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Studies on site-specifically modified human dihydrolipoamide dehydrogenase

Kim, Hakjung, Ph.D.
Case Western Reserve University (Health Sciences), 1992
STUDIES ON SITE-SPECIFICALLY MODIFIED HUMAN DIHYDROLIPOAMIDE DEHYDROGENASE

by

HAKJUNG KIM

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

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January, 1992
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Hajjung Kim
STUDIES ON SITE-SPECIFICALLY MODIFIED HUMAN DIHYDROLOPOAMIDE DEHYDROGENASE

Abstract
by
HAKJUNG KIM

The cDNA sequences encoding mature and precursor forms of human dihydrolipoamide dehydrogenase (E3) were expressed in E. coli using a λ PL promoter-driven prokaryotic expression vector. Since wild-type E. coli has its own endogenous E3 activity, the expression of human E3 was performed in a pyruvate dehydrogenase complex-deficient strain of E. coli JRG1342. The amino-terminal amino acid sequence analysis revealed that the recombinant mature E3 possessed an expected sequence while the recombinant precursor E3 lost 19 amino acid residues of its 35-amino acid leader sequence presumably due to a proteolytic cleavage. The recombinant mature E3 displayed comparable kinetic properties to those reported for highly purified mammalian E3s. The truncated precursor E3 showed about 36% of the mature E3 activity. The double reciprocal plot for the mature E3 in the direction of NAD⁺ reduction showed parallel lines (ping-pong mechanism) while that for the truncated precursor E3 displayed intersecting lines (sequential mechanism). In the direction of NADH oxidation, the kinetic mechanisms of both E3s were
apparently a ping-pong mechanism. This study indicated that the mature recombinant human E₃ was an active enzyme whose kinetic properties were similar to those of mammalian E₃s.

Two site-specifically mutated human E₃s (His-452 -> Gln and Glu-457 -> Gln) were expressed in *E. coli* JRG1342. The double reciprocal plot for the Gln-452 mutant E₃ in the direction of NAD⁺ reduction showed parallel lines, indicating that the mutant E₃, like wild-type enzyme, catalyzed E₃ reaction via a ping-pong mechanism. The specific activity of the Gln-452 mutant E₃ was about 0.2% of that of wild-type enzyme. Its *Kₘ* for dihydrolipoamide was dramatically increased by about 63-fold. The substitution of His-452 to Gln resulted in a destabilization of the transition state of human E₃ catalysis by about 6.4 kcal mol⁻¹. The Gln-452 mutant E₃ possessed about 2-fold higher activity at pH 8.5 than the activity at pH 8.0, indicating that the Gln-452 mutant E₃ probably catalyzed the reaction with the help of water molecules (hydroxyl ions) in assay solution. The Gln-457 mutant E₃, unlike wild-type enzyme, catalyzed the E₃ reaction via a sequential mechanism in the direction of NAD⁺ reduction based on the intersecting lines shown on a double reciprocal plot. Its specific activity decreased to 28% of that of wild-type enzyme. Its *Kₘ* for dihydrolipoamide increased about 4.3-fold. The substitution of Glu-457 to Gln resulted in a destabilization of the transition state by about 1.7 kcal mol⁻¹. These results indicate that His-452, which is
a possible proton acceptor/donor in E₃ reaction, is critical to E₃ catalysis and that the local environment around His-452 and Glu-457, which are suggested to be hydrogen-bonded, is important in the binding of dihydrolipoamide to the enzyme.
Dedication

This dissertation is dedicated to my parents, Yunsik and Youngja Kim, and my teachers.
Acknowledgements

I would like to give special thanks to my advisor, Dr. Mulchand S. Patel, for guiding me throughout this work. He is a nice and intelligent person. I am very grateful to him for his support of my research and career. I also thank my thesis committee members, Drs. Douglas S. Kerr, Joyce E. Jentoft and David Samols, for their helpful suggestions regarding my research. Dr. Kerr has a great deal of knowledge about the pyruvate dehydrogenase complex. He joined our lab group meetings and always gave me good suggestions regarding my research at the meetings. Dr. Jentoft provided me much knowledge about proteins through lectures and other educative meetings. Dr. Samols helped me to develop a human expression system which was a basis of this study. I thank Dr. Nelson Phillips for kindly serving as a member of my defense committee. He also gave me good suggestions about enzyme kinetics. I appreciate the support of all my committee members throughout this study. I am very fortunate to know them and hope these good relationships to continue for a long time.

It has been a nice experience to work with members of Dr. Patel's laboratory. I wish to thank Te-chung Liu for teaching me several recombinant techniques in the early stage of my research and for keeping a good relationship with me. I am very grateful to other members of the lab for providing me a pleasant life. We had
several enjoyable times at Dr. Patel's parties, at the amusement
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pyruvate dehydrogenase complex-deficient \textit{E. coli} strain JRG1342.
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List of Abbreviations.

dl : Dihydrolipoamide
E₁: α-Keto acid dehydrogenase
E₂: Dihydrolipoyl acyltransferase
E₃: Dihydrolipoamide dehydrogenase
PDC: Pyruvate dehydrogenase complex
GR: Glutathione reductase
TR: Thioredoxin reductase
kb: kilobase
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Chapter I
Introduction

1. General introduction.

Mammalian dihydrolipoamide dehydrogenase (E3) (EC 1.8.6.4) is a common component of three \( \alpha \)-keto acid dehydrogenase complexes (pyruvate, \( \alpha \)-ketoglutarate and branched-chain \( \alpha \)-keto acid dehydrogenase complexes) (Reed, 1974) and the glycine cleavage system (Walker and Oliver, 1986). It catalyzes the oxidation of the dihydrolipoyl moiety attached to the lysyl residue(s) of the acyltransferase components of the three \( \alpha \)-keto acid dehydrogenase complexes and to the hydrogen-carrier protein of the glycine cleavage system. It is a homodimeric flavoprotein. Each polypeptide consists of 474 amino acid residues with a molecular mass of 50,216 daltons calculated from the primary amino acid sequence deduced from human liver E3 cDNA sequence (Pons et al., 1988). One FAD is noncovalently bound to each subunit as a cofactor. E3 is a mitochondrial protein that is synthesized as a precursor form in the cytoplasm. The precursor E3 has a leader sequence composed of 35-amino acids at its amino-terminus which is cleaved off during its importation into the mitochondria matrix.

E3 is a member of a pyridine nucleotide-disulfide oxidoreductase family whose members include glutathione
reductase (GR), thioredoxin reductase (TR), and trypanothione reductase (Williams, 1976). They catalyze electron transfer reactions between pyridine nucleotides (NAD\(^+\) or NADPH) and their specific substrates. The electrons are transferred through FAD and the active disulfide center.

E3 has been isolated from a variety of prokaryotes including *Escherichia coli*, *Pseudomonas Bacillus*, *Halobacterium*, *Eubacteris Azotobacter vinelandii* and *Clostridium* (see reviews; Williams, 1976; Carothers et al., 1989). In *E. coli*, a single *lpd* gene codes for E3 which is a common component of the pyruvate and \(\alpha\)-ketoglutarate dehydrogenase complexes. Its expression is regulated by both a pyruvate dehydrogenase complex (PDC) operon promoter and an independent promoter of the *lpd* gene (Stephens, et al., 1983). Three E3s are found in *Pseudomonas* (Burns et al., 1989). One E3 is suggested to be for PDC, \(\alpha\)-ketoglutarate dehydrogenase complex, and the glycine cleavage system and another for the branched-chain \(\alpha\)-keto acid dehydrogenase complex. A third E3 reported from *Pseudomonas* is suggested to be associated with an unknown multienzyme complex (Burns et al., 1989). E3 has been also purified from various eukaryotic sources including yeast, pig heart, beef liver, rat liver and human liver (see reviews; Williams, 1976; Carothers et al., 1989). Two immunologically distinct E3s have been reported in rat liver mitochondria (Carothers et al., 1987). One is specific for \(\alpha\)-keto acid complexes and the other may
be specific for the glycine cleavage system.

2. **Three mammalian α-keto acid dehydrogenase complexes containing E3.**

The three mammalian α-keto acid dehydrogenases are composed of multiple copies of three catalytic components (Reed, 1974; Yeaman, 1986; Patel and Roche, 1990). The catalytic components consist of α-keto acid dehydrogenase (E₁), dihydrolipoyl acyltransferase (E₂) and E₃. They catalyze sequential reactions which are illustrated in Figure 1. PDC and the branched-chain α-keto acid dehydrogenase complexes also contain two specific regulatory subunits which control their activities. The regulatory components include E₁-kinase and phospho-E₁-phosphatase. The PDCs from mammals and yeast also contain protein X with a Mᵣ of 50,000 (De Marcucci and Lindsay, 1985; Jilka et al., 1986).

The E₂ component forms a core structure to which multiple copies of E₁, E₃, protein X and regulatory proteins bind noncovalently. There are two distinct polyhedral forms of the E₂ core (Oliver and Reed, 1982; Reed and Hackert, 1990). The cube form is found in the *E. coli* PDC and the mammalian α-ketoglutarate dehydrogenase complex and the branched-chain α-ketoacid dehydrogenase complex (Reed and Hackert, 1990).
Figure 1. Schematic representation of the sequence of partial reactions catalyzed by the three catalytic components. $E_1$ component catalyzes reactions 1 and 2. $E_2$ component catalyzes reaction 3. $E_3$ component catalyzes reactions 4 and 5.
Twenty-four E₂ subunits are arranged with octahedral (432) symmetry in this form. The pentagonal dodecahedron form is found in the PDCs from mammals, birds, fungi and the Gram-positive bacterium *Bacillus stearothermophilus* (Reed and Hackert, 1990). This form is consistent with 60 E₂ subunits arranged with icosahedral (532) symmetry.

The *E. coli* PDC has a molecular weight (Mₚ) of 4,600,000 and 12 E₁ dimers and 6 E₃ dimers are apparently bound on the 12 edges and in the 6 faces of the cube-like E₂ core (Reed et al., 1975; CaJacob et al., 1985). In the bovine heart PDC (Mₚ 8,500,000), about 30 E₁ tetramers bind near the 30 edges of the pentagonal dodecahedron-like E₂ core while 6 E₃ dimers are attached in the 12 faces (Barrera et al., 1972). About 3 E₁-kinases are also bound to the E₂ core. The phospho-E₁-phosphatase is loosely attached to the E₂ core in the presence of Ca²⁺. In the bovine kidney PDC, about 6 protein X are associated with the E₂ core. Protein X contains a lipoyl moiety that can be reductively acetylated. Studies on yeast protein X indicate that protein X is not involved in formation of the E₂ core structure (Lawson et al., 1991). From the deletion studies on yeast protein X gene (Lawson et al., 1991), it is revealed that the lipoyl domain (residues # 6-80) of protein X is not important to the overall activity of the PDC. However, the putative subunit binding domain (residues # 144-180) of protein X is important to the high-affinity binding of E₃ to the
E2 core structure which is important to the overall activity of the PDC.


The human erythrocyte glutathione reductase (GR) (E.C. 1.6.4.2) is a homodimeric flavoprotein with subunit $M_r$ of 52,400 (Worthington and Rosemeyer, 1975) (Table 1). It catalyzes the irreversible reduction of oxidized glutathione by NADPH in vivo (Williams, 1976). It maintains a high ratio of reduced to oxidized glutathione, which is estimated to be 300 (Akerboom, et al., 1982). Reduced glutathione is a substrate of a group of detoxification enzymes referred to as thiol S-transferases and of several transhydrogenases and glutathione peroxidase. It is, therefore, very important in many intracellular processes such as the recovery from oxygen induced cell damage and generation of deoxynucleotide precursors for DNA synthesis and maintenance of reduced thiols (Williams, 1976).

The kinetic mechanisms of human erythrocyte and yeast GRs in the physiological direction are mixed sequential and ping-pong mechanisms depending on the concentration of substrate (Staal and Veeger, 1969; Mannervik, 1973). Having distinct substrate binding sites for glutathione and NADPH, the structure of GR allows
# Table 1
Pyridine nucleotide-disulfide oxidoreductase family

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>MW of subunit</th>
<th>Pyridine nucleotide</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrolipoamide dehydrogenase</td>
<td>Pig heart</td>
<td>55000</td>
<td>NAD⁺</td>
<td>Dihydrolipoyl moiety (bound to protein)</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>Human erythrocyte</td>
<td>52400</td>
<td>NADPH</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td><em>E. coli</em></td>
<td>35400</td>
<td>NADPH</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>Trypanothione reductase</td>
<td><em>Trypanosoma cruzi</em></td>
<td>50000</td>
<td>NADPH</td>
<td>Trypanothione</td>
</tr>
</tbody>
</table>
this complicated kinetic mechanism. The two-electron-reduced intermediate (EH$_2$) of human GR exists as three possible species, EH$_2$, EH$_2$-NADP$^+$ or EH$_2$-NADPH to which oxidized glutathione binds (Williams, 1976). Since the relative concentrations of these intermediates will vary with substrate concentrations, simple kinetic mechanisms may not be appropriate. Assuming a ping-pong mechanism, the $K_m$ for NADPH of human erythrocyte GR has been determined as 13 uM, $K_m$ for oxidized glutathione as 125 uM, $k_{cat}$ as 238 s$^{-1}$ in 0.3 M phosphate buffer at pH 7, 25°C (Staal and Veeger, 1969). In low ionic strength, those parameters are altered. The $K_m$ for NADPH is lowered to 9.5 uM and $K_m$ for oxidized glutathione to 19 uM, $k_{cat}$ to 128 s$^{-1}$ in 0.03 M phosphate buffer.

Pre-steady state kinetic studies with yeast GR indicate that there are three kinetically detectable steps in the reductive half reaction (Huber and Brandt, 1980). The first step is attributed to the formation of a charge transfer complex in which NADPH is a donor and FAD is an acceptor. The second step is involved in reducing FAD. The third step is a rate limiting step in this reductive half reaction. In this step, electrons are transferred from the reduced FAD to the active site disulfide bond. This reductive half reaction (88 s$^{-1}$) of yeast GR is not a rate limiting step in the overall reaction of yeast GR (Williams, 1976). However, this step is much slower than that of pig heart E3 (> 800 s$^{-1}$).
The three dimensional structure of human erythrocyte GR has been established up to a resolution of 1.54 Å (Thieme et al., 1981; Karplus and Schulz, 1987). Thirty one percent of total amino acids (149 residues) in GR contribute to the formation of eleven α-helices. There are also 5 well defined pleated sheet structures comprising 152 residues (Thieme et al., 1981). The FAD domain is non-globular due to long α-helical structures (Schulz et al., 1982) (Figure 2). It contains the active site disulfide bond which is located adjacent to the flavin ring. The flavin moiety of FAD is buried inside the enzyme while the adenine moiety is partly accessible to the solvent. The disulfide bond is approximately perpendicular to the flavin ring and the sulfur atom of Cys-63 is located 3.6 Å from the C4a atom of flavin. The active disulfide bond is left-handed and unique among the known disulfide bonds (Schulz et al., 1982). A mechanical strain due to the unique conformation of the disulfide bond could be related to the catalytic mechanism. The distance between Ca atom of Cys-58 and that of Cys-63 is very short with distance of 4.6 Å.

NADPH is bound in a pocket on the surface of the enzyme (Pai et al., 1988; Karplus and Schulz, 1989). It has an extended conformation and its binding does not lead to a big conformational change. Most of its contacts are with residues of the NADPH domain except the nicotinamide moiety (Figure 3). The nicotinamide moiety stacks on the flavin ring with which it makes
Figure 2. Stereoviews of the chain fold of one complete subunit of human GR (upper) and of the chain fold of FAD domain (lower). The amino-terminal segment of residues 1 to 18 is omitted because it has no defined conformation. Bond between consecutive Cα atoms are represented by the lines. The numbers indicate the amino acid number of first and last residue. The molecular 2-fold axis with a dot as reference point is included (from Thieme et al., 1981)
Figure 3. Stereoview of the NADPH binding region of human GR. Amino acid sequence fragments included are 61-68 (VGCVPKKV), 160-163 (PSTP), 176-177 (TS), 193-202 (VGAGYIAVEM), 216-226 (MIRHKVLRSF), 249-251 (SQV), 287-291 (WAIQR), 336-340 (ALLTP), 368-372 (PTVVF), and 465'-468' (AIHP). Dots indicate the end of each fragment. Bound solvent sites are indicated by plus signs (+). The most tightly bound solvent molecules are indicated by the numbers (for example, S 3 = Sol-3) (from Pai et al., 1988).
extensive contacts. The C4 atom of nicotinamide and N5 atom of flavin are 3.2 Å apart and electron transfer is likely to occur between these two atoms (Karplus and Schulz, 1989). The nicotinamide moiety also contacts residues from the FAD, the central and the interface domains. The contact site of 2'-phosphate of NADPH is made up by the side chains of Arg-218, His-219 and Arg-224. These residues are not conserved in E3 which uses NADH lacking the 2'-phosphate. Crystallographic analysis of NADPH fragments and NADPH analogues bound to the enzyme suggests that the adenine moiety of the NADPH interacts with the enzyme first and this interaction then leads to the binding of 2'-phosphate moiety (Pai et al., 1988).

The dimer interface area is about 15% of the total surface of monomer. It can be divided into an upper and a lower part which are separated by a cavity with channel extensions to the solvent (Karplus and Schulz, 1987). The interface domain forms the upper part while the lower part is composed of the long helical extensions of the FAD binding domain (residues 70 to 110).

The glutathione binding site is formed by both subunits. One subunit provides residues 30-37, 59-64, 110-117 and 339-347 and the other subunit gives residues 406 and 467-476 (Karplus et al., 1989). Two reduced glutathiones bind to the enzyme. One reduced glutathione is bound to the enzyme in a V-shaped conformation. It
forms a disulfide bond to Cys-58 in the two-electron-reduced enzyme. The conformation of the other reduced glutathione is extended and its glutamyl end forms the most tight interaction with the enzyme (Karplus et al., 1989; Karplus and Schulz, 1989). The crystallographic and kinetic studies with synthetic glutathione analogues indicate that the enzyme avoids catalysis of incorrect substrates by reducing its binding strength to substrate analogues and/or by moving catalytically essential part(s) of substrate (Janes and Schulz, 1990).

The opening of the active disulfide bond is the only large change in the three-dimensional structure of the two-electron reduced enzyme at 3 Å resolution (Pai and Schulz, 1983). The higher 1.54 Å resolution structure shows that the thiolate anion of Cys-63 moves by 0.45 Å toward the flavin of FAD to form the charge transfer complex (Karplus and Schulz, 1989). The distance between the thiolate anion and the flavin is 0.2 Å in the charge transfer complex. The thiolate anion is stabilized by forming hydrogen bonds with Thr-333 and the O2' atom of FAD and by making the charge transfer interaction with the C4a atom of FAD (Karplus and Schulz, 1989).

4. Other pyridine nucleotide-disulfide oxidoreductases.

Other members of pyridine nucleotide-disulfide oxidoreductase
family are thioredoxin reductase and trypanothione reductase (Table 1). Thioredoxin reductase (TR) (EC 1.6.4.5) catalyzes the reduction of thioredoxin by NADPH (Williams, 1976). Thioredoxin is a small protein ($M_r$ 12,000) containing a redox active disulfide. The reduced thioredoxin is a substrate for ribonucleotide reductase, methionine sulfoxide reductase, adenosine 3'-phosphate-5'-pohosphosulfate reductase. *E. coli* TR consists of 320 amino acids with $M_r$ of 35,400 (Russell and Model, 1988). There are at least two forms of the two-electron reduced state of the enzyme: a form with reduced FAD and oxidized active disulfide center and a form with oxidized FAD and reduced active disulfide center (O'Donell and Williams, 1983). Unlike E3 and GR, there is no preference in the alkylation of active site disulfide thiols when two-electron-reduced enzyme is anaerobically alkylated (O'Donell and Williams, 1985). Another difference is that there is a two-amino acid bridge between the disulfide cysteines in TR while there is a four-amino acid bridge in E3 and GR (Williams, 1976).

Surprising results have been obtained from site-directed mutagenesis studies on *E. coli* TR. The disulfide active Cys-135 and Cys-138 have been each replaced by serines (Prongay et al., 1989). The Ser-135 mutant shows charge spectral patterns indicating that Cys-138 interacts with FAD. It has 10% of wild-type enzyme activity. The Ser-138 mutant is also active with 50% of wild-type enzyme activity. These activities are very surprising since other
enzymes such as E3 and GR become inactive when either one of the disulfide cysteines is mutated (Russell et al., 1989; Deonarain et al., 1990). It has been suggested that one remaining thiol can form a disulfide bond with thioredoxin and the resulting disulfide can be reduced by NADPH through the flavin. The replacement of FAD with 4-thio-FAD in the Ser-135 mutant results in a mixture of 4-thio-FAD and FAD (Prongay and Williams, 1990). This result suggests a direct interaction between Cys-138 and FAD since Cys-138 should react with the replaced 4-thio-FAD to generate FAD.

Trypanothione reductase catalyzes the reduction of trypanothione by NADPH in vivo. Trypanothione is \( N_1,N_8 \)-diglutathionyl spermidine, a glutathione analogue unique to the kinetoplastid parasites (Fairlamb et al., 1985; Shames, et al., 1986). The reductase has been purified from the insect Trypanosomatid Crithidia fasciculata (Shames et al., 1986) and the human parasite Trypanosoma cruzi (Krauth-Siegel et al., 1987). The reductase from T. cruzi has an \( M_r \) of 50,000. It has \( k_{\text{cat}} \) of 14,200 min\(^{-1}\) and \( K_m \) values for trypanothione and NADPH are 45 and 5 \( \mu \text{M} \), respectively.

5. Structural studies.

The three dimensional structure of mammalian E3 is not yet reported. However, the availability of primary amino acid sequences of E3s from several sources and of the three dimensional structures
of human erythrocyte GR and \textit{A. vinelandii} E3 provide a new insight to the prediction of human E3 structure. From detailed X-ray diffraction studies on human GR (Thieme et al., 1981; Karplus and Schulz, 1987), five structural domains are defined, namely, the flexible N-terminal segment (amino acid residues # 1 to 19 in human GR), the FAD domain (amino acid residues # 19 to 157), the NADPH domain (amino acid residues # 158 to 293), the central domain (amino acid residues # 294 to 365), and the interface domain (amino acid residues # 365 to 478).

Those domains are determined in human E3 by comparison of amino acid sequences (Carothers et al., 1989). There is a good homology (33%) between amino acid sequences of human E3 and GR with high identity around the disulfide active site, FAD binding site and NADPH binding site regions (Carothers et al., 1989). Structural comparisons of the E3s from \textit{E. coli} (Rice et al., 1984), yeast (Takenak et al., 1988) and \textit{A. vinelandii} (Schierbeek et al., 1989; Mattevi et al., 1991) with human GR have been reported. The detailed structural comparison between human GR and \textit{E. coli} E3 shows an excellent similarity with high conservation of amino acid residues at the disulfide active site and in the FAD and NADPH binding sites (Rice et al., 1984). The three dimensional structure of yeast E3 at 4.5 Å resolution reveals a good resemblance to that of human GR in overall structure (Takenak et al., 1988). This high degree of similarity in the three dimensional structures between
E3s and human GR is also supported by an X-ray diffraction study on *A. vinelandii* E3 at 2.8 Å resolution (Schierbeek et al., 1989). However, the refined structure of *A. vinelandii* E3 at 2.2 Å resolution reveals that the orientations of domain structures of *A. vinelandii* E3 are slightly deviated from those of human GR and that the interface domain has considerable tertiary structural changes (Mattevi et al., 1991). These changes lead to a shift (more than 4 Å) between two subunits with respect to each other in dimer structure of *A. vinelandii* E3 compared to that of human GR. This subunit shift results in a narrowing of the dihydrolipoamide binding cleft. However, these differences at tertiary and quaternary levels are compensated for by local conformational changes near the active site such that the active site structure of *A. vinelandii* E3 is very similar to that of human GR.

Extensive analysis on the putative secondary structure of human E3 has revealed a high degree of amino acid sequence conservation between the secondary structural elements of human GR and the corresponding putative elements of human E3 (Jentoft et al., in review). The internal β-sheet structures which form the core protein structure of E3 are especially conserved while most α-helical structures found on the surface show less similarity. The two putative α-helical regions in human E3 show many nonconservative substitutions, suggesting that these regions may provide interacting sites with E2 components and/or protein X. The structural analysis
of human E3 has been performed to test the similarity in tertiary structure with human GR. The putative active site and the dimeric interface region of E3s have been also compared with the corresponding residues of human GR. This study indicates that the structural scaffolding and overall tertiary structure of human E3 are very similar to those of human GR.

A model of the three dimensional structure of human E3 has been constructed using computer graphics system with the program FRODO (Jentoft et al., in review). It is built based on the structural coordinates for human GR and the extensive structural analysis of human E3. The model with a simplified substrate, lipoic acid, suggests the deprotonation of the sulfur at the 8 position of dihydrolipoamide as the first step of E3 catalysis. This model predicts a highly acidic nature for the E2 binding cleft, suggesting possible electrostatic interactions between E3 and E2 and/or between E3 and protein X.


Steady-state kinetics of mammalian E3 have been well studied (Williams, 1976). A series of parallel lines are observed on double reciprocal plots for initial velocities of pig heart E3 in the direction of the reduction of NAD$^+$ by dihydrolipoamide. On the basis of this observation, a bi-bi ping-pong mechanism (Figure 4) has been
Figure 4. Schematic representation of the E3 kinetic mechanism. The first substrate dihydrolipoamide (DL) binds to the oxidized state of the enzyme (E). The enzyme is reduced to the two-electron-reduced intermediate state (F), followed by the release of product lipoamide (LA). The second substrate NAD$^+$ binds to the enzyme intermediate (F). The electrons are transferred to NAD$^+$ resulting in the production of NADH and the transformation of the intermediate to the original oxidized form of enzyme. The product NADH is then released.
E3 Kinetic Mechanism

\[
\begin{align*}
& \text{DL} \quad \text{LA} \quad \text{NAD}^+ \quad \text{NADH} + \text{H}^+ \\
& \downarrow \quad \uparrow \quad \downarrow \quad \uparrow \\
& E (E \cdot DL \rightleftharpoons F \cdot LA) \quad F (F \cdot NAD^+ \rightleftharpoons E \cdot NADH) \quad E
\end{align*}
\]
proposed for pig heart E3 (Massey et al., 1960; Williams, 1976). The $k_{cat}$ is 555 s$^{-1}$ at 25°C pH 7.6. The $K_m$ for NAD$^+$ is 0.2 mM and $K_m$ for dihydrolipoamide is 0.3 mM (Massey et al., 1960). The pH dependence of pig heart E3 activity shows a bell shape with optimum pH of 7.9 in the direction of NAD$^+$ reduction. This pH dependence is suggested to occur in the second half reaction of the NAD$^+$ reduction by the two-electron-reduced E3 since the first half reaction of the reduction of the oxidized E3 by dihydrolipoamide is apparently independent of pH from 5.5 to 8.1 (Matthews et al., 1977). In the reverse direction, the pH optimum is broad with range of 5.5 to 6.5 in the presence of NAD$^+$ and the activity is decreased as pH is increased (Matthews et al., 1979).

The double reciprocal plots of initial velocities of rat liver E3 also show linear lines at 37°C pH 8.0 (Reed, 1973). The turnover number for NAD$^+$ reduction is 345 s$^{-1}$. The $K_m$s for NAD$^+$ and dihydrolipoamide are 0.52 mM and 0.49 mM, respectively. The double reciprocal plots for the reverse direction at pH 8.0 also show a series of parallel lines. The $K_m$ for NADH is 0.062 mM and for lipoamide is 0.84 mM. The $k_{cat}$ is 125 s$^{-1}$. At 25°C, the double reciprocal plots for the forward reaction are downwardly concave. The Hill coefficient, n, has been calculated to be 0.5 at 10°C, 0.6 at 25°C, 1.0 at 37°C. Isotopic exchange studies between NAD$^+$ and NADH support the proposed ping-pong mechanism (Reed, 1973). The product inhibition patterns are complex and consistent with a
"dead end" inhibition product of the two-electron-reduced enzyme and NADH complex. The pH dependence of log $V_{\text{max}}$ for NAD$^+$ reduction shows a bell shape with optimum pH of 8.2. These steady-state kinetic studies on pig heart and rat liver E3s indicate that mammalian E3 catalyzes the E3 reaction via a ping-pong mechanism.

The reduction rate of pig heart E3 by dihydrolipoamide has been monitored spectrophotometrically by the appearance of a charge transfer band at 530 nm. The charge transfer band is described in the following section. The rate is similar to the reported steady state turnover number (555 s$^{-1}$) at 25°C, pH 7.6 indicating that this half reaction is a rate limiting step in the pig heart E3 catalysis (Massey et al., 1960). Another study has reported the rate of 830 s$^{-1}$ between pH 5.5 and 8.1 (Matthews, et al., 1977). The reoxidation rate of the two-electron-reduced enzyme by NAD$^+$ is very rapid (> 833 s$^{-1}$) (Massey et al., 1960). In the reverse reaction, the reduction rate of the oxidized enzyme to the two-electron-reduced form by NADH is very fast (> 833 s$^{-1}$) at 25°C, pH 6.3 and the rate of reoxidation of two-electron-reduced enzyme by lipoamide is approximately 800 s$^{-1}$ (Massey et al., 1960). These pre-steady state kinetic studies indicate that the half reaction of the electron transfer between E3 and dihydrolipoamide or lipoamide is a rate-limiting step at optimum pH.
7. Spectral studies.

E3 contains an FAD prosthetic group in each subunit. It therefore has a spectrum characteristic of flavoproteins (Figure 5). The oxidized enzyme has peaks at 455 nm and 370 to 358 nm wavelengths. There is a unique shoulder between 465 nm and 485 nm which has been observed in many flavoproteins. The strong association of FAD with the flavoproteins may result in these characteristic features in the spectrum. When E3 becomes a two-electron-reduced form under anaerobic conditions, its absorption spectrum changes (Williams, 1976). The flavin absorbance around 455 nm is diminished and shifted to shorter wavelengths and one long wavelength band (maximum at about 530 nm) appears. These changes in spectrum have been attributed to the formation of a charge transfer complex between a thiolate anion and the flavin ring of FAD. This charge transfer complex has been demonstrated using model systems (Massey and Ghisla, 1974). E3 can be reduced further to four-electron-reduced state whose spectrum is similar to reduced FAD.

The spectra of oxidized pig heart E3 and four-electron-reduced enzyme are not pH dependent between pH 5.7 and 7.6 (Matthews and Williams, 1976). The spectrum of the two-electron-reduced enzyme is, however, pH dependent between pH 8 and pH 5.2 with a decreasing absorbance around 530 nm as pH decreases. The
Figure 5. Absorption spectra of pig heart E3. 1 is the oxidized state, 2 is the two-electron-reduced state and 3 is the four-electron reduced state (from Mattews and Williams, 1976).
dependence of macroscopic pKa values of two-electron-reduced pig heart E3 on pH change has been determined by monitoring changes in the flavin band near 455 nm and the charge transfer band at 529 nm (Sahlman and Williams, 1989). E3 has pKa values of 4.4 and 8.7 when monitored at 529 nm and at 3.9, 7.0, and 9.3 when monitored at 455 nm. Three active site amino acids (Cys-45 and Cys-50 and His-452) are suggested to be responsible for the observation (Sahlman and Williams, 1989).

8. Catalytic mechanism.

The catalytic mechanism of human GR has been postulated from the three dimensional structures of oxidized and two-electron-reduced enzymes (Pai and Schulz., 1983). Based on this and other biochemical evidence, the catalytic mechanism of E3 can be also postulated. E3 catalysis can be subdivided into two half reactions (Figure 6). The catalytic mechanism of the first half reaction can be better postulated than that of the second half reaction (Figure 7). One of two dihydrolipoamide sulfur atoms, presumably the sulfur atom at the position 8, is deprotonated by a possible proton acceptor/donor and becomes a nucleophile (Jentoft et al., in review). This electron-rich thiolate anion attacks the active disulfide center resulting in the formation of a disulfide bond with Cys-45 (Thorpe and Williams, 1976). Electrons are transferred to the sulfur atom of Cys-50 forming a thiolate anion which forms the
Figure 6. Schematic representation of the reaction mechanism of E3. The first substrate dihydrolipoamide binds to the enzyme (E: the active disulfide center is between Cys-45 and Cys-50, FAD and proton acceptor/donor (B) are shown). Then the first half reaction occurs to produce lipoamide and the two-electron-reduced intermediate form (EH2) of the enzyme. In the second half reaction, NAD+ binds to the intermediate form. Electrons are then transferred to NAD+ to produce NADH.
Figure 7. Schematic representation of the first half reaction of E3. The proton acceptor/donor (B) probably deprotonates the sulfur atom at position 8 of dihydrolipoamide (Jentoft et al., in review). The resultant thiol anion attacks the active disulfide center resulting in the formation of a disulfide bond between Cys-45 and dihydrolipoamide and a charge transfer complex between Cys-50 and FAD. The second deprotonation occurs resulting in the production of lipoamide and the two-electron-reduced state of the enzyme.
charge transfer complex with FAD.

The evidence for formation of a disulfide bond between dihydrolipoamide and Cys-45 comes from chemical modification studies of the two-electron-reduced pig heart E3 with iodoacetamide (Thorpe and Williams, 1976). Cys-45 is 13-fold more reactive than its disulfide bond partner Cys-50 at pH 7.6 when the two-electron-reduced E3 is chemically modified with iodoacetamide. Other evidence comes from the three dimensional structures of human GR (Thieme et al., 1981; Karplus et al., 1987) and A. vinelandii E3 (Schierbeek et al., 1989). Based on their locations, it is predicted that the more carboxy-terminal cysteine of the pair (Cys-50 in human E3), which is near to the FAD ring, forms the charge transfer complex and the more amino-terminal cysteine of the pair (Cys-45 in human E3) interacts with substrate.

The next step is the deprotonation of the other sulfur atom at the 6 position of dihydrolipoamide by a possible proton acceptor/donor. The resultant electron-rich thiolate anion attacks the sulfur atom at the 8 position of dihydrolipoamide, which is disulfide bonded to Cys-45 of E3. This results in the generation of lipoamide and the reduction of the sulfur atom of Cys-45. The lipoamide then dissociates from the two-electron-reduced E3. This dissociation may be driven by a conformational change of the enzyme upon the reduction. The two-electron-reduced E3 may
have a different conformation of a more open structure than the oxidized E3. The oxidized E3 from *A. vinelandii* is stable to trypsin digestion while the reduced E3 rapidly loses activity following the trypsin digestion (de Kok et al., 1982). The differences in the active site region of human GR between oxidized and two-electron-reduced forms are observed (Karplus and Schulz, 1989). The active disulfide center between Cys-58 and Cys-63 in human GR becomes opened upon reduction and Cys-63 moves close to the flavin ring of FAD.

The involvement of a possible proton acceptor/donor for the deprotonations of dihydrolipoamide is conceivable since the pK's for the thiol ionization of dihydrolipoamide is determined to have pK'1 of 9.35 and pK'2 of 10.65 (Matthews et al., 1977). A candidate for the possible proton acceptor/donor in E3 function is His-452 which is located near Cys-45 based on the three dimensional structure of human GR. This histidine is conserved in E3s and GRs from various sources (Carothers et al., 1989). The presence of an essential histidine at the active site of *E. coli* E3 has been suggested from bifunctional arsenoxyde modification studies (Adamson et al., 1984).

The importance of the corresponding His residues (His-444 in *E. coli* E3 and His-439 in *E. coli* GR) in *E. coli* E3 and GR catalyses have been studied by replacing the residues with
glutamine using site-directed mutagenesis (Williams et al., 1989; Berry et al., 1989). The *E. coli* mutant E3 has about 0.3 to 0.4% of wild-type enzyme activity and its two-electron-reduced form becomes unstable (Williams et al., 1990). The *E. coli* mutant GR possesses about 0.4% of wild type enzyme activity (Berry et al., 1989). The substitution of His-439 to Ala in *E. coli* GR also results in 0.1% of wild-type activity (Deonarain et al., 1989). From these studies, it has been concluded that the active site His is probably the proton acceptor/donor in *E. coli* E3 and GR catalyses.

The other half reaction covers electron transfer from the two-electron-reduced enzyme to NAD⁺. NAD⁺ binds to the two-electron-reduced enzyme and the deprotonated sulfur atom of Cys-45 attacks the sulfur atom of Cys-50 to reform the active disulfide center. Electrons are transferred to NAD⁺ by a very fast unknown pathway (> 800 s⁻¹) (Massey et al., 1960), resulting in the generation of NADH and oxidation of the enzyme. Since NAD⁺ is expected to bind at the re-face of the flavin of FAD based on the human GR structure, electrons should be accepted at the si-face of the flavin from the charge transfer complex and then transferred to NAD⁺. The flavin C4a-thiol adduct and the reduced flavin as possible intermediates are suggested from spectral analysis studies on Cys-45 monoalkylated pig heart E3 (Thorpe and Williams, 1976, 1981) and a homologous protein mercuric ion reductase (Miller et al., 1990). The addition of NAD⁺ to the monoalkylated pig heart E3
leads to a rapid and reversible spectral change. It is interpreted as the presence of a covalent adduct between Cys-50 and the C4a position of the flavin. The possible covalent interaction between NAD\(^+\) and the flavin adduct has been ruled out, based on double resonance proton nuclear magnetic studies (O'Donnell et al., 1983). The product NADH is dissociated from the oxidized enzyme, which then recatalyzes the reaction.


*E. coli* E3 has about 44% homology with human E3 at the amino acid sequence level (Carothers et al., 1989). The overall catalytic mechanism of *E. coli* E3 may be similar to that of mammalian E3. However, *E. coli* E3 is different from mammalian E3 in some aspects. *E. coli* E3, unlike mammalian E3, is very vulnerable to product inhibition by NADH (Wilkinson and Williams, 1981). This product inhibition may have physiological significance since *E. coli* PDC, unlike mammalian PDC, does not have a covalent modification control mechanism by a kinase and phosphatase. Due to this product inhibition, the kinetic studies in the direction of NAD\(^+\) reduction have been performed using the stopped-flow technique (Sahlman and Williams, 1989). The data have been analyzed assuming the positively cooperative binding of substrate with a Hill coefficient of 1.1-1.4. At pH 7.5, the \(k_{\text{cat}}\) is 420 s\(^{-1}\) and \(K_m\)s for NAD\(^+\) and dihydrolipoamide are 0.23 mM and 16 \(\mu\)M.
respectively. One interesting thing is the considerably low $K_m$ for dihydrolipoamide compared to those of mammalian E3s.

Even though the titration of *E. coli* E3 with excess dihydrolipoamide results in a four-electron-reduced state of enzyme, titrations with up to four moles of dihydrolipoamide lead to the two-electron-reduced state of *E. coli* E3 at equilibrium (Williams, 1965). While pig heart E3 has mainly one kind of two-electron-reduced form (charge transfer complex), the titration of *E. coli* E3 with dithionite leads to three spectrally distinct two-electron-reduced species; a fluorescent form with oxidized FAD and reduced active disulfide center, a form with an unstrained charge transfer similar to that of pig heart E3 and a form with reduced FAD and oxidized active disulfide center (Wilkinson and Williams, 1979). The anaerobic titration of *E. coli* E3 with the same mol of dihydrolipoamide results in the formation of the charge transfer complex in less than 1 s, which is transformed to the species observed in the dithionite titration in 12 s (Wilkinson and Williams, 1979). The catalytic mechanism of *E. coli* E3 is probably similar to that of mammalian E3. However, the contributions of certain amino acid residues in the mechanism of *E. coli* E3 may differ from those of mammalian E3 due to these differences.

E3 cDNA sequences from human liver (Pons et al., 1988) and small cell carcinoma (Otulakowski and Robinson, 1987) have been reported. E3 cDNA from human liver contains an open reading frame of 1527 bp encoding 509 amino acids of precursor E3. Mature E3 is composed of 474 amino acids with a calculated molecular mass of 50,216 daltons. The amino acid sequences from human liver and small cell carcinoma are identical with the exception of amino acids 69 and 119. Two E3 mRNA species (2.2 and 2.4 kb) hybridizes with human liver E3 cDNA probe (Pons et al., 1988). This is consistent with the presence of two polyadenylation signals located approximately 0.2 kb apart in the human liver E3 clone. The chromosomal location of E3 gene is determined as chromosome 7 (Olson et al., 1990). Pig E3 cDNA sequence has been also reported (Otulakowski and Robinson, 1987). It shows 96% homology with human E3 at the amino acid sequence level.


The intracellular synthesis and distribution of rat liver E3 have been studied (Matuda and Saheki, 1982). It has been observed that the rat liver E3 is mostly located in mitochondria. However, about 10% of E3 activity is found in cytosol. The E3s in mitochondria and cytosol are immunologically identical based on the Ouchterlony double diffusion test. However, the E3 activity in cytosol is more
sensitive to antibody inhibition than that in mitochondria indicating that they are slightly different from each other. The incorporation of $^{35}$S-methionine in vivo and immunoprecipitation reveal that the E3 is mainly synthesized on free ribosomes. Rat liver precursor E3, synthesized in a cell-free reticulocyte lysate system, is slightly larger than the mature E3 on SDS-PAGE gel (Matuda et al., 1983). Immunoprecipitation of ox heart cellular extracts treated with $^{35}$S-methionine and the following SDS-PAGE result in the detection of precursor E3 protein whose size is about 2 kDa larger than that of mature E3 (de Marcucci et al., 1986). These studies indicate that E3 is synthesized as a precursor protein on free ribosomes in cytosol and imported into mitochondria. In this process, a leader sequence (about 2 kDa) of the precursor protein is cleaved off and E3 exists as a mature protein in mitochondria.

12. Research objectives.

The objectives of this study are two-fold. The first goal is the development of an expression system in which wild-type and site-specifically modified human E3s can be expressed and purified. The second objective is the characterization of site-specifically modified human E3s to better understand the E3 mechanism. Recent developments in recombinant DNA technology make these objectives feasible. One popular heterologous expression system involves E. coli. To express human E3 in E. coli, it is necessary to
insert a DNA sequence encoding human E3 into an E. coli expression vector. Purification procedures for recombinant human E3 expressed in E. coli have to be developed. To produce site-specifically modified human E3s, specific strategies for the application of the site-directed mutagenesis to the human E3 expression system have to be developed.

Mammalian E3 catalytic mechanism can be postulated from studies on pig heart E3 (Williams, 1976) and human GR (Pai and Schulz, 1983). The important amino acid residues in human E3 catalysis have been predicted by comparison of primary amino acid sequences of E3s and GRs from several sources (Carothers et al., 1989; Jentoft et al., in review). The active disulfide center residues in human E3 have been predicted as Cys-45 and Cys-50. The active site proton acceptor/donor has been predicted as His-452 which is suggested to form a hydrogen bond to Glu-457 based on the structure of human GR (Karplus and Schulz, 1987). These predicted amino acid residues can be site-specifically substituted by other amino acids to better understand the contribution of these residues in human E3 catalysis. The contributions of the active site His-452 and Glu-457 in human E3 catalysis are of interest since the substitution of the corresponding His residues to Gln in both E. coli E3 and GR did not result in total loss of activity but very low activity. This property allows the quantitative analysis of the contributions of these residues in human E3 catalysis. This study
will provide evidence that His-452 is the proton acceptor/donor in human E₃ catalysis and also generate quantitative values for the contributions of the proton acceptor/donor in human E₃ catalysis. This study will also provide the contributions of Glu-457 as a stabilizing factor for the optimum function of His-452 in human E₃ catalysis.
Chapter II
Experimental procedures

1. Bacterial strains and plasmids.

*E. coli* strains MM294Cl+*, AR120 and a prokaryotic expression vector plasmid, pOTSV, the influenza NS1 gene expression vector, pAS1ΔEH801 were kindly provided by Dr. Allan Shatzman (Smith Kline French Laboratory). *E. coli* JRG1342, a pyruvate dehydrogenase complex deletion mutant, was a gift from Dr. John R. Guest (The University of Sheffield, Sheffield, U.K.). Plasmid PRK248CIts was purchased from American Type Culture Collection.

2. Enzymes and reagents.

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (BMB) or Bethesda Research Laboratories. T4 DNA ligase, dATP, dCTP, dGTP and dTTP were from BMB. The DNA sequencing kit containing Sequenase was purchased from U.S.Biochemicals. DL-6,8-thiociot acid amide (DL-lipoamide), pig heart lipoamide dehydrogenase (E₃), calcium phosphate gel-cellulose and DEAE-Sephadex were purchased from Sigma Chemical Co. Hydroxyapatite, Zeta-Probe membrane, affinity purified goat
anti-rabbit IgG-horseradish peroxidase conjugate, and horseradish peroxidase color development reagent were purchased from Bio-Rad. \(^{[1,2,5]}\)protein A was from ICN Radiochemicals. Dihydrolipoamide was made by reduction of lipoamide using sodium borohydride (Reed et al., 1958).

3. **DNA manipulations.**

The general methods of purification, restriction digestion and ligation of DNA and transformation of *E. coli* were performed as described elsewhere (Maniatis et al., 1982). DNA sequencing was done by the dideoxy nucleotide sequencing method using appropriate primers (Sanger et al., 1977).

4. **Site-directed mutagenesis.**

The mutagenic oligonucleotides (for precursor E3 construction: 5'-CGGAAAAATGGATCCTGGAGTCGT-3', for His-452->Gln: 5'-TAAGGTCGGCCTGTGCATGA-3', for Glu-457->Gln: 5'-CTAAAAGCTTGATGATAAGGT-3'; mismatched bases are underlined) were first phosphorylated by T4 polynucleotide kinase. Fifty nanograms of both phosphorylated mutagenic primer and universal -20 primer were annealed with 1 µg of the uracil-enriched single stranded DNA which was generated from *E. coli* CJ236 (ung- dut-). The polymerization and ligation reactions were performed with
Klenow fragment or Sequenase and T4 DNA ligase in ligation buffer containing 125 μM of each dATP, dTTP, dCTP and dGTP and 1 mM ATP. The reaction was done at 16°C overnight. This reaction solution was used for transformation of E. coli JM109. Screening of mutants was performed by DNA sequencing.

5. Construction of Bluescript-E₃ plasmid.

An E₃ coding sequence was obtained from two bacteriophage M13 E₃ cDNA subclones containing non-overlapping E₃ cDNA segments. These M13 subclones were from a 2.2 kb full-length E₃ cDNA which had been isolated from a human liver λgt 11 cDNA library and characterized in our laboratory (Pons et al., 1988). The 3' E₃ sequence from the phage M13 was first inserted into Bluescript plasmid with EcoRI and HincII, resulting in the construction of Bluescript-E₃c plasmid (Figure 8). The 5' EcoRI digest from the phage M13 subclone was then inserted into the Bluescript-E₃c plasmid, resulting in the construction of the Bluescript-E₃ plasmid.


The Bluescript-E₃ plasmid was digested with XbaI and XhoI restriction enzymes (Figure 9). Subsequently, the XbaI/XbaI
Figure 8. Construction of a Bluescript-E₃ plasmid. : 5'-untranslated region and leader sequence, : mature E₃ coding region, : 3'-untranslated region.
Figure 9. Construction of a mature E3 expression vector, pOTSV-E3.  
- : 5'-untranslated sequence and leader sequence region,  
- : mature E3 coding sequence region,  
- : 3'-untranslated sequence region.
Ligate fragments A, B, and C.
fragment (372 bp) was digested with Sau3A to yield a Sau3A/XbaI fragment that corresponded to the 5' end of the coding sequence of the mature protein. The Sau3A digestion generated the same cohesive end to that of BamHI digestion. This fragment together with the XbaI/XhoI fragment from the initial digest were ligated into a pOTSV vector which had been prepared by removing a small BamHI/XhoI fragment, resulting in the construction of the mature form of human E3 expression vector, pOTSV-E3 (Figure 9). The ligated DNA was used to transform E. coli MM294Cl+. The ligation resulted in a correct open reading frame for the mature E3.


The BamH I site at the multiple cloning site of the Bluescript-E3 plasmid was removed by digestion with BamHI and a following fill-in reaction with Klenow fragment. A new BamHI site was then introduced near the start site of the protein coding region of human E3 cDNA using site-directed mutagenesis as described in this chapter section 4. The DNA fragment encoding precursor E3 and 3'-untranslated region was released with BamHI and XhoI digestion. The fragment was ligated with the pOTSV, which had been previously digested with BamHI and XhoI, resulting in the precursor E3 expression vector, pOTSV-pE3 (Figure 10).
Figure 10. Construction of a precursor E3 expression vector, pOTSV-pE3.

- : leader sequence region,
- : mature E3 coding sequence region,
- : 3'-untranslated sequence region. The sequence region changed by site-directed mutagenesis is indicated in the box.

*E. coli* AR120 was grown in LB medium. JRG1342 was grown in LB medium containing 0.1% glucose (Allison et al., 1988). The pOTSV-E3 transformed AR120 was grown at 37°C until A650 reached 0.4 after inoculation by an overnight culture (200-fold dilution). Nalidixic acid was then added to a final concentration of 60 ug/ml to induce human E3 followed by further growth at 30°C (Shatzman and Rosenberg, 1987). Since JRG1342 did not contain λ repressor protein, a temperature sensitive λ repressor protein expression vector, PRK248Clts, was first introduced into JRG1342. The JRG1342 (PRK248Clts) was then transformed with the pOTSV-E3. The transformed JRG1342 (PRK248Clts) was grown to A650 of 0.4 and then transferred at 42°C for the induction.

9. Protein assay.

Protein concentration was measured by Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. To each of 0.6 ml samples was added 3 ml of a solution of 2% Na2CO3 in 0.1 N NaOH, 0.01% CuSO4 and 0.02% Na-K tartrate. The solution was incubated at room temperature for 10 min. Phenol reagent diluted with an equal amount of water (0.3 ml) was added to the solution and incubated at room temperature for 30 min. The absorbance at 650 nm was measured spectrophotometrically.
10. **SDS-polyacrylamide gel electrophoresis and Western blot analysis.**

For the SDS-PAGE and Western blot analysis, *E. coli* cells were pelleted and washed once with 50 mM potassium phosphate buffer, pH 7. Cell suspensions were sonicated twice for 20 sec at continuous pulse with a microtip. To obtain a soluble fraction, a portion of the sonicated sample was centrifuged at 12,000 g for 10 min. Samples were denatured by boiling for 5 min in SDS-PAGE loading buffer. SDS-PAGE (9%) was performed at 35 mA in discontinuous SDS-polyacrylamide gels (Laemmli, 1970). The gel was visualized by Coomassie brilliant blue. For Western blot analysis, SDS-PAGE was performed as described above. Proteins in the gel were electrophoretically transferred to a Zeta-Probe membrane using a Bio-Rad Western blot apparatus at room temperature at 75 mA for 8 h. The transfer buffer was 25 mM Tris, 192 mM glycine, pH 8.3. The membrane was incubated with rabbit anti-E3 antibody, which had been raised against pig heart E3. The membrane was treated either with $^{125}$I protein A or with horseradish peroxidase-conjugated anti-rabbit IgG-antibody (1:3000 dilution). Immunoreactive proteins were visualized either by exposing the membrane to X-Omat AR film in case of $^{125}$I Protein A incubation or by a color reaction using 4-chloro-1-naphthol in the latter case.
11. E₃ assay and kinetic studies.

E₃ activity was assayed spectrophotometrically by monitoring the reduction of NAD⁺ or the oxidation of NADH at 340 nm with a Gilford 250 or a Shimadzu UV160U spectrophotometer equipped with thermospacers. The temperature was maintained at 37°C using a constant-temperature circulating water bath (for the Gilford 250) or a CPS temperature controller (for the Shimadzu UV160). The detailed assay conditions are specified in the Figure legends and Tables. One unit of activity is defined as 1 umol of NAD⁺ reduced or NADH oxidized per min per mg of protein at 37°C. For kinetic studies, E₃ assays were performed at varying concentration of substrates. The mixed RS forms of dihydrolipoamide and lipoamide were used. The R form of the substrates is known to be a real substrate in vivo (Yang and Frey, 1989). The concentration of dihydrolipoamide was determined, based on the molecular weight of the substrate (207.3 daltons). Initial velocities were best-fitted using a non-linear regression data analysis Enzfitter program distributed by Elsevier-Biosoft (Cambridge, U.K.). The best-fitted reciprocal initial rates were plotted against reciprocal substrate concentrations using the Cricket Graph program (Version 3.0) on a Macintosh computer. For ping pong kinetic mechanism, kinetic parameters were calculated from the general equation:

\[ v = \frac{V_{\text{max}} [A][B]}{K_{\text{mB}} [A] + K_{\text{mA}} [B] + [A][B]} \]
For ordered sequential mechanism, kinetic parameters were obtained from the general equation:

\[ v = \frac{V_{\text{max}} [A] [B]}{K_{\text{ia}} K_{\text{KB}} + K_{\text{MB}} [A] + K_{\text{MA}} [B] + [A] [B]} \]

12. **Purification of mature human E3 expressed in JRG1342.**

Cells were harvested by centrifugation at 4,000 g for 10 min. The cells were suspended in 10 mM potassium phosphate buffer (pH 7.5) and were broken by passage through a French press. The supernatants were obtained after centrifugation at 12,000 g for 30 min and then fractionated with ammonium sulfate. The E₃ was precipitated between 0.55 and 0.80 ammonium sulfate saturation (Ide et al., 1967). The precipitates were collected by centrifugation at 12,000 g for 15 min and dissolved in 10 mM potassium phosphate buffer (pH 7.5). The solution was subjected to heat treatment at 75°C for 5 min (Lusty, 1963). After cooling in an ice-water bath for 10 min, the proteins were centrifuged at 12,000 g for 15 min and the pellet was discarded. The supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 7.5) and clarified by centrifugation at 12,000 g for 15 min. The solution was applied to a calcium phosphate gel-cellulose column which had been previously equilibrated with 100 mM potassium phosphate buffer (pH 7.5). The column was washed with 100 mM potassium phosphate buffer, pH 7.5 and the E₃ was eluted with the same
buffer containing 4% ammonium sulfate (w/v) (Ide et al., 1967).

13. **Purification of truncated precursor human E3 expressed in JRG1342.**

Cells were harvested and broken by sonication in 10 mM potassium phosphate buffer (pH 7.5). The supernatant was fractionated with ammonium sulfate. The precursor E3 was precipitated between 0.40 and 0.70 ammonium sulfate saturation. The remaining steps were identical to the procedures for the purification of the mature E3 except that the heat treatment was done at 70°C.

14. **Determination of amino acid sequence.**

The amino-terminal amino acid sequences of mature and truncated precursor E3s were determined with an Applied Biosystems 477A protein sequencer with on-line 120A phenylthiohydantoin derivatives analyzer at Molecular Biology Core Facility of Case Western Reserve University School of Medicine. Ten amino acids at the amino-terminus were sequenced for both E3s.

15. **Construction of human mutant E3 expression vector, pOTSV(ΔE3)-E3m.**
To remove an EcoRI site present near the P_L promoter of the E_3 expression vector sequence (Figure 11), we switched the EcoRI site containing part of pOTSV-E_3 with the corresponding part of pAS1ΔEH801 where the EcoRI site was deleted. The pOTSV-E_3 and pAS1ΔEH801 were each digested with BglII and PvuII to generate two fragments (Figure 11). The fragment containing the E_3 coding sequence from pOTSV-E_3 and the fragment containing the expression vector sequence lacking EcoRI and HindIII sites from pAS1ΔEH801 were ligated together, resulting in the construction of the EcoRI site deleted E_3 expression vector, pOTSV(ΔEH)-E_3 (Figure 11).

The 3' region of the E_3 cDNA sequence was isolated from Bluescript-E_3c plasmid with EcoRI and KpnI digestions and inserted into M13mp18 phage. The resulting M13mp18-E_3c was used for site-directed mutagenesis. The mutated 3' region of the E_3 cDNA sequence was isolated and ligated with the modified E_3 expression vector, pOTSV(ΔEH)-E_3, which had been digested with EcoRI and XhoI, resulting in the replacement of the corresponding region of the wild-type E_3 cDNA sequence (Figure 12).

16. Purification of mutant human E_3s from E. coli JRG1342.

Cells were harvested by centrifugation at 4,000 g for 10 min. They were suspended in 10 mM potassium phosphate buffer
Figure 11. Construction of a human E3 expression vector deleting EcoRI and HindIII sites, pOTSV(ΔEH)-E3. [Diagram]: E3 coding sequence, [Diagram]: 3'-untranslated sequence.
Figure 12. Construction of a human mutant E3 expression vector, pOTSV(ΔEH)-E3m. 

: E3 coding sequence.

: 3'-untranslated sequence.
(pH 7.5) containing 0.25 mM EDTA (Buffer A) and broken by 3 to 4 passages through a French press. The supernatants were obtained after centrifugation at 12,000 g for 30 min and subjected to ammonium sulfate precipitation. The precipitates between 55 and 85% ammonium sulfate saturation were collected by centrifugation at 12,000 g for 15 min and suspended in Buffer A. The suspension was dialyzed against Buffer A. The dialyzed solution was clarified by centrifugation at 12,000 g for 10 min and then loaded on a DEAE-Sephascel column which had been previously equilibrated with Buffer A. The column was washed with Buffer A and E₃ was eluted with 200 mM potassium phosphate buffer (pH 7.5) containing 0.25 mM EDTA (Buffer B). The eluants were diluted to 100 mM potassium phosphate solution by adding distilled water. The diluted solution was then loaded on a hydroxyapatite column which had been previously equilibrated with Buffer A. The column was washed with 100 mM potassium phosphate buffer (pH 7.5) containing 0.25 mM EDTA. The E₃ was eluted with Buffer B. The eluants were collected into a dialysis tube and concentrated by soaking in Buffer A containing 20% polyethylene glycol (MW 20,000). The concentrated eluants were resuspended with a small volume of Buffer A and clarified by centrifugation. They were then subjected to a heat treatment at 75°C for 5 min. The heat treated solution was centrifuged and the precipitated proteins were discarded.
Chapter III
Results


To develop an E. coli expression system in which wild-type and modified human E3s can be expressed and purified, we constructed an expression vector for the mature human E3, pOTSV-E3 (Figures 8 and 9), as described in Chapter II. This construction resulted in one amino acid substitution of alanine, the first amino acid of mature E3, by methionine because the first three bases of mature E3 nucleotide sequence were deleted by digestion with Sau3A while the initiation codon (AUG) was added during the construction of pOTSV-E3.

E3 is synthesized as a precursor form in the cytoplasm and imported into mitochondria. To study precursor form of E3, we constructed a precursor human E3 expression vector, pOTSV-pE3 (Figure 10) as described in Chapter II. Since a BamHI cohesive end was needed to insert a DNA sequence encoding precursor E3 into the pOTSV expression vector, we generated a new BamHI site using site-directed mutagenesis at the junction between the 5'-untranslated region and the leader sequence of E3. The uracil-
enriched single stranded Bluescript-E3 DNA was generated using a single stranded DNA inducing helper phage M13KO7 and *E. coli* CJ236 (ung- and dut-) strain (Kunkel et al., 1987). Site-directed mutagenesis was performed as described in Chapter II. The efficiency of the site-directed mutagenesis was 80%. As a result of the site-directed mutagenesis (Figure 10), the second (glutamine) and third (serine) amino acid residues in the precursor form of E3 were expected to be changed to glutamate and proline, respectively.

2. Expression of cDNA sequence encoding mature human E3 in *E. coli* AR120 and purification of mature human E3 from *E. coli* JRG1342.

Plasmids pOTSV-E3 and pOTSV were each transformed into wild-type *E. coli* AR120. The transformed cells were induced as described in Chapter II. Total cellular proteins from induced cells were analyzed for recombinant human E3 by Western blotting with anti-pig heart E3 antibody. As shown in Figure 13, five bands, with *M*<sub>r</sub>s of approximately 55,000 (full-length), 49,000, 47,000, 31,000, and 29,000, reacted to anti-pig heart E3 antibody in pOTSV-E3 transformed cells while no band appeared in pOTSV transformed cells, suggesting that this antibody preparation did not recognize *E. coli* E3. It should be noted that mature E3 migrates as a 55,000 Da polypeptide in SDS-PAGE though its calculated
Figure 13. Western blot analysis of expression of cDNA sequence encoding mature E₃ in *E. coli* AR120. Plasmids pOTSV-E₃ (+) and pOTSV (-) were transformed into *E. coli* AR120. The transformed cells were induced, harvested and total cellular proteins in 1 ml of cells (A₆₅₀ = 1) were separated by SDS-PAGE (9%). Proteins were electrotransferred to a Zeta Probe membrane and treated with anti-pig heart E₃ antibody followed by [¹²⁵I]protein A. E₃ (pig heart) was run as a standard.
molecular mass with one molecule of FAD is 50,919 Da (Pons et al., 1988). Western blot analysis of the soluble fraction from AR120 (pOTSV-E3) showed that the full-length E3 (Mr 55,000) was the most abundant species in the soluble fraction (Figure 14). To quantify the amount of the expressed full-length E3 in the soluble fraction, 40 μg of the soluble fraction from the AR120 (pOTSV-E3) harvested from 0 to 9 h following induction was subjected to SDS-PAGE with known amounts of pig heart E3 standard and analyzed by Western blotting (Figure 14). The comparison of the intensities of bands by densitometric scanning revealed that about 2% of the soluble fraction of chemically induced AR120 (pOTSV-E3) was the full-length E3.

It was difficult to assess whether or not the expressed human E3 had an enzymatic activity by just comparing the E3 activities of the pOTSV-E3 transformed AR120 and the pOTSV transformed AR120 cells because AR120 also had its endogenous E. coli E3 activity. We, therefore, expressed the pOTSV-E3 in E. coli JRG1342 which was deficient in its pyruvate dehydrogenase complex because of deletion of the gene sequences (for aceE, aceF and lpd genes) for this complex (Allison et al., 1988). The pOTSV-E3 transformed JRG1342 (PRK248Clts) was induced at 42°C and its E3 activity was assayed. The pOTSV-E3 transformed JRG1342 (PRK248Clts) showed E3 activity while pOTSV transformed
Figure 14. Time-dependent expression of cDNA sequence encoding mature E₃ in *E. coli* AR120. Cells were transformed with pOTSV-E₃ or pOTSV and E₃ expression was chemically induced for the time indicated. Forty µg of soluble proteins were separated by SDS-PAGE (9%). Proteins were electrotransferred to a Zeta Probe membrane and treated with anti-pig heart E₃ antibody followed by ¹²⁵I-protein A. The amount of expressed full-length E₃ (55 kDa) in the soluble fraction was determined by densitometric scanning using purified pig heart E₃ (1 and 2 µg) as a standard. For AR120 cells: 9⁻ was 9 h induction with pOTSV and 0, 1, 3, 5, 7 and 9 were induction times (h) with pOTSV-E₃.
<table>
<thead>
<tr>
<th>kDa</th>
<th>E3 [ug]</th>
<th>AR120 [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>9-0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1 3 5 7 9</td>
</tr>
</tbody>
</table>

55-
JRG1342 (PRK248CIt's) did not, demonstrating that the full-length human E3 expressed in *E. coli* JRG1342 was an enzymatically active protein (Table 2).

The mature form of human E3 expressed in *E. coli* JRG1342, a pyruvate dehydrogenase complex-deficient strain, was purified following the procedure used for the purification of mammalian E3 (Lusty, 1963; Ide et al., 1967). About 28-fold purification was achieved by an ammonium sulfate precipitation followed by heat treatment (Table 3). The partially purified E3 was subjected to calcium phosphate gel-cellulose chromatography. This step resulted in a further 15-fold purification. The purification at each step was analyzed by SDS-PAGE and Western blotting (Figure 15). After the calcium phosphate gel-cellulose column step, the recombinant E3 was nearly homogeneous as analyzed by SDS-PAGE. Based on the specific activity of the purified mature human E3, it was calculated that about 0.23% of the soluble proteins of JRG1342 was mature human E3 after induction. The amino-terminal amino acid sequence analysis provided the expected sequence of the recombinant mature E3 starting with methionine (Table 4).

3. Expression of cDNA sequence encoding precursor human E3 in *E. coli* AR120 and purification of truncated precursor E3 from *E. coli* JRG1342.
Table 2

Expression of cDNA sequence encoding mature human E₃ in a pyruvate dehydrogenase complex-deficient E. coli JRG1342 (PRK248CIts).

Cells were grown to an A₆₅₀ of 0.4 at 37°C and transferred to 42°C for the induction. A portion of the cells were harvested at 2, 3 and 16 h after induction and sonicated. E₃ activity in the crude supernatant solution was assayed in 50 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA, 0.4 mM dihydrolipoamide and 0.3 mM NAD⁺. This assay could detect E₃ activity higher than about 0.1 munit.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>Hours</th>
<th>E₃ activity (munits/mg of soluble protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRG1342</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(PRK248CIts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ pOTSV</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>+ pOTSV-E₃</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>400</td>
</tr>
</tbody>
</table>
Table 3
Purification of mature human E₃ expressed in E. coli JRG1342.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant solution after French press</td>
<td>1040.0</td>
<td>0.4 a</td>
<td>100</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.55-0.80) + Heat treatment (75°C, 5 min)</td>
<td>26.5</td>
<td>11.4 a</td>
<td>71</td>
</tr>
<tr>
<td>Calcium phosphate gel-cellulose column</td>
<td>0.9</td>
<td>172.5 a</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>406.7 b</td>
<td></td>
</tr>
</tbody>
</table>

a Activity was assayed in 50 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA, 0.4 mM dihydrolipoamide and 0.3 mM NAD⁺.

b Activity was assayed in 100 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA, 3.0 mM dihydrolipoamide and 3.0 mM NAD⁺.
Figure 15. SDS-PAGE analysis during purification of mature E₃ expressed in JRG1342 (A) and the corresponding Western blot analysis (B). Proteins were separated by SDS-PAGE (9%), electrotransferred to a Zeta Probe membrane and treated with anti-pig heart E₃ antibody followed by [¹²⁵I]Protein A. Lane 1: molecular weight markers (from the top; phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase), Lane 2: 1 µg of pig heart E₃ (Sigma), Lane 3: 150 µg of the supernatant solution after French press treatment, Lane 4: 15 µg of sample after heat treatment step, Lane 5: 1 µg of sample after a calcium phosphate gel-cellulose column.
Table 4

The amino-terminal sequences of recombinant mature and precursor E3s (Wild-type = sequence derived from cDNA sequence; Expected = sequence expected after insertion into expression vector; Recombinant = sequence from amino acid sequence analysis).

<table>
<thead>
<tr>
<th>E3</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mature form (Residue # 1 to 10)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>A-D-Q-P-I-D-A-D-V-T----------------------</td>
</tr>
<tr>
<td>Expected</td>
<td>M-D-Q-P-I-D-A-D-V-T----------------------</td>
</tr>
<tr>
<td>Recombinant</td>
<td>M-D-Q-P-I-D-A-D-V-T----------------------</td>
</tr>
</tbody>
</table>

Precursor form (Residue # -35 to -7)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>M-Q-S-W-S----R-I-S-H-G-L-Q-G-L-S-A--------</td>
</tr>
<tr>
<td>Expected</td>
<td>M-E-P-W-S----R-I-S-H-G-L-Q-G-L-S-A--------</td>
</tr>
<tr>
<td>Recombinant</td>
<td>I-S-H-G-L-Q-G-L-S-A--------</td>
</tr>
</tbody>
</table>
The procedures used for the expression of the cDNA sequence encoding precursor human E3 in *E. coli* were identical to those used for mature human E3. The expression pattern of the precursor E3 cDNA sequence in AR120 was similar to that of the mature E3 (Figure 16). The apparent full-length band of precursor E3, however, moved slightly slower than that of mature E3, indicating that the precursor E3 had a slightly higher molecular mass, presumably due to its additional amino-terminal leader sequence.

The precursor E3 expressed in JRG1342 was purified using similar procedures to those used in the purification of mature E3 (Table 5). Some of the precursor E3 was found in the cell pellet fraction after the French press treatment so that following centrifugation, sonication was applied to break the cells. The precursor E3 had a tendency to precipitate at a lower percent saturation of ammonium sulfate than did the mature E3. Most of the precursor E3 was precipitated between 0.40-0.70 ammonium sulfate saturation rather than between 0.55-0.80. This change in solubility presumably is due to the additional amino-terminus leader sequence. Purification steps were monitored by SDS-PAGE and the corresponding Western blot analysis (Figure 17). The results showed that the expressed precursor E3 was nearly homogeneous on SDS-PAGE. The amino-terminal amino acid sequence analysis revealed that the first 19 residues of the leader sequence were not
Figure 16. Western blot analysis of expression of cDNA sequence encoding precursor E₃ in AR120 and purified mature and precursor E₃s expressed in JRG1342. AR120 was transformed with pOTSV-pE₃, pOTSV-E₃ or pOTSV. The transformed cells were chemically induced for 7 h at 37°C. Total cell proteins were separated by SDS-PAGE, transferred to a Zeta Probe membrane and treated with anti-pig heart E₃ antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Lane 1: AR120 transformed with pOTSV-pE₃, Lane 2: AR120 transformed with pOTSV-E₃, Lane 3: AR120 transformed with pOTSV. Lane 4: purified mature human E₃ expressed in JRG1342 (after calcium phosphate gel-cellulose column step), Lane 5: partially purified precursor E₃ expressed in JRG1342 (after heat treatment step).
Table 5
Purification of truncated precursor human E3 expressed in *E. coli* JRG1342.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Specific activity a</th>
<th>Recovery of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>Supernatant solution after sonication treatment</td>
<td>312.0</td>
<td>1.8</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.40-0.70) + Heat treatment (70°C, 5 min)</td>
<td>34.5</td>
<td>10.5</td>
<td>64</td>
</tr>
<tr>
<td>Calcium phosphate gel-cellulose column</td>
<td>1.1</td>
<td>146.1</td>
<td>27</td>
</tr>
</tbody>
</table>

a Activity was assayed in 50 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA, 3.0 mM dihydrolipoamide and 3.0 mM NAD⁺.
Figure 17. SDS-PAGE analysis during purification of truncated precursor E3 expressed in JRG1342 (A) and the corresponding Western blot analysis (B). Proteins were separated by SDS-PAGE (9%), electrotransferred to a Zeta Probe membrane and treated with anti-pig heart E3 antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Lane 1: molecular weight markers (from the top: phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase), Lane 2: 81 μg of supernatant solution after sonication of cells, Lane 3: 13.9 μg of sample after heat treatment step, Lane 4: 1 μg of sample after a calcium phosphate gel-cellulose column, Lane 5: 1 μg of purified mature form of recombinant human E3.
present (Table 4).

4. **Comparison of kinetic properties between mature and truncated precursor human E₃s.**

The E₃ assay was performed at varying concentrations of substrates as described in Chapter II. The kₘₐₜ and Kₘs of mature E₃ were determined in the physiological direction using double reciprocal plots. The plot showed parallel lines (Figure 18 A) indicating that recombinant mature human E₃ like other mammalian E₃s catalyzed the reaction using a ping-pong mechanism (Massey, 1960; Reed, 1973; Williams, 1976). The kₘₐₜ and Kₘ values were obtained from replots (Figure 18 B and C). The kₘₐₜ was 421 s⁻¹ and Kₘs for dihydrolipoamide and NAD⁺ were 0.69 mM and 0.32 mM, respectively. The plot for the reverse direction showed parallel lines (Figure 19 A). Kinetic parameters were obtained from replots (Figure 19 B and C). The kₘₐₜ for the reverse direction was 153 s⁻¹ and Kₘs for lipoamide and NADH were 0.93 and 0.037 mM, respectively.

The double reciprocal plot for the truncated precursor E₃ in the direction of NAD⁺ reduction showed intersecting lines (Figure 20 A) indicating that the reaction proceeded via another kinetic mechanism than the ping-pong mechanism. Kinetic parameters were determined assuming an ordered sequential mechanism with
Figure 18. Double reciprocal plot (A) of initial velocities and replots (B and C) for recombinant mature human E3 in the direction of NAD$^+$ reduction. In A, NAD$^+$ concentrations from top to bottom were 0.1 (◇), 0.2 (■), 0.5 (▲) and 3.0 (□) mM. E3 activity was assayed at 37°C with 48 ng of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as µmol of NAD$^+$ reduced per min per µg of protein. * Here, dl is dihydrolipoamide.
Figure 19. Double reciprocal plot (A) of initial velocities and replots (B and C) for recombinant mature human E₃ in the direction of NADH oxidation. In A, lipoamide concentrations from top to bottom were 0.1 (◇), 0.2 (■), 0.5 (♦) and 1.0 (□) mM. E₃ activity was assayed at 37°C with 145 ng of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as μmol of NADH oxidized per min per μg of protein.
Figure 20. Double reciprocal plot (A) of initial velocities and replots (B and C) for truncated recombinant precursor human E₃ in the direction of NAD⁺ reduction. In A, NAD⁺ concentrations from top to bottom were 0.1 (○), 0.2 (■), 0.5 (◇) and 3.0 (◻) mM. E₃ activity was assayed at 37°C with 133 ng of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as µmol of NAD⁺ reduced per min per µg of protein.
dihydrolipoamide as the first binding substrate based on the order of the substrate binding of wild-type recombinant enzyme. The $k_{\text{cat}}$ and $K_m$ values were obtained from replots (Figure 20 B and C). The $k_{\text{cat}}$ was 152 s$^{-1}$ which was about 36% of the $k_{\text{cat}}$ of the mature E3. The $K_m$ values for dihydrolipoamide and NAD$^+$ were 0.51 and 0.54 mM, respectively. In the direction of NADH oxidation, parallel lines were observed in the reciprocal plot (Figure 21 A). Kinetic parameters were obtained from replots (Figure 21 B and C). The $k_{\text{cat}}$ was 69 s$^{-1}$ and $K_m$ values for lipoamide and NADH were 1.10 mM and 0.053 mM, respectively.

5. Construction of mutant E3 expression vector, pOTSV(AEH)-E3m and site-directed mutagenesis. Since we wished to introduce mutations at residues 452 (His -> Glu) and 457 (Glu -> Gln) in the carboxy terminal region of E3, we took advantage of the presence of an EcoRI site in the 3' region of the E3 cDNA sequence. We removed an EcoRI site in the E3 expression vector, pOTSV-E3 (Figure 11), by switching the EcoRI containing part of pOTSV-E3 with the corresponding part of the influenza NS1 gene expression vector pASΔEH801, in which the EcoRI site and nearby HindIII site had been deleted (Young et al., 1983), as described in Chapter II.

The 3' region of the E3 cDNA sequence was inserted into
Figure 21. Double reciprocal plot (A) of initial velocities and replots (B and C) for truncated recombinant precursor human E3 in the direction of NADH oxidation. In A, lipoamide concentrations from top to bottom were 0.1 (▲), 0.2 (●), 0.5 (♦) and 1.0 (□) mM. E3 activity was assayed at 37°C with 200 ng of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as μmol of NADH oxidized per min per μg of protein.
A

$\frac{1}{\text{Velocity}}$

$\frac{1}{\text{[NADH] mM}^{-1}}$

B

$\frac{1}{\text{Vmax}}$

$\frac{1}{\text{[Lipoamide] mM}^{-1}}$

C

$\frac{1}{\text{Km NADH}}$

$\frac{1}{\text{[Lipoamide] mM}^{-1}}$
M13mp18 phage. The uracil-enriched E3 3' region single stranded DNA was generated using E. coli CJ265 (ung- and dut-) (Kunkel et al., 1987). Site-directed mutagenesis was performed as described in Chapter II. The mutated sequence was screened by DNA sequencing (Figure 22). The efficiency of the site-directed mutagenesis was about 20%. The 3' region of the wild-type E3 cDNA sequence in pOTSV(ΔEH)-E3 was replaced by the mutated sequence by EcoRI and Xhol digestions and ligation (Figure 12). Sequencing of the mutated 3' regions of E3 cDNA was performed and no mutations other than the intended mutations were found.

6. Purification of mutant human E3s expressed in E. coli JRG1342.

Mutant human E3s were purified from JRG1342 by modifying the procedure used to purify the recombinant wild-type enzyme (Table 6), as use of exactly the same procedure resulted in the presence of an additional protein band on SDS-PAGE. This protein was removed by DEAE-Sephacel chromatography. Instead of the previously used calcium phosphate gel-cellulose column, a hydroxyapatite column with similar properties was used and heat treatment was performed at the last step of purification. The purification was analyzed by SDS-PAGE. After the heat treatment step, the mutant E3s were virtually homogeneous on the gel (Figure 23). The overall yield of the Gln-457 mutant E3 purification was 35%. About 1.4 mg of the Gln-457 mutant E3 was obtained from 15
Figure 22. DNA sequences of wild-type and mutant E₃s in the mutated region. The DNA sequences around the mutation sites are shown and the mutation sites are indicated by horizontal lines (Left: Glu-457 to Gln; Right: His-452 to Gln).
Table 6
Purification of the human Gln-457 mutant E3 expressed in *E. coli* JRG1342.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant solution after French press treatment</td>
<td>1925</td>
<td>0.13</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.55-0.85)</td>
<td>260</td>
<td>0.52</td>
<td>54</td>
</tr>
<tr>
<td>DEAE-Sephacel + Hydroxyapatite columns</td>
<td>4.35</td>
<td>20.6</td>
<td>35</td>
</tr>
<tr>
<td>Heat treatment (75°C, 5 min)</td>
<td>1.43</td>
<td>62.6</td>
<td>35</td>
</tr>
</tbody>
</table>

*a*  Activity was assayed at 37°C in 100 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA, 3 mM dihydrolipoamide and 3 mM NAD⁺.
Figure 23. SDS-PAGE analysis during purification of human mutant E3s expressed in JRG1342. A: His-452 -> Gln mutant E3 B: Glu-457 -> Gln mutant E3. Lane 1: molecular weight markers (from the top: phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase), Lane 2: supernatant, Lane 3: after ammonium sulfate precipitation, Lane 4: after DEAE-Sephadex and hydroxyapatite columns, Lane 5: after heat treatment, Lane 6: pig heart E3 (obtained from Sigma Co.).
liters of cell culture (Table 6). The purification of the Gln-452 mutant E₃ was performed with the same procedure (Table 7). Since this mutant E₃ had very low enzyme activity, the purification was followed by measuring the yellow color of eluants with spectrophotometer. Based on the experience of purifying the Gln-457 mutant E₃, this color was nearly coincident with E₃ elution. About 0.6 mg of the Gln-452 mutant E₃ was obtained from 15 liters of cell culture. The purified mutant E₃s had the bright yellow color characteristic of flavoproteins. The absorption spectra of the mutant E₃s observed from 340 nm to 700 nm wavelength (Figure 24) were similar to that of reported pig heart E₃ (Williams, 1976).


Parallel lines were obtained on the double reciprocal plot of the Gln-452 mutant E₃ indicating that the mutant E₃, like the recombinant wild-type enzyme (His-452), catalyzed the E₃ reaction via a ping-pong mechanism (Figure 25 A). Kinetic parameters were obtained from the replots (Figure 25 B and C). The $k_{\text{cat}}$ was determined as 0.77 s⁻¹ which was about 0.2% of that of wild-type enzyme (Table 8). The $K_m$ for dihydrolipoamide was 43.6 mM. This value was 63-fold higher than that of wild-type enzyme. The $K_m$ for NAD⁺ was 0.38 mM which was slightly higher than that of wild-type enzyme. The free energy difference for the transition state between the Gln-452 mutant and wild-type E₃s was calculated as about 6.4
Table 7

Purification of the human Gln-452 mutant E3 expressed in *E. coli* JRG1342.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>after French press treatment</td>
<td>2000</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.55-0.85)</td>
<td>221.6</td>
<td>9</td>
</tr>
<tr>
<td>DEAE-Sephacel + Hydroxyapatite columns + heat treatment (75°C, 5 min)</td>
<td>0.6</td>
<td>3333</td>
</tr>
</tbody>
</table>
Figure 24. Absorption spectra of mutant E3s. A: His-452 \(\rightarrow\) Gln (0.44 mg/ml), B: Glu-457 \(\rightarrow\) Gln (0.97 mg/ml). The spectra were measured with a Shimadzu UV160 spectrophotometer. The buffer was 10 mM potassium phosphate (pH 7.6) containing 0.25 mM EDTA.
Figure 25. Double reciprocal plot (A) for initial velocities and replots (B and C) for the His-452 → Gln mutant E₃ in the direction of NAD⁺ reduction. In A, NAD⁺ concentrations from top to bottom were 0.1 (■), 0.2 (▲) and 0.5 (□) mM. E₃ activity was assayed at 37°C with 34.7 µg of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as µmol of NAD⁺ reduced per min per µg of protein. * Here, dl is dihydrolipoamide.
Table 8
Kinetic parameters and the free energy difference ($\Delta G$) for recombinant human wild-type and mutant E3s.

Activity was assayed at 37°C in 100 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA. Kinetic parameters are mean ± standard deviation of two (mutants) to three (wild-type) independent determinations.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$</th>
<th>$K_m$ dl $^a$</th>
<th>$K_m$ NAD$^+$</th>
<th>$k_{cat}/K_m$ dl $^a$</th>
<th>$\Delta G$ $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s$^{-1}$</td>
<td>mM</td>
<td>mM</td>
<td>s$^{-1}$ M$^{-1}$</td>
<td>kcal mol$^{-1}$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>421±24</td>
<td>0.69±0.02</td>
<td>0.32±0.03</td>
<td>6.08±0.27 x 10$^5$</td>
<td>N/A $^c$</td>
</tr>
<tr>
<td>His-452-&gt;Gln$^d$</td>
<td>0.77±0.22</td>
<td>43.6±18.5</td>
<td>0.38±0.11</td>
<td>19.1±1.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Glu-457-&gt;Gln</td>
<td>118±4</td>
<td>2.94±0.18</td>
<td>0.32±0.08</td>
<td>4.01±0.11 x 10$^4$</td>
<td>1.7</td>
</tr>
</tbody>
</table>

$^a$ Mixed RS dihydrolipoamide.

$^b$ Calculated from the equation:

$$\Delta G = -RT \ln \left( \frac{(k_{cat}/K_m)_{mutant}}{(k_{cat}/K_m)_{wild-type}} \right)$$

Using the mean $k_{cat}/K_m$ values for dihydrolipoamide as described by Wilkinson et al. (1983) and Lowe et al. (1985).

$^c$ Not applicable.

$^d$ These parameters are apparent values since they are determined at low concentrations of dihydrolipoamide compared to its $K_m$ value due to low solubility of the substrate.
8. **Characterization of the human Gln-457 mutant E_3.**

The double reciprocal plot of the Gln-457 mutant E_3 showed intersecting lines (Figure 26 A) indicating that this mutant E_3 catalyzed the E_3 reaction via a different kinetic mechanism. Kinetic parameters were determined assuming an ordered sequential mechanism with dihydrolipoamide as the first binding substrate based on the order of substrate binding of recombinant wild-type enzyme. The $k_{cat}$ and $K_m$ values were obtained from replots (Figure 26 B and C). The $k_{cat}$ was 118 s$^{-1}$ which was about 28% of that of wild-type enzyme. The $K_m$ for NAD$^+$ was 0.32 mM which was identical to that of wild-type enzyme. The $K_m$ for dihydrolipoamide was, however, 2.94 mM which was 4.3-fold higher than that of wild-type enzyme (Table 8). The free energy difference between the mutant and wild-type E_3s was estimated as about 1.7 kcal mol$^{-1}$ (Table 8).

9. **pH dependence of E_3 activity of recombinant human wild-type and the Gln-452 mutant E_3s.**

The recombinant wild-type human E_3 had an optimum pH around pH 8.0 (Table 9). This pH optimum is similar to those of pig heart (pH 7.9) and rat liver (pH 8.2) E_3s. Its $K_m$ for NAD$^+$ was
Figure 26. Double reciprocal plot (A) for initial velocities and replots (B and C) for the Glu-457 -> Gln mutant E₃ in the direction of NAD⁺ reduction. In A, NAD⁺ concentrations from top to bottom were 0.1 (◊), 0.2 (■), 0.5 (◆) and 3.0 (□) mM. E₃ activity was assayed at 37°C with 1.4 µg of enzyme in 100 mM potassium phosphate buffer, pH 8.0 containing 1.5 mM EDTA. The assay volume was 1 ml. Velocity is expressed as µmol of NAD⁺ reduced per min per µg of protein.
Table 9

Kinetic parameters of recombinant human wild-type and the Gln-452 mutant E3s at different pHs.

Activity was assayed at 37°C in 100 mM potassium phosphate buffer containing 1.5 mM EDTA. Kinetic parameters at pH 8.0 are mean of two (Gln-452 mutant) to three (wild-type) independent measurements while the parameters at other pHs are from one measurement.

<table>
<thead>
<tr>
<th>pH</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>260</td>
<td>365</td>
<td>421</td>
<td>235</td>
</tr>
<tr>
<td>$K_m$ dl $^a$ (mM)</td>
<td>0.49</td>
<td>0.56</td>
<td>0.69</td>
<td>0.40</td>
</tr>
<tr>
<td>$K_m$ NAD$^+$ (mM)</td>
<td>0.50</td>
<td>0.47</td>
<td>0.32</td>
<td>0.23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Gln-452</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>0.77</td>
<td>1.46</td>
</tr>
<tr>
<td>$K_m$ dl $^a$ (mM)</td>
<td>43.6</td>
<td>67.4</td>
</tr>
<tr>
<td>$K_m$ NAD$^+$ (mM)</td>
<td>0.38</td>
<td>0.86</td>
</tr>
</tbody>
</table>

$^a$ Mixed RS dihydrolipoamide
slightly decreased as pH increased. Its $K_m$ for dihydrolipoamide was slightly increased until pH 8.0 and then was decreased at pH 8.5. The activity of the Gln-452 mutant E3 at pH 8.5 was 1.46 s$^{-1}$ which was about 2-fold higher than the activity at pH 8.0. The double reciprocal plot showed parallel lines (Figure 27 A). The $k_{cat}$ and $K_m$ values were obtained from replots (Figure 27 B and C). The $K_m$s for dihydrolipoamide and NAD$^+$ were 67.4 mM and 0.86 mM respectively.
Figure 27. Double reciprocal plot (A) for initial velocities and replots (B and C) for the His-452 -> Gln mutant E₃ in the direction of NAD⁺ reduction at pH 8.5. In A, NAD⁺ concentrations from top to bottom were 0.1 (■), 0.2 (♦) and 0.5 (□) mM. E₃ activity was assayed at 37°C with 19.2 mM of enzyme in 100 mM potassium phosphate buffer containing 1.5 mM EDTA. The assay volume was 0.5 ml. Velocity is expressed as µmol of NAD⁺ reduced per min per µg of protein. * Here, dl is dihydrolipoamide.
Chapter IV
Discussion

1. Expression of mature and precursor forms of human E3 in E. coli.

The expression vector pOTSV has been chosen to express human E3 because it has several characteristics leading to high expression levels for an inserted gene sequence (Shatzman and Rosenberg, 1987). It has λ phage P_L promoter which provides efficient transcription and the CII gene ribosome binding site which gives efficient translation initiation. In addition to these, phage λ antitermination function utilization sites and a transcription terminator are provided to maximize transcription and plasmid stability. A unique BamHI site is located adjacent to the CII gene ribosome binding site and provides a translation start codon (AUG) to any inserted gene sequence.

The expression of human E3 in E. coli resulted in a full-length E3 and four additional short-length E3 polypeptides (Figure 13). The short-length polypeptides were mainly located in the insoluble fraction of E. coli. The reasons for the formation of the insoluble E3 polypeptides are not clear. One possible reason is digestion by E. coli proteases. Possible slow translation processes
due to a rare codon usage could expose protease target sites on newly synthesized E3 polypeptide. The new environment could slow down the proper folding or cofactor FAD binding processes of E3 so that the protease vulnerable sites may be exposed and improper association between E3 polypeptides may occur.

Since wild-type *E. coli* has its own endogenous *E. coli* E3, human E3 was expressed in a PDC-deficient *E. coli* strain JRG1342. The PDC operon gene sequence of JRG1342 was totally deleted resulting in the absence of *E. coli* E3 activity. JRG1342 was successfully used for the characterization of *E. coli* mutant E3s which showed no activity (two active site Cys substitution mutants; Russell et al., 1989) or a decreased activity (His-444 to Gln mutant; 0.3-0.4% of wild-type activity; Williams et al., 1989). The soluble fraction (17.5 μg) after sonication and centrifugation from JRG1342 (pRK248CIts) transformed with the expression vector (pOTSV) did not possess any detectable activity after thermal induction. The assay could detect E3 activity higher than about 0.1 munit. However, the same amount of the soluble fraction from JRG1342 (pRK248CIts) transformed with E3 expression vector (pOTSV-E3) did have 7 munits of E3 activity after the induction (Table 2). Due to a combined deficiency of PDC and α-ketoglutarate dehydrogenase complex because of deletion of the lpd gene responsible for E3 component, *E. coli* JRG1342 grew poorly and hence may have expressed human E3 at a relatively low levels. However, due to a
lack of *E. coli* E₃ in JRG1342, it was preferable to express and purify human E₃ in this strain.

The amino-terminal amino acid sequencing revealed that the recombinant mature E₃ possessed an expected amino-terminal sequence while the first 19 amino acids of the recombinant precursor E₃ was deleted (Table 4). This deletion was presumably due to a cleavage by a trypsin-like protease since the cleavage site was right after arginine residue leaving isoleucine as the first residue of the truncated precursor E₃. This result indicated that the leader sequence of human E₃ might hang out from the core structure of mature E₃ and become a target site for a protease attack.

The recombinant mature human E₃, like other mammalian E₃, catalyzed the E₃ reaction via a ping-pong mechanism. The kinetic parameters of the recombinant mature human E₃ (Table 10) were comparable to those reported for mammalian E₃ considering differences in assay conditions and enzyme sources (Massey et al., 1960; Reed, 1973). Human liver E₃ had a $K_m$ for dihydrolipoamide of 0.26 mM and the presumed $k_{cat}$ was 97 s⁻¹ at pH 8.3 and 25°C (Ide et al., 1967). It should be emphasized that these values probably did not represent the true values since they were obtained at a fixed low concentration of NAD⁺ (Ide et al., 1967). The kinetic parameters for rat liver E₃ were reported
Table 10
Comparison of kinetic parameters for recombinant mature and precursor forms of human E₃ with those for reported mammalian E₃s.

Activity for the recombinant E₃ was assayed at 37°C in 100 mM potassium phosphate buffer, pH 8.0, containing 1.5 mM EDTA. The references for reported mammalian E₃s are the following: human liver from Ide et al., 1967; rat liver from Reed, 1973; pig heart from Massey et al., 1960.

<table>
<thead>
<tr>
<th>E₃</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>$K_m$ dl a (mM)</th>
<th>$K_m$ NAD⁺ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature form</td>
<td>421</td>
<td>0.69</td>
<td>0.32</td>
</tr>
<tr>
<td>Truncated precursor form</td>
<td>152</td>
<td>0.51</td>
<td>0.54</td>
</tr>
<tr>
<td>Human liver</td>
<td>97 b</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>Rat liver</td>
<td>345 b</td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td>Pig heart</td>
<td>555 b</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Reverse direction

<table>
<thead>
<tr>
<th>E₃</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>$K_m$ lipoamide c (mM)</th>
<th>$K_m$ NADH (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature form</td>
<td>153</td>
<td>0.93</td>
<td>0.037</td>
</tr>
<tr>
<td>Truncated precursor form</td>
<td>69</td>
<td>1.10</td>
<td>0.053</td>
</tr>
<tr>
<td>Rat liver</td>
<td>124 b</td>
<td>0.84</td>
<td>0.062</td>
</tr>
</tbody>
</table>

a Mixed RS dihydrolipoamide.
b Recalculated from the references assuming the molecular mass of E₃ is 50 kDa.
c Mixed RS lipoamide
(Reed, 1973). The $K_m$s for dihydrolipoamide and NAD$^+$ were 0.49 and 0.52 mM, respectively. The $k_{cat}$ was 345 s$^{-1}$ at 37°C, pH 8.0. These values were very similar to our data and the assay conditions were nearly identical (Table 10). Kinetic data for pig heart E$_3$ were reported (Massey et al., 1960). The $K_m$s for dihydrolipoamide and NAD$^+$ were 0.3 and 0.2 mM and $k_{cat}$ was 555 s$^{-1}$ at 25°C, pH 7.6.

The truncated recombinant precursor E$_3$ had about 36% of the recombinant mature E$_3$ activity (Table 10). The low catalytic efficiency of the truncated recombinant precursor E$_3$ may result from a possible conformational change due to the additional aminoterminal 16 amino acid sequence of E$_3$. Since this sequence was not removed by E. coli proteases, it probably became a part of the core structure of E$_3$. This would result in the creation of new interactions between the additional sequence and the core structure of E$_3$ which could lead to the possible conformational change. The different conformational states of precursor and mature mitochondrial malate dehydrogenase have been reported (Chien and Freeman, 1986). The conformational change may be responsible for altered kinetic mechanism of the truncated precursor E$_3$ in the forward reaction which is the topic of the following section.

One interesting observation in the kinetic properties of the truncated precursor E3 and the Gln-457 mutant E3 is the altered kinetic mechanism. There are two general steady state kinetic mechanisms for enzymes involving two substrates and two products. One is a ping-pong mechanism and the other is a sequential mechanism. In the ping-pong mechanism, one substrate binds first to enzyme (Figure 28). The first half reaction then occurs, resulting in the catalytic intermediate state of enzyme (F in Figure 28). The release of the first product from the intermediate state of the enzyme is followed by the binding of the second substrate. The second half reaction occurs and the catalytic intermediate state of enzyme returns to the original state, followed by the release of the second product. In the sequential mechanism, the second substrate binds to enzyme before the release of the first product, resulting in a ternary complex (E·S1·S2<-->E·P1·P2 in Figure 28). The order of substrate binding could be ordered or random. The difference between ping-pong and sequential mechanism is basically whether enzyme can form the ternary complex during catalysis or not.

Glutathione reductase has two spatially distinct substrate binding sites which are separated by flavin ring of FAD (Schulz et al., 1982). One site is for NADPH and the other is for glutathione. Glutathione binds to the si-face of FAD while NADPH binds to the re-face as shown in Figure 29 which is built based on the structure
Figure 28. Two general kinetic mechanisms for enzymes involving two substrates and two products. In ping-pong mechanism, the first substrate (S1) binds to enzyme (E). The first half reaction occurs. Enzyme becomes the intermediate form (F) and the first product (P1) is released. The second substrate (S2) binds to the intermediate form. The second half reaction occurs. Enzyme returns to the original state and the second product (P2) is released. In a sequential mechanism, the two substrates (S1 and S2) bind consecutively to enzyme. A ternary complex forms during catalysis (E·S1·S2 <-> E·P1·P2). The products (P1 and P2) are released.
Ping-pong Mechanism

\[
\begin{array}{cccc}
S1 & P1 & S2 & P2 \\
\downarrow & \uparrow & \downarrow & \uparrow \\
\end{array}
\]

E \ (E \cdot S1 \not\carrow F \cdot P1) \quad F \ (F \cdot S2 \not\carrow E \cdot P2)

Ordered Sequential Mechanism

\[
\begin{array}{c}
S1 & S2 & P1 & P2 \\
\downarrow & \downarrow & \uparrow & \uparrow \\
\end{array}
\]

E \ E \cdot S1 \ (E \cdot S1 \cdot S2 \not\carrow E \cdot P1 \cdot P2) \quad E \cdot P2
Figure 29. Predicted active site of human E3 built by molecular graphic modeling based on the three dimensional structure of human GR. Lipoic acid and NAD$^+$ are added. FAD and the active disulfide center between Cys-50 and Cys-45 and the amino acid residues predicted to interact with substrates are included. His-452' and Glu-457' are from other subunit (from Jentoft et al., in review).
of human GR and where glutathione binding site is indicated by lipoic acid. This structural feature of GR allows a ternary complex formation in certain circumstances. GR catalyzes physiologically a similar but reverse reaction of E₃, the oxidation of NADPH and the reduction of oxidized glutathione. The kinetic mechanism of yeast GR in the physiological direction is a mixed sequential and ping-pong mechanism depending on the concentration of oxidized glutathione (Mannervik, 1973). In low concentration of oxidized glutathione, GR catalyzes the reaction via a ping-pong mechanism. In high concentration of the substrate, GR catalyzes the reaction via an ordered sequential mechanism (Figure 30). It has been suggested that this mixed kinetic mechanism could be applied to other homologous flavoproteins such as E₃ (Mannervik, 1973). GRs from yeast and E. coli display a Ter-Bi sequential mechanism in the direction of NADP⁺ reduction which is the physiological direction of E₃ (Icen, 1971; Berry et al., 1989). It has been reported that the kinetic mechanism of E. coli GR has been changed from a ping-pong to a sequential mechanism in the direction of NADPH oxidation by a one amino acid change of Tyr-177 to Ser or Gly (Berry et al., 1989). One explanation for this phenomenon is that a possible slowed step of the first half reaction, enzyme-NADPH to the two-electron-reduced intermediate, and the coincident lower Kₘ for oxidized glutathione may force the enzyme reaction via the ordered sequential mechanism. An alternative explanation is based on the mixed sequential and ping-pong mechanism of yeast GR
Figure 30. The kinetic mechanism of yeast GR. In low concentration of oxidized glutathione, GR catalyzes the reaction via a ping-pong mechanism (counterclockwise). In high concentration of oxidized glutathione, GR catalyzes the reaction via a ordered sequential mechanism (clockwise) (from Mannervik, 1973).
(Figure 30). The lower $K_m$ for oxidized glutathione of the mutant GRs might have effect of favoring the ordered sequential mechanism.

Based on the extensive structural comparison between human E₃ and GR and the molecular graphic modeling (Jentoft et al., in review) and the three dimensional structure of A. vinelandii E₃ (Schierbeek et al., 1989), human E₃ is expected to have the same two spatially distinct substrate binding sites (Figure 29). Based on the structure of human GR (Schulz et al., 1982), dihydrolipoamide is thought to be bound to the si-face of FAD while NAD⁺ binds to the re-face (Figure 29). The intrinsic property of alterable kinetic mechanism of GR could be true of E₃ since they have similar structural and catalytic characteristics (Williams, 1976; Carothers et al., 1989; Thekkumkara et al., 1989).

One feasible explanation for the altered kinetic mechanism of the modified human E₃s is their possible slowed first half reaction. This slowed half reaction may allow the binding of NAD⁺ to the enzyme before the release of product lipoamide. The decreased activity of modified E₃s may be due to the slowed first half reaction which is a rate limiting step in wild-type mammalian enzyme. The Gln-457 mutant E₃ may have a possible increased affinity to product lipoamide. The possible increased affinity to lipoamide may slow the releasing step of lipoamide to allow the binding of NAD⁺ to
the Gln-457 mutant enzyme before the release, resulting in the formation of a ternary complex which is a characteristic of the sequential mechanism. It is plausible since the substitution of Glu-457 to Gln, which removes one negative charge may make the dihydrolipoamide binding site more hydrophobic. The more hydrophobic substrate binding site of the Gln-457 mutant E₃ may have the possible increased affinity to product lipoamide which is very hydrophobic, resulting in the slowed release of lipoamide.

3. Involvement of His-452 and Glu-457 in human E₃ catalysis.

The presence of a proton acceptor/donor in the active site of E₃ has been previously postulated (Williams, 1976). Since the pK's for the thiol ionization of dihydrolipoamide has been determined as pK'₁ of 9.35 and pK'₂ of 10.65 (Matthews et al., 1977), E₃ requires a proton acceptor/donor which can deprotonate the substrate when it catalyzes the reaction in the physiological condition. Two electrons and two protons from dihydrolipoamide are transferred to E₃ to form the two-electron-reduced E₃ (Matthews and Williams, 1976). One proton is thought to be accepted by Cys-45. The two-electron-reduced E₃ can be characterized as a charge transfer complex between a thiolate anion (Cys-50) and the flavin of FAD (Williams, 1976). Therefore, it is necessary to have a proton acceptor/donor which can accept the proton that could otherwise protonate Cys-50 to make the thiolate anion interaction with FAD
feasible (Figure 6). The positively charged proton acceptor/donor can also stabilize the thiolate anion. The three dimensional structures of human GR (Thieme et al., 1981) and A. vinelandii E3 (Schierbeek et al., 1989) indicated one His residue near the active disulfide center as a possible candidate for the proton acceptor/donor. The His residue can form a strong hydrogen bond to a nearby Glu residue (Figure 29). These two residues are conserved in all known E3s and GRs and trypanothione reductases (Table 11).

The substitution of the corresponding His-444 to Gln in E. coli E3 (Williams et al., 1989) resulted in very low enzyme activity (about 0.3 to 0.4% activity of wild-type enzyme) and destabilization of the thiolate anion. The detailed kinetic properties of this mutant are not available. The corresponding His-439 in E. coli GR was also substituted to Gln (Berry et al., 1989). The k$_{cat}$ of the Gln-439 mutant GR was about 0.4% of that of wild-type enzyme and the K$_{m}$ for oxidized glutathione was increased about 3-fold. Since it was possible that the amide group of Gln might form a hydrogen bond to a water molecule which could act as an alternative proton acceptor/donor, His-439 was also replaced by Ala whose side chain could not form a hydrogen bond to a water molecule (Deonarain et al., 1989). The Ala-439 mutant GR showed similar kinetic properties to those of the Gln-439 mutant GR with 0.1% of wild-
Table 11
Comparison of amino acid sequence around active site His and Glu. The conserved His (H) and Glu (E) residues are highlighted by bold letters.

<table>
<thead>
<tr>
<th>Enzyme/Source</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dihydrolipoamide dehydrogenase</strong></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>F S H P T L S E A</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>H A H P T L G E A</td>
</tr>
<tr>
<td><em>A. vinelandii</em></td>
<td>F A H P A L S E A</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>H A H P T L H E S</td>
</tr>
<tr>
<td>Yeast</td>
<td>H A H P T L S E A</td>
</tr>
<tr>
<td>Pig</td>
<td>H A H P T L S E A</td>
</tr>
<tr>
<td>Human</td>
<td>H A H P T L S E A</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione reductase</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>A I H P T A A E E</td>
</tr>
<tr>
<td>Human</td>
<td>A I H P T S S E E</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trypanothione reductase</strong></td>
<td></td>
</tr>
<tr>
<td><em>T. congolense</em></td>
<td>G V H P T S A E E</td>
</tr>
<tr>
<td><em>C. fasciculata</em></td>
<td>G V H P T S A - -</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
type activity. Another possible proton acceptor/donor (Tyr-99) in *E. coli* GR was also substituted to Phe (Deonarain et al., 1989). Since this Phe-99 mutant GR showed similar activity to that of wild-type enzyme, it was concluded that His-439 was probably the proton acceptor/donor in *E. coli* wild-type GR. It was explained that the residual activities of the Gln-439 and Ala-439 mutant enzymes could be due to yet unidentified alternative functional protein side chains or water molecules in solution.

In the present study, the corresponding His-452 in human E₃ was substituted to Gln. The Gln-452 mutant E₃ possessed about 0.2% activity of wild-type enzyme. The degree of reduction in activity of the Gln-452 mutant E₃ was similar to that seen in the corresponding mutants, *E. coli* E₃ (His-444 to Gln) and GR (His-439 to Gln or Ala). The *Kₘ* for dihydrolipoamide was dramatically increased by about 63-fold, indicating that His-452 was important in the binding affinity of dihydrolipoamide to the active site of the enzyme. This increase was much higher than that observed in the corresponding *E. coli* mutant GR. The binding energy of dihydrolipoamide to the Gln-452 mutant E₃ was determined as about 1.9 kcal mol⁻¹ which was decreased by 2.6 kcal mol⁻¹ compared to that (about 4.5 kcal mol⁻¹) of wild-type enzyme as shown in Figure 31. The large changes in *kₐₜ* and *Kₘ* for dihydrolipoamide resulted in a dramatic decrease in the catalytic efficiency of the Gln-452 mutant E₃ toward dihydrolipoamide. The
Figure 31. The simplified energy diagrams of wild-type (1), Gln-452 (2), and Gln-457 (3) mutant E3s from the viewpoint of dihydrolipoamide. Here, E is the ground state of enzyme. ES is the dihydrolipoamide bound enzyme and [ES]* is the transition state of enzyme. The dihydrolipoamide binding energy was calculated using the equation: $G = RT \ln K_m$ for dihydrolipoamide. The energy state of the transition state was calculated using the equation: $G = RT \ln \left( \frac{k_B T}{h} \right) - RT \ln \left( \frac{k_{cat}}{K_m} \right)$ where $R$ is the gas constant, $T$ is the absolute temperature, $k_B$ is Boltzmann's constant and $h$ is Planck's constant. Energy states are relative to the ground state of free enzyme and substrate whose energy state is assumed as 0 kcal mol$^{-1}$. 
$k_{\text{cat}}/K_m$ for dihydrolipoamide was decreased about $3.2 \times 10^4$ times providing an estimate of the decrease in efficiency of the mutant enzyme. This corresponded to a destabilization of the transition state of the human E3 catalysis by about $6.4 \text{ kcal mol}^{-1}$ (Figure 31). These results indicated that His-452 was critical to human E3 catalysis.

It is worthwhile to discuss a possible local environmental change due to the substitution of His to Gln. This substitution replaces the imidazole group of the His side chain with an $\alpha$-amidomethyl group (-CH$_2$CONH$_2$). Even though this substitution in the human E3 active site is expected to cause only a smallest local environmental change, this substitution affects the local environment around His-452 in three aspects, namely, charge distribution, occupied area and hydrogen bonding network. The substitution abolishes the potential of His-452 to be positively charged upon protonation. The occupied areas of the imidazole group and the $\alpha$-amidomethyl group are different. The potential to form hydrogen bonds of the imidazole group differ from that of the $\alpha$-amidomethyl group. Based on the three dimensional structure of human GR, the $\pi$-nitrogen atom of His-452 forms a hydrogen bond to oxygen atom of Glu-457 in wild-type human E3 (Figure 32). In the Gln-452 mutant E3, Gln-452 may still form a similar hydrogen bond to Glu-457 as shown in Figure 32. But the strength of hydrogen bond could be different from that of the corresponding
Figure 32. Schematic representation of the possible interactions among dihydrolipoamide and amino acid residues 452 and 457 in wild-type, Gln-452 and Gln-457 mutant E₃s.
Wild-type

His-452 -> Gln   Glu-457 -> Gln
hydrogen bond of wild-type E3 since hydrogen bonding is usually affected by the distance and orientation in addition to the electronegativity of participating atoms. Besides the hydrogen bond to Glu-457, the nitrogen atom of Gln-452 may form another hydrogen bond to a possible hydrogen bonding partner such as a water molecule. These factors may cause a local environmental change in the active site of the Gln-452 mutant E3 which may contribute to the dramatic decrease in the binding affinity of dihydrolipoamide to the enzyme. This change is probably small and located to this region of active site since the binding of FAD as measured by spectral analysis is not affected (Figure 24).

There are two possible explanations for the residual activity of the Gln-452 mutant E3. One possibility is the existence of alternative proton acceptor/donor(s) which may partially replace the role of His-452. A water molecule or other alternative functional group(s) may be located near the active site of Gln-452 mutant E3 and function as the proton acceptor/donor in the Gln-452 mutant E3 catalysis. In the refined three dimensional structure of human GR (Karplus and Schulz, 1987), a water molecule, which is hydrogen-bonded to the corresponding active site His-467 in human GR, has been suggested to function as a proton acceptor/donor in human GR catalysis. Since the side chain of Gln-452 in the Gln-452 mutant E3 can form a hydrogen bond to a water molecule, this is a good possibility. His-439 in human E3, located
near the active site (Figure 29), may function as an alternative proton acceptor/donor. In molecular graphic modeling structure of human E₃ (Jentoft et al., in review), the distance from His-439 to the sulfur atom at 8 position of dihydrolipoamide is 6.08 Å which is slightly longer than the corresponding distance (4.45 Å) from His-452 to dihydrolipoamide. The possible local environmental change may bring this residue close enough to dihydrolipoamide in the Gln-452 mutant enzyme to act as the alternative proton acceptor/donor.

The other possibility is that the Gln-452 mutant E₃ itself may be able to catalyze the E₃ reaction with the help of water molecules in assay solution. This possibility assumes interactions between dihydrolipoamide bound to the Gln-452 mutant E₃ and water molecules in assay solution. This possibility has been tested by measuring E₃ activity of the Gln-452 mutant at pH 8.5. If water molecules in assay solution act as a proton acceptor/donor in the Gln-452 mutant E₃, the activity of the Gln-452 mutant E₃ is expected to increase at pH 8.5 compared to the activity at pH 8.0. At pH 8.5, the concentration of hydroxyl ion (OH⁻) is increased so that the thiol ionization of dihydrolipoamide occurs more easily. The activity of the Gln-452 mutant E₃ at pH 8.5 is about 190% of that at pH 8.0 as shown in Figure 33, indicating that the Gln-452 mutant E₃ is likely to catalyze the E₃ reaction with the help of water molecules in assay solution. This result also suggests the
Figure 33. pH dependence of activities of wild-type and the Gln-452 mutant E₃s. Activities are expressed % values relative to activity at pH 8.0 which is 100%. The $k_{cat}$ of wild-type enzyme at pH 8.0 is 421 s⁻¹ while that of the Gln-452 mutant is 0.77 s⁻¹. Activities were assayed at 37°C in 100 mM potassium phosphate buffer containing 1.5 mM EDTA in the direction of NAD⁺ reduction.
accessibility of water molecules in assay solution to the active site of Gln-452 mutant E₃. This concept that water molecules in assay solution act as a proton acceptor/donor in the absence of the active site proton acceptor/donor has been suggested for the corresponding *E. coli* GR mutant (Deonarain et al., 1989) and is probably responsible for the residual activity of the corresponding *E. coli* E₃ mutant. Wild-type E₃ has an optimum pH around 8.0 as shown in Figure 33. At pH 8.5, the activity of wild-type E₃ decreases to about 56% of its activity at pH 8.0.

The suggestion that His-452 is the proton acceptor/donor in wild-type human E₃ catalysis is supported by the following facts from this study. First, the degree of the reduction in catalytic rate due to the substitution of His-452 to Gln is similar to the corresponding *E. coli* E₃ and GR mutants. Since the contribution of His-452 to catalytic rate of human E₃ is nearly similar to those of the corresponding His residues to catalytic rates of *E. coli* E₃ and GR, it should be a common critical element in E₃ and GR reactions by functioning as the proton acceptor/donor. The residual activity of the Gln-452 mutant E₃ can be explained by the thiol ionization of dihydrolipoamide with the help of water molecules in assay solution. This explanation could be also responsible for the residual activities of the corresponding *E. coli* E₃ and GR mutants. Secondly, the large increase in *Kₘ* for dihydrolipoamide in the Gln-452 mutant E₃ indicates the closeness and possible
interactions between His-452 and dihydrolipoamide upon binding of the substrate to human E3. This result supports the conclusion that His-452 is located close to dihydrolipoamide in human E3 to deprotonate it.

The contribution of the carboxylic anion group (-COO\(^-\)) of Glu-457, the hydrogen bonding partner of His-452, in human E3 catalysis was investigated by substituting Glu-457 to Gln. The suggested roles of Glu-457 were the fixation of His-452 for optimum function and the stabilization of positively charged His-452 upon protonation. The Gln-457 mutant E3 showed 28% of wild-type E3 activity. The 4.3-fold increased \(K_m\) for dihydrolipoamide indicated again the importance of the local environment around Glu-457 and His-452 in the binding of dihydrolipoamide to the enzyme. The catalytic efficiency for dihydrolipoamide of the Gln-457 mutant E3 was decreased to 6.7% of that of wild-type enzyme, corresponding to a destabilization of the transition state of human E3 catalysis by about 1.7 kcal mol\(^{-1}\) (Figure 31).

The substitution of Glu to Gln is a modest isosteric change. The substitution replaces the carboxylic anion group (-COO\(^-\)) of the Glu-457 side chain with an amine group (-NH\(_2\)). A hydrogen bond between His-452 and Gln-457 may exist as shown in Figure 32. The strength of the hydrogen bond could be weaker than that of the
corresponding one in wild-type enzyme since a charged hydrogen bonding partner is not involved in the hydrogen bond of the Gln-457 mutant E3. Also, neutral Gln-457, unlike negatively charged Glu, can not stabilize positively charged His-452 upon protonation which may lead to a less efficient function of His-452 as the proton acceptor/donor. The nitrogen atom of Gln-457 may form hydrogen bond(s) with possible hydrogen bonding acceptor(s) in the Gln-457 mutant E3, resulting in a possible local environmental change. The weakened hydrogen bond, inability to stabilize the positively charged His-452 and/or the possible local environmental change may partly contribute to the decreased catalytic efficiency of the Gln-457 mutant E3.

This study strongly indicates that His-452 is critical to human E3 catalysis by functioning as the proton acceptor/donor. Human E3 can catalyze the reaction with the help of the water molecules in assay solution in the absence of the proton acceptor/donor. The carboxylic anion group (\(-\text{COO}^-\)) of Glu-457 is not critical to human E3 catalysis but contributes to E3 catalytic efficiency stabilizing the transition state by about 1.7 kcal mol\(^{-1}\). The local environment around His-452 and Glu-457 is important in the binding of dihydrolipoamide to the enzyme. This study on two site-specifically modified human E3 provides the first quantitative assessments of the contribution of His-452 and the carboxylic anion group (\(-\text{COO}^-\)) of Glu-457 in human E3 catalysis. This study also implies the
accessibility of water molecules in assay solution to the $E_3$ active site during the catalysis.
Chapter V
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