the NAD binding site.

4. The subunit interaction

The components of the active center of E3 are assembled from two polypeptides, joined together by hydrogen bonds between the subunits. There are 3 regions of hydrogen bonding: group 1 consists of the amino acid in the FAD domain of one polypeptide and the amino acid of the FAD domain in the other polypeptide: group 2 consists of the amino acid in the FAD domain of one polypeptide and the amino acid of the interface domain in the other polypeptide: and group 3 consists of the amino acid in the interface domain of one polypeptide and the amino acid of the interface domain in the other polypeptide. The proposed H-bond of the FAD-FAD domain interaction involves S-60(Oδ)-R-74'(Ne1), G-75(O)-N-86'(N), E-77(N)-R-82(O), E-77(O)-R-82(N), and S79-(N)-S-79'(N). The proposed H-bond of the FAD-interface domain interaction involves Q-91(Nε2)-T-396'(O), Q-27(Nε1)-K-
5. The active center of the E3

The active center of the E3 is composed of the active disulfide, an active base, the FAD, the NAD, and the dihydrolipoamide. The active center includes the FAD domain, the NAD domain, the central domain, and the interface domain (Table 6). In creating the active base, the H-452 and E-457 form a H-bond. The imidazole N3 forms an ion pair with C-45, which donates its proton to dihydrolipoamide. The active disulfide is formed between C-45 and C-50. Three factors are necessary for the formation of the disulfide bond. They are the hydrogen bond of C-45(O)-I-51(N), the G-49 with a unusual φ ψ combination (-132°, -111°) and the P-52 turn. After the charge transfer between thio-disulfide interchange, the electron is transferred to the FAD through C-50. The K-54 forms an ion pair with E-192 which withdraws the electron from the FAD and transfers the electron to the NAD+

F. Reaction mechanism, and mutation of E3

1. The studies of the reaction mechanism of E3

The first kinetic studies of the reduction of NAD+ by dihydrolipoamide from pig heart E3 which was obtained by Lineweaver & Burk extrapolation (7) showed that the reaction catalyzed by E3 followed the 'Ping Pong' mechanism as described on page 11. The $K_m$ for DL-dihydrolipoamide is 0.3 mM, while the $K_m$ for NAD+ being 0.2 mM. The $V_{max}$ (the limiting rate with infinite concentrations of both substrates) is $3.33 \times 10^4$ moles of NAD+ reduced/min/mole of enzyme (or the $K_{cat}$ is 555 S^{-1}) (7).

From the E3 visible spectral data, the enzyme can be separated into three different states which are the oxidized form (E, active form), the two electron state
(EH2, active form) and the four electron state (EH4, inactive form). The addition of NADH or Na2S2O4 by different amounts and time can drive the enzyme from the oxidized form through EH2 to the EH4 state. Dihydrolipoamide can only drive the enzyme to the EH2 state. Arsenite has little effect on this driving process but in the presence of the NADH, this process sped up and inhibited the enzyme activity. Because arsenite reacted with dithiols, it appears that the intermediate state of the disulfide of the E3 converted to the dithiol by NADH is involved in the reaction. Therefore, the stable form of the EH2 state may be maintained by an interaction between the FADH and a sulfur radical, in the active center disulfide.

The active disulfide center is identified by amino acid sequence and iodoacetamide alkylation analysis in *Escherichia coli* E3 (85). Meanwhile, Williams et al. also found that the two sulfur molecules in the two electron reduced state are chemically inequivalent, and that the N-terminal C-45 is more reactive (13 fold) toward iodoacetamide (86). The monoalkylated EH2 blocks its catalytic activities toward lipoamide and dihydrolipoamide. But the monoalkylated EH2 doubles the transhydrogenase activity which transfers the proton or electron to an electron acceptors such as NAD+ or acetylpyridine adenine dinucleotide by comparison to the native E3 (87). These observations support the concept of the reversible sequential flow of electrons in E3 from NADH to FAD to active disulfide and to the disulfide substrate. The fact of the difference between C-45 and C-50 in the active disulfide shows that the C-45 may participate in the transient formation of the protein-lipoamide bond, while the C-50 may interact with the FAD forming the thio-flavin charge transfer complex (the existence of 530 nm absorbency). This phenomenon suggests that two covalent bonds exist: a transient covalent bond between the thiolate of the C-45 and the thiol of the lipoamide and a transient covalent intermediate between thiolate moiety of C-50 and the
C(4a) position of the isoalloxazine ring. Upon treatment the monoalkylated EH2 with NAD$^+$ at pH 8.3, the spectrum decreased at 448 and 380 nm. This pattern is similar to the partial formation of an adduct at the C(4a) portion of the flavin (88). The NMR data also suggest a transient covalent intermediate between thiolate moiety of cys-50 and the C(4a) (89). However the NMR data pointed out that the electron transferred between FADH and NAD$^+$ is not directly through the covalent bond. In summary, the entire route of the electron transfer of the E3 is established, which is described below.

\[ \text{DHL} \rightarrow \rightarrow \rightarrow \text{Active disulfide} \rightarrow \rightarrow \rightarrow \text{FAD} \rightarrow \rightarrow \rightarrow \text{NAD}^+ \]

When studying the EH2 form of E3, the spectrum of EH2 is pH dependent. The molar extinction at 530 nm changed from 3250 M$^{-1}$ cm$^{-1}$ at pH 8 to 2050 M$^{-1}$ cm$^{-1}$ at pH 5.2. The pH dependence which is observed in the absorption properties of EH2 is consistent with the disappearance of a charge transfer complex between an amino acid side chain and the oxidized flavin at the low pH value (90). The apparent pKa of the side chain is 5. The reduced E3 accepts two protons (or two electrons). One proton creates the charge transfer complex between the thiolate anion (C-50) and the oxidized flavin, and the other acts as a protonated base to stabilize the thiolate anion (C-45). Therefore, the bound FAD alternates between the oxidized (FAD) and the semiquinone (FADH) level, turning over once per catalytic cycle. In the overall two electron oxidoreduction reaction, only one of the electrons is accepted and redonated to NAD$^+$.

The reduction of E3 by dihydrolipoamide is pH dependent. The reduction rate is constant between the range of pH 5.5 to 8.1. The actual absorbance changes observed ranged from 0.02 to 0.07 optical density; half-times for the observed reactions ranged from 1.5 to 10 ms, first order rate constant rate $= 830 \pm 80 \text{ S}^{-1}$ (90). The rapid reaction kinetic data (90) also provide evidence for the allotment of a base in the active center of E3. Therefore, the pH-independent rate of E3 reduction by
dihydrolipoamide occurs at the pH values above the pK of the base on oxidized E₃.
The pH above the pK of this base, the deprotonized active base deprotonated
dihydrolipoamide through nucleophilic attack, the deprotonated dihydrolipoamide
served as a nucleophile activating the E₃ active disulfide.

In summary, the spectrum of EH₂ is pH dependent, the reduction rate is
constant between the range of pH 5.5 to 8.1 and a thiolate/imidazolium ion pair is
formed at the active center of papain. Williams (91) proposed that the reaction
mechanism of E₃ includes an ion pair in the active center (Figure 9). Scheme 1-1
represents the Michaelis complex of the oxidized enzyme and dihydrolipoamide. The
first thiol of the bound dihydrolipoamide undergoes a nucleophilic attack by a base. In
scheme 1-2, the deprotonated hydrolipoamide and protonated base are formed. The
deprotonated hydrolipoamide attacks the C-45 and the protonated base transfers the
proton to C-50 where the disulfide bond between the enzyme and substrate are formed
(scheme 1-3). The protonated base is then deprotonated again. The second thiol of the
hydrolipoamide undergoes nucleophilic attack by the same base which deprotonates the
second thiol and protonates the base (scheme 1-4). Finally the deprotonated thiol
attacks the first thiol of the lipoamide which forms the lipoamide and protonated base
(scheme 1-5). The protonated base forms an ion pair with the C-45 to stabilize the
structure of ion pair between S⁺ and HB⁺ (scheme 2). The deprotonated C-45
protonates through the charge transfer to C-50 (scheme 3). Finally, the FAD
undergoes nucleophilic attacked by deprotonated C-50 which forms an adduct at the
C(4a) portion of the flavin.

The X-ray diffraction analyses of E₃ and GR show that the active base is the
imidazole of H-452. The pK of the imidazole ring of His is 6. In the E₃ reaction, the
pH independent range from 5 is because the γ-carboxy group of the E-457 from the
hydrogen bound with the N³ of the H-452 which withdrew the electron resonance between the N¹, C², and N³ of the imidazole ring of the H-452 lowered the pK of the side chain. The three dimensional structure of GR also suggests that the K-54 serves as an electron acceptor between FADH and NADP⁺. Thus, this completes the entire electron flow pathway of the E3 reaction.

2. The studies of mutant protein

Site-directed mutagenesis of the active disulfides of thioredoxin changed C-136, C-139 to S-136, C-139 and C-136, S139. Steady state kinetic analyses of the proteins show that the S-136, C-139 and C-136, S-139 decreased the turnover number to 10 and 50 % of the value of the wild type TR, respectively. A charge transfer complex (e530 = 1300 M⁻¹ cm⁻¹) has been observed. This result confirmed that the C-139, C(4a)FAD-interaction was formed in the reduction of thioredoxin by NADPH through TR (92).

In mercuric ion reductase, two disulfides are involved in the reaction are between C-135, C-140 and C-558, C-559. The mutant proteins produced by the site-directed mutagenesis, A-135, C-140 and A-558, A-559, destroyed the Hg(II) binding site and knocked out the MR activity. But under the reduction of NADPH, an MR-FADH⁺ form accumulated. Furthermore, a flavin C(4a), C-140 thiol adduct accumulated by adding NADP⁺ to the mutant protein (93). This result supports the hypothesis that the electron flows through NADPH to FADH⁺ to C(4a)-thiol adduct to the FAD/dithiol form.

In Escherichia coli E3, the single amino acid substitution of Y-184 to I caused the decrease in turnover rate of dihydrolipoamide to the NAD⁺ by 90 %; the NADH to lipoamide decreased by 50 %. This altered protein showed remarkable changes in the fine structure of the visible absorption and circular dichroism spectra, and nearly
complete quenching of FAD fluorescence were observed. However, the spectra showed no change in the charge-transfer stabilization at the EH2 level. Stop-flow data indicate that the electron transfer from NADH to FAD was only slightly inhibited while the EH2 to disulfide was markedly inhibited. Reduction by dihydrolipoamide to the EH2 stage is not altered. Y-184 appear to be important in modulating the properties of the flavin in E3 (94).

The E3 mutants involving H-450, E-455 diad in *Azotobacter vinelandii* show that three (to F, S, and Y) have an apparent $pK_{a1}$ near pH 5.5 and a second apparent $pK_{a2}$ which are 6.9, 7.5, and 7.1, respectively, and significantly to the deproton of the tautomeric equilibrium between interchange and charge-transfer thiol. However the pH dependence EH2 level of the E-455 mutated enzymes (D, and Q) follows the model for the wild type enzyme except for the E mutants shifting the tautemic equilibrium of EH2 in favor of charge-transfer species. This effect is explained by an impaired in-pair formation between the protonated H and the interchangeable thiolate and a lowering of the $pK_{a}$ of H-450 (95).

Human E3 mutants of H-452 to Q and E-457 to Q both destroyed the active base and the specific activity is decreased to 0.5 % and 6 %, respectively (96). In this study, two natural mutations in the E3 deficient patient have been identified. One of the mutations replaced P-453 to Leu which results in a protein which has significantly different spectral, circular dichroism and fluorescence properties. The dimerization was reduced by 50 % of the mutant enzyme and the FAD binding decreased to 30 %, the protein had only 0.04 % specific activity when compared to that of the wild type E3. This observation suggests that P-453 involved in the formation of the active base, the subunit interaction and FAD binding. The other K-37 to E mutant did not change its optical properties, nor the dimerization. The FAD binding decreased 30 % as well as
specific activity of the mutant E3. Therefore, K-37 is involved in the FAD binding of the E3.

In summary, the mutations studied in this work thus far appear to affect formation of the active disulfide, the active base, and the FAD binding site, thus supporting the concepts that E3 reaction mechanism involves two elements: the active disulfide and the active base. The existence of P-453 shows that the H-bond formation between H-452 and E-457 not only reduces the pK of E3 reaction but also is essential for the formation of the active base and the conformation for subunit interaction and FAD binding of the E3 molecules.

G. Physical properties of E3

1. Absorption spectra

The typical absorption spectrum of E3 is shown in Figure 10. In the oxidized form (E form) of E3, the absorption of violet (380 to 435 nm) and blue (435 to 480 nm) light is caused by FAD which incidentally imports a yellowish color to purified E3 protein. Dihydrolipoamide or NADH can drive E3 from the E form to the EH2 form (reduced form). In the EH2 form, the violet and blue absorption of the FAD shifts toward the lower wavelength and a new absorption of a bluish-green (490 to 560 nm) light occurs. This absorption in the 490 to 560 nm range (or 530 nm absorption) represents the formation of the thiol-FAD charge transfer state. The EH2 state can return to the E state by treatment with lipoamide (Figure 11) or can be further reduced to the EH4 state by treatment with dithionite (Figure 12). In the EH4 state, the blue color absorption disappears, and E3 shows a yellow-green color. The change in the reduction state from EH2 to E can be induced by adding acetylpyridine adenine dinucleotide (APyAD+, an analog of NAD+) (Figure 13) or NAD+ (Figure 14).

Therefore, the visible spectrum presents evidence for the existence of an intermediate
state (EH2 or charge-transfer state) during the electron transfer between dihydrolipoamide and NAD$^+$. 

2. Fluorescence spectrum

The fluorescence spectrum of E3 is shown (Figure 15). Excitation at 296 nm of amino acid Y causes the emission at 300 to 360 nm, the emission energy can excite the neighboring FAD and the excited FAD emits at 480 to 550 nm. Adding NAD$^+$, then dihydrolipoamide causes the emission at 460 nm and decreases the emission at 480 to 550 nm. This 460 nm emission represents the formation of NADH which then decreases as the NADH leaves.

3. Circular dichroism

The circular dichroism spectrum at 190 to 240 nm is shown in Figure 16. This spectrum detects aspects of secondary structure of E3 including $\alpha$-helix and $\beta$-sheets. The spectra at 350 to 480 and 420 to 520 nm represents the FAD in E3 environment (Figure 17). In the EH2 state, the absorption from 350 to 480 nm decreases and shifts toward the lower wavelengths and the absorption from 420 to 520 increases and shifts toward the longer wavelength (Figure 18).

H. Research objective

The objective of this research is to identify and characterize E3 mutation(s) in E3 deficient patient's fibroblasts. To identify the mutation(s) in cDNA from an E3 deficient patient, two questions will be investigated. The two questions are as follows: (1) where is the location of the E3 mutation(s) in the patient's E3 cDNA, and (2) what is the mutation causing an E3 deficiency. In order to answer these two questions, the patient's specific cDNA will be amplified and sequenced to locate the mutation(s). To characterize the mutation(s) in cDNA from an E3 deficient patient, the mutant E3 protein(s) will be expressed in bacteria. After bacterial expression, the initial rate
kinetics and physical properties of both the normal and mutant E3's will be compared to test the functional significance of the mutation(s).

Several cases of E3 deficiency have been reported, among one of them is studied in this dissertation (73). In this case, the patient's fibroblast showed 6% specific activity, a normal amount of mRNA, and 60% protein compared to the control fibroblasts (77). Two point mutations have been identified which caused the K-37 to E and P-453 to L mutations. The E3 K-37 to E and E3 P-453 to L mutations together with two other active base point mutation, E3 H-452 to Q and E3 E-457 to Q (96), offered us the opportunity to study the physical properties of these mutant proteins in the E3 reaction mechanism. Previously, the role of K-37 in FAD binding had not been studied extensively, but x-ray crystallographic studies suggested that K-37 is only one of the FAD contact sites (see Table 3). As a result, K-37 was not expected to change much of the FAD binding in the E3 K-37 to E mutation protein. Thus, the nature of this E3 K-37 to E mutation offered us a chance to reevaluate the FAD binding site more specifically.
Chapter II
Experimental Procedures

A. Materials

1. Biochemicals and reagents

The following reagents were purchased from Sigma Chemical Company (St. Louis, Missouri): NAD\textsuperscript{+}, NADH, D,L-6.8-thiolic acid amide, sodium borohydride, streptomycin sulfate, phenoxyemethylpenicillin acid, oxaloacetate, and 5,5\textsuperscript{'}-dithio-bis(2-nitrobenzoic acid). GIBCO Laboratories (Grand Island, New York) was the source for fetal bovine serum, and Minimum essential medium (MEM). Trypsin, Bacto-trypton, Bacto-yeast extract, Bacto-aga were from DIFCO Laboratories (Detroit, Michigan). Acrylamide, bis acrylamide, Coomassie Brilliant Blue R-250, TEMED, bromophenol blue, and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Rockville Center, New York).

2. Enzymes, Kits, Plasmid, and Bacteria Strain

Restriction enzymes and random primed labeling kits were purchased from Boehringer Mannheim Biochemical (Indianapolis, Indiana). The DNA sequencing kit was purchased from United State Biochemicals (Cleveland, Ohio). The enhanced chemiluminescence Western blotting detection system was purchased from Amersham (Arlington highs, Illinois). His bind resin in Sepharose 6B were purchased from Novagen (Madison, Wisconsin). The GeneAmp PCR kit and GeneAmp RNA PCR kit were purchased from Perkin Elmer Cetus (Norwalk, Connecticut). The pQE-9 plasmid and Escherichia coli M15 strain was a gift from Dr. Stuart Le Grice.

B. Subject

The clinical features of the patient investigated in this study were reported elsewhere (86). The patient was a poorly nourished boy who exhibited E3 deficiency and neurological dysfunction. He had elevated plasma levels of pyruvate, \(\alpha\)-
ketoglutarate, branched-chain amino and α-keto acids, and died at the age of 21 months, following a ketoacidotic episode. The total PDC activity of the patient's cells was 10-30 % of that of control cells. Pyruvate dehydrogenase (E1) and dihydrolipoamide acetyltransferase (E2) activities were normal in the patient's muscle cells but E3 activity was not detected in either muscle or liver specimens obtained at autopsy.

C. Cell Culture of Fibroblast cells

1. Cell growth, maintenance, and harvest

Both patient and control skin fibroblast were grown in a minimum essential medium supplemented with 20 % fetal calf serum, streptomycin (100 mg/ml), penicillin (60 mg/ml) and amphotericin B (1.5 mg/ml). The fibroblast cells were maintained in a 37 °C, humidified, water-jacketed incubator with an atmosphere of 5 % CO₂ and 95 % air. The cells were given fresh medium every four days until they reached confluence, as determined by examination under phase-contrast microscopy. At confluency, the medium was aspirated from the dishes, and the cells were washed with phosphate buffered saline (PBS). Five ml of the trypsin solution (made of 8 g NaCl, 0.4 g KCl, 1 g glucose, 0.35 g NaHCO₃, 0.2 g EDTA and 0.4 g Trypsin per liter) was added to each dish at room temperature. After the cells detached from the culture dish, the trypsin was inactivated by the addition of 5 ml medium, and the cells were collected by centrifugation of the suspension in a 50 ml conical plastic tube in a table top, clinical centrifuge. The cells were either passaged or prepared for freezing. For passaging, each dish cell was resuspended in 8 ml medium, and distributed 2 ml to each one of the 4 culture dishes (100 mm diameter) containing 8 ml of medium.

2. Preparation of Frozen Cell stocks

Cells were harvested from the dishes as described above. Pelleted cells were resuspended in a freezing medium made of 5 ml DMSO, 10 ml fetal bovine serum, and
35 ml MEM. Cells from one dish (100 mm diameter) were resuspended in two ml freezing medium and were dispensed into a sterile Nunc cryotube (Vangard International, Inc., Neptune, New Jersey). The tubes were frozen at -70 °C for 4 hours, and were transferred to liquid nitrogen for long-term storage.

3. Preparation of Cellular Extracts

The cells were harvested and washed twice in phosphate buffered saline (PBS) and scraped into PBS by using rubber policeman. The cells were washed three times in PBS, and resuspended in PBS containing 0.05 % Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, and 0.7 mg/ml pepstatin. Cell suspensions were frozen and thawed three times, then sonicated three times for 10 seconds each. Sonicated cell lysates were used for enzyme assays.

4. Preparation of Mitochondrial fraction

The cells were harvested, washed twice in PBS and an aliquot was saved for the protein assay. The cells were resuspended in 0.4 ml of STM solution ( made of 2.14 g sucrose, 65 ul 1 M Tris-HCl pH 7.4, and 2 μl β-mercaptoethanol per 25 ml) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, and 0.7 mg/ml pepstatin. The cell suspensions were homogenized using six passes in a motor-driven Fisher Dyna-Mix homogenizer and kept on ice. Unbroken cells and nuclei were removed by a 650 x g centrifugation, 4 °C, for 10 minutes and the pellet was discarded. The supernatant was centrifuged at 12,000 x g, 4 °C, for 10 minutes. The supernatant fraction was discarded, and the mitochondrial pellet was suspended in the loading buffer of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

D. Enzymatic analysis

1. Dihydrolipoamide dehydrogenase
E3 activity was assayed spectrophotometrically by following the reduction of NAD\(^+\) at 340 nm at 37 °C, in a Shimadzu UV160U spectrophotometer equipped with thermospacers. D,L-6,8-thiociolic acid amide (D,L-lipoamide) was converted to the reduced form (dihydrolipoamide) chemically. Four hundred mg of D,L-lipoamide were dissolved in 8 ml methanol and 2 ml distilled water. Four hundred mg of sodium borohydride in 2 ml distilled water was added and incubated at 4 °C for 6 hours. After 6 hours, the reaction was terminated by adjusting the pH to 2 with HCl. The dihydrolipoamide was extracted by chloroform, and dried under vacuum. The reaction mixture for the E3 assay contained 50 mM potassium phosphate, pH 8, 1.5 mM EDTA, pH 7, 3 mM NAD\(^+\), 3 mM dihydrolipoamide, and 0.05 % Triton X-100, in a total volume of 1 ml. The reaction was started by the addition of dihydrolipoamide. Blank reactions contained no added enzyme or cell extract. One unit of enzyme activity was defined as 1 mmol of NADH or mercaptide ion produced per min per mg of protein. Enzyme assays were performed by using seven different concentrations of both dihydrolipoamide and NAD\(^+\) (0.1, 0.15, 0.2, 0.3, 1.3, and 6 mM). The initial velocity was processed by the Graphic fit program. In this program, the 'Ping Pong' mechanism was calculated from the general equation as shown below.

\[
v = \frac{V_{\text{max}} [A] [B]}{K_{\text{mB}} [A] + K_{\text{mA}} [B] + [A][B]}
\]

The order sequential mechanism was calculated from the equation as shown below.

\[
v = \frac{V_{\text{max}} [A] [B]}{K_{\text{iA}} K_{\text{mB}} + K_{\text{mA}[A]} + K_{\text{mA}}[B] + [A][B]}
\]

2. **Citrate synthase**

Citrate synthase activity was assayed by measuring the production of mercaptide ion at 412 nm at 37 °C. The reaction mixture for the citrate synthase assay contained 100 mM Tris-HCl, pH 7.8, 10 mM dithionitrobenzoic acid, 0.1 mM acetyl
CoA, 0.5 mM oxaloacetate and 0.05 % Triton X-100, in a total volume of 1 ml. The reaction was started by the addition of oxaloacetate. Blank reactions contained no added enzyme or cell extract.

E. Protein assays

Protein concentration was determined by Lowry's method using bovine serum albumin as the standard, with or without 0.05 % Tx-100. To each sample was added 1.5 ml of a solution of 2 % Na₂CO₃ in 0.1 N NaOH, 0.01 % CuSO₄·5H₂O, 0.02 % Na-K-Tartrate, 1 % SDS. The solution was incubated at room temperature for 10 minutes. Phenol reagent diluted with an equal amount of water was added (0.3 ml), and the samples were kept at room temperature for 30 minutes. The absorbency at 500 nm was measured spectrophotometrically.

F. Western blot Analysis

For Western blot analyses, the mitochondrial fraction for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used as mentioned above. After SDS-PAGE, the gels were electrotransferred to a nitrocellulose membrane in transfer buffer (25 mM Tris-HCl and 192 mM glycine, pH 8.3) at 75 mA for 18 hours. After the transfer, the membrane was incubated with 5 % bovine serum albumin in Tris-buffered saline at room temperature overnight and then reacted with rabbit anti-porcine E₃ antiserum for 3 hours. In a separate experiment, the membrane incubated with rabbit antiserum against bovine heart PDC-E₂ served as a positive control. Specific E₃ protein was detected using the enhanced chemiluminescence Western blotting detection system. Levels of protein were quantitated using a scanning densitometer (United States Biochemical, Cleveland, Ohio).

G. Northern blot Analysis

Total RNA was extracted from cultured skin fibroblast cells using the guanidine thiocyanate method. About 25 mg of total RNA were separated on a 0.8 % agarose gel
containing 50 % formaldehyde, and subsequently transferred to a GeneScreen membrane. The membrane was pre-hybridized at 42 °C overnight in a solution of 20 mM PIPES pH 6.4, 2 mM EDTA, 0.8 M NaCl, 50 % formaldehyde and 100 mg/ml salmon sperm DNA. The pre-hybridized membrane was hybridized at 65 °C for 30 min, and then at 42 °C for a further 48 hours, with a 1.1 kb fragment of E3 cDNA located at the 5' end of the previously reported E3 cDNA sequence. This probe was labeled prior to hybridization by the random primer reaction to a specific activity of 100 cpm/pg using [α-32P]dCTP. After hybridization, the membrane was washed at 50 °C first with 2X SSC containing 1 % SDS, then with 1X SSC buffer containing 0.1 % SDS, and finally with 0.1X SSC buffer containing 0.1 % SDS. The 1X SSC solution contained 150 mM NaCl and 15 mM citrate, pH 7.0. The washed membrane was air-dried and autoradiographed.

H. Polymerase Chain Reaction

1. Reverse transcription and PCR

E3 cDNA fragments were obtained from the patient's total RNA using reverse transcription and the polymerase chain reaction (PCR). Three pairs of sequence specific primers (A5-7, A3-521; B5-417, B3-1088; and C5-1034, C3-1671; Table 7) were used for PCR as sense and antisense primers synthesized in the Core Laboratory on site. These three fragments overlapped each other and covered the entire coding region of E3 cDNA. The reverse transcription reaction was performed at 42 °C for 45 minutes with 1 mg of total RNA in 20 ml of reaction mixture containing 10 mM Tris-HCl pH 8.4, 2.5 mM MgCl₂, 5 pmol of the 3' primer, 200 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 200 mg/ml gelatin, and 2 units of Maloney murine leukemia virus reverse transcriptase. The newly generated E3 cDNAs was further amplified for 35 cycles using Thermus aquaticus DNA polymerase (Perkin Elmer-Cetus) and 20 pmol of the 5' and 3' primers. Each cycle was performed at 94 °C for 1 minute, 55 °C
for 1 minute, and 72 °C for 2 minutes. The amplified cDNA fragments were purified by electrophoresis in low melting temperature agarose. The purified fragments were then either used for asymmetric PCR or subcloned into Bluescript plasmid (digested with Smal) using blunt end ligation. Plasmids used for sequencing were purified using the protocol described by Maniatis et al. (97).

2. Asymmetric PCR

Asymmetric PCR was performed to obtain single-stranded DNA for direct DNA sequencing. The amplification conditions for the asymmetric PCR were the same as described above except the molar ratio of the two primers was 100:1 (50 pmol to 0.5 pmol).

I. DNA sequencing

The amplified single-stranded DNA and the Bluescript E3 cDNA subclones were denatured with 0.5 % soft agar (New sieve) at 98 °C for 10 minutes and annealed with appropriate primers (as internal primers shown in Table 8) at 37 °C for at least 10 minutes. The sequencing kit was purchased from United States Biochemical (Cleveland, Ohio) and the sequencing reaction was performed as suggested by the manufacturer.

I. Construction of Human E3 expression Vector

1. Construction of pQE-9-E3 expression Vector

The E3 cDNA (33) was isolated from a human liver λgt 11 cDNA library. The E3 cDNA was subcloned into a Bluescript plasmid by two steps. The first step involved constructing of the c-terminal fragment of E3 cDNA into EcoRI and HindII sites and named BS-E3c. The BS-E3c was constructed with the N-terminal cDNA into the EcoRI site. The right orientation was chosen by the sequencing of the flanking region of the restriction site and named BS-E3 (Figure 19). The E3 cDNA was
amplified by PCR with the primer with BamHI linker at the 5' end (L1 and L2, see Table 8) and further subcloned into the BamHI site of the pQE-9 expression vector and named pQE-9-E3 (constructed by Dr. L. G. Korotchkina, Figure 20). All primers used for site-directed mutagenesis are listed in Table 9.

2. Construction of pQE-9-E3 K-37 to E expression Vector

The pQE-9-E3-K-37 to E expression vector was constructed from site-directed mutagenesis PCR by a two-step procedure. The first step used the site-directed mutagenesis primer (K-37 to E) and the c-terminal primer, L2, to amplify the first mutant fragment. The mutant fragment, served as the primer, with the N-terminal primer, L1, to amplify the whole E3 K-37 to E mutant cDNA. This E3 K-37 to E mutant cDNA was constructed into pQE-9 and named pQE-9-E3 K to E (Figure 21).


The E3 mutant cDNA, E3 P-453 to L, E3 H-452 to Q (both constructed by Dr. Kim) and E3 E-457 to Q (constructed by Dr. H. Kim) were constructed by using the site-directed mutagenesis technique with the single stranded DNA induced from BS-E3c. The mutated C-terminal E3 cDNA was released by EcoRI and XhoI and replaced the normal E3 cDNA from pOTSV-E3 (Figure 22). These three C-terminal E3 mutants were amplified by PCR with the L1 and L2 primers and further subcloned into the BamHI site of the pQE-9 expression vector. These vectors are pQE-9-E3-P-453 to L (Figure 23), pQE-9-E3-H-452 to Q (constructed by S. Hyatt, Figure 24), pQE-9-E3-E-457 to Q (constructed by S. Hyatt, Figure 25).

K. Purification of E3, E3 K-37 to E and E3 P-453 to L

The expression vectors pQE-9-E3, pQE-9-E3 K-37 to E, and pQE-9-E3-P-453 to L were transformed separately into an Escherichia coli M15 strain, containing
pDM1.1 plasmid. The transformed cells were inoculated into 10 ml LB (made of 10 g bactotryptone, 5 g yeast extract, and 10 g NaCl per liter) containing 100 μg/ml ampicillin, 25 μg/ml kanamycin at 37 °C for overnight. The 10 ml overnight growth mixture was inoculated into 1 liter LB containing 100 μg/ml ampicillin, 25 μg/ml kanamycin until OD600 nm = 0.7. IPTG was added to a final concentration of 200 μg/ml and the cells was transferred to 30 °C for growth overnight. The cells were harvested by centrifugation at 4000 x g for 20 minutes. The cell pellets were resuspended in 50 mM sodium phosphate pH 8, and 0.25 mM EDTA. The lysozyme was added to a final concentration of one mg/ml at 4 °C for 30 minutes. The cells were broken by French Press at 20,000 pound per square inch. The unbroken cells were removed by centrifugation at 10,000 x g for 30 minutes. NaCl was added to the supernatant to a final concentration of 100 mM and applied to a Nickel column. The column was washed with the solution containing 50 mM sodium phosphate, pH 8, 0.25 mM EDTA, and 100 mM NaCl. The bound proteins were eluted out by the 0 to 100 mM imidazole gradient containing 50 mM sodium phosphate, pH 8, 0.25 mM EDTA and 100 mM NaCl.

L. Quantitation of FAD

The protein (0.5 mg/ml, 0.75 mg/ml, and 1 mg/ml) was denatured by boiling in a water bath for 5 minutes. The denatured proteins were removed by centrifugation. The supernatants were scanned from 220 nm to 500 nm spectrophotometrically and the peak of the optical density at 448 nm was used for FAD quantitation. The standard curve of the FAD after boiling 5 minutes was obtained in using four different concentrations (5, 10, 15, and 20 μM)

M. Determination of molecular weight by molecular sieving
The experiment was performed by using the method of molecular sieving of High Performance Liquid Chromatography (HPLC). The column used for molecular sieving was TSK sw3000G. The mobile phase of HPLC was 50 mM potassium phosphate, pH 8, 0.25 mM EDTA with 0.5 % trifluoroacetate. The pump was Shimadzu LC-6A. The affluent of the proteins was detected at UV 220 nm by a UV-VIS spectrophotometric detector. The recorder used was C-R5A chromatopac micro processor.

N. Spectrophotometric analysis of E3 and its mutant proteins

The scanning of optical density was performed in a Varian UV-Vis-NIR spectrophotometer Carry 2300. Ten µM (dimer) of the oxidized proteins were scanned from 220 to 700 nm. The reduced form of protein is driven by adding dihydrolipoamide to a final concentration of 2.5 mM. The data were transferred to the ASCII file by Spectra Calc program and the scans were drawn by origin program.

O. Fluorescence analysis of E3 and its mutant proteins

The fluorescence spectra were performed in a PTI fluorescence instrument. One µM (dimer) of the proteins was excited at 296 nm and the emission pattern was recorded from 300 to 560 nm. The enzymes assay was monitored by the adding of NAD⁺ to a final concentration of 50 µM for 5 minutes incubation. Then dihydrolipoamide was added to a final concentration of 250 µM and monitored with the time interval (0, 5, 10 and 30 minutes). The data were transferred to the ASCII file by PTI program and the scans were drawn by the origin program.

P. Circular Dichroism (CD) analysis of E3 and its mutant proteins

The CD spectra were performed in a Jasco J-600 spectropolarimeter. One µM (dimer) proteins were scanned from 190 nm to 250 nm and 300 to 600 nm. The reduced forms of the enzyme driven by dihydrolipoamide (5 mM final concentration) and NADH (0.5 mM final concentration) were scanned from 300 to 600 nm. These
reduced forms of the enzymes were driven back to the oxidized form by NAD\(^+\) (0.5 mM final concentration) and lipoamide (5 mM final concentration) respectively. The data were transferred to the ASCII file by Jasco program and the pictures were drawn by the origin program.
A. Enzymatic assay

E3 and citrate synthase activities were determined in solubilized extracts of cultured skin fibroblasts (Figure 26). The patient's cells had about 6% of the E3 activity of control cells while the citrate synthase activity of the patient's cells was comparable to that of control cells. The low E3 and normal citrate synthase activities in the patient cells indicate that the E3 deficiency of the patient resulted from a defect specific to E3 rather than a generalized defect in mitochondrial function.

B. Western and Northern Blot analyses

Western blot analysis using anti-porcine E3 antibody (Figure 27 A), indicated a 40% reduction in E3 protein in the patient's cells compared to control cells. In separate experiments, when the blots were analyzed for the presence of the E2 component of the PDC using rabbit antiserum against bovine heart E2. (Figure 27B), a near normal level of this protein was observed in the patient's cells compared to control cells.

Northern blot analysis, using a 5' E3 cDNA-specific fragment (about 1.1 kb) (Figure 27 C), indicated that E3 mRNA was present in normal amounts in the patient's cells. These results indicate that the E3 deficiency in the patient did not result from a deficit in production or stability of the E3 mRNA. The observed moderate reduction in the E3 protein level could not account for a near absence of enzyme activity. Therefore, the mutation(s) are likely to have occurred to affect the coding sequence of the E3 message resulting in inactivity of the E3 protein.

C. Sequencing of Patient's specific E3 cDNA

Two substitution mutations were identified by amplifying three E3 cDNA fragments covering the entire coding region of the E3 cDNA (515,672 and 638 bp fragments which covered base pairs 7 to 521, 417 to 1088 and 1034 to 1671,
respectively, of the E3 cDNA). These two mutations were initially identified using three independent asymmetrical amplifications of the appropriate cDNA fragments followed by direct sequencing. In these experiments both normal and mutant nucleotides were identified simultaneously (results not shown). To confirm these findings, these two regions were amplified (two independent amplifications for each region) and the cDNA fragments were subcloned into BlueScript for sequencing (Figure 28). One mutation changed a single nucleotide (#246) in the fragment 7 to 521, from A to G, resulting in the substitution of E(GAA) for K-37(AAA). The other mutation in the fragment 1034 to 1671 replaced C with T (#1513), resulting in the substitution of Le(CTG) for P-453(CCG). For the K-37 to E mutation, a total of 13 subclones derived from two separate amplifications were sequenced with 9 mutant and 3 normal sequences. For the P-453 to L mutations, a total of 8 subclones derived from two separate amplifications were sequenced with 5 mutant and 3 normal sequences.

D. Expression and Purification of E3, E3 K-37 to E and E3 P-453 to L mutant proteins

In this experiment, the eluent pattern of the Nickel column had three peaks (Figure 29). The E3 activity and/or the flavin absorbency was detected in peak 3. Peak 3 consisted of two pool fractions, fractions I and II. The recovery rates of E3 activity in peak 3, fraction II, for E3, E3 K-37 to E, and E3 P-453 to L mutant proteins were 81.3 %, 69.3 %, and 0.3 %, respectively. The specific activity of E3 K-37 to E and E3 P-453 to L mutant proteins in peak 3, fraction II, decreased to 50 % and 0.04 % of wild-type E3, respectively (Table 9). The purified proteins were used for further analyses as described below.

E. Kinetic studies

The kinetic studies on both the E3 and E3 K-37 to E mutant proteins were performed in parallel. The kinetic studies were performed using 7 different
concentrations of both dihydrolipoamide (DHL) and NAD\(^+\) (0.1, 0.15, 0.2, 0.3, 1.0, 3.0, and 6.0 mM). The initial velocity was processed by the Graphic fit program.

Both E3 and E3 K-39 to E mutant proteins showed the 'Ping-Pong' catalytic mechanism. The \(K_{\text{cat}}, K_m\) of NAD\(^+\) (\(K_m\text{NAD}\)), and \(K_m\) of DHL (\(K_m\text{DHL}\)) of E3 were 570 S\(^{-1}\), 0.29 mM, and 0.81 mM, respectively. The \(K_{\text{cat}}, K_m\text{NAD}\), and \(K_m\text{DHL}\) of E3 K-37 to E mutant protein were 315 S\(^{-1}\), 0.25 mM, and 0.76 mM, respectively. The \(K_{\text{cat}}/K_m\text{NAD}\) and \(K_{\text{cat}}/K_m\text{DHL}\) of E3 were 1966 and 706 S\(^{-1}\)/mM, respectively. While the \(K_{\text{cat}}/K_m\text{NAD}\) and \(K_{\text{cat}}/K_m\text{DHL}\) of E3 K-37 K to E mutant protein were 1260 and 414 S\(^{-1}\)/mM, respectively. The efficiency of E3 K-37 to E mutant protein for the usage of substrates NAD\(^+\) and DHL (\(K_{\text{cat}}/K_m\text{NAD}\) and \(K_{\text{cat}}/K_m\text{DHL}\) ratios) decreased to 64 and 59 %, respectively, when compared to that of the wild-type E3.

F. Determination of the molecular weight of each purified protein, the E3 and its mutant proteins (E3 K-37 to E, E3 P-453 to L, E3 H-452 to Q and E3 E-457 to Q)

Previously, the E3 H-452 to Q and E3 E-457 to Q were constructed and expressed in pOTSV expression vectors by Dr. H. Kim. These mutant cDNAs were reconstructed in pQE-9 vector and expressed by S. Hyatt. E3 H-452 to E has 0.1 % and E3 E-457 to Q has 6 % of wild-type E3 specific activity.

To examine whether the mutation affected the ability of self- dimerization of mutant proteins, the proteins were sieved using the method of molecular sieving of HPLC. The column for molecular sieving was TSK SW3000G. The mobile phase of HPLC was 50 mM KPO\(_4\) pH 8, 0.25 mM EDTA with 0.5 % trifluoroacetate. The affluence of the proteins was detected by UV 220 nm. The results are shown in the Figure 30. E3 P-453 to L mutant showed 50 % monomer and 50 % dimer; the other mutants and E3 showed only dimers. This result demonstrated that the P-453 to L
mutation affected the ability to self-dimerize while other mutant proteins were unaffected.

G. Quantitation of FAD in the E3 and its mutant proteins

This experiment was to test whether the mutation decreased the FAD binding of the mutant proteins. The proteins were denatured by boiling in a water-bath for 5 minutes. The denatured proteins were removed by centrifugation. The supernatants were scanned from 220 nm to 500 nm spectrophotometically and the peak of the optical density at 448 nm was used for FAD quantitation. The molar ratio of FAD to E3 monomer is: E3 = 1; E3 K-37 to E= 0.76; E3 P-453 to L= 0.32; E3 H-452 to Q = 0.94 and E3 E-457 to Q = 0.9. These results suggest that the K-37 to E and the P-453 to L mutations decrease the affinity for FAD of the mutant proteins. The H-452 to Q and the E-457 to Q mutations caused little change of FAD binding when compared to the wild-type E3.

H. Spectrophotometry analysis of E3 and its mutant proteins

The scanning of optical density of those five proteins was performed in a Varian UV-Vis-NIR spectrophotometer Carry 2300. The proteins were scanned from 220 to 700 nm. The scanning spectra from 300 to 600 nm are shown in Figure 31. These proteins were reduced by the addition of dihydrolipoamide (DHL) and the scanning spectra are shown in Figure 32. The results showed that the absorbency at 453 nm of the E3 P-453 to L mutant had 70 % reduction while the other mutant proteins decreased this peak by 10 % to 30 %. The E3 P-453 to L mutant protein did not absorb at 350 nm. The spectrum of E3 K-37 to E mutant protein in 300 to 600 nm was very similar to the wild type E3 except that the peaks were reduced to 70 % of those seen with the normal protein. All proteins except the E3 P-453 to L mutant showed an absorbency increase in the 530 to 550 nm range when reduced. This increase represents a charge
transfer complex involving the reduced active disulfide. It is implied that all proteins except the E3 P-453 to L mutant protein could be reduced by DHL.

I. Fluorescence analysis of E3 and its mutant proteins

The fluorescence spectrum of the E3 when excited at 296 nm is shown in Figure 42 and the mechanism of excitation is described below. Tryptophan residues are excited at 296 nm and emitted at 330 nm. The emission energy at 330 nm will excite the FAD and the excited FAD will emit at 510 nm. If the DHL is added to the E3, the emission peak at 330 nm will not change, but the emission peak at 510 nm will decrease and then recover as the FAD cycles between oxidized and reduced forms (data not shown). If NAD$^+$ was added first, the spectrum will not change much except that the peak at 510 nm will decrease a little (Figure 33). If I add NAD$^+$ first and wait, the peak at 510 nm reaches from (1) to (2) (Figure 33), then when we add DHL and record the spectral change by time, I find that the peak at 510 nm decreases immediately with the formation of a peak at 460 nm. The peak at 460 nm represents the formation of NADH. In Figure 33, the spectrum showed that after both substrates (NAD$^+$ and DHL) were added the FAD peak disappeared and the NADH peak formed. The NADH peak disappeared later and the FAD peak was restored. It was observed that the peak at 330 nm decreased first then recovered. The E3 K-37 to E mutant protein had a similar spectrum as the E3 but the peak height at 510 nm is about 80 % compared to the normal E3 (Figure 34). The E3 P-453 to L mutant protein lost the peak at 510 nm (Figure 35). However, work discussed previously showed that the E3 P-453 to L mutant protein still binds some FAD (32 % compared to E3). The lack of the 510 nm emission therefore indicates that the distance between the tryptophan and FAD is too long in this mutant protein and that the FAD cannot be excited. The E3 H-452 to Q mutant protein showed the slow reduction of FAD and without the formation of NADH (Figure 36). The E3 E-457 to Q mutant protein showed the smaller peak at 510 nm, after the
substrates were added, and the formation of NADH at peak 460 nm was maintained with a slow decreasing rate (Figure 37).

I. Circular dichroism analysis of E3 and its mutant proteins

The proteins were scanned from 190 nm to 250 nm and 300 nm to 600 nm. The scanning at 190 to 250 nm showed the change of the composition of α helix and β sheets (Figure 38). This result showed that each mutant protein changed its secondary structure to different degrees. The scanning range between 300 to 600 nm represents the protein-FAD interaction. The proteins except the E3 P-453 to L mutant had the same pattern as the wild-type E3 which included a major peak at 350 nm and a minor peak at 430 to 500 nm (Figure 39). After the addition of DHL, the peak at 350 nm shifted to the left and the peak at 430 to 500 nm increased (Figure 40). At this stage, the second substrate, NAD⁺, was added, and the spectra of the E3 and the E3 K-37 to E mutant proteins showed similarity but the E3 H-452 to Q mutant protein lost the peak at 430 to 500 nm while the E3 E-457 to Q mutant protein had a higher peak at this region (Figure 41). The reverse reaction of the proteins was observed, when NADH was added (Figure 42) and then added lipoamide (L) (Figure 43). I found the peak at 430 to 500 nm increased in E3, and the same spectrum as the E3 K-37 to E. The E3 E-457 to Q had a higher peak at 430 to 500 nm while the E3 P-453 to L and the E3 H-452 to Q mutant proteins had no peak (Figure 43). After the addition of L, the peak at 430 to 500 nm decreased (Figure 43).
Chapter IV
Discussion

Enzymatic, Western and Northern blot analyses indicated that the very low E3 activity of the patient likely resulted from defects in the E3 protein. Results from Western blot analysis (Figure 36A and 36B) differ from the results previously reported for this patient by Matuda et al. (73) who used Ouchterlony double diffusion analysis to detect E3 protein in the liver and muscle biopsies. Since they could not detect any E3 precipitation band by anti-rat E3 antibody, they concluded that the E3 deficiency was due to the absence of E3 protein in the patient's cells. In contrast, we detected a reduced amount of the E3 protein in skin fibroblasts by Western blot analysis using anti-porcine heart E3 antibody. The double diffusion method recognizes non-denatured E3 protein and requires larger amounts of protein to form precipitates while Western blot analysis detects denatured E3 protein and is able to detect smaller quantities of antigen. This difference in the detection methods may be responsible for the different results. Alternatively, the mutant E3 in the autopsy specimens (84) may have been degraded.

Two substitution mutations were identified by sequencing E3 cDNA fragments generated from the total RNA of the patient's cells via reverse transcription and PCR. One mutation substituted E(GAA) for K-37(AAA). The other is L(CTG) for P-453(CCT). Direct sequencing of the patient's specific E3 cDNA showed two bands corresponding to both normal and mutant sequences at the same position on the sequencing gel (data not shown). Furthermore, some cDNA subclones from the patient contained the normal sequence, while others contained the mutant sequence (Figure 37). The two mutations in the patient's E3 gene are likely to be located on different alleles. The human E3 gene is located on chromosome #7(34). Furthermore, E3 activities in cultured skin fibroblasts from the parents of another E3-deficient patient
were approximately 30% and 42% of control values, indicating a possible heterozygous state. In our patient, presumably each of the E3 mutations is derived from one of the parents and together are responsible for the compound heterozygous state of the patient. Unfortunately, cells from the parents are not available to confirm this assumption.

The E3 P-453 to L and E3 K-37 to E mutant proteins showed 0.04% and 50%, respectively, of the specific activity of the wild-type E3. The patient's fibroblasts had only 6% of E3 activity. To account for this observations, two factors will be considered. First, the amount of the patient's E3 protein is 60% of the control E3 protein. Second, E3 is a homodimer. Therefore, three possible recombinations of the E3 mutant protein may exist in the patient. If the E3 P-453 to L and E3 K-37 to E mutant proteins are expressed equally, then the E3 P-453 to L homodimer should show no (or very low) activity, while E3 K-37 to E homodimer should show 25% of the total mutated E3 protein which represents 7.5% of the E3 activity (25% x 60% x 50%). The E3 K-37 to E and E3 P-453 to L heterodimer has 50% of the total mutated E3 protein which may represent no E3 activity.

This study indicates that two substitution mutations in the E3 coding region are consistent with the existence of a state of compound heterozygosity in an E3-deficient patient. This is the first documentation of specific mutations in this protein. The identification of specific mutations adds to the increasing understanding of structure-function relationships of the E3 protein.

The K-37 of human E3 is conserved in E3's from several sources (human, porcine, yeast, A. vinelandii and B. aureus) and is conservatively substituted by R in E. coli E3 (Table 10). P. putida (val) and P. putida (3) E3's are the only exception without a match at amino acid #37 by sequence comparison. Based on both the three
dimensional structure of human glutathione reductase, A. vinelandii E3 and P. putida (val) E3, and extensive structural comparison of E3’s from several species, K-37 is suggested to be located in the FAD-binding region of human E3. Its main chain nitrogen atom is expected to form a hydrogen bond to the adenine NA3 of FAD. The modification of K-37 to E would alter the charge distribution of the local environment around K-37 in human E3, in as much as the positively charged K side chain is replaced by a negatively charged E side chain. This residue is conserved in most E3’s, suggesting that positively charged amino acid residues at this position in E3 plays a critical role in the structure and/or function of E3.

The kinetic study shows that the reaction mechanism of both E3 and E3 K-37 to E mutant proteins is the ‘Ping Pong’ mechanism. The KmNAD and KmDHL values for the E3 K-37 to E mutant protein remained unchanged compared to the wild-type E3. The Kcat of the E3 K-37 to E mutant protein decreased 50% as compared to the wild-type E3. The Kcat/KmNAD and Kcat/KmDHL ratio of the E3 K-37 to E mutant protein decreased 36% and 41%, respectively, when compared to the E3. These results showed that the mutated protein reduced its efficiency of substrate utilization but did not change its reaction mechanism.

The other mutation is the substitution of L(CTG) for P-453(CCG). P-453 is highly conserved in E3’s (Table 2) and other homologous enzymes such as glutathione reductase and trypanothione reductase. P-453 is adjacent to the active base H-452 in human E3. H-452 is a suggested proton acceptor/donor in catalysis by human E3. Based on the three dimensional structure of human glutathione reductase, A. vinelandii E3 and P. putida (val) E3, H-452 is predicted to form a hydrogen bond to E-457. These two residues are also highly conserved in E3, glutathione reductase and trypanothione reductase. The change of H-452 to E in the recombinant human E3 by
site-directed mutagenesis resulted in dramatic decreases in both E3 activity (0.1% of wild-type activity) and binding affinity of dihydrolipoamide to the enzyme. The site-directed mutation of E-457 to Q resulted in moderate decreases in both E3 activity (6% of wild-type activity) and binding affinity of dihydrolipoamide to the enzyme. X-ray crystallographic studies of human glutathione reductase, A. vinelandii E3 and P. putida (val) E3, indicated that a bend is expected at P-453 in human E3. This bend is crucial for the formation of a hydrogen bond between H-452 and E-457. Therefore, P-453 may be important in maintaining the local structure around active base residues H-452 and E-457 in human E3. Substitution of A for P-451 (corresponding to P-453 in human E3) at the active site of the A. vinelandii E3 by site-directed mutagenesis causes almost complete loss of enzyme activity, further indicating the importance of this residue in E3 function. Based on modeling studies of the crystal structure of A.

vinelandii E3, it is suggested that the mutation at the site of proline would lead to a change in the backbone structure in or near the active base. In vitro studies of recombinant human E3 P-453 to L mutant protein shows only 0.04% of wild-type activity further confirming this point.

The E3 K-37 to E mutant protein formed the dimer, contained 72% of normal levels of FAD, and maintained about 50 specific activity. The spectra of spectrophotometric, fluorescence and CD data of the E3 K-37 to E mutant protein showed that it was very similar to the E3 data. This implied that the decrease of the specific activity was due to the lower affinity for FAD in the mutant protein. Therefore in conclusion, the K-37 is implicated in the binding of FAD. The K-37 to E mutant protein decreased the FAD binding and decreased its specific activity.

The P-453 is very important in maintaining the E3 conformation. The E3 P-453 to L mutant protein decreased the self-dimerization of E3 by 50% and decreased FAD
binding by 70%. This mutant changed its physical properties in spectrophotometry, fluorescence and CD, and showed nearly complete loss of E3 activity.

The E3 H-452 to Q mutant protein had only 0.1% activity remaining. The mutation did not change its self-dimerization, slightly decreased the FAD binding, but the mutation destroyed the active base of the E3. The spectrophotometric data are very similar to the wild type protein. In the reduced form of the protein it even exhibits similarity for the charge transfer between the active center (peak 530 to 550 nm) and the CD data of DHL treatment. Also these data indicated that the reduced form of the protein existed. But the fluorescence data indicated that the reaction proceeded very slowly, even though the DHL treatment spectra of CD were similar to E3 at 300 to 600 nm; but after addition of NAD+ the spectra is different from normal E3. This implied that the electron did not transfer to NAD+. The fluorescence data confirmed that point; even after 5 min of reaction time, NADH did not form. The CD spectra of the reverse reaction which was driven by NADH had a different spectra in this mutant protein, implying that the reverse reaction also does not proceed.

The E3 E-457 to Q mutant protein had normal self-dimerization, a slightly reduced amount of FAD, and 6% of the specific activity. In addition, the mutation destroyed the active base of the E3. This mutant protein had similar spectrophotometric data to E3, but the fluorescence data showed that NADH accumulated during the reaction. The CD spectra of this protein showed that it had a higher peak formed at 430 to 500 nm than the E3 (Figure 51), and after addition of lipoamide, the peak was still higher than the E3 peak (Figure 52). It is possible that the binding ability of NADH to the protein is reduced or the reverse reaction occurred at a slow rate.

The proposed active center of human E3 was based on the three-dimensional structure of human GR (Figure 44) and was built by molecular graphic modeling. The
active center includes an active disulfide (C-45 and C-50), an active base (H-452' and E-457 loop), FAD, NAD$^+$, and lipoic acid. The functional significance of the mutant proteins in the E3 reaction mechanism is listed in Table 11. The E3 K-37 to E mutant protein decreased 30% of FAD binding, decreased 50% of the specific activity, without changing the reaction mechanism and self-dimerization. The E3 P-453 to L mutant protein destroyed the protein conformation. This modified protein conformation inactivated the active base by destroying the H-452 and E-457 loop, reduced the FAD binding to 30%, and reduced the self-dimerization to 50%. The E3 H-452 to Q mutant protein replaced the H-452 to E. This amino acid residue replacement destroyed the active base and caused the decrease of specific activity to 0.1%. The E3 E-457 to Q mutant protein inhibited the release of NADH from the NAD binding site (see Figure 37) and may have caused the competitive inhibition for the E3 reaction resulting in the decrease of specific activity to 6%.

Besides the observation mentioned above, in the entire reaction mechanism of E3, two points remain unclear. First, the electron is not transferred from FAD to NAD$^+$ directly. The possible amino acid to accept the electron between FAD and NAD$^+$ is K-54, but this hypothesis needs to be further investigated. Secondly, since W-197 is located near both FAD and NAD$^+$, its role in electron transfer needs to be further investigated.
Chapter V

Bibliography


Figure 1. The reduction of ribonucleotides via the glutaredoxin system.
Figure 2. The catalytic reactions for the α-keto acid dehydrogenase complex
Figure 3. Scheme for the overall reaction of reversible glycine cleavage.

Figure 4. The catalytic reactions for the 2,6-Dichlorophennolindophenol oxidoreductase complex.

A). This enzyme complex cleaves acetoin to acetaldehyde and acetyl CoA, methyl acetoin to acetone and acetyl CoA, and 2,3-butanedione to acetate and acetyl CoA in *Pelobacter carbinolicus*.

B). The structure of methyl acetoin and 2,3-butanedione.
Figure 5. Schematic diagram illustrating the arrangements of strands and helices in the NAD-binding domain of dehydrogenase. This diagram is designed by B. Furugren (79).
Figure 6. The hypothetical model of the FAD-binding domain of human E3.

The model is established base on the sequence comparison of human E3 to *Azotobacter vinelandii*, (80) *Pseudomonas putida* (81), and *Pseudomonas fluorescens* (82). The β-sheets are shown as arrows and α-helices as rectangles with Roman numbers (83).
Figure 7. The hypothetical model of the NAD-binding, the central and the interface domain of human E3.

The model is established base on the sequence comparison of human E3 to *Azotobacter vinelandii,* (80) *Pseudomonas putida* (81), and *Pseudomonas fluorescens* (82). The β-sheets are shown as arrows and α-helices as rectangles with Roman numbers(83).
Figure 8. The hypothetical model of whole dimer of human E3.

The model is established base on the sequence comparison of human E3 to *Azotobacter vinelandii* (80) *Pseudomonas putida* (81), and *Pseudomonas fluorescens* (82). The β-sheets are shown as arrows and α-helices as rectangles with Roman numbers (83).
Figure 9. Scheme of the mechanism of E₃ catalyzed reactions.

B represents the active base. This diagram is designed by Sahlman, L. and Williams, C. H. Jr., (91).
Oxidized enzyme (1.2 mg/ml), ---; after addition of 1.9 moles of NADH/mole of enzyme, x---x; after addition of 18 moles of dihydrolipoamide/mole of enzyme, o---o.
Figure 11. Effect on the absorption spectrum of E3 of NADH followed by lipoic acid (7).

Oxidized enzyme, x; after addition of 3.0 moles of NADH/mole of enzyme, o; after further addition of 40 moles of D,L-α-lipoic acid/mole of enzyme, Δ.
Figure 12. Effect of NADH and dithionite on the absorption spectrum of E3 (7).

Oxidized enzyme, ●; after addition NADH (read 7 to 21 minutes), x; after addition of dithionite to x (read after 130 minutes), closed triangle.

Forty eight µM of EH2 produced by addition dithionite; ----, 2 mini second after addition 2 mM APyAD⁺, o---o; 12 mini second after addition 2 mM APyAD⁺, x—x; final spectrum of the enzyme after mixing with 2 mM APyAD⁺.

Fifty $\mu$M of EH2 produced by addition dithionite; ----, in the dead time after addition 50 mM NAD$^+$, x—x; 2 mini second after addition 50 mM NAD+, ——.
Figure 15. The fluorescence spectra of the E3 (In this study).

The protein was excited at 296 nm by PTI fluorescence instrument. (1): 1 μM of the E3 (dimer). (2) 1 μM of the E3 plus NAD$^+$ added to a final concentration of 50 μM. (3) the same as (2) in the present of dihydrolipoamide (DHL) added to a final concentration of 250 μM. (4): same as (3) plus 5 minutes incubation. (5): same as (3) plus 10 minutes incubation. (6): same as (3) plus 30 minutes incubation.
Figure 16. The CD spectra of *Azotobacter vinelandii* E3 at 190 to 250 nm (95).
Figure 17. The CD spectra of oxidized *Azotobacter vinelandii* E3 at 300 to 600 nm (95).

Wild-type E3, ---; E3 E-455 to D mutant protein, ----; E3 H-450 to F, -----.
Figure 18. The CD spectra of reduced *Azotobacter vinelandii* E3 at 300 to 600 nm (95).

Wild-type E3, ---; E3 E-455 to D mutant protein, ----; E3 H-450 to F, ----.
Figure 19. The construction of the BS-E3c and the BS-E3 vector.
Figure 20. The construction of the pQE-9-E3 expression vector
Figure 21. The construction of the pQE-9-E3 K-37 to E expression vector
Figure 22. The construction of the pOTSV-E3 expression vector
Figure 23. The construction of the pQE-9-E3 P-453 to L expression vector
Figure 24. The construction of the pQE-9-E3 H-452 to Q expression vector
Figure 25. The construction of the pQE-9-E3 E-457 to Q expression vector
Figure 26. Activities of E3 and citrate synthase (CS) in fibroblasts from a control subject and the E3-deficient patient. Results are means ± SE of six culture dishes.
Figure 27. Western blot analysis (A and B) of the mitochondrial fraction prepared from cultured skin fibroblasts (75 µg of protein) of control and patient. (A) Lanes: 1, 4 ng of purified porcine E3; 2, patient; 3, control. This membrane was reacted with an epitope-selected anti-porcine heart E3 antibody. (B) Lanes: 1, control; 2, patient. E1α, E1β, E2, and E3 proteins were identified by using a combined antibody preparation. (C) Northern blot analysis of total RNA (25 µg) from cultured skin fibroblasts of the control (lane 1) and patient (lane 2).
Figure 28. DNA sequencing of the patient's specific E3 cDNA. (Upper) 5' normal and mutant sequence of subcloned cDNAs. (Lower) 3' normal and mutant sequences of subcloned cDNAs.
Figure 29. The Nickel column chromatographic profile of E3 (A), E3 K-37 to E (B) and E3 P-453 to L mutant proteins (C). The gradient is from 0 to 100 mM imidazole. The square represents the reading of OD_{273} nm. The diamond represents the reading of OD_{455} nm. The close circle represents the E3 activity. The close triangle represents the imidazole gradient. I represents the pool I in table 9. II represents the pool 2 in table 9.
Figure 30. Molecular sieving on HPLC of E3 and E3 mutant proteins. A: Bovine serum albumin fraction V, dimer, molecular weight 134 K dalton. B: E3; C: E3 K-37 to E; D: E3 H-452 to Q; E: E3 E-457 to Q; F: E3 P-453 to L mutant protein. The mobile phase is 50 mM potassium phosphate, pH 8, 0.25 mM EDTA and 0.5 % Trifluoroacacetate. The outlet of the protein was monitored at UV 220 nm.
Figure 31. The absorption spectra from 300 to 600 nm of 10 μM (dimer) of oxidized E3 and E3 mutant proteins. (1) E3; (2) E3 H-452 to Q (HQ); (3) E3 E-457 to Q (EQ); (4) E3 K-37 to E (KE); (5) E3 P-453 to L (PL).
Figure 32. The absorption spectra from 300 to 600 nm of 10 µM (dimer) of reduced E3 (10 mM of dimer plus dihydrolipoamide (DHL) to a final concentration of 2.5 mM) and E3 mutant proteins. (1) E3; (2) E3 H-452 to Q (HQ); (3) E3 E-457 to Q (EQ); (4) E3 K-37 to E (KE).
Figure 33. The fluorescence spectra of the E3.

The protein was excited at 296 nm by PTI fluorescence instrument. (1) 1 \mu M of the E3 (dimer). (2) 1 \mu M of the E3 plus NAD\(^+\) added to a final concentration of 50 \mu M. (3) the same as (2) in the present of dihydrolipoamide (DHL) added to a final concentration of 250 \mu M. (4) same as (3) plus 5 minutes incubation. (5) same as (3) plus 10 minutes incubation. (6) same as (3) plus 30 minutes incubation.
Figure 34. The fluorescence spectra of the E3 K-37 to E mutant protein (KE).

The protein was excited at 296 nm by PTI fluorescence instrument. (1) 1 μM of the E3 K-37 to E (dimer). (2) 1 μM of the E3 K-37 to E plus NAD$^+$ added to a final concentration of 50 μM. (3) the same as (2) in the presence of dihydrolipoamide (DHL) added to a final concentration of 250 μM. (4) same as (3) plus 5 minutes incubation. (5) same as (3) plus 10 minutes incubation. (6) same as (3) plus 30 minutes incubation.
(1) KE  
(2) KE + NAD⁺  
(3) KE + NAD⁺ + DHL  
(4) KE + NAD⁺ + DHL + 5min  
(5) KE + NAD⁺ + DHL + 10min  
(6) KE + NAD⁺ + DHL + 30min
Figure 35. The fluorescence spectra of the E3 P-453 to L mutant protein (PL).

The protein was excited at 296 nm by PTI fluorescence instrument. (1) 1 μM of the E3 P to L(dimer). (2) 1 μM of the E3 P to L plus NAD+ added to a final concentration of 50 μM. (3) the same as (2) in the present of dihydrolipoamide added to a final concentration of 250 μM. (4) same as (3) plus 5 minutes incubation.
Figure 36. The fluorescence spectra of the E3 H-452 to Q mutant protein (HQ).

The protein was excited at 296 nm by PTI fluorescence instrument. (1) 1 μM of the E3 H-452 to Q (dimer). (2) 1 μM of the E3 H-452 to Q plus NAD⁺ added to a final concentration of 50 μM. (3) the same as (2) in the present of dihydrolipoamide (DHL) added to a final concentration of 250 μM. (4) same as (3) plus 5 minutes incubation.
(1) HQ
(2) HQ + NAD^+
(3) HQ + NAD^+ + DHL
(4) HQ + NAD^+ + DHL + 5min

Fluorescence x 10^3

Wavelength (nm)
Figure 37. The fluorescence spectra of the E3 E-457 to Q mutant protein (EQ).

The protein was excited at 296 nm by PTI fluorescence instrument. (1) 1 µM of the E3 E to Q (dimer). (2) 1 µM of the E3 E to Q plus NAD+ added to a final concentration of 50 µM. (3) the same as (2) in the present of dihydrolipoamide (DHL) added to a final concentration of 250 µM. (4) same as (3) plus 5 minutes incubation. (5) same as (3) plus 10 minutes incubation. (6) same as (3) plus 30 minutes incubation.
Fluorescence x 10^{-5}

Wavelength (nm)

(1) EQ
(2) EQ + NAD^+
(3) EQ + NAD^+ + DHL
(4) EQ + NAD^+ + DHL + 5 min
(5) EQ + NAD^+ + DHL + 10 min
(6) EQ + NAD^+ + DHL + 30 min
Figure 38. The CD spectra of the E3 and E3 mutant proteins at 190 to 250 nm. Each protein concentration is 10 μM (dimer). (1) E3; (2) E3 K-37 to E (KE); (3) E3 H-452 to Q (HQ); (4) E3 E-452 to Q (EQ); (5) E3 P-452 to L (PL).
Figure 39. The CD spectra of the oxidized E3 and E3 mutant proteins at 300 to 600 nm. Each protein concentration is 10 μM (dimer). (1) E3; (2) E3 K-37 to E (KE); (3) E3 H-452 to Q (HQ); (4) E3 E-457 to Q (EQ); (5) E3 P-453 to L (PL).
Figure 40. The CD spectra of the reduced E3 and E3 mutant proteins (driven by DHL) at 300 to 600 nm. Each protein concentration is 10 μM (dimer). The dihydrolipoamide (DHL) was added to a final concentration of 5 mM. (1) E3; (2) E3 K-37 to E (KE); (3) E3 H-452 to Q (HQ); (4) E3 E-457 to Q (EQ); (5) E3 P-453 to L (PL).
Figure 41. The CD spectra of the reduced to oxidized E\textsubscript{3} and E\textsubscript{3} mutant proteins (driven by DHL, then N) at 300 to 600 nm. Each protein concentration is 10 µM (dimer). The dihydrolipoamide (DHL) was added to a final concentration of 5 mM. The NAD\textsuperscript{+}(N) was added to a final concentration of 500 µM. (1) E\textsubscript{3}; (2) E\textsubscript{3} K-37 to E (KE); (3) E\textsubscript{3} H-452 to Q (HQ); (4) E\textsubscript{3} E-457 to Q (EQ); (5) E\textsubscript{3} P-453 to L (PL).
Figure 42. The CD spectra of the reduced E3 and E3 mutant proteins (driven by NH) at 300 to 600 nm. Each protein concentration is 10 μM (dimer). The NADH (NH) was added to a final concentration of 500 μM. (1) E3; (2) E3 K-37 to E (KE); (3) E3 H-452 to Q (HQ); (4) E3 E-457 to Q (EQ); (5) E3 P-453 to L (PL).
Figure 43. The CD spectra of the reduced to oxidized E3 and E3 mutant proteins (driven by NH, then L) at 300 to 600 nm. Each protein concentration is 10 μM (dimer). The NADH (NH) was added to a final concentration of 500 μM. The lipoamide (L) was added to a final concentration of 5 mM. (1) E3; (2) E3 K-37 to E (KE); (3) E3 H-452 to Q (HQ); (4) E3 E-457 to Q (EQ); (5) E3 P-453 to L (PL).
Figure 44. The proposed active center of human E3 built by molecular graphic modeling based on the three-dimensional structure of human GR (83).
Table 1. The substrates and products of the pyridine nucleotide oxidoreductase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Products</th>
</tr>
</thead>
</table>
| E₃     | \[
  \begin{array}{c}
  \text{CH}_2 \\
  \text{CH}_2(CH_2)_4\text{C}NH_2 \\
  \text{RS} \\
  \text{SH}
  \end{array}
\] | \[
  \begin{array}{c}
  \text{CH}_2 \\
  \text{CH}_2(CH_2)_4\text{C}NH_2 \\
  \text{S} \\
  \text{S}^{-}
  \end{array}
\] | Dihydrolipoamide |
|        | \[
  \begin{array}{c}
  \text{Gly} \\
  \text{Gly} \\
  \gamma\text{Glu-Cys-S-Cys-Gly}
  \end{array}
\] | \[
  \begin{array}{c}
  \text{Gly} \\
  \text{Gly} \\
  \gamma\text{Glu-Cys-SH}
  \end{array}
\] | Oxidized Glutathione |
|        | \[
  \begin{array}{c}
  \text{Oxidized Thioredoxin}
  \end{array}
\] | \[
  \begin{array}{c}
  \text{Reduced Thioredoxin}
  \end{array}
\] | Reduced Thioredoxin |
| MR     | Hg(II)     | Hg⁰      |
| TTR    | \[
  \begin{array}{c}
  \text{(CH}_2\text{)}_3\text{NH}(\text{CH}_2)_4 \\
  \text{NH} \\
  \text{gly}
  \end{array}
\] | \[
  \begin{array}{c}
  \text{(CH}_2\text{)}_3\text{NH}(\text{CH}_2)_4 \\
  \text{NH} \\
  \text{gly}
  \end{array}
\] | Oxidized Trypanothione |
|        | \[
  \begin{array}{c}
  \gamma\text{Glu-Cys-S-Cys-Gly}
  \end{array}
\] | \[
  \begin{array}{c}
  \gamma\text{Glu-Cys-SH} \text{HS-Cys-Gly}
  \end{array}
\] | Reduced Trypanothione |
| AD     | \[
  \begin{array}{c}
  \text{COOH} \\
  \text{CH}_2 \\
  \text{SH} \\
  \text{SH}
  \end{array}
\] | \[
  \begin{array}{c}
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  \text{CH}_2 \\
  \text{SH} \\
  \text{SH}
  \end{array}
\] | Dihydroaspartic acid |
| GSR    | \[
  \begin{array}{c}
  \gamma\text{Glu-Cys-S-Cys-Gly}
  \end{array}
\] | \[
  \begin{array}{c}
  \gamma\text{Glu-Cys-SH}
  \end{array}
\] | Bis-γ-glutamylcysteine |

See references 16, 19, 22, 24, and 25.
Table 2. The substrates, products, and precursor amino acids of α-keto acid dehydrogenase complexes and glycine cleavage system

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<th>Substrates</th>
<th>Products</th>
<th>Precursor Amino acids</th>
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<td>Pyruvate</td>
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<td>α-Ketoglutarate</td>
<td>Succinyl CoA</td>
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<td>α-Ketoisovalerate</td>
<td>2-Methyl propionyl CoA</td>
<td>Val</td>
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<td>α-Ketoisocaproate</td>
<td>3-methyl butyryl CoA</td>
<td>Leu</td>
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<td>α-Keto β-methyl valerate</td>
<td>2-Methyl butyryl CoA</td>
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<td>α-Ketobutyrate</td>
<td>Propionyl CoA</td>
<td>Thr</td>
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<td>α-Keto γ-methyl thiobutyrate</td>
<td>3-Methyl thiopropionyl CoA</td>
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<tr>
<td>GCS</td>
<td>Glycine</td>
<td>CH$_2$ and NH$_3$</td>
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See references 26, 27, 28, and 34.
Table 3. The amino acids in FAD binding site

<table>
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<th>Domain</th>
<th>Amino Acid</th>
<th>Description</th>
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<td></td>
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<td>αIF (15-29)</td>
<td>G17:</td>
<td>main chain, amide NH, H-bond to pyrophosphate 0 OF1</td>
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<tr>
<td>βF3 (32-36)</td>
<td>E36:</td>
<td>side chain, link to ribose hydroxyl O2' A and O3' A</td>
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<tr>
<td></td>
<td>K37:</td>
<td>main chain, amide NH, H-bond to adenine N3</td>
</tr>
<tr>
<td>αII (50-66)</td>
<td>T44:</td>
<td>main chain, amide NH, H-bond to pyrophosphate OA2</td>
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<td></td>
<td>T44:</td>
<td>side chain, H-bond to phosphate OA1, OA2, and O4'</td>
</tr>
<tr>
<td></td>
<td>K54:</td>
<td>side chain, link to FAD 4x and form ion-pair (salt bridge) with E192 side chain</td>
</tr>
<tr>
<td>βM3 (119-122)</td>
<td>I121:</td>
<td>main chain, carboxyl O, H-bond to adenine 6x A</td>
</tr>
<tr>
<td></td>
<td>I122:</td>
<td>main chain, amide NH, H-bond to adenine IA</td>
</tr>
<tr>
<td>βF4 (143-148)</td>
<td>G149:</td>
<td>main chain, amide NH, H-bond to OF1 and OF2 via HOH</td>
</tr>
<tr>
<td>NAD domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αV (187-201)</td>
<td>V188:</td>
<td>side chain, non-polar interaction with flavin ring</td>
</tr>
<tr>
<td></td>
<td>E192:</td>
<td>side chain, ion-pair (salt bridge) with K34</td>
</tr>
<tr>
<td>βN2(277-277)</td>
<td>R280:</td>
<td>side chain, interact with benzene ring</td>
</tr>
<tr>
<td>Center domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>βM4 (287-289)</td>
<td>N286:</td>
<td>main chain, amide NH, H-bond to adenine 6x A via HOH</td>
</tr>
<tr>
<td></td>
<td>N286:</td>
<td>main chain, amide NH, H-bond to adenine 7x A via HOH</td>
</tr>
<tr>
<td>βF5 (314-318)</td>
<td>I314:</td>
<td>main chain, carboxyl O, H-bond to pyrophosphate OF1 and OF2 via HOH</td>
</tr>
<tr>
<td></td>
<td>G315:</td>
<td>main chain, amide NH, H-bond to pyrophosphate OF1 and OF2 via HOH</td>
</tr>
<tr>
<td></td>
<td>D316:</td>
<td>main chain, amide NH, H-bond to pyrophosphate OF1 and OF2 via HOH</td>
</tr>
<tr>
<td></td>
<td>D316:</td>
<td>side chain, H-bond to riboflavin hydroxyl O3'</td>
</tr>
<tr>
<td>αII (329-345)</td>
<td>A328:</td>
<td>main chain, amide NH, H-bond to FAD 2x</td>
</tr>
<tr>
<td>Interface domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αXI (455-461)</td>
<td>A452':</td>
<td>main chain, carboxyl O, H-bond FAD N3</td>
</tr>
</tbody>
</table>

See reference 80, 81, 82, and 83.
Table 4. The amino acids in NAD binding site

<table>
<thead>
<tr>
<th>NAD domain</th>
<th>Amino Acid</th>
<th>Interaction Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>αV (187-210)</td>
<td>Glu187</td>
<td>main chain, amide NH, H-bond to O2'</td>
</tr>
<tr>
<td></td>
<td>Gln188</td>
<td>main chain, amide NH, H-bond to O2'</td>
</tr>
<tr>
<td>βN4 (203-208)</td>
<td>Glu208</td>
<td>main chain, amide NH, H-bond to O3' via HOH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>side chain, H-bond to O2'A and O3'A</td>
</tr>
<tr>
<td>αV1 (219-233)</td>
<td>Lys232</td>
<td>main chain, carbonyl O, H-bond to O4' and O4 via HOH</td>
</tr>
<tr>
<td>βMN1 (240-249)</td>
<td>Val243</td>
<td>main chain, amide NH, H-bond to adenine N1A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>main chain, carbonyl O, H-bond to adenine N6A</td>
</tr>
<tr>
<td>βN2 (272-277)</td>
<td>Arg280</td>
<td>side chain, H-bond to O3' via HOH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>main chain, carbonyl O, H-bond to Nicotinamide 3nN</td>
</tr>
<tr>
<td>αVII (327-343)</td>
<td>Met327</td>
<td>main chain, carbonyl O, H-bond to O2'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interface domain</th>
<th>Amino Acid</th>
<th>Interaction Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>αVIII (370-377)</td>
<td>Gln373</td>
<td>main chain, carbonyl O, H-bond to O2'A via HOH</td>
</tr>
</tbody>
</table>

See reference 80, 81, 82, and 83.
Table 5 The amino acid involved in intersubunit hydrogen bonding

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Amino Acid 1</th>
<th>Amino Acid 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD domain-FAD domain interaction</td>
<td>S60 (O5)</td>
<td>R74' (Nη1)</td>
</tr>
<tr>
<td></td>
<td>G75 (O)</td>
<td>N86'(N)</td>
</tr>
<tr>
<td></td>
<td>E77(N)</td>
<td>R82'(O)</td>
</tr>
<tr>
<td></td>
<td>E77(O)</td>
<td>R82'(N)</td>
</tr>
<tr>
<td></td>
<td>S79(N)</td>
<td>S79'(N)</td>
</tr>
<tr>
<td>FAD domain-Interface domain interaction</td>
<td>Q91(Nε2)</td>
<td>T396'(O)</td>
</tr>
<tr>
<td></td>
<td>Q27(Nε2)</td>
<td>K470'(O)</td>
</tr>
<tr>
<td></td>
<td>N57(Oδ1)</td>
<td>R393'(Nη1)</td>
</tr>
<tr>
<td>Interface domain-Interface domain interaction</td>
<td>Y359(Oη)</td>
<td>P453'(O)</td>
</tr>
<tr>
<td></td>
<td>E427(Oε1)</td>
<td>S456'(N)</td>
</tr>
<tr>
<td></td>
<td>N430(Oε1)</td>
<td>A451'(N)</td>
</tr>
<tr>
<td></td>
<td>N430(Nε2)</td>
<td>C449'O</td>
</tr>
</tbody>
</table>

See reference 80, 81, 82, and 83.
Table 6. The amino acids in the active site.

<table>
<thead>
<tr>
<th>Active disulfide</th>
<th>C45: disulfide link with C50, main chain, carbonyl O, H-bond to I51 N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G49: (q, w) = (-132°, -119°)</td>
</tr>
<tr>
<td></td>
<td>C50: disulfide link with C45</td>
</tr>
<tr>
<td></td>
<td>I51: main chain, amide NH, H-bond to C45 O</td>
</tr>
<tr>
<td></td>
<td>P52: proline turn</td>
</tr>
<tr>
<td></td>
<td>K54: side chain, form ion-pair with E192' side chain, withdraw electron</td>
</tr>
<tr>
<td></td>
<td>from FAD, transfer electron to NAD</td>
</tr>
<tr>
<td></td>
<td>E192': side chain, ion pair with K54</td>
</tr>
<tr>
<td>Active base</td>
<td>H457: imidazole N1 form H-bond with E457' side chain, N3 form ion pair</td>
</tr>
<tr>
<td></td>
<td>with C45</td>
</tr>
<tr>
<td></td>
<td>P453': P turn</td>
</tr>
<tr>
<td></td>
<td>E457': side chain, form H-bond with H452'N1</td>
</tr>
<tr>
<td>Isoalloxazine</td>
<td>see Table 4</td>
</tr>
<tr>
<td>NAD binding site</td>
<td>see table 5, re-face of the flavin ring</td>
</tr>
<tr>
<td>Lipoamide binding site</td>
<td>s-face of the flavin ring</td>
</tr>
</tbody>
</table>

See reference 80, 81, 82, and 83.
Table 7. Primers for PCR amplification and sequencing.

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5-7</td>
<td>AGCGGAGGTGAAAGTATTTGCGGA</td>
<td>Sense</td>
</tr>
<tr>
<td>A3-521</td>
<td>AGTTATCTTTCTATATCCATTGAC</td>
<td>Antisense</td>
</tr>
<tr>
<td>B5-417</td>
<td>ATGATGGAGCAGAAGAGTACTGCA</td>
<td>Sense</td>
</tr>
<tr>
<td>B3-1088</td>
<td>TTTAGTTTGAAATCTGATTGAC</td>
<td>Antisense</td>
</tr>
<tr>
<td>C5-1034</td>
<td>AATTTGACTAGATCCACAGGATGAG</td>
<td>Sense</td>
</tr>
<tr>
<td>C3-1671</td>
<td>TCTTTGAGCTGAGAATATCCT</td>
<td>Antisense</td>
</tr>
<tr>
<td>E3-274</td>
<td>CACTTGTTGGTACCTCCCTGAATGTTG</td>
<td>IP sense</td>
</tr>
<tr>
<td>E3-365</td>
<td>ATCTTTTCCATGGGCCCATAT</td>
<td>IP antisense</td>
</tr>
<tr>
<td>E3-768</td>
<td>CAGCAGTTGAACGTTTAGGTCATG</td>
<td>IP sense</td>
</tr>
<tr>
<td>E3-835</td>
<td>GAAAGTTTTAGATATCTCC</td>
<td>IP antisense</td>
</tr>
<tr>
<td>E3-1282</td>
<td>TCTTTCAACTGCTCTTCTGA</td>
<td>IP antisense</td>
</tr>
<tr>
<td>E3-1462</td>
<td>TGGAATATGGAGCATCCTGT</td>
<td>IP sense</td>
</tr>
<tr>
<td>E3-1522</td>
<td>TAAGGTCCGCTGTCATGA</td>
<td>IP antisense</td>
</tr>
</tbody>
</table>

IP, internal primer.

*Number represents location of 5' end of the primer, which corresponds to the E3 cDNA numbering system published by Pons et al. (33).
Table 8. The primers for site-directed mutagenesis PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>GCGCGCGGATCCGAGATCAGCCGATT</td>
<td>Sense</td>
</tr>
<tr>
<td>L2</td>
<td>GCGCGCGGATCCTCAAAAAGTTGATTGATTTGCC</td>
<td>Antisense</td>
</tr>
<tr>
<td>E3K-37 to E</td>
<td>TGCATTGAGGAAAATGAAACA</td>
<td>Sense</td>
</tr>
<tr>
<td>E3P-453 to L</td>
<td>CATGCACATCTGACCTTATC</td>
<td>Sense</td>
</tr>
</tbody>
</table>
Table 9. Purification table of E3, E3 K to E, and E3 P to L mutant protein.

Purification of human E3 (wild-type) from *Escherichia coli* M15 strain with 'pQE-9-E3' expression vector

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>380</td>
<td>34.6</td>
<td>100</td>
</tr>
<tr>
<td>Flow through</td>
<td>248</td>
<td>3.5</td>
<td>6.6</td>
</tr>
<tr>
<td><strong>Imidazole gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>22</td>
<td>7</td>
<td>1.2</td>
</tr>
<tr>
<td>Peak 3 (pool I)</td>
<td>3.4</td>
<td>406</td>
<td>10</td>
</tr>
<tr>
<td>(pool 2)</td>
<td>14</td>
<td>776</td>
<td>81.3</td>
</tr>
</tbody>
</table>

Purification of human E3 K-37 to E mutant protein from *Escherichia coli* M15 strain with 'pQE-9-E3 K-37 to E' expression vector

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>376</td>
<td>15.7</td>
<td>100</td>
</tr>
<tr>
<td>Flow through</td>
<td>224</td>
<td>3.4</td>
<td>12.9</td>
</tr>
<tr>
<td><strong>Imidazole gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>20</td>
<td>17.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Peak 3 (pool I)</td>
<td>7.2</td>
<td>81</td>
<td>9.8</td>
</tr>
<tr>
<td>(pool 2)</td>
<td>11</td>
<td>377</td>
<td>69.3</td>
</tr>
</tbody>
</table>

Purification of human E3 P-453 to L mutant protein from *Escherichia coli* M15 strain with 'pQE-9-E3 P-453 to L' expression vector

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>344</td>
<td>2.8</td>
<td>100</td>
</tr>
<tr>
<td>Flow through</td>
<td>212</td>
<td>3.7</td>
<td>81.6</td>
</tr>
<tr>
<td><strong>Imidazole gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>25</td>
<td>1.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Peak 3 (pool I)</td>
<td>5.6</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>(pool 2)</td>
<td>14</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Table 10. Comparison of E3 amino acid sequence from various sources around mutated residues K-37 and P-453 in human E3.

<table>
<thead>
<tr>
<th>Source</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>VERYN . . . HAPTLSSE</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>IEKYI . . . PAPTLSSEA</td>
</tr>
<tr>
<td>A. vinelandii</td>
<td>IEKYK . . . PARPALSEA</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>VEKGN . . . HAPTLSGE</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>VEKAT . . . HAPTLGEX</td>
</tr>
<tr>
<td>P. putida (valine)</td>
<td>VEGQA . . . HAPTLSGEA</td>
</tr>
<tr>
<td>P. putida (glucose)</td>
<td>IEKTY . . . HAPTLSSEA</td>
</tr>
<tr>
<td>P. putida (3)</td>
<td>VEGRS . . . HAPTRSEA</td>
</tr>
<tr>
<td>Yeast</td>
<td>VEKRG . . . HAPTLSSEA</td>
</tr>
<tr>
<td>Pig</td>
<td>IEKNE . . . HAPTLSSEA</td>
</tr>
<tr>
<td>Human</td>
<td>IEKNE . . . HAPTLSSEA</td>
</tr>
<tr>
<td>E3 patient</td>
<td>IEENE . . . HAHLTLSSEA</td>
</tr>
<tr>
<td>Mutant Protein</td>
<td>Functional Significance</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>E3 K-37 to E</td>
<td>Decreased the FAD Binding</td>
</tr>
<tr>
<td>E3 P-453 to L</td>
<td>Destroyed Protein Conformation</td>
</tr>
<tr>
<td>E3 H-452 to Q</td>
<td>Destroyed the Active Base</td>
</tr>
<tr>
<td>E3 E-457 to Q</td>
<td>Inhibited NADH Release</td>
</tr>
</tbody>
</table>