Substrate Recognition and Catalytic Mechanism of Phenylalanine Dehydrogenase

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1994

Summary

Amino acid dehydrogenases, belonging to EC class 1.4.1., catalyze the reversible deamination of amino acids to the corresponding keto acids in the presence of $NAD(P)^+$. Recently, Soda and co-workers have cloned and sequenced the genes of two thermostable NAD^+-dependent amino acid dehydrogenases, phenylalanine dehydrogenase (PheDH) from *Thermoactinomyces intermedius* and leucine dehydrogenase (LeuDH) from *Bacillus stearothermophilus*. Although the two enzymes show a significant sequence similarity, they have different substrate specificities; PheDH acts preferentially on L-phenylalanine and L-tyrosine, whereas LeuDH acts almost exclusively on L-leucine and some other branched-chain L-amino acids. Hence, the enzymes are good models for studying substrate recognition and catalytic mechanism of amino acid dehydrogenases. This thesis describes the studies of substrate recognition and catalytic mechanism of PheDH in the following three sections.

1) Amino acid residues involved in catalysis and substrate binding has been identified to shed light on the catalytic mechanism of PheDH. A monoanionic reagent, methyl acetyl phosphate, has been used to acetylate lysyl residues of the recombinant PheDH from *T. intermedius*. The enzyme was inactivated irreversibly with the reagent in a time- and dose-dependent manner. Simultaneous addition of substrate and coenzyme markedly protected the enzyme from inactivation. Acetylated lysyl residues possibly occurring at the active site were determined by the differential modification; the enzyme was first modified with the cold reagent in the presence of both substrate and coenzyme and, after removal of the added substances by gel filtration, was then labeled with the radioactive reagent. At least 7 lysyl residues per enzyme subunit were radiolabeled by this method. To further

specify the lysyl residue(s) whose modification results in the enzyme inactivation, 5 lysyl residues highly conserved in various amino acid dehydrogenase sequences were replaced with Ala by site-directed mutagenesis. Although all of the single mutant enzymes were inactivated with the reagent as effectively as the wild-type enzyme, a double mutant enzyme in which Lys-69 and Lys-81 are both replaced with Ala was found to be inactivated very slowly. These results suggest that the reagent can acetylate both of these two lysyl residues and inactivate the enzyme. Kinetic analyses of the single Lys-69 and Lys-81 mutant enzymes revealed their roles in substrate binding and catalysis, respectively, like the corresponding residues in the homologous LeuDH from *B. stearothermophilus*.

2) PheDH from *T. intermedius* and LeuDH from *B. stearothermophilus* show a 59% sequence similarity in their substrate-binding region including two catalytic lysyl residues, although their substrate specificities are different. Aiming at elucidation of the mechanism of substrate recognition by the two amino acid dehydrogenases, a chimeric enzyme consisting of an N-terminal domain of PheDH containing the substrate-binding region and a C-terminal domain of LeuDH containing the NAD^+-binding region was constructed. The chimeric enzyme purified to homogeneity acted on phenylalanine with a specific activity of 6% of that of the parental PheDH and showed a broad substrate specificity in the oxidative deamination like PheDH. It, however, acted much more effectively on isoleucine and valine than PheDH. Its K_m values for L-phenylalanine and L-leucine were similar to those of PheDH. The substrate specificity of the chimeric enzyme in the reductive amination was an admixture of those of the two parent enzymes. These results suggest that

the two domains of PheDH and LeuDH probably can fold independently. Accordingly, their chimera forms a new active enzyme which consists of their N- and C-terminal domains containing the substrate- and coenzyme-binding regions, respectively. However, the two domains of chimeric enzyme interact and communicate with each other to form a new active site and consequently show the new substrate specificity.

3) A PheDH mutant enzyme whose inherent hexapeptide segment (^{124}Phe-Val-His-Ala-Ala-^{129}Arg) in the substrate-binding domain was replaced by the corresponding part of LeuDH (^{123}Met-Asp-Ile-Ile-Tyr-^{128}Gln) was prepared in order to futher elucidate the mechanism of substrate recognition by PheDH. The catalytic efficiencies (k_{cat}/K_m) of the mutant enzyme with aliphatic amino and keto acids as substrates were 0.5 to 2% of those of the wild-type enzyme. In contrast, the efficiencies for L-phenylalanine and phenylpyruvate decreased to 0.008 and 0.035 % of those of the wild-type enzyme, respectively. These results suggest that the hexapeptide segment plays an important role in the substrate recognition by PheDH.

Contents

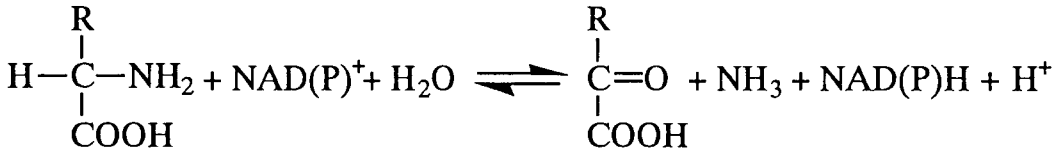
List of Abbreviations

CD	circular dichroism
DEAE-	dietylaminoethyl
DH	dehydrogenase: For example, PheDH represents phenylalanine dehydrogenase.
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
(k)bp	(kilo)base pairs
(k)Da	(kilo)daltons
MAP	methyl acetyl phosphate
$NAD(P)^+$, NAD(P)H	nicotinamide adenine dinucleotide (phosphate) and its reduced form
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PLP	pyridoxal 5'-phosphate
SDS-PAGE	sodium dodecyl sulfate / polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminoethane

Mutant enzymes: For example, Lys-81→Ala denotes a mutant enzyme in which Lys-81 is replaced by Ala.

Chapter I
General Introduction

General Aspects of Amino Acid Dehydrogenases. ——Amino acid dehydrogenases, belonging to EC class 1.4.1., catalyze the reversible deamination of amino acids to the corresponding keto acids in the presence of a pyridine nucleotide coenzyme, $NAD(P)^+$:



The dehydrogenation and the reverse hydrogenation are accompanied by deamination and amination, respectively, and in this respect the enzymes are different from many other $NAD(P)^+$-dependent dehydrogenases such as lactate dehydrogenase and alcohol dehydrogenase, catalyzing the simple oxidoreduction of a hydroxyl group of substrates. Virtually all amino acid dehydrogenases act on the L-enantiomer of the substrate amino acids; *meso*-diaminopimelate dehydrogenase [EC 1.4.1.16] is the sole exception acting on the D-configuration of the *meso*-diaminodicarboxylic substrate. So far, more than ten kinds of amino acid dehydrogenases have been found in various organisms, glutamate dehydrogenase (GluDH) (*1-10*), leucine dehydrogenase (LeuDH) (*11-17*), alanine dehydrogenase (AlaDH) (*18, 19*), and phenylalanine dehydrogenase (PheDH) (*20-28*) being the representatives (Table I-I). Although GluDH [EC 1.4.1.2-4] is found ubiquitously in various organisms including animals, insects, plants, and microorganisms except for the majority of the bacilli, the other amino acid dehydrogenases occur in microorganisms and/or plants.

Amino acid dehydrogenases provide a route for interconversion between inorganic and organic nitrogen compounds and, in other words,

link the amino acid and organic acid metabolisms. The equilibrium constants, K, defined by the following equation,

K = [keto acid] [NH_3] [NAD(P)H] [H^+]/[amino acid] [$NAD(P)^+$] [H_2O],

are in the order of 10^{-15} M for the reactions catalyzed by GluDH, AlaDH, LeuDH, and PheDH (*30-32*). However, the metabolic roles of amino acid dehydrogenases differ with the individual enzymes and organisms that contain each enzyme. For example, *Neurospora crassa* produces two distinct GluDHs; one is specific for $NADP^+$ and has a biosynthetic role, and the other requires NAD^+ and functions biodegradatively (*33, 34*). Because of their extremely small equilibrium constants as described above, the enzymes are useful as catalysts for the production of amino acids from keto analogs. $NAD(P)^+$-dependent dehydrogenases show either pro-*R* or pro-*S* stereospecificity for hydrogen transfer from the C-4 position of the nicotinamide moiety of NAD(P)H to the substrate. Most amino acid dehydrogenases except for AlaDH and lysine dehydrogenase (LysDH) show pro-*S* stereospecificity (Table I-I).

TABLE I-I. **$NAD(P)^+$-dependent amino acid dehydrogenases**

Enzyme	EC number	Coenzyme	Stereospecificity
Alanine dehydrogenase (AlaDH)	1.4.1.1	NAD^+	Pro-*R*
Glutamate dehydrogenase (GluDH)	1.4.1.2	NAD^+	Pro-*S*
Glutamate dehydrogenase (GluDH)	1.4.1.3	$NAD(P)^+$	Pro-*S*
Glutamate dehydrogenase (GluDH)	1.4.1.4	NAD^+	Pro-*S*
Serine dehydrogenase (SerDH)	1.4.1.7	NAD^+	
Valine dehydrogenase (ValDH)	1.4.1.8	$NAD(P)^+$	Pro-*S*
Leucine dehydrogenase (LeuDH)	1.4.1.9	NAD^+	Pro-*S*
Glycine dehydrogenase (GlyDH)	1.4.1.10	NAD^+	
3,5-Diaminohexanoate dehydrogenase	1.4.1.11	NAD^+	
2,4-Diaminopentanoate dehydrogenase	1.4.1.12	$NAD(P)^+$	
Lysine Dehydrogenase (LysDH)	1.4.1.15	NAD^+	Pro-*R*
Diaminopimelate dehydrogenase	1.4.1.16	$NADP^+$	
Phenylalanine dehydrogenase (PheDH)	1.4.1.20	NAD^+	Pro-*S*
Tyrosine dehydrogenase (TyrDH)	1.4.1.-	$NAD(P)^+$	

Some Features of Leucine Dehydrogenase. ——LeuDH [EC 1.4.1.9] catalyzes the reversible deamination of L-leucine and several other aliphatic amino acids to their keto analogs. It occurs widely in *Bacillus* species (*12*) and functions catabolically in bacterial metabolisms of L-branched chain amino acids (*35*). It has been suggested that the enzyme plays an important role in spore germination in cooperation with AlaDH (*36, 37*). LeuDH has been purified from *B. sphaericus* (*11, 12*), *B. cereus* (*13*), *B. stearothermophilus* (*14*), and *Clostridium thermoaceticum* (*15*). The enzymes from *B. sphaericus*, *B. stearothermophilus*, and *C. thermoaceticum* are hexamers of identical subunits, while the *B. cereus* enzyme is an octamer (*12, 15, 38, 39*). The substrate specificity of LeuDH is much lower than those of GluDH and AlaDH. The low specificity for keto acid substrates is advantageous for the enzymatic synthesis of various L-amino acids. The reaction catalyzed by LeuDH proceeds *via* the ordered Bi-Ter mechanism, in which NAD^+ and L-leucine are bound and NH_4^+, α-keto-*iso*-caproate and NADH are released in this order (*5, 6*). Thiol regents such as *p*-chloromercuribenzoate and $HgCl_2$, and pyridoxal 5'-phosphate (PLP) (*40, 41*) inhibit the enzyme. The structural genes coding for the enzymes of *B. stearothermophilus* (*16*) and *T. intermedius* (*17*) have been cloned, and their nucleotide sequences have been determined. Matsuyama *et al.* of this laboratory identified an active-site lysine (Lys-80) in the recombinant LeuDH from *B. stearothermophilus* by chemical modification with PLP (*42*). This lysyl residue is contained in the consensus Gly-Gly-Gly-Lys sequence conserved in most of the amino acid dehydrogenase sequences and has been shown to bear a general-base function in catalysis by site-directed mutagenesis studies (*43*). Furthermore, another conserved active-site lysyl residue (Lys-68) of the same enzyme has been identified by site-directed mutagenesis (*44*). This

Lys-68 participates in substrate binding through an ionic interaction between its protonated ε-amino group and the negatively charged α-carboxyl group of substrate (*44*).

Some Features of Phenylalanine Dehydrogenase. ——PheDH [EC 1.4.1.20] was first found in *Brevibacterium* sp. isolated from soil (*20*) and could be used as a catalyst for the enzymatic synthesis of L-phenylalanine, which is a starting material of an artificial sweetener, L-aspartyl-L-phenylalanine methyl ester (aspartame). The enzyme occurs in several gram-positive, aerobic spore-forming bacteria and actinomycetes, and has been purified to homogeneity from *Sporosarcina ureae* (*21*), *B. sphaericus* (*21*), *B. badius* (*22*), *Nocardia* sp. (*23*), *Rhodococcus maris* (*24*), and *T. intermedius* (*25*). The production of bacterial PheDH is induced by the addition of L-phenylalanine to the culture medium (*20*), although the metabolic role of the enzyme is unknown yet. The enzymes from *S. ureae*, *B. sphaericus*, and *B. badius* are octamers of identical subunits, while monomeric, dimeric, and hexameric structures have been suggested for the *Nocardia* sp., *R. maris,* and *T. intermedius* enzymes, respectively. The structural genes encoding the enzymes from *B. sphaericus* (*26*), *S. ureae* (*27*), *T. intermedius* (*28*), and *Rhodococcus* sp. (*29*) have been cloned and sequenced. As observed with other amino acid dehydrogenases, the enzyme is highly active in an extremely alkaline pH region. The substrate specificity of PheDH is higher than that of LeuDH, acting preferentially on L-phenylalanine and L-tyrosine.

Three-Dimensional Structure of Amino Acid Dehydrogenases. —— A recent X-ray crystallographic study of GluDH from *Clostridium symbiosum* has revealed that the enzyme is composed of structurally independent coenzyme- and substrate-binding domains, like other $NAD(P)^+$-dependent dehydrogenases such as lactate and alcohol

dehydrogenases (*45*). However, the three-dimensional structures of other amino acid dehydrogenases have been yet unresolved, although significant similarities were noted among the primary structures of the amino acid dehydrogenases (*14, 17, 28, 46*). The coenzyme-binding domains of $NAD(P)^+$-dependent dehydrogenases including GluDH have a similar βαβ-nucleotide-binding fold (*47, 45*), and there are some basic residues that interact with the 2'-phosphate group of $NADP^+$ and thereby allow the enzyme to distinguish $NADP^+$ from NAD^+ (*48-54*). Indeed, the coenzyme specificity of glutathione reductase was changed from $NADP^+$ to NAD^+ by substitution of a neutral amino acid residue for the basic residues (*55*). Thus, the structures and functions of the coenzyme-binding domains have been studied in some detail. However, little is known about the substrate-binding domains except for that of GluDH from *C. symbiosum*. Although active-site structure models for LeuDH and PheDH have been proposed based on the GluDH structure, the mechanism of substrate recognition and the residues participating in determination of the substrate specificity of amino acid dehydrogenases remain to be elucidated.

Purpose of This Study.——The primary structures of PheDH from *T. intermedius* (*28*) and LeuDH from *B. stearothermophilus* (*16*), both of which are thermostable (*14, 25*), have been deduced from the nucleotide sequences of the coding genes, as described above. These two enzymes show a considerable sequence similarity (overall identities, 47%), and particularly in the region from Asp-58 to Glu-130 of PheDH containing the conserved Gly-Gly-Gly-Lys sequence, a 59% homology has been noted (42 out of 73 matchable residues: Fig. I-1). The two enzymes, however, show markedly different substrate specificities and hence are suitable as

models for studying the differences in the mechanisms of catalysis and substrate recognition by two homologous enzymes.

In the forthcoming chapter (Chapter II) of this thesis, chemical modification with methyl acetyl phosphate (MAP) combined with site-directed mutagenesis has been performed to identify the active-site residue(s) of PheDH. The results of the modification of mutant enzymes have shown that MAP inactivates PheDH by acetylating mainly the two highly conserved active-site lysyl residues. Steady-state kinetic analyses of the mutant enzymes for the two lysyl residues revealed their roles in substrate binding and catalysis, exactly like the corresponding residues in the homologous LeuDH. In Chapter III, to unravel the mechanism of substrate recognition by the two amino acid dehydrogenases, PheDH and LeuDH, a chimeric enzyme consisting of each domain of the two enzymes has been constructed. Examination of molecular and catalytic properties of the purified chimeric enzyme with a considerable enzymatic activity has led to the conclusion that both enzymes are composed of two structurally independent domains involved in binding of substrate and coenzyme, as demonstrated for the GluDH structure by X-ray crystallography. The substrate specificities of this class of amino acid dehydrogenases appear to be determined by the structural interactions of the two domains. Subsequently in Chapter IV, to examine the function of the unconserved hexapeptide segment in the substrate-binding domain of PheDH, a PheDH mutant enzyme whose inherent hexapeptide segment (^{124}Phe-Val-His-Ala-Ala-^{129}Arg) is replaced by the corresponding part of LeuDH (Met-Asp-Ile-Ile-Tyr-Gln) has been prepared. The results obtained with the mutant enzyme suggest that the hexapeptide segment plays an important role in the substrate recognition, and the substitution affects the gross conformation of the enzyme. Finally in Chapter V, a comprehensive discussion is given to

the mechanisms of catalysis and substrate recognition by these amino acid dehydrogenases on the basis of the results obtained in the present studies.

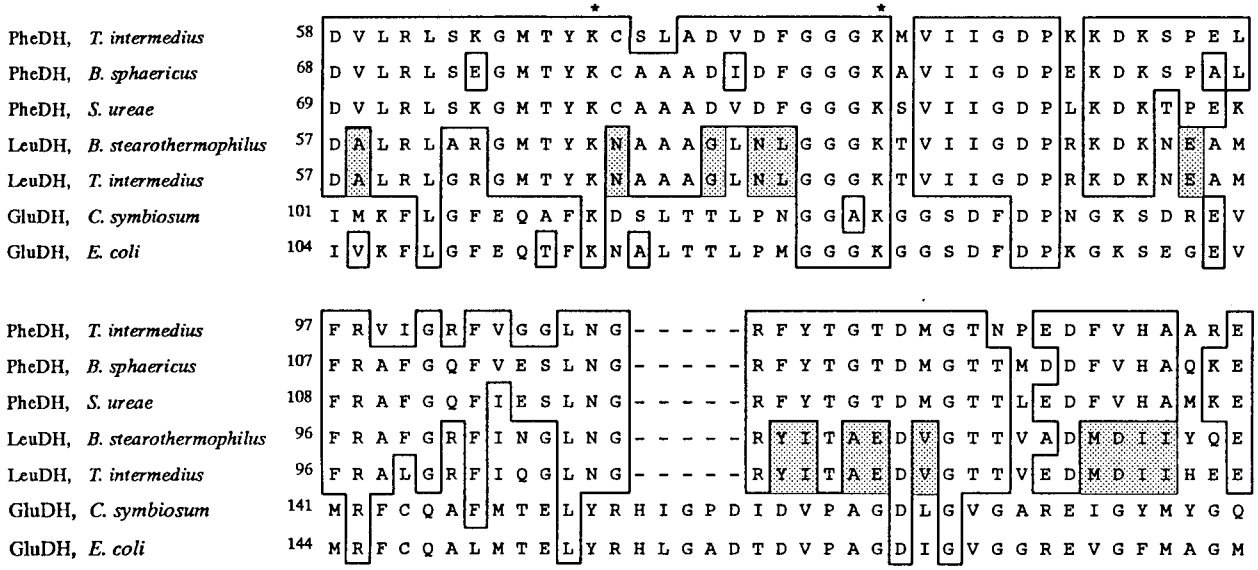


Fig. I-1. **Sequence comparison of the substrate-binding region containing the catalytic lysyl residues in several amino acid dehydrogenases.** The catalytic lysyl residues in the region are shown by *asterisks* and the conserved residues in PheDHs are *boxed*. The residues conserved in three PheDHs but not in LeuDHs are indicated by *shadows*.

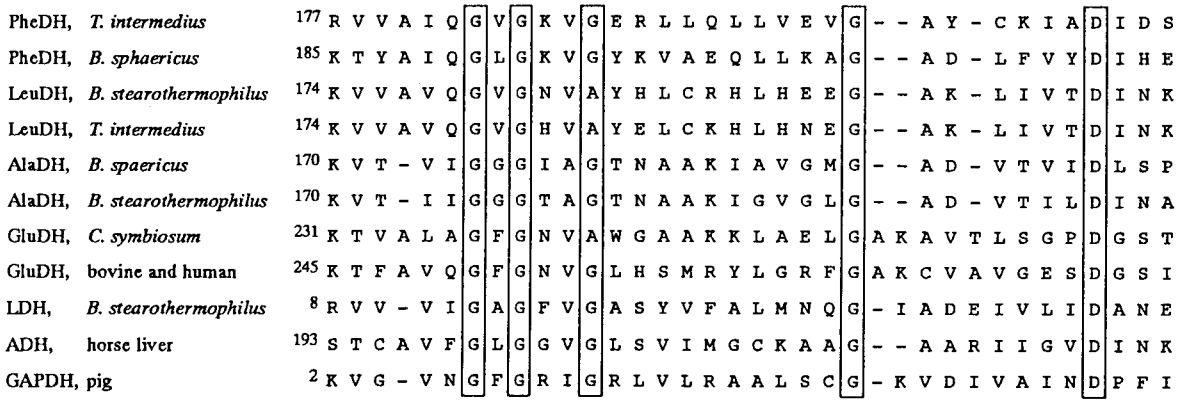


Fig. I-2. **Sequence comparison of the coenzyme-binding domain of several NAD(P)$^+$-dependent dehydrogenases.** Conserved residues that are thought to be important for coenzyme binding are *boxed*.

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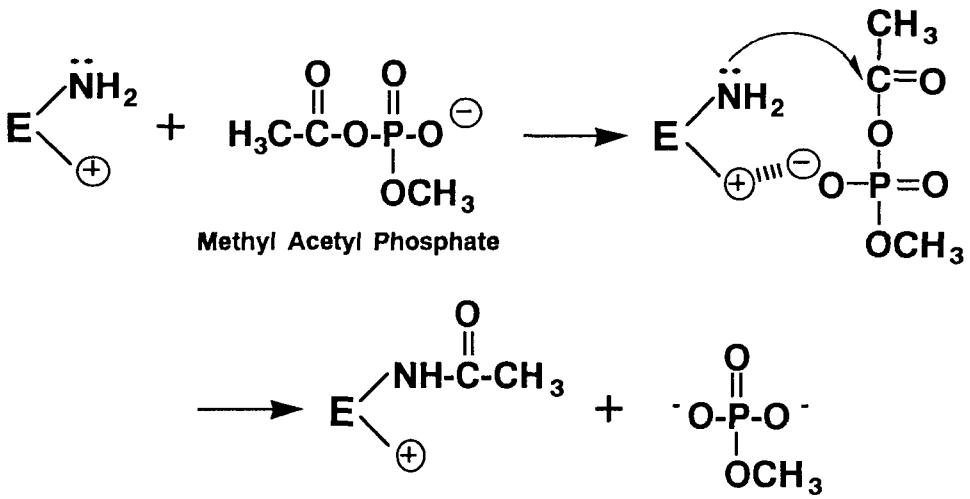
Chapter II

Exploring the Active Site by Chemical Modification with Methyl Acetyl Phosphate Combined with Site-Directed Mutagenesis

II-1. Introduction

PheDH catalyzes the reversible oxidative deamination of L-phenylalanine and, to a lesser extent, L-tyrosine using NAD^+, functions in the metabolic degradation of L-phenylalanine (*1*). The enzymes from various sources have been purified to homogeneity, and physicochemically and enzymologically characterized (*1-7*). Primary structures of the enzymes from *Bacillus sphaericus* (*8*), *Sporosarcina ureae* (*9*), *Thermoactinomyces intermedius* (*10*), and *Rhodococcus* sp. M4 (*11*) have been deduced from the nucleotide sequences of their cloned genes. Sequence comparison with the homologous LeuDH from *B. stearothermophilus* (*12*) and GluDH from various sources (*13-16*) has pointed out several conserved residues that might bear functional importance at the active site (*10, 11*). Furthermore, an active-site structure model has been proposed for PheDH (and also for LeuDH) (*17*) based on the three-dimensional structure of GluDH from *Clostridium symbiosum* determined at 1.96 Å resolution (*18*). However, active-site residues of PheDH remain to be elucidated experimentally.

In order to identify the catalytic residues of PheDH, methyl acetyl phosphate (MAP), a monoanionic acetylation reagent that has been utilized in the modification of amino groups located close to an anion-binding site in proteins (Scheme II-1, Ref. *19*), has been used for the modification of lysyl residues of PheDH. In this chapter, identification of active-site lysyl



Scheme II-1. **Acetylation of amino groups of proteins with MAP.**

residues of PheDH by modification with MAP combined with site-directed mutagenesis is described. Although the differential modification of the enzyme protected with a substrate and NAD^+ versus the unprotected one indicated the presence of multiple lysyl residues at the active site, a mutant enzyme in which two conserved lysyl residues are simultaneously replaced with Ala was found to be inactivated only slightly by incubation with MAP, suggesting that both of these residues may be responsible for the enzyme inactivation by acetylation with MAP. Steady-state kinetic analyses of the single mutant enzymes for Lys-69 and Lys-81 revealed their roles in substrate binding and catalysis, respectively, like the corresponding residues in the homologous LeuDH (*20, 21*).

II-2. Experimental Procedures

Materials.——The reagents for DNA synthesis and fluorescent dye-primers (m13(-21), m13rp-1) for DNA sequencing were obtained from Applied Biosystems. A site-directed mutagenesis kit (Mutan-K) was

purchased from Takara Shuzo. The recombinant wild-type PheDH was overproduced in *Escherichia coli* JM109 carrying pKPDH2 and purified to homogeneity, as described previously (*10*). MAP and [acetyl-^{14}C]MAP (550 cpm/nmol) were synthesized according to the methods reported previously (*19, 22*).

Enzyme and Protein Assays. ——The oxidative deamination of amino acids and reductive amination of α-keto acids by PheDH were determined by spectrophotometric measurements of the reduction of NAD^+ and the oxidation of NADH, respectively, as described previously (*10*). Apparent steady-state kinetic parameters were determined by varying the concentration of a substrate (coenzyme) in the presence of constant, saturating concentrations of other substrates (coenzyme); 1.25 mM NAD^+ for determination of K_m for L-phenylalanine, 0.1 mM NADH and 1 M ammonia for determination of K_m for phenylpyruvate, 10 mM L-phenylalanine for determination of K_m for NAD^+, and 10 mM phenylpyruvate and 1 M ammonia for determination of K_m for NADH. Protein concentrations of the wild-type and mutant PheDH were estimated by using an absorbance ($A^{0.1\%}$) of 0.63 at 280 nm.

Modification with MAP. ——The reaction mixture for the enzyme modification with MAP contained various concentrations (0.2-200 mM) of MAP in 0.2 M potassium phosphate buffer (pH 7.0) in a total volume of 1 ml. The reaction was started by the addition of enzyme (20 μM subunit, M_r 41,000) and performed at 37°C. During the incubation, 10-μl aliquots were withdrawn at various times for assaying the residual activity. For protection studies, the compound to be examined was added to the reaction mixture before starting the reaction. Pseudo-first-order rate constants for the inactivation were calculated from the slope of the semilogarithmic plot

of residual activity versus reaction time or from the slope of a Guggenheim plot.

Differential Modification and Isolation of Labeled Peptides. ——The enzyme (4 mg, 0.1 μmol of subunit) was first modified with 20 mM MAP in the presence of 1 mM NAD^+ and 10 mM L-phenylalanine to allow acetylation of unprotectable lysyl residues. After incubation at 37°C for 4 h, the reaction mixture was passed through a Pharmacia PD-10 column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) to remove the added substances. The enzyme was concentrated by ultrafiltration in an Amicon Centricon-10 cartridge and then labeled with 20 mM [^{14}C]MAP at 37°C for 7 h (until the residual activity decreased below 10% of the initial). The unreacted [^{14}C]MAP was thoroughly removed by gel filtration of the reaction mixture as above, in which solid urea was added to a final concentration of 8 M. The radioactivity incorporated into the enzyme protein was measured with a Hewlett Packard liquid scintillation counter. The labeled enzyme was dissolved in 0.2 ml of 0.2 M ammonium acetate buffer (pH 8.0) containing 6 M guanidine HCl, and then diluted with 0.4 ml of 0.1 M ammonium acetate. *N*-Tosyl-L-phenylalanine chloromethylketone-treated trypsin (Millipore) was added to the turbid solution in a 1:50 (mol/mol) ratio of protease to substrate. Digestion was performed at 37°C for 24 h. The peptides were separated on a Tosoh HPLC system equipped with a Capcell Pak C18 reverse-phase column (Shiseido) using a solvent system of 0.1% trifluoroacetic acid (A) and 0.095% trifluoroacetic acid containing 90% acetonitrile (B). A 60-min linear gradient from 0 to 65% B was used to elute peptides at a flow rate of 1.0 ml/min with continuous monitoring of the absorbance at 215 nm.

Site-Directed Mutagenesis.——A 1.1-kbp *Eco*RI-*Hin*dIII fragment excised from pKPDH2 (*10*), which contains the entire coding region for PheDH, was subcloned into phage M13mp18 DNA. *E. coli* CJ236 (*dut*$^-$ *ung*$^-$) cells were transfected with the M13 phage, and the uracil-containing, single-stranded phage DNA was purified from the culture supernatant. Individual substitutions of Ala for Lys-69, Lys-81, Lys-89, Lys-90, and Lys-173 of PheDH were performed by the method of Kunkel *et al.* (*23*), using a commercial kit (Mutan-K). The following 5 oligonucleotide primers were synthesized with an Applied Biosystems DNA synthesizer Model 381 to contain suitable mismatching bases (asterisked) in the complementary codon for each lysyl residue (underlined).

Lys-69→Ala: 5'-CAGACTGCA<u>TGC</u>ATAGGTCATG-3'
**

Lys-81→Ala: 5'-GATAACCAT<u>TGC</u>TCCCCCG-3'
**

Lys-89→Ala: 5'-GATTTATCTTT<u>TGC</u>CGGATCGCCG-3'
**

Lys-90→Ala: 5'-GGCGATTTATC<u>TGC</u>TTTCGGATCGC-3'
**

Lys-173→Ala: 5'-CACACGCCC<u>TGC</u>CAGCTGATCCG-3'
**

After confirming the nucleotide sequences of 312-bp *Sac*II-*Spl*I fragments (for Lys-69, -81, -89, -90 mutants) or a 284-bp *Spl*I-*Eco*RV fragment (for Lys-173 mutant) containing the mutated sites, each fragment was excised from the replicative form M13mp18 phage DNA, and ligated into the corresponding site of pKPDH2 (*10*). In construction of the double mutant (Lys-69/81→Ala), the uracil-containing, single-stranded phage DNA obtained from the Lys-81→Ala mutant plasmid and the oligonucleotide designed for the Lys-69→Ala mutant described above were used as a

template and a mutagenic primer, respectively. The single Lys-69→Ala and Lys-81→Ala mutant enzymes were purified to homogeneity by the same method as that for the wild-type enzyme (*10*). However, for purification of the double mutant enzyme (Lys-69/81→Ala), which had much decreased thermal stability as compared with the wild-type and other single mutant enzymes, an affinity chromatography on a Blue Sepharose CL-6B column was used instead of the heat treatment at 70°C for 30 min (*10*). The enzyme solution was applied to a Blue Sepharose CL-6B column equilibrated with 10 mM potassium phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.02% 2-mercaptoethanol, under the conditions of which the enzyme passed through the column.

II-3. Results

Inactivation of Wild-Type PheDH by MAP. ——When incubated with various concentrations of MAP at 37°C and pH 7.0, the purified wild-type PheDH was inactivated in a time-dependent manner (Fig. II-1). The extent of inactivation depended also on the concentration of MAP; more than 90% of the original activity was lost by incubation with 200 mM MAP for 30 min. The inactivation was found to be irreversible, no activity being restored upon extensive dialysis of the enzyme inactivated to various extents (data not shown). In the presence of much excess MAP (2-200 mM) over the enzyme (20 μM), the inactivation reaction proceeded in a pseudo-first-order kinetics, giving straight lines in the semilogarithmic plots of residual activity versus reaction time or the Guggenheim plots [logarithm of the difference in activities at time t and at time $(t+\Delta t)$ plotted against t]. Hence, a pseudo-first-order rate constant

(k_{obs}) of the inactivation with 20 mM MAP was calculated to be 0.014 min^{-1} from the slope of the Guggenheim plot.

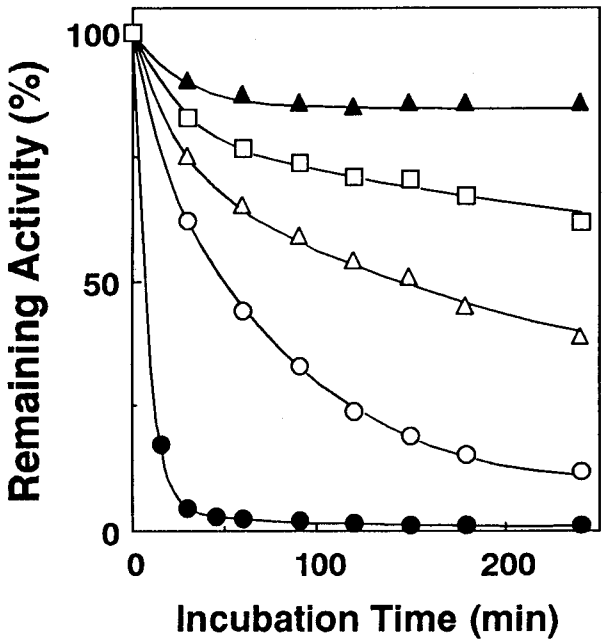


Fig. II-1. **Inactivation of PheDH with MAP.** The purified wild-type enzyme (20 μM) was incubated at 37°C with 0.2 (▲), 2 (□), 5 (△), 20 (○), and 200 mM (●) MAP in 0.1 M potassium phosphate buffer (pH 7.0) in a final volume of 1.0 ml, and 10-μl aliquots of the reaction mixture were withdrawn at indicated times for assaying the residual activities.

Effect of Substrates and Coenzymes. ——The protective effect of substrates and coenzymes on the enzyme inactivation by MAP was investigated to examine whether the modifiable lysyl residues are present at the active site or not. Among various combinations of the substrates and coenzymes added, 10 mM L-phenylalanine plus 1 mM NAD^+ or 5 mM phenylpyruvate plus 1 mM NADH provided marked protection of the enzyme against inactivation by 20 mM MAP, although their single addition offered little protection, except for the moderate protection by 1 mM NADH (Fig. II-2). Accordingly, the protective effects observed are not due to the consumption of MAP by the added compound (in particular, the

substrate having an α-amino group which could react with MAP), but can be explained by the binding of substrate and coenzyme according to the ordered sequential mechanism, in which L-phenylalanine is bound to the enzyme following NAD^+ in the oxidative deamination, and NADH is bound to the enzyme before the others in the reductive amination (*7*). Thus, the marked protection observed suggests that the lysyl residue(s) responsible for the enzyme inactivation is(are) located at the active site and thus unreactive with MAP in the enzyme/amino acid/NAD^+ or enzyme/keto acid/NADH ternary complex.

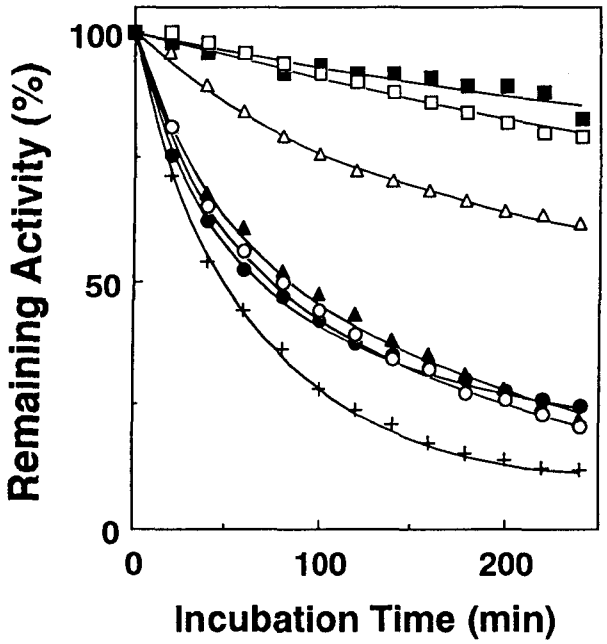


Fig. II-2. **Effect of substrates and coenzymes on inactivation of PheDH with MAP.** The wild-type enzyme (20 μM) was incubated with 20 mM MAP in the absence (+) or presence of 10 mM L-phenylalanine (●), 5 mM phenylpyruvate (○), 1 mM NAD^+ (▲), 1 mM NADH (△), 10 mM L-phenylalanine plus 1 mM NAD^+ (■), and 10 mM phenylpyruvate plus 1 mM NADH (□). At indicated times, 10-μl aliquots of the reaction mixture were withdrawn for assaying the residual activities.

Table II-I compares the protective effects more quantitatively in terms of $t_{1/2}$, the time (min) needed for 50% inactivation by 20 mM MAP, calculated from the slopes of the semilogarithmic or Guggenheim plots.

Again, the concomitant addition of substrate and coenzyme provided more than 10-fold extension of $t_{1/2}$, whereas the addition of substrate or coenzyme alone extended only slightly. L-Glutamate, a non-substrate amino acid, also did not extend $t_{1/2}$ significantly even in the co-presence of NAD^+. It should be noted, however, that over 40-fold extension of $t_{1/2}$ was observed with 1 mM NAD^+ plus 10 mM L-leucine, a very poor substrate for PheDH with a relative activity of only 4% of that of L-phenylalanine (*7*). The reason for this marked protective effect by NAD^+ plus L-leucine is unknown at present.

TABLE II-I. **Extension of inactivation time by substrates and coenzymes.** The values of $t_{1/2}$ were calculated from the slopes of the semilogarithmic or Guggenheim plots of the inactivation time course.

Addition (mM)	$t_{1/2}$ (min)	Extension of $t_{1/2}$ (-fold)
None	48	(1)
L-Phenylalanine (10)	55	1.1
L-Leucine (10)	68	1.4
Phenylpyruvate (5)	65	1.4
α-Keto-*iso*-caproate (10)	60	1.3
NAD^+ (1.0)	75	1.6
NADH (1.0)	230	4.8
L-Phe (10) + NAD^+ (1.0)	660	14
L-Tyr (10) + NAD^+ (1.0)	450	9.4
L-Leu (10) + NAD^+ (1.0)	2,000	42
L-Glu (10) + NAD^+ (1.0)	70	1.5
Phenylpyruvate (5) + NADH (1.0)	650	14
α-Keto-*iso*-caproate (10) + NADH (1.0)	590	12

Differential Modification. ——To determine the total number of amino groups labeled with MAP, the enzyme was modified with 20 mM [^{14}C]MAP at 37°C and pH 7.0 for 6 h until the residual activity decreased below 10% of the initial. Counting of the enzyme-bound radioactivity indicated a total of about 12 mol of the label incorporated per mol of the enzyme subunit (Table II-II). On the other hand, when the enzyme was

first modified with the cold reagent in the presence of 1 mM NAD^+ and 10 mM L-phenylalanine, which showed the highest protection of the enzyme from inactivation as described above, to allow modification of unprotectable lysyl residues, and then modified with [^{14}C]MAP after removal of the added substances, the amount of the enzyme-bound radioactivity decreased to about 7 mol/mol of subunit (Table II-II). These results show that at least 7 amino groups are protected from the modification in the concomitant presence of substrate and coenzyme.

TABLE II-II. **Incorporation of [^{14}C]MAP into protected and unprotected enzymes.** The experimental conditions are described in the text.

Condition	Amount of subunit used (nmol)	Total radioactivity incorporated (cpm)	Amount of MAP incorporated (nmol)	Stoichio-metry (mol/mol)
Unprotected	1.7	11,116	20.2	11.9
Protected	0.85	3,240	5.9	6.9

To locate the ^{14}C-labeled amino groups in the primary structure of PheDH (*10*), the differentially radiolabeled enzyme was digested completely with trypsin, and the tryptic peptides were separated by reverse-phase HPLC according to the procedure described under "Experimental Procedures." As predicted from the rather low site specificity of MAP toward proteinyl amino groups, more or less in virtually all of the peptides eluted were found to contain the radioactivity (background counts subtracted, Fig. II-3). Among them, 7 peptide peaks (designated as P_1~P_7 in order of elution), contained more than the averaged radioactivity (25 cpm/100 μl). Incidentally, this number of peptide peaks corresponded probably to the total molar number of [^{14}C]acetyl groups incorporated per mol of subunit. These tryptic peptides were further

purified by rechromatography on a reverse-phase column. Automated Edman degradation of peptides P5 and P6 revealed their sequences starting from Phe-165 and Met-82, respectively, suggesting that the labeled site in

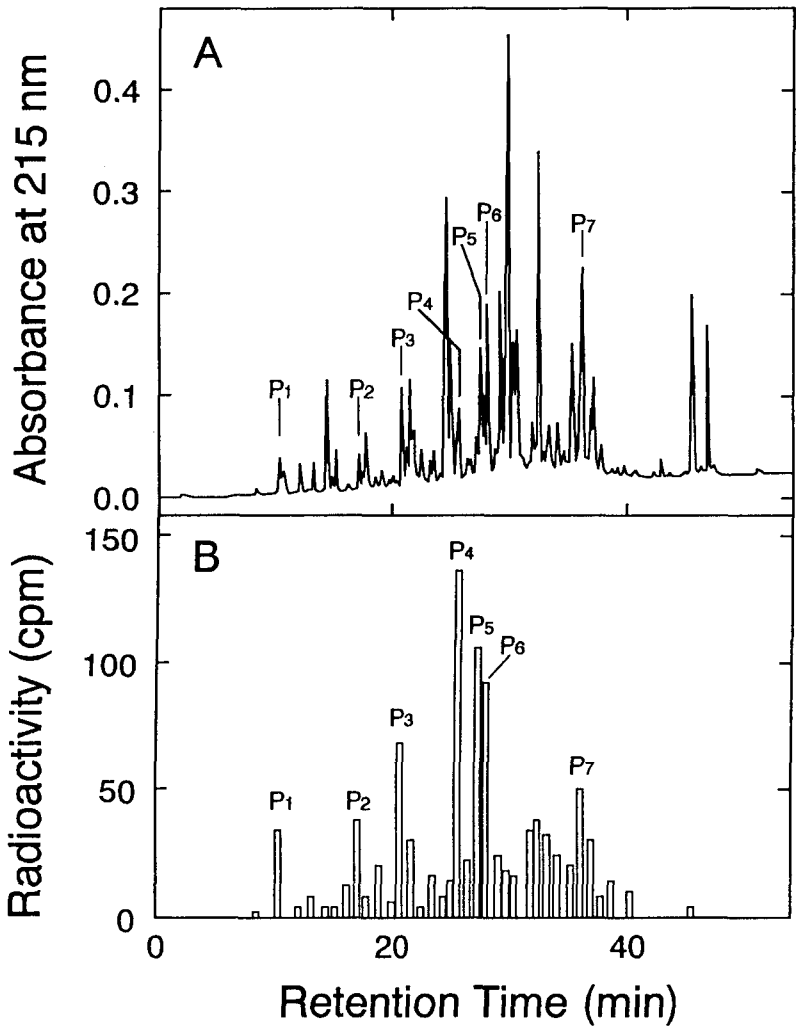


Fig. II-3. **HPLC elution profile of tryptic digest of [^{14}C]MAP labeled enzyme.** (A) The tryptic digest of the enzyme differentially labeled with [^{14}C]MAP as described in the text was chromatographed on a reverse-phase column with continuous monitoring of the absorbance at 215 nm. (B) Radioactivity in 100-μl each of the peptide peaks collected in fraction tubes was measured with a liquid scintillation counter. The peptide fragments containing significant radioactivities were designated as P1~P7 in order of elution.

P_5 is Lys-173 and that in P_6 is Lys-89 and/or Lys-90 (data not shown). However, the labeled sites in other tryptic peptides could not be determined because of the insufficient amounts.

Inactivation of Mutant Enzymes by MAP. ——Since it appeared difficult to specify lysyl amino group(s) whose modification results in the enzyme inactivation, even if all the 7 labeled sites could have been identified, other approach has been undertaken. Thus, the 5 lysyl residues totally or highly conserved in various amino acid dehydrogenase sequences reported to date (*8-16*) including Lys-89, Lys-90, and Lys-173 (above identified) were substituted for Ala by site-directed mutagenesis, and the sensitivity of the mutant enzymes for inactivation with MAP was examined. The two invariant lysyl residues, Lys-69 and Lys-81 of PheDH, corresponding to Lys-68 and Lys-80 of LeuDH that are involved in substrate binding and catalysis, respectively (*20, 21*), were also mutated into Ala individually or simultaneously. The crude extracts of *E. coli* cells transformed with the Lys-89→Ala, Lys-90→Ala, and Lys-173→Ala mutant plasmids thus constructed exhibited considerable activities comparable to those carrying the wild-type plasmid pKPDH2 (4.3 units/mg protein) (*10*). However, the cells carrying the Lys-69→Ala, Lys-81→Ala, and double Lys-69/81→Ala mutant plasmids showed very low activities, even though the amounts of enzyme protein produced were found to be significantly large by SDS-PAGE (data not shown). Therefore, the latter 3 mutant enzymes had to be purified to homogeneity for accurate measurements of the residual activities during incubation with MAP.

The 5 single mutant enzymes and one double mutant enzyme of PheDH were subjected to the MAP inactivation with the crude extracts (for Lys-89→Ala, Lys-90→Ala, and Lys-173→Ala) or the purified

samples (for Lys-69→Ala, Lys-81→Ala, and Lys-69/81→Ala). For comparison, inactivation of the wild-type, Lys-68→Ala, and Lys-80→Ala mutant enzymes of LeuDH (*20, 21*) with MAP were also examined. As shown in Fig. II-4 and Table II-III, all of the single mutant enzymes of PheDH as well as the wild-type LeuDH were inactivated with the reagent at 20 mM in a similar manner with the wild-type PheDH, whereas the Lys-68→Ala and Lys-80→Ala mutant enzymes of LeuDH were inactivated extremely slowly. Interestingly, the double mutant enzyme (Lys-69/81→Ala) of PheDH was also inactivated very slowly with $t_{1/2}$ extended nearly 10 folds (Table II-III).

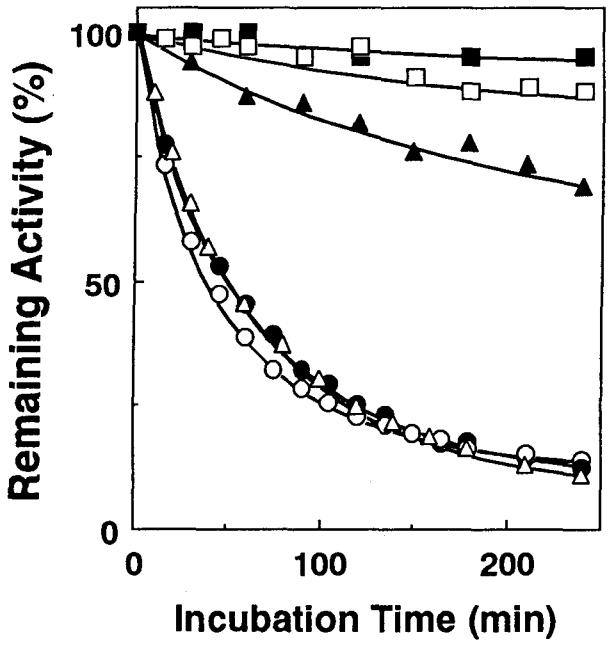


Fig. II-4. **Inactivation of mutant enzymes of PheDH and LeuDH with MAP.** The purified Lys-69→Ala (●), Lys-81→Ala (○), and Lys-69/81→Ala (▲) mutant enzymes of PheDH, and the purified wild-type (△), Lys-68→Ala (■), and Lys-80→Ala (□) mutant enzymes of LeuDH all at 20 μM subunit were incubated at 37°C with 20 mM MAP in 0.1 M potassium phosphate buffer (pH 7.0) in a final volume of 1.0 ml. At indicated times, 10-μl aliquots of the reaction mixture were withdrawn for assaying the residual activities.

These results altogether suggest that MAP inactivates both of PheDH and LeuDH by acetylating mainly the two active-site lysyl residues (Lys-69 and Lys-81 of PheDH, Lys-68 and Lys-80 of LeuDH). However, the reactivities of these two lysyl residues with MAP are different between PheDH and LeuDH; in PheDH both of the two residues are reactive with MAP even when either one is absent, while in LeuDH they are unreactive if either one is absent. In addition, it is evident that the three highly conserved lysyl residues, Lys-89, Lys-90, and Lys-173 of PheDH are not responsible for the enzyme inactivation by modification with MAP.

TABLE II-III. **Inactivation of mutant enzymes of PheDH and LeuDH with MAP.** The values of $t_{1/2}$ were calculated from the slopes of the semilogarithmic or Guggenheim plots of the inactivation time courses shown in Fig. II-4. The Lys-89→Ala, Lys-90→Ala, and Lys-173→Ala mutant enzymes of PheDH were the crude extracts of each transformant and the other wild-type and mutant enzymes were homogeneous preparations.

Enzyme	$t_{1/2}$ (min)	Extension of $t_{1/2}$ (-fold)
PheDH		
Wild type	48	(1)
Lys-69→Ala	50	1.0
Lys-81→Ala	38	0.8
Lys-89→Ala	75	1.6
Lys-90→Ala	80	1.7
Lys-173→Ala	60	1.3
Lys-69/81→Ala	450	9.4
LeuDH		
Wild type	49	(1)
Lys-68→Ala	3,000	61
Lys-80→Ala	1,200	24

Roles of Lys-69 and Lys-81. ——Finally, to elucidate the functional roles of Lys-69 and Lys-81 of PheDH, steady-state kinetic analyses were performed with the mutant enzymes purified (Table II-IV). The rate constants (k_{app}) of the Lys-69→Ala, Lys-81→Ala, and Lys-69/81→Ala

mutant enzymes were only 0.15, 0.23, and 0.09% in the oxidative deamination of L-phenylalanine, and 2.3, 2.4, and 1.1% in the reductive amination of phenylpyruvate, respectively, of those of the wild-type enzyme, showing that the two lysyl residues play very important roles in catalysis. The K_m values of the Lys-69→Ala and Lys-69/81→Ala mutant enzymes for both substrates were markedly larger than those of the wild-type and Lys-81→Ala mutant enzymes, whereas the K_m values of the three mutant enzymes for NAD^+ and NADH were similar to or even smaller than those of the wild-type enzyme. These results demonstrate that Lys-69 and Lys-81 of PheDH are involved in substrate binding and catalysis (presumably as a general base), respectively, exactly like the corresponding residues (Lys-68 and Lys-80) of the homologous LeuDH (*20, 21*).

TABLE II-IV. **Steady-state kinetic parameters of the wild-type and mutant enzymes of PheDH.** The assay conditions are described in the text.

	k_{app} (s^{-1})		K_m (mM)			
Enzyme	Deamination	Amination	NAD^+	L-Phe	NADH	Phenylpyruvate
Wild type	22	200	0.17	0.10	0.083	0.065
Lys-69→Ala	0.032	4.6	0.16	140	0.051	8.3
Lys-81→Ala	0.050	4.8	0.057	0.09	0.026	0.052
Lys-69/81→Ala	0.020	3.3	0.081	20	0.017	7.0

II-4. Discussion

In the present studies aimed at identification of the active-site lysyl residue(s) of PheDH, MAP, an acetylation reagent for amino groups of proteins (*19*) was used. Although the site specificity of MAP may be rather low, modifying not only ε-amino groups of lysine but also N-terminal amino groups, the negative charge on its phosphate group is expected to facilitate binding of the reagent to anion-binding sites in

proteins. If a nonprotonated amino group having sufficient nucleophilicity is present close to the anion-binding positive charge, it would be readily acetylated by the reagent according to the reaction scheme depicted in Scheme II-1. Indeed, the functional Cl^--binding site in hemoglobin has been identified by modification with MAP (*24*), in which only 3 lysyl groups are acetylated (*25*). Inactivation of several $NAD(P)^+$-dependent dehydrogenases such as D-3-hydroxybutyrate, lactate, malate, and alcohol dehydrogenases with MAP also has been reported (*19, 26*).

However, in the reaction of PheDH with MAP, not less than 12 amino groups were modified among a total of 23 amino groups derived from 22 lysyl residues and one N-terminal amino group (*10*), even though the incubation with MAP was done at pH 7 where most lysyl amino groups are probably protonated. This represents the low specificity of MAP for the labeled sites. Nevertheless, the concomitant addition of substrate and coenzyme resulted in the marked protection of the enzyme from inactivation (Fig. II-2) and prevented as many as 7 amino groups from acetylation with MAP (Table II-II). Thus, it is suggested that multiple lysyl residues are present at the active site of PheDH. Alternatively, some of these lysyl residues might be unreactive in the ternary complex formed with substrate and coenzyme. Singh *et al.* (*27*) have shown that a very large conformational change takes place in the bovine liver GluDH upon binding of substrate and coenzyme. If such a large conformational change also occurs during the ternary complex formation of PheDH, some lysyl residues exposed to the solvent in the uncomplexed state would move to the protein interior upon binding of substrate and coenzyme and thereby become unreactive with MAP. The three highly conserved lysyl residues, Lys-89, Lys-90, and Lys-173, are the candidates for the residues being

masked by the conformational change and may not be necessarily located at the active site. Significant activities of the mutant enzymes for the 3 lysyl residues also support this interpretation.

Since, only by chemical modification with MAP, it is difficult to specify active-site lysyl residue(s) whose modification with MAP leads to the enzyme inactivation, the 5 lysyl residues totally or highly conserved in various amino acid dehydrogenases were substituted for Ala by site-directed mutagenesis and the susceptibilities of the mutant enzymes of PheDH as well as the wild-type and two active-site mutant enzymes of LeuDH to MAP were examined. The results indicated that MAP inactivates both of PheDH and LeuDH by acetylating mainly the two lysyl residues, Lys-69 and Lys-81 of PheDH, and Lys-68 and Lys-80 of LeuDH, which are involved in binding of the substrate α-carboxyl group and in catalysis as a general base, respectively (*20, 21*). Unexpectedly, however, all the single mutant enzymes of PheDH were inactivated as effectively as the wild-type enzyme. This observation markedly contrasts with the extremely slow inactivation of the Lys-68→Ala and Lys-80→Ala mutant enzymes of LeuDH. Thus, both of Lys-68 and Lys-80 of LeuDH are responsible for the enzyme inactivation with MAP, but they are unreactive if either one is absent. On the other hand, both of the two residues of PheDH are reactive with MAP even when either one is absent. These results suggest that Lys-68 and Lys-80 of LeuDH are located within the distance of hydrogen-bonding interactions with each other, elevating the mutual nucleophilicity, but the corresponding Lys-69 and Lys-81 of PheDH are rather independent and may be neighbored with another positive charge assisting the binding of MAP to the active site.

In conclusion, the results in present study have shown that the use of the acetylation reagent MAP combined with site-directed mutagenesis is useful not only for identification of active-site lysyl residues but also for elucidation of the electrostatic environment around the active site.

II-5. References

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Chapter III

Construction and Characterization of Chimeric Enzyme Consisting of an Amino-terminal Domain of Phenylalanine Dehydrogenase and a Carboxy-terminal Domain of Leucine Dehydrogenase

III-1. Introduction

As described in Chapter I, many kinds of amino acid dehydrogenases including GluDH, LeuDH, and PheDH have been reported so far. The X-ray crystallographic study of GluDH from *Clostridium symbiosum* has revealed that the enzyme is composed of structurally independent coenzyme- and substrate-binding domains like other $NAD(P)^+$-dependent dehydrogenases such as lactate and alcohol dehydrogenases (*1*). However, the three-dimensional structures of other amino acid dehydrogenases have been unresolved, although significant similarities were noted among the primary structures of the amino acid dehydrogenases catalyzing the *pro-S* hydrogen transfer (*2-5*). The structures and functions of the coenzyme-binding domains of $NAD(P)^+$-dependent dehydrogenases have been extensively studied (*6-12*), and based on these studies the coenzyme specificity of glutathione reductase could have been altered (*13*). In marked contrast, little is known about the substrate-binding domains of amino acid dehydrogenases. It has been suggested that the active-site lysyl residue plays an important role as a general acid-base catalyst (*14, 15*), and another conserved lysyl residue about 10 residues apart from the catalytic lysine in the N-terminal side participates in the binding of substrate in LeuDH (*16*). The corresponding residues of PheDH have been revealed to have the same roles as those of LeuDH (Chapter II). However, the mechanism of substrate recognition and the residues responsible for

determination of substrate specificity of amino acid dehydrogenases still remain unknown.

PheDH from *T. intermedius* (*4*) and LeuDH from *B. stearothermophilus* (*2*) share a significant sequence homology (overall identities, 47%), and in particular the region from Asp-58 to Glu-130 of PheDH shows a 59% homology with the corresponding region of LeuDH (42 out of 73 matchable residues; see Chapter I, Fig. I-1). The two enzymes, however, have different substrate specificities: PheDH acts preferentially on L-phenylalanine and L-tyrosine, whereas LeuDH acts almost exclusively on L-leucine and some other branched-chain L-amino acids (*17*). In the present studies, a chimeric enzyme consisting of two domains from both enzymes has been constructed to unravel the mechanism of substrate recognition by the two amino acid dehydrogenases. Studies of molecular and catalytic properties of the chimeric enzyme with a considerable activity has led to the conclusion that both enzymes are also composed of two structurally and functionally independent domains binding the substrates and the coenzymes. The substrate specificities of amino acid dehydrogenases probably are determined based on the structural interaction of the two domains.

III-2. Experimental Procedures

Materials.——All restriction enzymes, *Taq* DNA polymerase, a T4 DNA ligation kit, and a DNA blunting kit were purchased from Takara Shuzo; plasmid pKK223-3 and Blue Sepharose CL-6B were from Pharmacia; standard proteins for SDS-PAGE were from Bio-Rad; SeaKem GTG agarose was from FMC; the reagents for DNA synthesis and fluorescent dye-primers (m13(-21), m13rp-1) for DNA sequencing were

from Applied Biosystems; and standard proteins used for molecular size determination by gel filtration were from Oriental Yeast. Preparations of plasmid pKPDH2 containing the PheDH gene (*4*), plasmid pICD2 containing the LeuDH gene (*2*), and rabbit polyclonal antisera against LeuDH and PheDH were described previously (*18*).

Construction of Plasmid pKPSLN Encoding Chimeric Enzyme. —— A 681-bp DNA fragment coding for the C-terminal domain of the LeuDH polypeptide was amplified by polymerase chain reaction (PCR) (*19*) with the following two oligonucleotides as primers, which were synthesized with an Applied Biosystems 381A DNA synthesizer. Primer P1 (5'-AAATCGTACGGCTCATCCGGCAA-3', 23-mer) is a coding sequence corresponding to the region from Gly-141 to Gly-144 of LeuDH (*2*) preceded by the region from Lys-139 to Tyr-141 of PheDH (*4*), linked by a new *Spl*I site (*underlined*), and primer P2 (5'-GTTTTATATTGCCGAA-GCACC-3', 21-mer) is a complementary sequence corresponding to the C-terminal region of LeuDH (*2*). PCR was performed in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mg/ml gelatin, 0.2 mM each dNTP, 2.5 units of *Taq* DNA polymerase, 0.3 ng of the template pICD2, and 1 μM each P1 and P2 primers with a program consisting of the reaction cycle (94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min) repeated 35 times. The DNA fragment amplified was inserted into the *Sma*I site of vector pKK223-3, followed by digestion with *Spl*I and *Hin*dIII and substitution for the same restriction fragment in pKPDH2 to form the final plasmid pKPSLN encoding the chimeric amino acid dehydrogenase (see Fig. III-1). The nucleotide sequence of the coding region of pKPSLN was confirmed, after

subcloning into pUC118, by the dideoxy chain termination method with an Applied Biosystems 373A DNA sequencer.

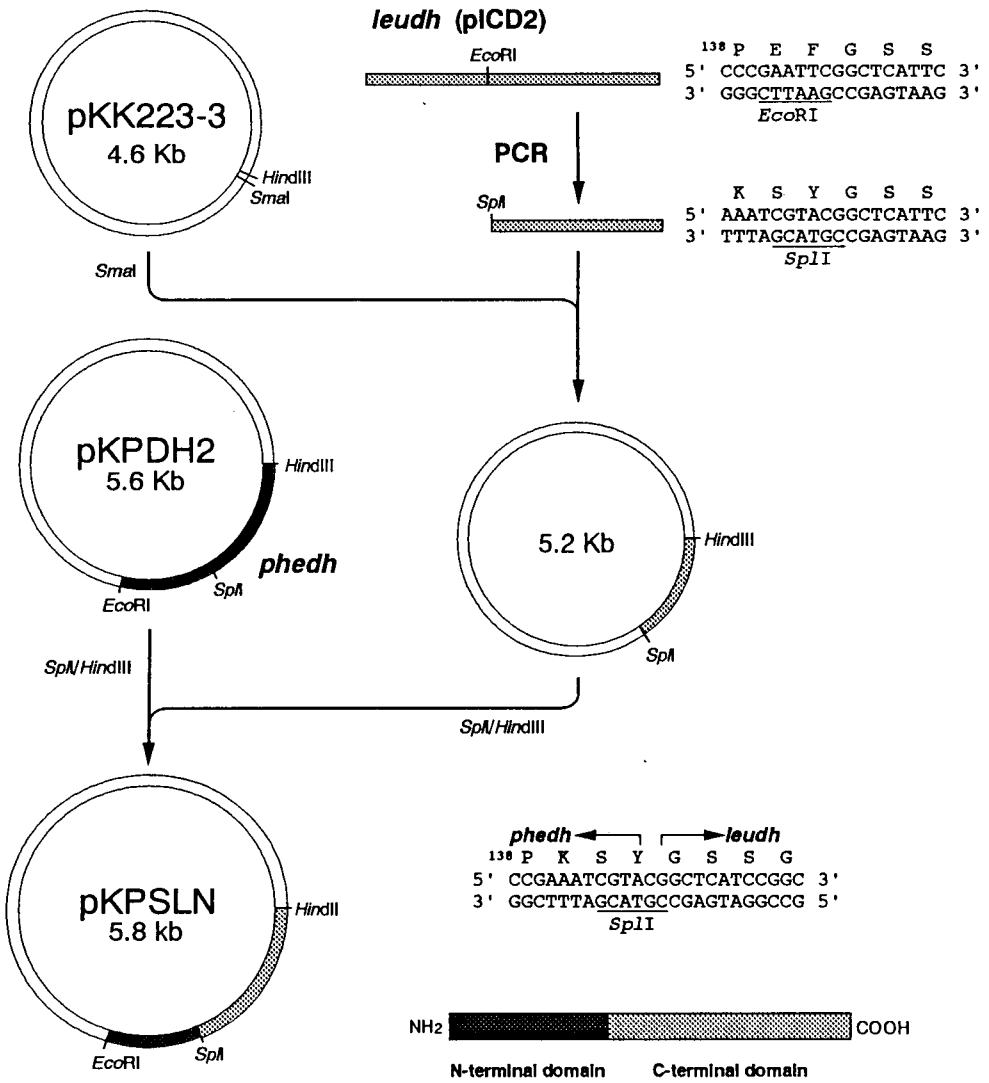

Fig. III-1. **Construction scheme for plasmid pKPSLN.** The *Eco*RI site in the LeuDH gene (*leudh*) was changed into a *Spl*I site by PCR, and the amplified fragment cloned into pKK223-3 was substituted for the corresponding fragment in the PheDH gene (*phedh*) to form pKPSLN encoding the chimeric enzyme shown schematically at the bottom (see under "Experimental Procedures" for details).

Purification of Chimeric Enzyme.——*E. coli* JM109 cells carrying pKPSLN were cultured at 37 °C for 16 h in 6 liters of Luria-Bertani medium supplemented with 0.1 mg/ml ampicillin, harvested by

centrifugation, and washed with 0.85% NaCl. The cells (wet weight, 32 g) suspended in 100 ml of 10 mM potassium phosphate buffer (pH 8.0) containing 0.01% (v/v) 2-mercaptoethanol (buffer A) were disrupted with a Biomic 7040 ultrasonic oscillator. After removal of the cell debris by centrifugation and dialysis of the supernatant solution against 250 volumes of buffer A, the enzyme solution (107 ml) was applied onto a Blue-Sepharose CL-6B column (2.5 x 15 cm) preequilibrated with buffer A. Unabsorbed proteins were washed out with buffer A supplemented with 0.3 M KCl, and the enzyme was eluted with the same buffer containing 0.5 M KCl. The active fractions were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. Finally the enzyme solution (11 ml) was applied onto a HiLoad 26/60 Superdex 200 preparative scale column (Pharmacia) equipped on a Pharmacia fast protein liquid chromatography system and preequilibrated with 10 mM potassium phosphate buffer (pH 6.7) containing 0.5 M KCl at a flow rate of 1.5 ml/min. Active fractions were pooled and concentrated by ultrafiltration, and the chimeric enzyme thus purified was used for subsequent experiments.

Other Methods.——The amino acid dehydrogenase activity was measured as described previously (*4*). The protein concentration of the chimeric enzyme was determined as reported previously for PheDH (*20*) using bovine serum albumin as a standard. Amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems 470A gas-liquid phase protein sequencer.

Immunoblotting of the chimeric enzyme separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane was carried out by first incubation with an antiserum against LeuDH or PheDH followed by the

second incubation with the anti-rabbit goat IgG conjugated with peroxidase and staining with 4-chloro-1-naphthol.

Stereospecificity of the chimeric enzyme for the hydrogen transfer from NADH was determined by the consecutive enzymatic method with alanine racemase and alanine dehydrogenase in ^2H_2O, monitoring the fate of the deuterium in NAD^{2}H by ^{1}H NMR operated at 200 MHz, as described previously (*21*).

Limited proteolysis of the chimeric enzyme (0.75 mg/ml) by subtilisin was done at 30 °C in 20 mM potassium phosphate buffer (pH 7.0) at a protease-to-substrate ratio of 1:100 (w/w). At various times of incubation, a 10-μl aliquot was taken and mixed with 0.4 M *p*-aminophenylmethyl-sulfonyl fluoride to stop the digestion, and analyzed by SDS-PAGE (15%). Two major polypeptides detected were further purified by SDS-PAGE, electroeluted from the gel, and subjected to N-terminal amino acid sequence analysis.

III-3. Results

Construction of Chimeric Amino Acid Dehydrogenase. ——In the amino acid sequences of PheDH from *T. intermedius* and LeuDH from *B. stearothermophilus*, identical residues are clustered in rather contiguous manners. In particular, the region from Asp-58 to Glu-130 of PheDH (residue numbers are referred to those of the *T. intermedius* enzyme; see Chapter I, Fig. I-1) is matched to the region from Asp-57 to Glu-129 of LeuDH, which contains the two essential residues, Lys-80 and Lys-68, involved in catalysis (*14*) and substrate binding (*15*), respectively.

Accordingly, this region probably constitutes the substrate-binding domain of these amino acid dehydrogenases.

Another noteworthy region with high sequence similarity is that from Arg-177 to Asp-206 of PheDH, which is homologous to the corresponding regions of not only other amino acid dehydrogenases but also other $NAD(P)^+$-dependent enzymes such as lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (*4*). X-ray crystallographic studies of a large number of $NAD(P)^+$-dependent enzymes have revealed that this region folds into a characteristic βαβ structure (Rossmann fold) with conserved residues at β-turn positions (*22*), constituting the coenzyme-binding domain.

Based on the sequence decipherment described above, a pKK223-3-derived plasmid pKPSLN encoding a chimeric enzyme consisting of an N-terminal domain of PheDH including the substrate-binding region and a C-terminal domain of LeuDH including the coenzyme-binding region was constructed, according to the procedure depicted in Fig. III-1. The structure of pKPSLN was confirmed by restriction mapping and nucleotide sequencing (data not shown).

Gene Expression and Purification of Chimeric Enzyme.——*E. coli* JM109 cells transformed with pKPSLN were found to produce the chimeric enzyme in the soluble fraction when analyzed by SDS-PAGE (data not shown). The chimeric enzyme was therefore purified to homogeneity from the crude extract of the clone cells by the procedure described under "Experimental Procedures," and a typical result of the purification is summarized in Table III-I (final yield, 64%).

The N-terminal amino acid sequence of the chimeric enzyme determined up to the 15th cycle of automated Edman degradation

TABLE III-I. **Purification of the chimeric enzyme from *E. coli* cells carrying pKPSLN.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	2067	889	0.43	100
Blue Sepharose CL-6B	220	683	3.1	77
Superdex-200	108	570	5.3	64

coincided with that of PheDH (*4*). With L-phenylalanine as a substrate, the chimeric enzyme exhibited a specific activity of 5.3 units/mg protein, which is 6% of that of the parental PheDH (86.2 units/mg protein) (*20*).

Molecular Properties. ——Molecular mass of the native chimeric enzyme was measured to be about 72,000 daltons by gel filtration on a Tosoh G-3000 SW_{XL} column (0.75 x 60 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4 and calibrated with GluDH (290,000 daltons), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000), and cytochrome *c* (12,400). The analysis by SDS-PAGE indicated the molecular weight of the polypeptide to be about 40,000 (see Fig. III-3), suggesting that the chimeric enzyme is composed of two identical subunits. The presumed dimeric structure of the chimeric enzyme contrasts with the hexameric structures of LeuDH (*17*) and PheDH (*20*).

The antigenic structure of the chimeric enzyme was studied by immunoblotting with rabbit antisera raised against the parent enzymes, PheDH from *T. intermedius* and LeuDH from *B. stearothermophilus* (Fig. III-2). The chimeric enzyme reacted strongly with both of the polyclonal antibodies specific for each parent enzyme, indicating that it has antigenic determinants derived from both parent enzymes. In addition, clear precipitation lines were formed between the nondenatured chimeric

enzyme and each antiserum on an Ouchterlony double-diffusion analysis (data not shown), also showing the identity of the antigenic structures on its folded protein surface with those of the parent enzymes.

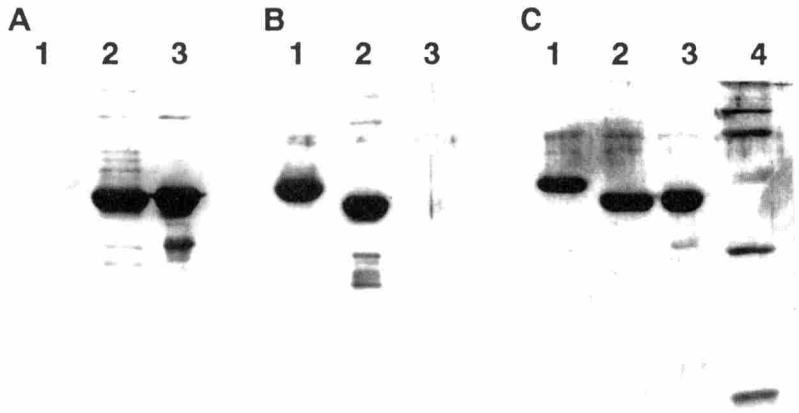

Fig. III-2. **Immunoblotting of the chimeric enzyme.** Proteins separated by SDS-PAGE (stained with Coomassie brilliant blue in *C*) were electrotransferred onto a nitrocellulose membrane and reacted with the antiserum against PheDH (in *A*) or LeuDH (in *B*). Lane 1, LeuDH purified from *B. stearothermophilus*; lane 2, the purified chimeric enzyme; lane 3, PheDH purified from *T. intermedius*; and lane 4, standard proteins.

The chimeric and two parent enzymes showed essentially identical profiles of CD spectra in the far-UV regions (data not shown), and the mean residue ellipticities (θ) at 220 nm were estimated to be -13.2, -12.9, and -12.1 deg·cm^2·dmol^{-1} for LeuDH, PheDH, and the chimeric enzyme, respectively. This suggests that their secondary structure contents are about the same.

Both of LeuDH of *B. stearothermophilus* (*17*) and PheDH of *T. intermedius* (*20*) are thermostable. Accordingly, the thermostability of the chimeric enzyme was examined by heating at various temperatures for 60 min. The chimeric enzyme retained its full activity on heating up to 54 °C between pH 7.0 and 9.5, but lost the activity by heating above 58 °C.

Therefore, the chimeric enzyme is less thermostable by about 10 °C than the parent enzymes, which are stable up to 65-70 °C.

The chimeric enzyme was subjected to controlled proteolysis with subtilisin in order to investigate the domain structure. As shown in Fig. III-3, the enzyme rapidly suffered the limited proteolysis forming two polypeptide fragments of dissimilar molecular sizes. On automated sequence analysis for the polypeptides eluted from the gel, the smaller fragment with a molecular weight of about 15,000 had an N-terminal sequence identical with that of PheDH, and the larger fragment with a molecular weight of about 27,000 had Gly at its N-terminus followed by residues 143 and thereafter in the designed sequence of the chimeric

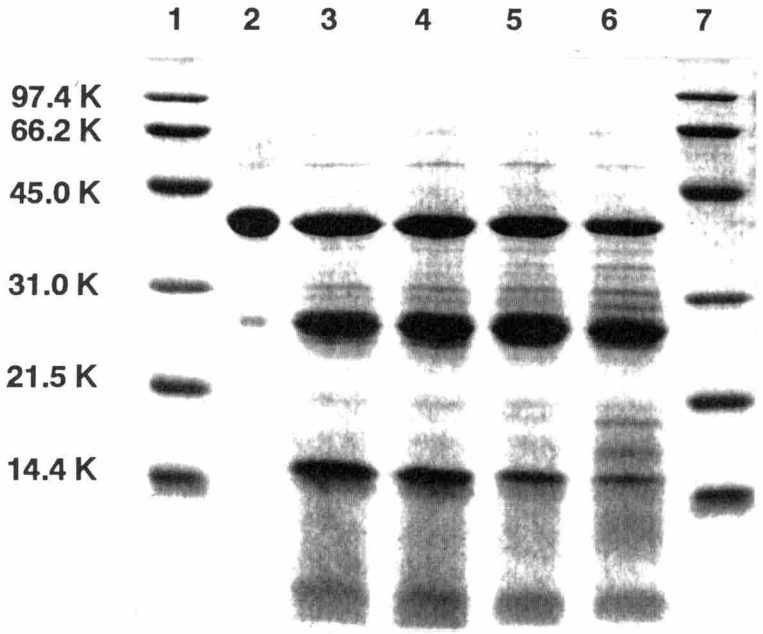

Fig. III-3. **Time course of limited proteolysis of the chimeric enzyme with subtilisin.** Lanes 1 and 7, standard proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme, from top to bottom; numbers on the left are in daltons); lanes 2-6, the chimeric enzyme digested for 0 (undigested), 5, 10, 30, and 60 min.

enzyme (see Fig. III-1). These results clearly show that subtilisin cleaved the peptide bond between Tyr-141 and Gly-142 of the chimeric enzyme,

which is located at the position connecting the N-terminal domain of PheDH with the C-terminal domain of LeuDH.

Catalytic Properties. ——The substrate specificity of the chimeric enzyme was studied in the oxidative deamination with various amino acids as a substrate at 10 mM, and the results are compared with those of PheDH and LeuDH in Table III-II. As a whole, the chimeric enzyme has broader substrate specificity than the parent enzymes. It acts on, in addition to the preferred substrates of PheDH, poor substrates of both parent enzymes

TABLE III-II. **Substrate specificity in the oxidative deamination.**

Amino acid	Relative activity (%)[a]		
	Chimera	PheDH	LeuDH
L-Phenylalanine	100 (2.7)[b]	100 (45)[b]	0
D-Phenylalanine	0	0	0
L-Tyrosine	22	40	0
L-Tryptophan	8.5	1.2	0
L-Histidine	0.4	0.2	0
L-Methionine	29	2.2	0.7
L-Ethionine	17	4.0	0
L-Valine	42	1.3	39
L-Leucine	10	3.9	100 (50)[b]
L-Isoleucine	53	0.4	54
L-*allo*-Isoleucine	0.3	26	47
L-Alanine	3.4	0.4	0.8
L-Phenylglycine	12	0	4.5
L-2-Aminobutyrate	4.2	1.8	14
L-Norvaline	21	28	56
L-Norleucine	38	30	14
p-Amino-L-phenylalanine	68	61	0
o-Fluoro-L-phenylalanine	51	65	0
m-Fluoro-L-phenylalanine	108	100	0
p-Fluoro-L-phenylalanine	117	118	0

[a]The specific activities of PheDH and the chimeric enzyme with L-phenylalanine as substrate and that of LeuDH with L-leucine as substrate were taken as 100, and relative activities of each enzyme for various amino acids are listed.
[b]The values in parentheses are the k_{cat} values (sec^{-1}) measured with each amino acid as substrate.

such as L-methionine, L-tryptophan, and L-phenylglycine. Furthermore, it is interesting to note that the chimeric enzyme also utilizes branched-chain

amino acids (L-valine, L-isoleucine, and L-leucine), which are good substrates for LeuDH.

TheK_m values of the chimeric enzyme were calculated to be 0.12 mM for L-phenylalanine and 0.06 mM for L-leucine from the double reciprocal plots of the initial rates against the substrate concentrations measured at a fixed concentration of NAD^+ (0.5 mM). The K_m values of the chimeric enzyme were similar to those of PheDH (0.11 mM for L-phenylalanine and 0.09 mM for L-leucine) and much smaller than the value of LeuDH for L-leucine (5.1 mM), whereas the value for NAD^+ was determined to be 0.09 mM, which is comparable to those of LeuDH (0.06 mM) and PheDH (0.17 mM).

The substrate specificity of the chimeric enzyme in the reductive amination was also studied (Table III-III), and it was found that a large variety of α-keto acids can be substrates for the chimeric enzyme. The specificity in the reductive amination appears closely similar to both substrate specificities of the parent enzymes. The K_m value for phenylpyruvate of the chimeric enzyme was calculated to be 0.02 mM from the double reciprocal plots of the initial rates against the substrate concentrations measured at a fixed concentration of NADH (0.2 mM) and NH_4Cl (200 mM), which is much smaller than that of PheDH (0.06 mM) and the value for α-ketoisocaproate of LeuDH (0.88 mM).

The pH optimum for the reactions of the chimeric enzyme was determined to be around pH 10.7-11.0 in the oxidative deamination of L-phenylalanine and at pH 10.6 in the reductive amination of phenylpyruvate. The pH optimum of the chimeric enzyme in the oxidative deamination is similar to those of the parent enzymes, but that in the reductive amination is higher by about 1 pH unit than those of the parent enzymes.

TABLE III-III. **Substrate specificity in the reductive amination.**

Keto acid	Relative activity (%)[a]		
	Chimera	PheDH	LeuDH
Phenylpyruvate	100 (4.0)[b]	100 (91)[b]	0
p-Hydroxyphenylpyruvate	576	80	0
α-Keto-γ-methylthiobutyrate	362	55	15
α-Ketoisovalerate	593	16	167
α-Ketoisocaproate	143	47	100 (280)[b]
α-Keto-β-methylvalerate	461	16	100
α-Ketovalerate	395	37	86
α-Ketocaproate	415	130	22
α-Ketobutyrate	30	5.5	47
α-Ketoglutarate	0	0	0
α-Ketoadipate	0	0	0
Oxalacetate	3	0	0
Pyruvate	0	0	0

[a]The specific activities of PheDH and the chimeric enzyme with phenylpyruvate as substrate and that of LeuDH with α-ketoisocaproate as substrate were taken as 100, and relative activities of each enzyme for various keto acids are listed.
[b]The values in parentheses are the k_{cat} values (sec^{-1}) measured with each α-keto acid as substrate.

The stereospecificity of the hydrogen transfer from NADH was investigated by the consecutive enzymatic method (*21*) with the reaction system containing the chimeric enzyme, alanine racemase, alanine dehydrogenase, D-alanine, phenylpyruvate, NAD^+, ammonia, and potassium phosphate in ^2H_2O (p^2H 8.5, uncorrected). In the ^1H NMR spectrum of the reaction mixture, the doublet resonance peak at δ8.8 ppm due to the C-4 proton of NAD^+ was not observed, indicating that NAD^+ retained a deuterium at the C-4 position of the nicotinamide ring. Because alanine dehydrogenase (belonging to the *pro-R* specific dehydrogenases) transfers hydrogen at the *pro-R* position of NADH (*23*), the chimeric enzyme catalyzes the *pro-S* stereospecific hydrogen transfer with regard to the coenzyme. The chimeric enzyme thus belongs to the *pro-S* specific dehydrogenases like the two parent enzymes (*20, 21*).

III-4. Discussion

Genetic construction of a chimeric enzyme from two functionally-related proteins sharing extensive sequence similarity and assessment of its catalytic properties is expected to provide valuable information on the structure-function relationship of the parent proteins. Enzymatic activities are one of the most sensitive criteria in judging for the correct folding of engineered proteins. As described above, an active chimeric enzyme consisting of an N-terminal domain of PheDH and a C-terminal domain of LeuDH has been prepared to elucidate the mechanism of substrate recognition of the two enzymes. The antigenic structures on the surface of the folded chimeric enzyme were shown to be common with those of both parent enzymes, and the secondary structures of the chimeric and parent enzymes are probably very similar, as predicted from their far-UV CD spectra.

However, several lines of evidence suggest that the stereostructure of the chimeric enzyme is to a certain extent distorted in the limited regions as compared to the parent enzymes: (i) the catalytic efficiency of the chimeric enzyme with L-phenylalanine as a substrate is much lower than that of PheDH, although the chimeric enzyme shows similar K_m values for L-phenylalanine and the same stereospecificity of a hydrogen transfer at the C-4 position of the coenzyme nicotinamide ring, (ii) the thermostability of the chimeric enzyme is decreased by about 10 °C, and (iii) the chimeric enzyme has a dimeric subunit structure, unlike hexameric structures of the parent enzymes.

Nevertheless, it seems likely that the chimeric enzyme maintains the correct two-domain structure of the parent enzymes, as envisaged from the results of limited proteolysis (Fig. III-3). Thus, the N- and C-terminal domains of the two amino acid dehydrogenases seem to correspond to two

structurally independent domains involved in binding of substrate and coenzyme. According to the X-ray crystallographic structure of GluDH from *Clostridium symbiosum* determined at 1.96 Å resolution (*1*), the N-terminal domain is connected with the C-terminal domain via a loop structure joining β_f and α_9 of each domain. The loop is composed of residues from Gly-194 to Ser-202, which correspond to residues from Gly-136 to Lys-144 of PheDH from *T. intermedius* in the sequence alignment reported recently (*24*) and thus to the connecting region in the chimeric enzyme (see Fig. III-1), where the limited proteolysis occurred.

Although the substrate-binding region is most probably contained in the N-terminal domain of PheDH, the chimeric enzyme shows roughly both specificities of its parent enzymes, particularly in the reductive amination (Table III-III). In the three-dimensional structure of GluDH, the substrate-binding region is composed of not only the N-terminal domain but also a part (α_{15}, formed by residues from Pro-369 to Arg-390) of the C-terminal domain (*1*), rendering Val-377 and Ser-380 to interact with the side chain of the bound L-glutamate (*5*). Assuming that the structure of the substrate-binding region in the chimeric enzyme is similar to that of GluDH (*24*), its low specificity probably is derived at least partly from the contribution of the C-terminal domain originated from LeuDH. Therefore, the substrate specificities of amino acid dehydrogenases may be determined by structural interaction of the two domains.

In conclusion, an active chimeric enzyme consisting of two domains containing the substrate- and coenzyme-binding regions of the two homologous amino acid dehydrogenases has been constructed successfully. Domain interchangeability as demonstrated here strongly suggests that the domain shuffling plays an important role in the molecular evolution of

dehydrogenases acting on various amino acids. Construction of another chimeric enzyme consisting of the substrate-binding domain of LeuDH and the NAD^+-binding domain of PheDH has also been attempted by molecular-genetic manipulation. However, this chimeric enzyme was produced in an insoluble fraction of the *E. coli* cell extract. Although the chimeric enzyme could be solubilized by denaturation with 8 M urea and the following dialysis, it showed a very weak activity with L-leucine as a substrate, insufficient for the detailed kinetic analysis.

III-5. References

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Chapter IV

Site-directed Mutagenesis of a Hexapeptide Segment Involved in Substrate Recognition

IV-1. Introduction

As other $NAD(P)^+$-dependent dehydrogenases, GluDH from *Clostridium symbiosum*, whose tertiary structure was first determined among amino acid dehydrogenases, consists of two domains, a coenzyme-binding domain and a substrate-binding domain (*1*). The results of the preceding chapter showed that LeuDH and PheDH also consist of two structurally independent domains. The N-terminal and C-terminal domains of both enzymes function as a substrate-binding domain and a coenzyme-binding domain, respectively. However, the mechanism of substrate recognition and the residues responsible for determination of substrate specificity remain to be identified.

Although PheDH from *Thermoactinomyces intermedius* (*2*) and LeuDH from *Bacillus stearothermophilus* (*3*) show a significant sequence homology especially in the substrate-binding region (see Chapter I, Fig. I-1), it is noted that 15 amino acid residues in this region are different between LeuDH and PheDH, suggesting that some of these residues may be involved in substrate recognition by the two enzymes. The largest difference in the compared sequences is found in the C-terminal side of this region: ^{124}Phe-Val-His-^{127}Ala in PheDH and ^{123}Met-Asp-Ile-^{126}Ile in LeuDH. In this chapter, in order to examine the function of the tetrapeptide sequence of PheDH, a mutant enzyme of PheDH, in which the segment from Phe-124 to Arg-129 containing the tetrapeptide sequence is replaced by the corresponding segment from Met-123 to Gln-128 of

LeuDH, has been constructed. The results described in this chapter show that the replacement affects the gross conformation of PheDH in such a way as to change the molecular recognition of substrates.

IV-3. Experimental Procedures

Materials.——All restriction enzymes, *Taq* DNA polymerase, a T4 DNA ligation kit, and a DNA blunting kit were purchased from Takara Shuzo (Kyoto). Plasmid pKK223-3 and Blue Sepharose CL-6B were obtained from Pharmacia (Sweden); a protein assay reagent and SDS-PAGE standards were from Bio-Rad (U.S.A.); all reagents for DNA synthesis and dye primer [M13(-21), M13RP-1] for DNA sequencing were from Applied Biosystems, Inc. (ABI) (U.S.A.); and standard proteins for gel filtration were from Oriental Yeast (Osaka). All other chemicals were of analytical grade. The plasmid pKPDH2 carrying the PheDH gene was prepared as described previously (*2*).

Enzyme and Protein Assays.——The oxidative deamination of amino acids and the reductive amination of keto acids by PheDH were determined by spectrophotometric measurement of the reduction of NAD^+ and the oxidation of NADH, respectively, as described previously (*2*). Protein concentrations were determined by the dye staining method with a Bio-Rad protein assay reagent or estimated by using an absorbance coefficient ($A^{0.1\%}$) at 280 nm of 0.63 for the wild-type enzyme and 0.68 for the mutant enzyme of PheDH.

Construction of Plasmid pKCS2 Carrying the Mutant Gene.——The plasmid pKCS2 coding for the hexapeptide-substituted PheDH was constructed by PCR (*4*), as shown in Fig. IV-1.

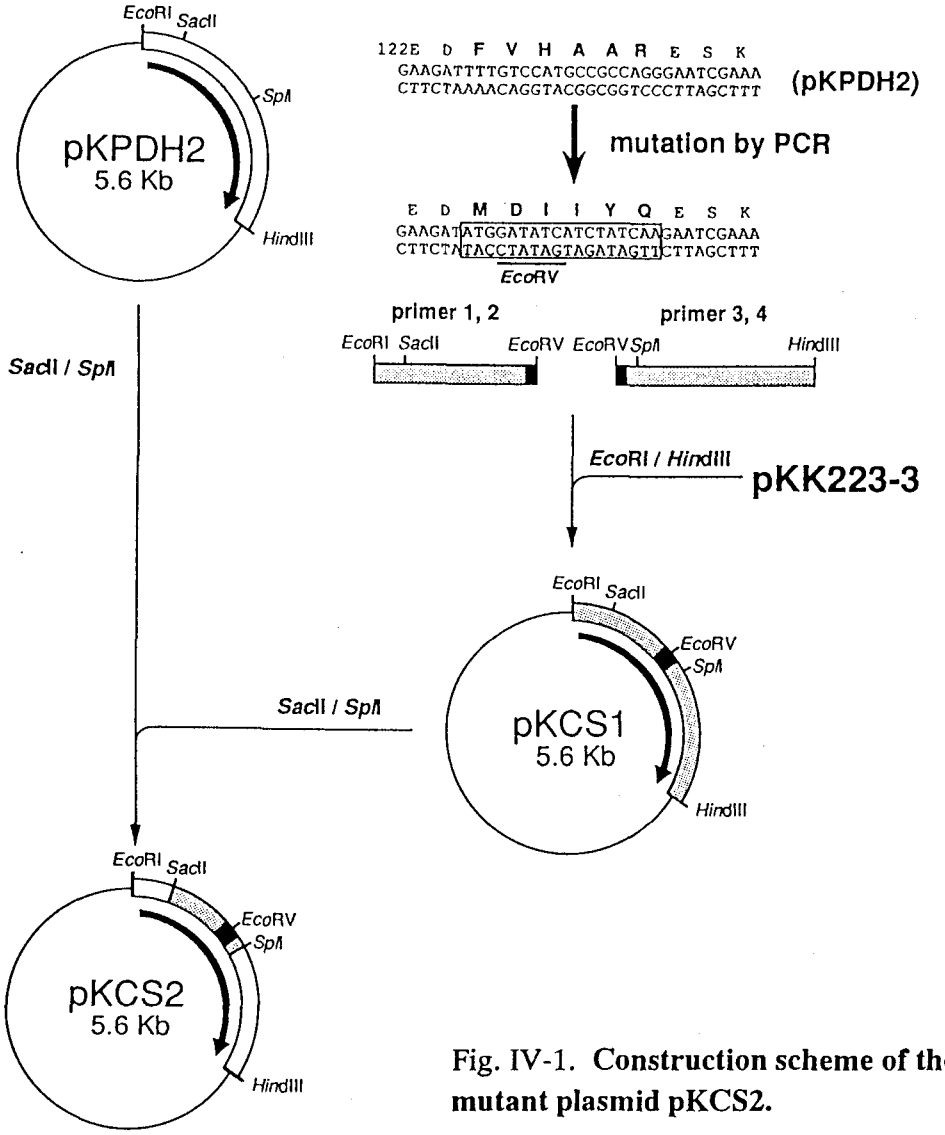

Fig. IV-1. **Construction scheme of the mutant plasmid pKCS2.**

For PCR, the following four primers were synthesized by the phosphoamidite method with an ABI 381A DNA synthesizer. Primer 1 encoded the N-terminal sequence of PheDH and contained an SD sequence and an *Eco*RI site (5'-GGAATTCGGAGGAAGCGAAGATGC-3', 24 mer); primer 2 encoded the C-terminal sequence of the N-terminal half region of PheDH and a substituted hexapeptide sequence and contained an *Eco*RV site (5'-TTGATAGATGATATCCATATCTTCCGGATTGGTT-3', 34 mer); primer 3 encoded the N-terminal sequence of the C-terminal half region of PheDH and contained an *Eco*RV site (5'-GAAGATATGGAT-

ATCATCTATCAAGAATCG-AAATCTTTT-3', 39 mer); and primer 4 encoded the C-terminal sequence of PheDH and contained a *Hin*dIII site (5'-AAACAGACAAGCTTTTTACCTCCTTGCGCT3', 30 mer). Two DNA fragments were amplified with a pair of primers, 1 and 2 or 3 and 4, and pKPDH2 as a template. The reaction mixture (10 μl) for PCR consisted of 1 μmol of Tris-HCl buffer (pH 8.3), 5 μmol of KCl, 0.15 μmol of $MgCl_2$, 0.01% of gelatin, 20 μmol each of dNTP, 2.5 units of *Taq* DNA polymerase, 0.3 ng of plasmid pKPDH2, and 100 pmol each of primers 1 and 2, or primers 3 and 4. The reaction mixture was heated at 94 °C for 1 min (for denaturation), cooled rapidly to 50 °C with a 2-min hold (for annealing), then incubated at 72 °C for 3 min (for extension). The programmed temperature shift was repeated 30 times. Each amplified fragment was digested with *Eco*RI-*Eco*RV or *Eco*RV-*Hin*dIII and ligated into the pKK223-3 digested with *Eco*RI and *Hin*dIII to yield pKCS1. The hexapeptide substitution was confirmed by DNA sequencing of the *Eco*RI-*Spl*I fragment of pKCS1 by the dideoxy chain termination method (*5*) with a DNA sequencer. The final plasmid pKCS2 was constructed by replacement of the *Sac*II-*Spl*I fragment of pKPDH2 by the corresponding fragment excised from pKCS1.

Purification of the Mutant Enzyme from E. coli JM109/pKCS2.—— The mutant enzyme designated as CS2 was purified from cells of *E. coli* JM109 transformed with pKCS2. During purification, the activity was assayed by determination of phenylpyruvate formed from L-phenylalanine. All operations were performed at room temperature unless otherwise stated.

Step 1: Cells of *E. coli* JM109/pKCS2 were cultured at 37 °C for 8 h in 40 liters of Luria-Bertani's broth containing 4.0 g of ampicillin, harvested by centrifugation, and washed with 0.85% NaCl. The cells (wet

weight, 130 g) suspended in 200 ml of 10 mM Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 0.02% 2-mercaptoethanol (buffer A) were disrupted with an Auto Chaser 300 ultrasonic processor (Kaijo Denki, Tokyo). After centrifugation, the supernatant solution was dialyzed against 1,000 volumes of the same buffer.

Step 2: The enzyme solution (480 ml) was applied to a DEAE Toyopearl 650M (Tosoh, Tokyo) column (9 X 16 cm) equilibrated with buffer A. The column was washed with the same buffer, then the enzyme was eluted with buffer A containing 0.1 M NaCl. The active fractions were concentrated by ultrafiltration with an Amicon PM-10 membrane and dialyzed against 1,000 volumes of 10 mM potassium phosphate buffer (pH 6.0) containing 1 mM EDTA and 0.02% 2-mercaptoethanol (buffer B).

Step 3: The enzyme solution (360 ml) was applied to a Blue Sepharose CL-6B column (5 X 15 cm) equilibrated with buffer B. The column was washed with the same buffer, then the enzyme was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1 mM EDTA and 0.02% 2-mercaptoethanol. The active fractions were concentrated and dialyzed against 1,000 volumes of buffer A.

Step 4: The enzyme solution (37 ml) was applied to a DEAE-TOYOPEARLPAK 650M (Tosoh, Tokyo) column in a Pharmacia fast protein liquid chromatography system. The column was developed at a flow rate of 3 ml/min with 400 ml of a linear gradient of NaCl concentration (0-0.2 M) in buffer A. The purity of the enzyme was estimated by SDS-PAGE.

Immunoblot Analysis.——Immunoblot analysis was carried out with the anti-PheDH rabbit serum. CS2 was electrophoresed on an SDS-polyacrylamide gel and transferred onto a nitrocellurose membrane. After incubation with the serum, the membrane was incubated with a peroxidase-

conjugated goat anti-rabbit IgG. Protein bands were visualized with 4-chloro-1-naphthol.

Molecular Weight Determination. ——The molecular weight of CS2 was determined by gel filtration on a Tosoh TSK-GEL G3000SW$_{XL}$ column (0.75 X 60 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 8.0) containing 0.1 M Na_2SO_4 at a flow rate of 0.5 ml/min. A calibration curve was made with the following proteins: GluDH (M_r, 290,000), lactate dehydrogenase (M_r, 142,000), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), and chymotrypsinogen (M_r, 25,000). The molecular weight of subunits was determined by SDS-PAGE with the following standard proteins: myosine (M_r, 200,000), β-galactosidase (M_r, 116,250), phosphorylase *b* (M_r, 97,400), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,400).

Cross-linking Experiment. ——To study the subunit structure of CS2, the enzyme was treated with bifunctional reagents, bis-imidoesters, which have an imidoester group at both ends of the molecule and react highly specifically with amino groups of proteins (*6*). Before the cross-linking reaction, the enzyme was concentrated, and the buffer was replaced by 10 mM potassium phosphate buffer (pH 8.0) by ultrafiltration. The reaction mixture (30 μl) containing 33 mM Tris-HCl (pH 8.9), 3 mM dimethyl suberimidate (DMS) or dimethyl adipimidate (DMA), and enzyme was kept at room temperature for 3 h, then subjected to SDS-PAGE.

CD and Fluorometric Measurements. ——CD measurements were carried out with a JASCO J-600 recording spectropolarimeter at 25 °C with a 1-mm light-path cell. The CD spectra were taken at the protein concentration of 0.5 mg/ml in the far-UV region (190-300 nm) under

nitrogen atmosphere. Fluorometric measurements were carried out with a Hitachi MPF-4 spectrofluorophotometer with a 5-mm light-path cell.

IV-4. Results

Construction and Expression of the Plasmid pKCS2.——The plasmid pKCS2 coding for the mutant PheDH with the sequence of ^{123}Met-Asp-Ile-Ile-Tyr-^{128}Gln of LeuDH from *B. stearothermophilus* instead of its inherent sequence of ^{124}Phe-Val-His-Ala-Ala-^{129}Arg was constructed as described under "Experimental Procedures" (Fig. IV-1). *E. coli* JM109 was transformed with pKCS2, and a low activity of PheDH was found in recombinant cells.

Purification of the Mutant Enzyme.——The gene product of pKCS2, CS2, was determined by measurement of the oxidative deamination activity of L-phenylalanine as an indicator, and purified to near homogeneity by three column chromatographies with a final yield of 34%. The purification is summarized in Table IV-I. The specific activities of CS2 for the oxidative deamination of L-phenylalanine (1.2 units/mg) and the reductive amination of phenylpyruvate (17 units/mg) were 1.8 and 13% of those of the wild-type enzyme, respectively. CS2 showed a strong reactivity with the anti-PheDH serum (data not shown).

TABLE IV-I. **Summary of the purification of CS2 from *E. coli* JM109/pKCS2.**

Steps	Protein (mg)	Total act. (U)	Sp. act. (U/mg)	Yield (%)	Fold
Crude extract	2,940	247	0.08	100	1.0
DEAE-TOYOPEARL 650M	1,300	260	0.20	105	2.5
Blue Sepharose CL-6B	693	210	0.30	85	3.6
DEAE-TOYOPEARLPAK 650M	70	84	1.2	34	15

Molecular Weight and Subunit Structure of CS2. ——SDS-PAGE of CS2 gave a molecular weight of about 41,000, which is in agreement with that calculated from the amino acid sequence (40,612). The chromatogram of gel filtration of CS2 on TSK-GEL G3000SW$_{XL}$ showed two peaks indicating the apparent molecular weight of 55,000 (major) and 110,000 (minor), though that of the wild-type enzyme was about 340,000 (Fig. IV-2A). The CS2 enzyme of both peaks showed the same specific activity and the identical mobility on SDS-PAGE. These results suggest that CS2 exists as a monomer and a dimer which are in an equilibrium, whereas the wild-type PheDH is a hexamer (*7*).

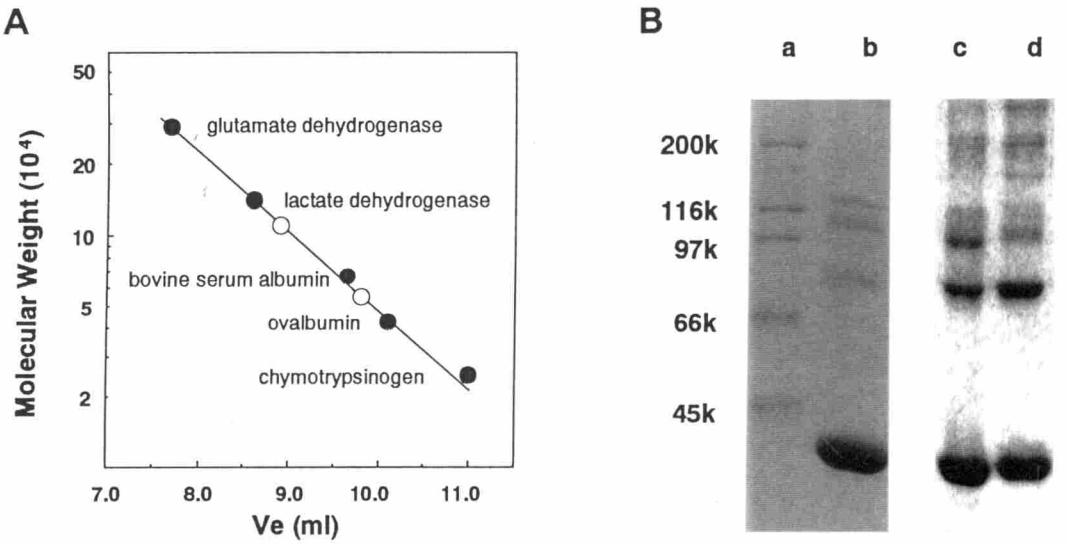

Fig. IV-2. **Determination of the molecular weight of CS2 (A) and the cross-linking experiments of the wild-type enzyme and CS2 (B).** Lane a, standard proteins; lane b, CS2 (10 mg/ml); lane c, PheDH (10 mg/ml); lane d, PheDH (1 mg/ml).

The subunit structure of CS2 was also studied by a cross-linking experiment. The SDS-PAGE of CS2 treated with the cross-linking reagents showed molecular weights of about 41,000, 80,000, 110,000 and 140,000 (Fig. IV-2B), which correspond to a monomer, dimer, trimer, and tetramer, respectively. In contrast, cross-linking of the wild-type enzyme

results in formation of monomer, dimer, trimer, tetramer, and hexamer. This also suggests a change in the subunit structure caused by the mutation.

CD and Fluorescence Spectra of CS2. ——CD spectra of CS2 and the wild-type PheDH in the far-UV region shown in Fig. IV-3 indicate that the secondary structure content is lower in CS2 than in the wild-type enzyme.

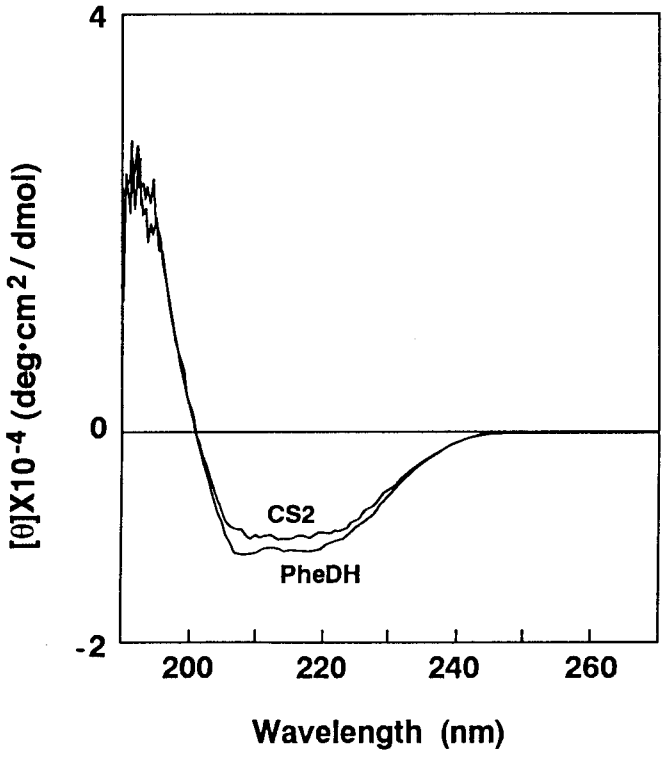

Fig. IV-3. **CD spectra of CS2 and the wild-type PheDH.** CD spectra were taken at the enzyme concentration of 0.5 mg/ml.

The emission fluorescence of the enzymes was measured by excitation at 285 nm. The fluorescence intensity of CS2 at 335 nm was 10% lower than that of the wild-type enzyme (data not shown). Both the wild-type enzyme and CS2 have two tryptophanyl residues (Trp-167 and Trp-282). One of the tryptophanyl residues of CS2 is more highly quenched than the

corresponding residue of the wild-type enzyme. At least one of the tryptophanyl residues of CS2 probably occupies a more hydrophilic environment than the corresponding residue in the wild-type enzyme, due to either a conformational change or the absence of a closely interacting hydrophobic residue.

Effect of pH on the CS2 reaction.——The pH optima for the oxidative deamination of L-phenylalanine of the wild-type enzyme and CS2 were 11.0 and 9.7-10.1, respectively. The pH optimum for the reductive amination of phenylpyruvate was 9.0, similar to that of the wild-type enzyme. The change in the pH optimum also suggests a difference in the active site environment between CS2 and the wild-type enzyme.

Thermostability of CS2.——The wild-type PheDH is thermostable, retaining full activity when heated at 70°C for 60 min (*7*). CS2 lost 50% of its initial activity on incubation at 50°C for 60 min, and its thermostability was thus lowered by the mutation.

Substrate Specificity of CS2.——The substrate specificity of CS2 for the oxidative deamination was different from that of the wild-type PheDH. As shown in Table IV-II, the relative activities of CS2 toward aliphatic amino acids such as L-leucine, L-isoleucine and L-norleucine were higher than those of the wild-type enzyme. In contrast, its relative activities toward aromatic amino acids, especially L-tyrosine, were lower than those of the wild-type enzyme. Table IV-II also shows the relative activity of CS2 and wild-type enzyme in the reductive amination of keto acids: CS2 is inert toward *p*-hydroxyphenylpyruvate. The relative activities of CS2 toward aliphatic keto acids were closely similar to those of the wild-type enzyme.

Both CS2 and the wild-type enzyme required NAD^+ (NADH) as a

TABLE IV-II. **Substrate specificities of the wild-type PheDH and CS2.** The oxidative deamination reaction was carried out in the reaction mixture containing 10 μmol of each amino acid, 0.5 μmol of NAD^+, 200 μmol of Gly-KCl-KOH buffer (pH 10.8), and the enzyme (0.021 mg for the wild-type enzyme, 0.13 mg for CS2) in a total volume of 1.0 ml. The reductive amination reaction was carried out in the reaction mixture containing 10 μmol of each keto acid, 200 μmol of NH_4Cl, 0.2 μmol of NADH, 200 μmol of Gly-KCl-KOH buffer (pH 9.5), and the enzyme in a total volume of 1.0 ml.

Substrates	Relative activity	
(10 mM)	CS2	PheDH
Deamination		
L-Phenylalanine	100	100
D-Phenylalnine	ND[a]	ND
L-Tyrosine	1.0	40
L-Tryptophane	ND	1.2
L-Histidine	ND	0.2
L-Methionine	43	2.2
L-Ethionine	35	4.0
L-Valine	6.0	1.3
L-Leucine	64	3.9
L-Isoleucine	65	0.4
L-*allo*-Isoleucine	12	26
L-Alanine	ND	0.4
L-2-Aminobutyrate	3.5	1.8
L-Norvaline	36	2.1
L-Norleucine	65	6.3
Amination		
Phenylpyruvate	100[b]	100[b]
p-Hydroxyphenylpyruvate	ND	80[c]
α-Keto-γ-methylthiobutyrate	86	55
α-Keto-*iso*-valerate	5.2	16
α-Keto-*iso*-caproate	54	47
DL-α-Keto-β-methylvalerate	19	16
α-Ketovalerate	18	37
α-Ketocaproate	171	130
α-Ketobutyrate	1.6	5.5
α-Ketoadipate	ND	ND
α-Ketoglutarate	ND	ND
Oxalacetate	ND	ND
Pyruvate	ND	ND

[a]Not detectable. [b]Measured at 5 mM. [c]Measured at 2.5 mM, NADH 0.1 mM.

coenzyme, and $NADP^+$ (NADPH) was inert. The K_m values of CS2 for NAD^+ and NADH were 0.14 and 0.025 mM, respectively, comparable to those of the wild-type enzyme ($K_{m\text{-}NAD^+}$; 0.17 mM, $K_{m\text{-}NADH}$; 0.083 mM).

Kinetic Studies of CS2 Reaction.——Table IV-III summarizes the apparent K_m and k_{cat} values of CS2 and the wild-type enzyme. CS2 has higher K_m values than the wild-type enzyme for all substrates. In the oxidative deamination, the K_m values of CS2 for straight-chain amino acids

such as L-norvaline and L-ethionine are 10- to 25-fold higher, while those for L-leucine and L-isoleucine are greater 50 to 100 folds. The K_m value for L-phenylalanine was increast most by the mutation. The K_m values for α-keto acids were similarly increased by the mutation, the largest change being a 400-fold increase in the value for phenylpyruvate. The k_{cat} values of CS2 for all substrates except L-isoleucine were lower than those of the wild-type enzyme. The k_{cat} value for L-phenylalanine was most markedly reduced by the mutation.

TABLE IV-III. **Kinetic constants of CS2 and the wild-type PheDH.** K_m and k_{cat} values were determined from double reciprocal plots at fixed concentrations of NAD^+ (0.5 mM in the oxidative deamination), NADH and NH_4Cl (0.1 and 200 mM in the reductive amination, respectively).

Substrates	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1}s^{-1}$)		
	PheDH	CS2	PheDH	CS2	PheDH	CS2	(%PheDH)
L-Phenylalanine	0.11	23	45	0.75	410	0.033	(0.008)
L-Leucine	0.09	4.7	3.6	0.21	40	0.045	(0.11)
L-Isoleucine	0.09	10	0.24	0.28	2.6	0.028	(1.1)
L-Norvaline	0.53	10	0.59	0.15	1.1	0.015	(1.4)
L-Norleucine	0.22	2.1	1.9	0.17	8.6	0.081	(0.94)
L-Methionine	0.34	7.5	0.84	0.16	2.5	0.021	(0.84)
L-Ethionine	0.27	4.0	1.1	0.10	4.0	0.025	(0.63)
Phenylpyruvate	0.06	23	91	12	1,500	0.52	(0.035)
α-Keto-*iso*-caproate	9.1	56	57	7.4	6.2	0.13	(2.1)
α-Keto-β-methylvalerate	13	50	26	2.5	2.0	0.050	(2.5)
α-Ketovalerate	35	107	82	4.8	2.3	0.045	(2.0)
α-Ketocaproate	2.5	47	79	22	31	0.47	(1.5)
α-Keto-γ-methylthiobutyrate	2.8	87	37	18	13	0.21	(1.6)

The catalytic efficiencies (k_{cat}/K_m) of CS2 and the wild-type enzyme are also shown in Table IV-III. The efficiencies of CS2 for both aliphatic amino acids and aliphatic keto acids were 0.5 to 2% of those of the wild-type enzyme. In contrast, k_{cat}/K_m values for L-phenylalanine and phenylpyruvate were 0.008 and 0.035% of those of the wild-type enzyme, respectively. Thus, the hexapeptide substitution brought about the most

marked reduction of the catalytic efficiency for phenylalanine and phenylpyruvate.

III-5. Discussion

In this work, the hexapeptide segment (^{124}Phe-Val-His-Ala-Ala-^{129}Arg) of PheDH from *T. intermedius* was replaced by Met-Asp-Ile-Ile-Tyr-Gln, which is the corresponding sequence of LeuDH from *B. stearothermophilus*. It was suggested that the segment plays a significant role in substrate recognition, because this is the largest non-homologous segment in the substrate-binding domains of these two enzymes, which show high sequence homology.

The K_m values of the mutant enzyme (CS2) were higher for all substrates than those of the wild-type enzyme (Table IV-III). In particular, the K_m values for L-phenylalanine and phenylpyruvate showed the highest increments: 210 and 380 times, respectively. The mutation also brought about the highest reduction of the catalytic efficiencies (k_{cat}/K_m) for L-phenylalanine and phenylpyruvate. These results suggest that the hexapeptide segment (^{124}Phe-Val-His-Ala-Ala-^{129}Arg) is implicated in the recognition of the most preferable substrates of PheDH, phenylalanine and phenylpyruvate.

The region from Leu-62 to Gly-143 of PheDH containing the hexapeptide segment shows a 27.7% sequence similarity with that from Leu-106 to Gly-186 of GluDH from *C. symbiosum*. A crystallographic study of GluDH revealed that the region corresponds to α-helix-6, β-strand-d, α-helix-7, β-strand-e and α-helix-8 of the substrate-binding domain (*1*). The side chain of Ala-163 and the main chain carbonyl of Gly-164 of GluDH are suggested to interact with the substrate L-glutamate.

These residues correspond to Pro-121 and Glu-122 of PheDH, which are adjacent to the substituted hexapeptide segment. This also suggests that the structure around the hexapeptide segment is significant in the substrate recognition.

The hexapeptide replacement affects the whole enzyme structure as well as the catalytic functions, probably owing to an increase in acidity of this segment. The difference in the CD spectrum between CS2 and the wild-type enzyme shows reduction of secondary structures by the mutation. The fluorescence spectral change suggests a difference between CS2 and the wild-type enzyme in the local environments around tryptophanyl residue(s). The subunit structure of the enzyme was also changed from hexamer to monomer by the mutation. This gross conformational change probably affects the catalytic efficiency of the mutant enzyme. As shown in Table IV-III, k_{cat}/K_m values of CS2 for branched-chain amino acids, aliphatic amino acids, and their keto analogues were also diminished by the mutation, though less effectively than those for phenylalanine and phenylpyruvate by two orders of magnitude. This reduction of the catalytic efficiency probably results from a conformational change of the catalytic site by the mutation.

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Chapter V

Comprehensive Discussion

The structure and function of the coenzyme-binding sites of $NAD(P)^+$-dependent dehydrogenases have been extensively investigated (*1-9*). However, the catalytic sites including the substrate-binding loci of amino acid dehydrogenases have not been fully elucidated. For understanding the catalytic mechanism of the enzymes, it is prerequisite to identify amino acid residues involved in catalysis and substrate binding at the active site. In Chapter II of this thesis is described the identification of active-site residues of PheDH from *Thermoactinomyces intermedius* by chemical modification with a monoanionic acetylation reagent (MAP). Although MAP is nonspecific for the labeled sites, unlike affinity labeling regents which are specific for the active sites of enzymes (*10*), it is expected to bind to the anion-binding sites in proteins through its negatively charged phosphate group.

As shown in Chapter II, PheDH was inactivated irreversibly with MAP in a time- and dose-dependent manner and was markedly protected from inactivation by the concomitant addition of substrate and coenzyme. In the differential chemical modification, at least 7 lysyl residues per enzyme subunit were found to be protected by the concomitant addition of substrate and coenzyme, suggesting the presence of multiple lysyl residues at the active site of PheDH. To specify the lysyl residue(s) whose modification results in the enzyme inactivation, the mutant enzymes for several lysyl residues were modified with MAP. All the single mutant enzymes of PheDH, in which 5 totally or highly conserved lysyl residues

(Lys-69, Lys-81, Lys-89, Lys-90, and Lys-173) were replaced with Ala, were inactivated with the reagent as effectively as the wild-type enzyme. However, a double mutant enzyme, in which Lys-69 and Lys-81 were both replaced with Ala, was found to be considerably resistant to the inactivation. In contrast, the corresponding single mutant enzymes of LeuDH from *Bacillus stearothermophilus* (Lys-68→Ala and Lys-80→Ala) were almost completely resistant to the inactivation. Assuming that Lys-69 and Lys-81 of PheDH correspond to the active-site residues, Lys-68 and Lys-80 of LeuDH, respectively, these results suggest that the reagent can discriminate subtle differences in the electrostatic environment around the two active-site lysyl residues of these enzymes. Thus, it is likely that Lys-68 and Lys-80 of LeuDH are located in close vicinity with each other, elevating the mutual nucleophilicity. On the other hand, in the active site of PheDH, there may be other positive charge(s) besides Lys-69 and Lys-81, assisting the binding of MAP to the active site. Despite these differences, the kinetic analyses of the single mutant enzymes revealed that Lys-69 and Lys-81 of PheDH play important roles in substrate binding and catalysis, respectively, like the corresponding residues (Lys-68 and Lys-80) of the homologous LeuDH.

Based on the reaction mechanism of LeuDH proposed by Sekimoto *et al.* of this laboratory (*11,12*) and referred to that of the synonymous GluDH (*13-15*), the reaction mechanism of PheDH is proposed as depicted in Fig. V-1. In the oxidative deamination, the α-carboxyl group of L-phenylalanine is bound to the protonated amino group of Lys-69. Following hydrogen bonding of the unprotonated ε-amino group of Lys-81 to a water molecule and hydride transfer from the substrate α-hydrogen atom to the C4 atom of the coenzyme nicotinamide ring, a nucleophilic attack by the Lys-81-bound water on the resulting α-imino acid intermediate produces an α-

carbinolamine, which then decomposes to the keto acid, liberating ammonia. In the reductive amination, although binding of the α-carboxyl group of phenylpyruvate to the protonated Lys-69 is necessary as in the oxidative deamination, the ε-amino group of Lys-81 would also be protonated and is hydrogen-bonded to the α-carbonyl oxygen of keto acid. The reaction proceeds with free ammonia attacking the α-carbonyl carbon because of the electron deficiency produced by the interaction with Lys-81. The ε-amino group of Lys-81 acts in this case as a general acid providing a proton to the carbonyl oxygen to form the α-carbinolamine intermediate. After proton transfer from the amino group to the hydroxyl group, loss of a water molecule results in the formation of the imine intermediate, reduction of which by NADH then yields the amino acid. This general acid-base function of the conserved lysyl residue is probably common to LeuDH, PheDH, and GluDH that share significant sequence similarity as described below.

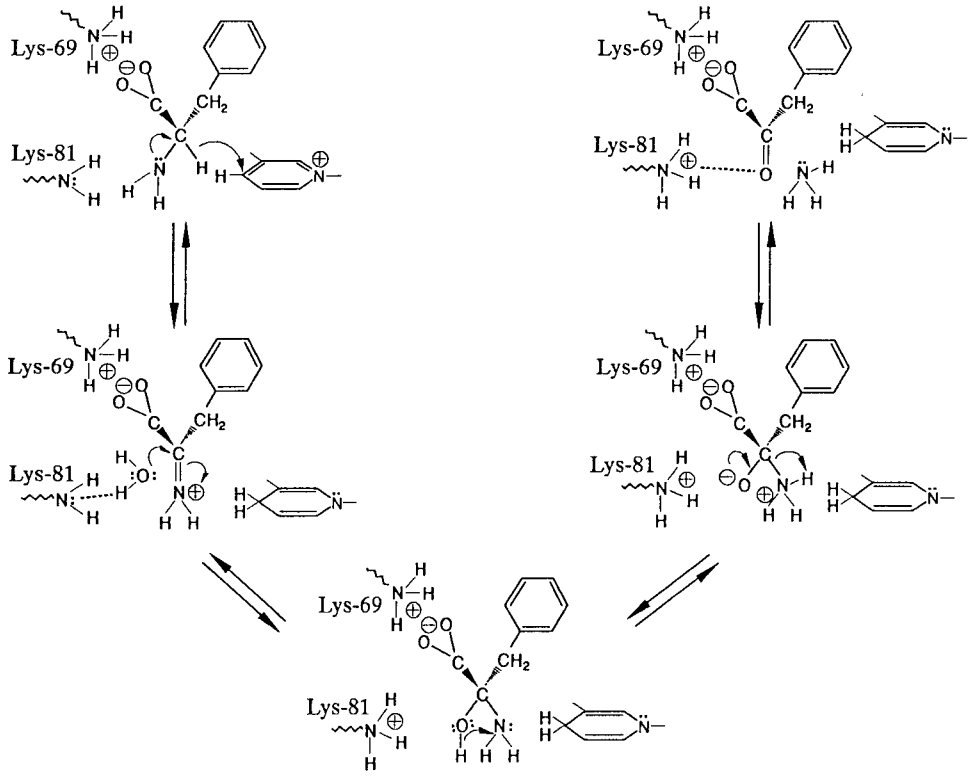

Fig. V-1. **A postulated reaction mechanism of phenylalanine dehydrogenase.**

PheDH from *T. intermedius* and LeuDH from *B. stearothermophilus* show low overall sequence similarities (20 and 17 %, respectively) with GluDH from *Clostridium symbiosum*, whose three-dimensional structure has been determined. However, 32 and 35 amino acid residues among 68

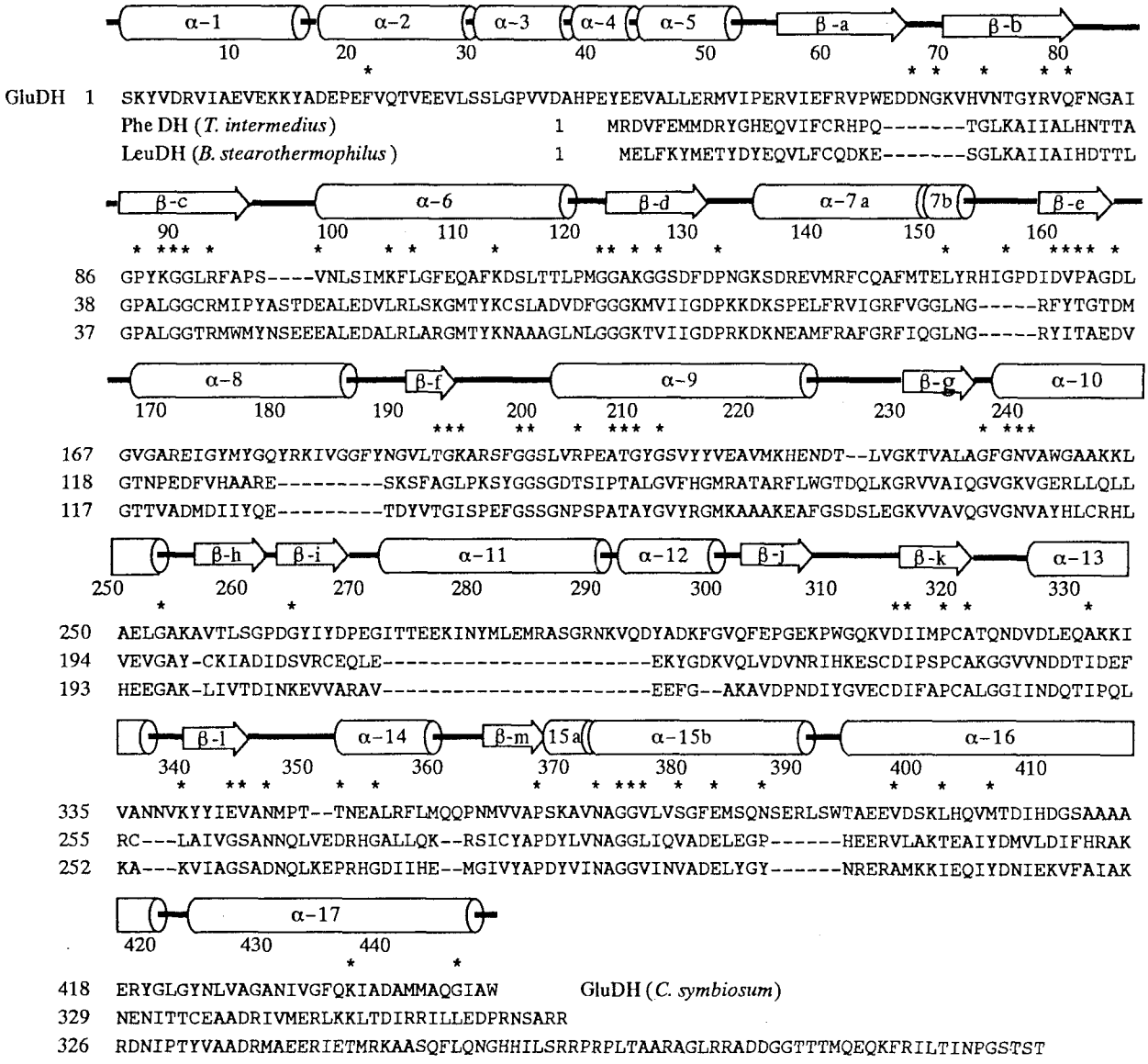

Fig. V-2. **Sequence alignment of GluDH, PheDH, and LeuDH (modified from Ref. *17*).** The secondary structural elements in the three-dimensional structure of GluDH from *C. symbiosum* with helices represented by cylinders and strands represented by arrows are shown above the aligned sequences together with the residue numbers. Residues indicated by asterisks above the sequences represent the set of 68 residues which are regarded as strongly conserved ones in the GluDH family (*16*).

residues, which have been considered to be important for the maintenance of the three-dimensional structure and for the catalysis of the GluDH family (*16*), are conserved in PheDH from *T. intermedius* and LeuDH from *B. stearothermophilus*, respectively (Fig.V- 2; see also Ref. *17*). The strong conservation of these important residues indicates the close relationship among the structures (and also the catalytic mechanisms) of PheDH, LeuDH, and GluDH. In Chapter III, a chimeric enzyme consisting of an N-terminal half of PheDH and a C-terminal half of LeuDH has been genetically constructed to elucidate the mechanism of substrate recognition by PheDH and LeuDH. Structural analyses of the chimeric enzyme demonstrated that PheDH and LeuDH are both composed of two domains corresponding to independent folding units, and suggested that their N- and C-terminal halves comprise the substrate- and coenzyme-binding domains, respectively, as shown for the X-ray crystallographic structure of GluDH (*13*).

The chimeric enzyme thus constructed had a dimeric subunit structure, unlike hexameric structures of the parent enzymes. GluDH assembles into a hexamer with 3,2-symmetry (a dimer of trimers or a trimer of dimers, Fig. V-3) (*13, 16, 17*). The dimer interface is formed mainly between β_a in the N-terminal region and its 2-fold symmetry related mate ($\beta_{a'}$) from the adjacent subunit. In contrast, the trimer interface is extensive and involves α_{17} in the C-terminal region, lying over the substrate- and coenzyme-binding domains, which also contact with each domain of the 3-fold symmetry related adjacent subunit. If these modes of subunit interactions are applicable to the hexameric LeuDH and PheDH, it is reasonable to assume that the chimeric enzyme forms a dimeric structure between the same N-terminal domains derived from one parent (PheDH), but does not form a trimeric (and hence hexameric) structure between the heterologous domains derived from each parent enzyme; the polypeptide of LeuDH from

B. stearothermophilus is longer by 60 residues than that of PheDH at the C-terminus, when aligned for maximum matching (see Fig. V-2). The mutant enzyme of PheDH, whose inherent hexapeptide segment is replaced by the corresponding part of LeuDH, also changed to a monomeric structure in slight equilibrium with a dimeric structure (Chapter IV). The hexapeptide replacement resulted in reduction of the secondary structure content of the enzyme and hence might cause a conformational change not only in the catalytic site but also in the subunit interface.

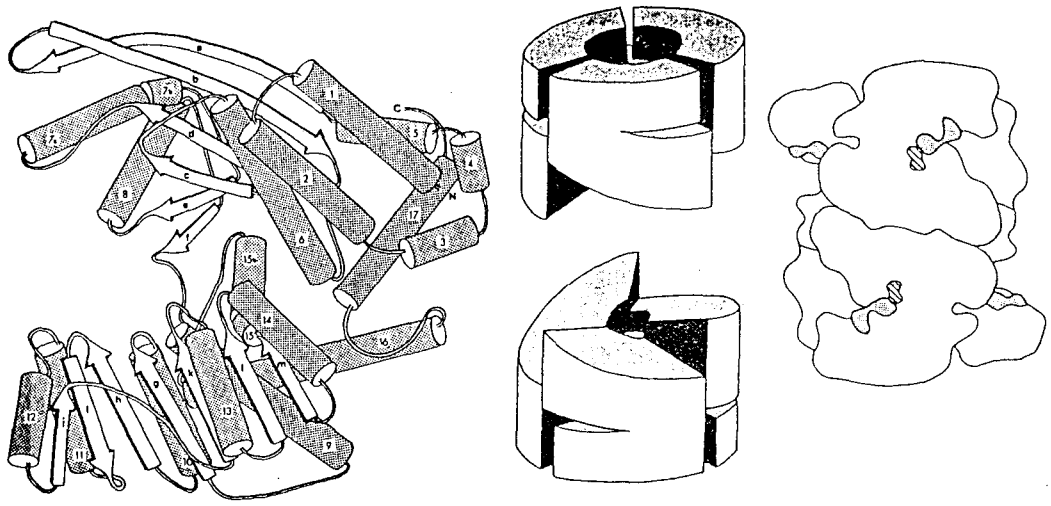

Fig. V-3. **A schematic representation of the GluDH structure (modified from Ref. *13*).** (*Left*), the subunit connectivity is shown with α-helices represented by cylinders and β-sheets by arrows; (*middle*), the positive interlocking of the subunits around the 2-fold axis is illustrated; and (*right*), the assembly of six subunits into the active hexamar is portrayed with the shaded segments representing the bound NAD^+ and hatched areas representing the model of the bound glutamate.

The chimeric enzyme of PheDH and LeuDH had K_m values for L-phenylalanine and L-leucine similar to those of PheDH and showed a broad substrate specificity, especially in the reductive amination, as compared to those of the two parent enzymes (Chapter III). This low specificity probably is derived at least partly from the contribution of the C-terminal domain originated from LeuDH. Therefore, the substrate specificities of

amino acid dehydrogenases may be determined by the structural interaction of the two domains. In the case of the hexapeptide-substituted mutant PheDH, the catalytic efficiencies (k_{cat}/K_m) for L-phenylalanine and phenylpyruvate decreased to 0.008 and 0.035 % of those of the wild-type enzyme, respectively, in marked contrast with those for aliphatic amino and keto acids (0.5~2 % of those of the wild-type enzyme). Accordingly, it is suggested that the hexapeptide segment plays an important role in the substrate recognition by PheDH. In GluDH, the principal interactions that determine the substrate specificity are of the glutamate side-chain carboxyl group with the amino group of Lys-89 and with the side-chain hydroxyl group of Ser-380, with Gly-90, Ala-63 and Val-377 forming interactions with the hydrophobic component of the side-chain (*17*). In the sequence alignment (Fig. V-2), Lys-89 and Ser-380 of GluDH are coordinately replaced by Leu-41(40) and Val-294(291) in PheDH (LeuDH), respectively, producing a pocket with a significantly greater hydrophobic character. Britton *et al.* (*17*) have modeled the active sites of PheDH and LeuDH based on the structure of GluDH, altering only the nature and conformation of the side-chains in the amino acid specificity pocket. In their models, the difference in substrate specificity between PheDH and LeuDH arises only from unacceptable steric interactions of the methyl group of Ala-113 of LeuDH with the meta position of the substrate phenylalanine benzene ring, which are relieved in PheDH by the critical replacement of this residue (Ala-113) by Gly-114. To examine the validity of the models, a mutant enzyme of LeuDH, in which Ala-113 is replaced by Gly, has been constructed and characterized. This mutant enzyme showed a considerable activity toward L-phenylalanine (35 % of V_{max} for L-leucine), even though it showed only 4 % of the original activity (Kataoka, K. *et al.*, unpublished results). Although the K_m value of this

mutant for L-leucine was comparable to that of the wild-type LeuDH, its K_m value for L-phenylalanine was 30 mM, being 300-fold higher than that of the wild-type PheDH (see Chapter IV). Furthermore, a mutant enzyme of PheDH, in which Gly-114 is replaced by Ala, also acted on L-phenylalanine (Kataoka, K. *et al.*, unpublished results). These results indicate that the steric hindrance of the side-chain methyl group of Ala-113 of LeuDH for the substrate benzene ring may be responsible for the specificity of LeuDH, but there should be other residues (including those in the hexapeptide segment) giving the high specificity for L-phenylalanine to PheDH. Based on the findings described in this thesis and referring to the structure of GluDH, the active-site model of PheDH is proposed as shown in Fig. V-4.

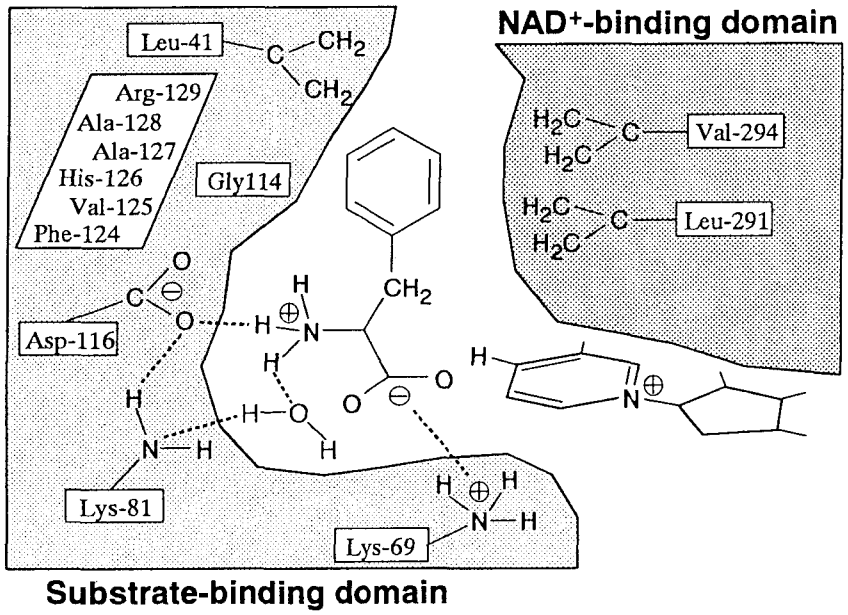

Fig. V-4. **A hypothetical model for the active-site of *T. intermedius* PheDH.**

In this model, the phenylalanine side-chain is bonded by hydrophobic interactions with Leu-41, Gly-114, Leu-291, and Val-294, and the shape

and character of the pocket provide an explanation for the specificity for the hydrophobic, bulky side-chain amino acid substrates. The two catalytically important lysyl residues (Lys-69 and Lys-81) (see Chapter II) and Asp-116, the corresponding residue of which is thought to be involved in the proton transfer to and from the amino acid substrate in GluDH (*17*), are also shown in this model.

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Acknowledgments

The present work has been performed at Institute for Chemical Research, Kyoto University, and Institute of Scientific and Industrial Research, Osaka University.

I wish to express my sincere thanks to Dr. Kenji Soda, Professor of Institute for Chemical Research, Kyoto University, and Dr. Toshio Fukui, Professor of Institute of Industrial and Scientific Research, Osaka University, for their kind guidance, valuable advice and encouragement throughout the course of this study.

I am also greatly indebted to Dr. Katsuyuki Tanizawa, Associate Professor of Institute of Industrial and Scientific Research, Osaka University, Dr. Nobuyoshi Esaki, Associate Professor of Institute for Chemical Research, Kyoto University, and Dr. Toshihisa Ohshima, Professor of Kyoto University of Education, for their valuable discussion, clear instruction and supports.

I express my deep gratitude to Dr. Hiroshi Ueno, Associate Professor of Kyoto University, Dr. Tohru Yoshimura, Institute for Chemical Research, Kyoto University, and Dr. Harumi Takada, Showa Denko Ltd., for their clear suggestion, warm encouragement and supports.

I would also thank all the members of Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, and Laboratory of Food Chemistry, Institute of Industrial and Scientific Research, Osaka University, both past and present, for sharing ideas with me and countless other things they have done for me.

List of Publications

I. Papers Related to the Thesis:

Exploring the Active Site of Phenylalanine Dehydrogenase by Chemical Modification with Methyl Acetyl Phosphate Combined with Site-Directed Mutagenesis.
Kunishige Kataoka, Katsuyuki Tanizawa, Toshio Fukui, Hiroshi Ueno, Tohru Yoshimura, Nobuyoshi Esaki, and Kenji Soda (1994) *J. Biochem.*, submitted for publication.

Construction and Characterization of Chimeric Enzyme Consisting of an Amino-terminal Domain of Phenylalanine Dehydrogenase and a Carboxy-terminal Domain of Leucine Dehydrogenase.
Kunishige Kataoka, Harumi Takada, Katsuyuki Tanizawa, Tohru Yoshimura, Nobuyoshi Esaki, Toshihisa Ohshima, and Kenji Soda (1994) *J. Biochem.*, **106**, in press.

Site-Directed Mutagenesis of Hexapeptide Segment Involved in Substrate Recognition of Phenylalanine Dehydrogenase from *Thermoactinomyces intermedius*.
Kunishige Kataoka, Harumi Takada, Tohru Yoshimura, Setsuo Furuyoshi, Nobuyoshi Esaki, Toshihisa Ohshima, and Kenji Soda (1993) *J. Biochem.* **114**, 69-75.

II. Other Published Paper:

The Purification, Characterization, Cloning and Sequencing of the Gene for a Halostable and Thermostable Leucine Dehydrogenase from *Thermoactinomyces intermedius*.
Toshihisa Ohshima, Norikazu Nishida, Sundararaju Bakthavatsalam, Kunishige Kataoka, Harumi Takada, Tohru Yoshimura, Nobuyoshi Esaki, and Kenji Soda (1994) *Eur. J. Biochem.*, in press.