CHAPTER I

INTRODUCTION

Amino acid dehydrogenases (EC 1.4.1.-) catalyze the reversible deamination of L-amino acids to their corresponding keto acids in the presence of the pyridine nucleotide coenzymes, NAD^+ and / or $NADP^+$. They are important enzymes that exist at the interface of nitrogen and carbon metabolism and provide a route for interconversion of inorganic nitrogen with organic nitrogen, and, in other words, serve as a connecting link between amino acid and organic acid metabolism. The enzymes are considerably different from alcohol and lactate dehydrogenases in their structures and properties. As shown in Table 1.1, amino acid dehydrogenases are catagorized based on the specificity they display toward their amino acid substrate and more than ten kinds of them have been so far found in various kind of organisms [1-3]. The metabolic role of amino acid dehydrogenase consists of regulation of the synthesis of amino acids and keto acids. In spite of their metabolic roles, the equilibrium of amino acid dehydrogenase reactions lies far to the amination of keto acid: the K_{eq} values are 10^{-14} - 10^{-18}. Therefore, the reactions are favorable for asymmetric synthesis of amino acids from their prochiral keto analogs and ammonia. The amino acid dehydrogenases have been studied intensively because of their ubiquitous distribution and a number of potential industrial applications. In addition, they have been used for analysis of amino acids and keto acids as well as assay of some enzymes acting on the substrate L-amino acids, which are important for pharmaceutical and dietary consumption purposes [1-6].

One of the most interesting aromatic amino acid dehydrogenases is phenylalanine dehydrogenase (PheDH) which has been focused on numerous studies since its discovery in 1984. Much attention is being paid to the enzyme not only because its occurrence was completely unknown until the discovery by Hummel *et al.* [7], but because it appears to be useful as an commercial catalyst in the industrial production of optical pure L-phenylalanine and related L-amino acids from their keto analogs and also in the clinical and pharmaceutical applications.

Table 1.1 NAD(P)-dependent amino acid dehydrogenases [6]

EC number	Enzyme	Coenzyme	Major source
1.1.4.1	AlaDH	NAD	Bacteria (*Bacillus, Strptomyces, Anabena, Pseudomonas, Rhodobacter, Arthrobacter, Thermus, Enterobacter, Phormidium*), chrorella
1.4.1.2	GluDH	NAD	Plants, fungi, yeasts, bacteria
1.4.1.3	GluDH	NAD(P)	Animals (bovine liver, chicken liver), tetrahymena, bacteria (*Clostridium, Thiobacillus*)
1.4.1.4	GluDH	NADP	Plants, *Euglena gracilis*, *Chrorella sarokiniana*, fungi, yeasts, bacteria
1.4.1.5	L-Amino acid DH	NADP	Bacteria (*Clostridium sporogenes*)
1.4.1.7	SerDH	NAD	Plants (parsley)
1.4.1.8	ValDH	NAD, NADP	Bacteria (*Streptomyces, Alcaligenes faecalis, Planococcus*), plants (pea, wheat)
1.4.1.9	LeuDH	NAD	Bacteria (*Bacillus, Clostridium, Thermoactinomyces*)
1.4.1.10	GlyDH	NAD	Bacteria (*Mycobacterium tuberculosis*)
1.4.1.11	DAHDH	NAD, NADP	Bacteria (*Clostridium, Brevibacterium*)
1.4.1.12	DAPDH	NAD(P)	Bacteria (*Clostridium*)
1.4.1.15	LysDH (cyclizing)	NAD	Human liver
1.4.1.16	DAPMDH	NADP	Bacteria (*Corynebacterium glutamicum, Brevibacterium* sp., *Bacillus sphaericus*)
1.4.1.17	MethylalaDH	NADP	Bacteria (*Pseudomonas* sp.)
1.4.1.18	LysDH (Lys-6-DH)	NAD	Bacteria (*Agrobacteria tumefaciens, Klebsiella pneumoniae*)
1.4.1.19	TrpDH	NAD(P)	Plants (*Nicotiana tabacum, Pisum sativum, Spinacia oleracea*)
1.4.1.20	PheDH	NAD	Bacteria (*Brevibacterium* sp., *Sporosarcina ureae, Bacillus sphaericus, Bacillus badius, Rhodococcus maris, Thermoactinomyces intermedius, Microbacterium* sp.)
1.4.1.-	AspDH	NADP	Bacteria (*Klebsiella pneumoniae*)

DH; dehydrogenase, NAD(P); NAD and NADP-nonspecific, DAHDH; L-erythro-3,5-diaminohexanoate dehydrogenase, DAPDH; 2,4-diaminopentanoate dehydrogenase, DAPMDH; meso-2,6-diaminopimelate dehydrogenase, MethylalaDH; *N*-methyl-L-alanine dehydrogenase

1.1 Isolation and purification of phenylalanine dehydrogenase

At the beginning of the 1980s , the wide screening of aromatic amino acid dehydrogenases led to the discovery of PheDH in *Brevibacterium* species [7]. The enzyme has been isolated from several gram-positive and spore-forming aerobic mesophiles such as *Rhodocccus* sp. M4 [8], *Sporosarcina ureae* [9-10], *Bacillus sphaericus* [10], *B. badius* [11], *R. maris* [12] and *Nocardia* sp. 239 [13]. In addition, Ohshima *et al.* found thermostable PheDH in many thermophilic actinomycetes and purified it from *Thermoactinomyces intermedius* IFO14230 [14]. The last thermostable PheDH was found in non-spore forming mesophilic bacteria, *Microbacterium* sp. DM 86-1, by Asano and Tanetani [15].

The study on physical and biochemical properties of PheDH requires separation technique to purify enzyme. How pure enzymes should be used is dependent on the purpose of the enzyme application, as well as the properties and purity of the substrates and products. Usually screening is carried out for the purpose of finding microorganisms that produce the enzyme effectively, and the medium and growth conditions that lead to abuntdant enzyme production are searched. The addition of L-phenylalanine, as a PheDH inducer, to the medium is usaully very effective for promoting the enzyme production [7-12]. Moreover, enzyme activity can also be induced by other amino acids such as L-histidine [7-8], L-tyrosine [14] and L-methionine [15]. In most cases, the isolation procedures used are rather straightforward and employ various chromatographic procedures. Typical methods reported so far for PheDH purification include heat treatment, protamine and ammonium sulfate precipitation, ion exchange chromatography (mostly by DEAE-Toyopearl column), adsorption chromatography (mostly by hydroxyapatite column), hydrophobic interaction chromatography (mostly by Butyl-Toyopearl column) and gel filtration chromatography. During 1987-88, Hummel *et al.* successfully used an aqueous two-phase system composed of polyethylene glycol, potassium phosphate and sodium chloride in the industrial purification of PheDH from *Brevibacterium* sp. and *Rhodococcus* sp. M4 for phenylalanine production because of its advantage in cell debris removal and enzyme enrichment [7-8]. The affinity chromatography, in particular with Red-sepharose CL 4B column, is also applicable to the PheDH

purification from *R. maris* (12) and *T. intermedius* (14). In addition, very efficient purification of PheDH from *B. badius* was achieved on ion exchange high performance liquid chromatography using DEAE-5PW column whereas those of *R. maris* and *Microbacterium* sp. DM 86-1 were successful on FPLC, mono Q column (11-12,15). Recently, Mulcahy *et al.* described a novel kinetic locking-on strategy for bioaffinity purification of NAD^+-dependent dehydrogenases including PheDH based on immobilized cofactor derivatives through use of enzyme-specific substrate analogues in irrigants to promote selective and biospecific adsorption (18). Substituted N^6-linked immobilized NAD^+ derivatives and D-phenylalanine were used as a matrix and a locking-on ligand, respectively.

1.2 Basic molecular and catalytic properties of phenylalanine dehydrogenase (7-17)

Phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating: EC 1.4.1.20), which is speculated to play a role in the metabolic degradation of L-phenylalanine, catalyzes the reversible oxidoreduction of L-phenylalanine to phenylpyruvate and ammonia in the presence of NAD^+ (Figure 1.1). Basic molecular and catalytic properties of various microbial PheDHs are summarized in Table 1.2. PheDHs do not share a common quaternary structure. The enzyme from *S. ureae*, *B. sphaericus*, *B. badius* and *Microbacterium* sp. DM86-1 were shown to be octamer whereas monomeric, dimeric, tetrameric and hexameric structures are found in *Nocardia* sp., *R. maris*, *Rhodococcus* sp. M4 and *T. intermedius* enzymes, respectively. All PheDHs are NAD^+-specific enzymes. Alternate nucleotide coenzymes have been tested with the *R. maris* enzyme and found that 3-acetylpyridine-NAD^+, an analog of NAD^+, was a much better coenzyme than NAD^+ while thionicotinamide-NAD^+ and deamino-NAD^+ were similar to NAD^+ in coenzyme activity. The substrate specificity for amino acids and keto acids differs markedly among the various enzyme sources. The *B. sphaericus* enzyme acts on L-tyrosine as well as L-phenylalanine, whereas the *T. intermedius* enzyme is highly specific for L-phenylalanine. In reductive amination, PheDHs show relatively generous substrate specificity. Interestingly, *p*-hydroxyphenylpyruvate served as a

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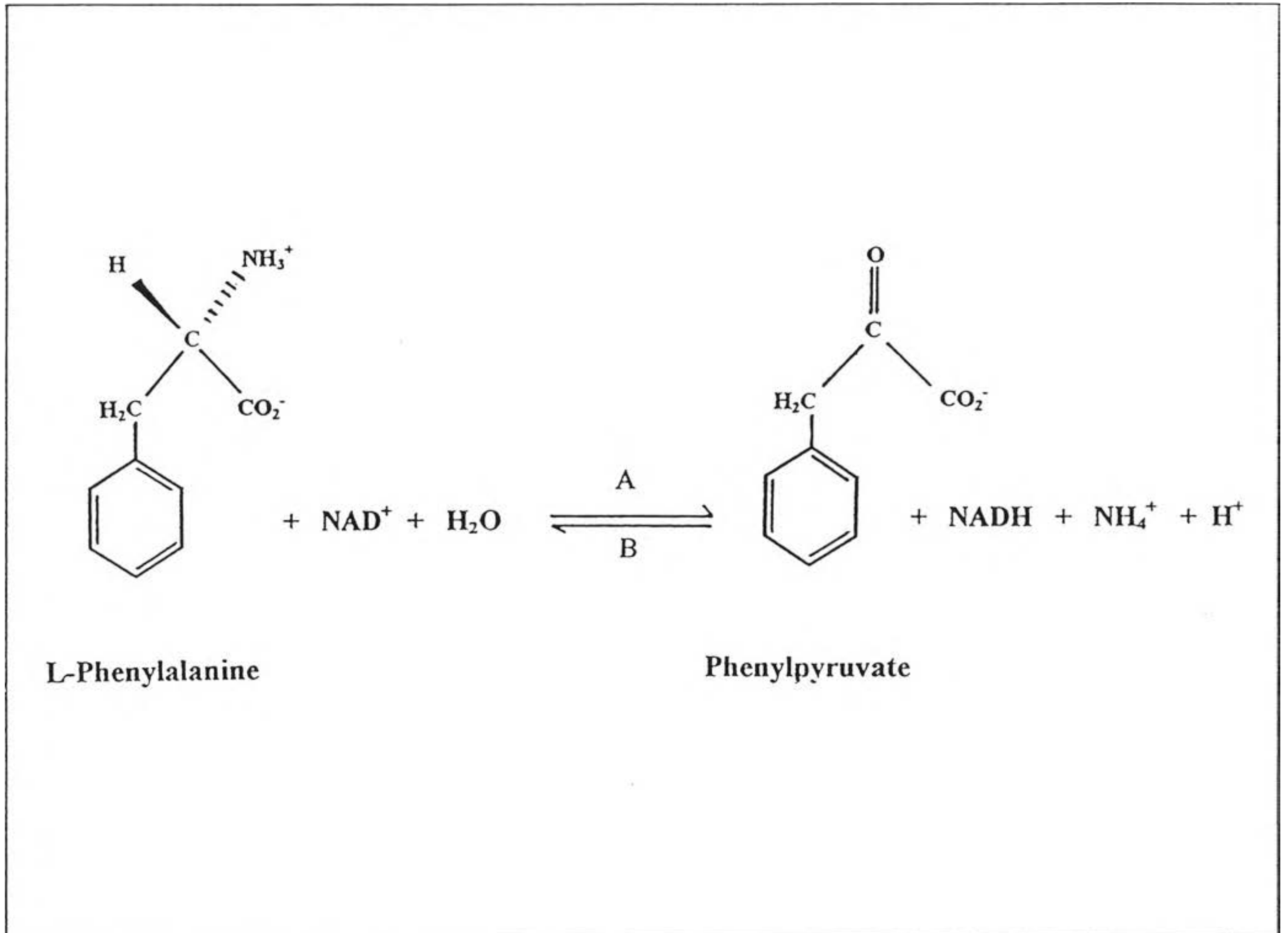

Figure 1.1 Reaction of phenylalanine dehydrogenase
(A) oxidative deamination (B) reductive amination

Table 1.2 Comparison of properties of phenylalanine dehydrogenases from various microorganisms [a]

Properties	*Brevibacterium* sp.	*Rhodococcus* sp.M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.
Native Mr									
from gel filtration	-	150,000	310,000	340,000	335,000	70,000	42,000	270,000	330,000
from deduced amino acid sequence	-	-	330,608	331,480	330,800	-	-	249,928	-
Subunit Mr	-	39,500	41,326	41,435	41,350	36,000	42,000	40,488	41,000
Number of subunit	-	4	8	8	8	2	1	6	8
Isoelectric focusing point (pI)	-	5.6	5.3	4.3	3.5	-	-	-	5.8
pH optimum									
Oxidative deamination	10.5	10.1	10.5	11.3	10.4	10.8	-	11.0	12.0
Reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0
Thermostability (% remaining activity after incubation for 10 min)	-	-	75 (40°C, pH 9)	100 (55°C, pH 9)	50 (55°C, pH 8)	100 (35°C, pH 7.4)	50 (53°C, pH 9.5-10, 2h)	100 (70°C, pH 7.2, 1h)	100 (55°C, pH 9)
Equilibrium constant (M^2)	-	4.5×10^{-14}	-	1.4×10^{-15}	-	-	3.2×10^{-18}	-	-

Table 1.2 Comparison of properties of phenylalanine dehydrogenases from various microorganisms [a] (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp.M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.
Stereochemistry of hydrogen transfer	-	Pro-S	-	Pro-S	-	-	-	Pro-S	-
Inhibitors [b]	-	-	Ag^{+}, *p*CMB	Ag^{+}, Hg^{2+}, *p*CMB	Ag^{+}, Hg^{2+}, *p*CMB	Hg^{2+}, *p*CMB	-	-	Hg^{2+}, *p*CMB
Substrate specificity [c]									
Oxidative deamination									
L-phenylalanine	100	100	100	100	100	100	100	100	100
L-tyrosine	-	12	5	72	9	2	-	0	4
L-tryptophan	-	2	5	1	4	8	-	0	0
L-methionine	-	4	4	3	8	5	-	0	7
L-valine	-	-	3	1	4	0	-	0	5
L-leucine	-	-	2	1	3	2	-	4	3
L-isoleucine	-	-	1	0.5	0.2	3	-	0	0
L-norvaline	-	-	6	1	5	0	-	-	6
L-norleucine	-	-	15	4	19	16	-	-	16
L-ethionine	-	-	7	3	7	13	-	-	-
L-α-aminobutyrate	-	-	2	-	1	1	-	-	2
L-phenylalaninamide	-	-	9	3	9	-	-	-	-
L-phenylalaninol	-	-	9	0.6	9	-	-	-	-

Table 1.2 Comparison of properties of phenylalanine dehydrogenases from various microorganisms [a] (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp.M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.
L-*p*-aminophenylalanine	-	-	-	-	-	-	-	7	-
L-phenylalanine methyl ester	-	-	38	10	38	-	-	-	-
L-tyrosine methyl ester	-	-	0.4	7	0.4	-	-	-	-
p-fluoro-DL-phenylalanine	-	62	-	-	34	8	-	-	-
m-fluoro-DL-phenylalanine	-	-	-	-	11	8	-	-	-
o-fluoro-DL-phenylalanine	-	-	-	-	2	2	-	-	-
D-phenylalanine	-	0	0	0	0	0	-	0	-
Reductive amination									
phenylpyruvate	100	100	100	100	100	100	100	100	100
p-hydroxyphenylpyruvate	96	5	24	136	53	91	28	0	21
indole-β-pyruvate	24	3	1	0	-	5	54	-	-
α-ketovalerate	-	-	9	6	12	0	-	-	-
α-ketocaproate	-	-	32	0	31	9	-	-	-
α-ketoisovalerate	-	-	2	6	13	0	-	6	-
α-ketoisocaproate	-	-	13	8	-	1	240	-	-
α-ketobutyrate	-	-	-	-	3	0	-	1	6

Table 1.2 Comparison of properties of phenylalanine dehydrogenases from various microorganisms [a] (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp.M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.
α-keto-γ-methylthiobutyrate	59	33	27	11	16	9	-	14	39
α-keto-β-methylbutanoate	-	-	-	-	-	-	-	-	17
α-keto-γ-methylpentanoate	-	-	-	-	13	-	-	6	20
α-ketohexanoate	-	-	-	-	31	-	-	-	48
Apparent K_m (mM)									
L-phenylalanine	0.385	0.87	0.096	0.22	0.088	3.8	0.75	0.22	0.10
NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20
NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072
phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02
ammonia	431	387	85	78	127	70	9.6	106	85
Reference	3, 7	4, 8, 16-17	9-10	10	11	12	13	14	15

[a] S.; *Sporosarcina*, B.; *Bacillus*, R.; *Rhodococcus*, T.; *Thermoactinomyces*

[b] *p*CMB = *p*-chloromercuribenzoate

[c] Substrate specificity expressed as relative activity (%)

\- = data was not detected

good substrate for the *Brevibacterium* sp., *B. sphaericus* and *R. maris* enzymes. The pH optima of microbial PheDHs were reported in Table 1.2. Like in other amino acid dehydrogenases, a high activity is found at highly alkaline pH range. The optimal reaction conditions for the PheDHs mirror those for the amino acid dehydrogenase family as a whole. Where they were tested, sulfhydryl-modifying agents have been shown to inhibit the enzymatic reaction while metal chelating or carbonyl reagents have been not. In addition, Misono *et al.* reported that D-phenylalanine and D-tyrosine inhibited the reaction of the *R. maris* enzyme competitively against L-phenylalanine. As with other amino acid dehydrogenases, PheDHs have equilibrium constants that lie far toward amino acid synthesis, despite their predominantly catabolic roles. The equilibrium constant (K_{eq}) reported for the *B. sphaericus* enzyme was 1.4×10^{-15} M^2 whereas the *Nocardia* and *Rhodococcus* sp. M4 enzymes were reported to have K_{eq} of 3.2×10^{-18} M^2 and 4.5×10^{-14} M^2, respectively.

1.3 Catalytic mechanism and structure of phenylalanine dehydrogenase

1.3.1 Catalytic mechanism

Amino acid dehydrogenases, like other NAD(P)H-dependent dehydrogenases and reductases, show either pro-R (A-) or pro-S (B-) stereospecificity for hydrogen transfer from the C-4 position of the nicotinamide moiety of the reduced coenzyme, NAD(P)H, to the substrate amino acids as shown in Figure 1.2. A-stereospecific enzymes tranfer hydrogen to or from the pro-R position of the nicotinamide, while B-stereospecific enzymes transfer at opposite site (pro-S side) [19]. The stereospecificity is an inherent characteristic of individual NAD(P) dehydrogenases and depends on the catalytic reaction and enzyme source. GluDH, LeuDH, ValDH and DAPDH are pro-S-specific enzymes whereas AlaDH and LysDH are pro-R-specific enzymes [1-6]. For PheDH, the stereochemistry of hydrogen transfer has been determined for the *B. sphaericus* [10], *T. intermidius* [14]

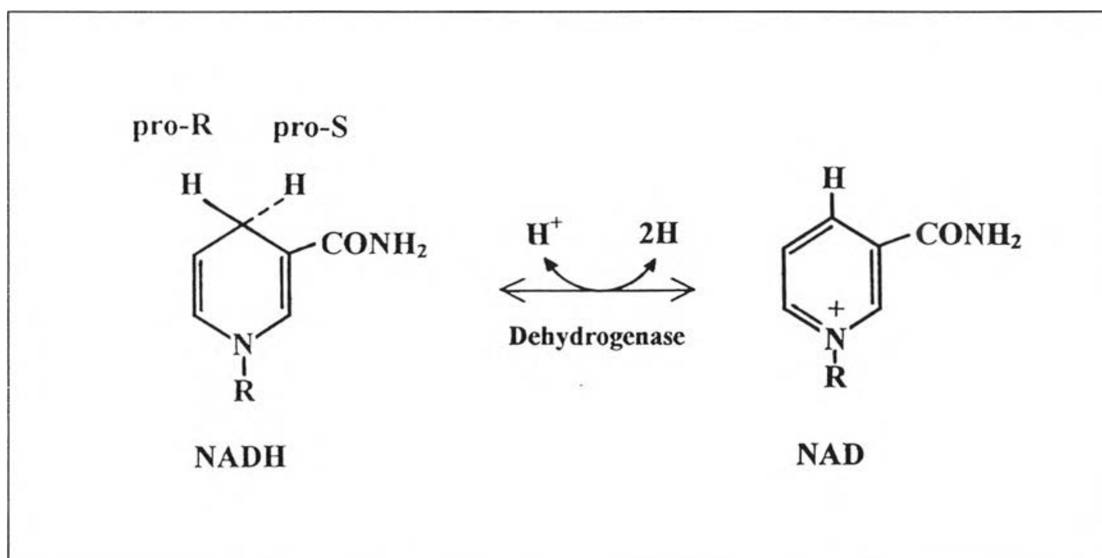

Figure 1.2 Stereospecificity of hydrogen transfer of NADH catalyzed with dehydrogenases [2]

R represents ADP-ribosyl

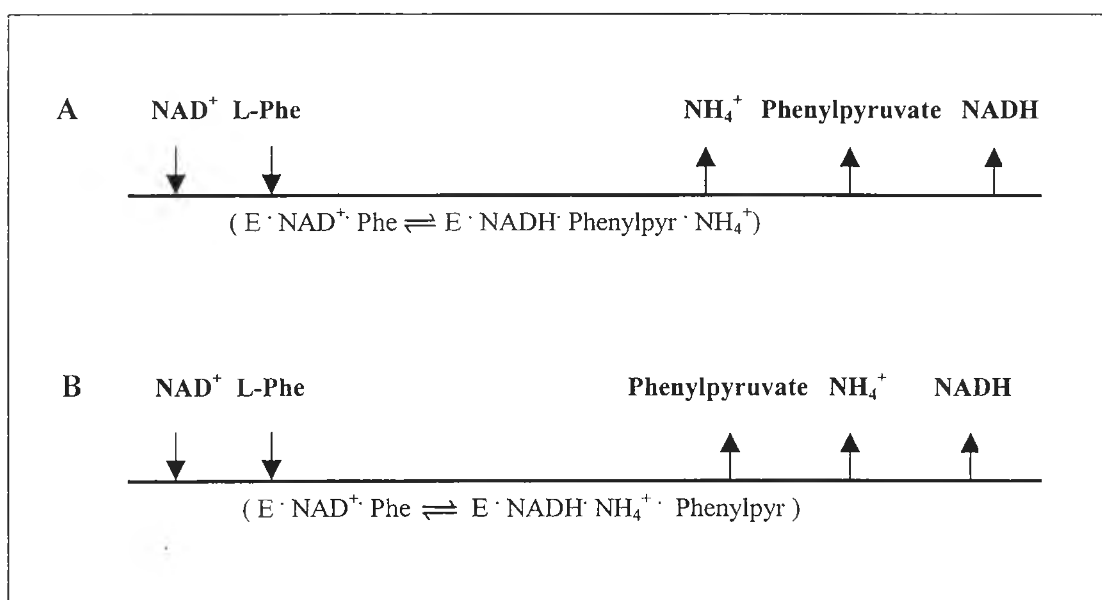

Figure 1.3 Kinetic mechanisms of phenylalanine dehydrogenases

(A) PheDH from *Rhodococcus maris* [12] and *Rhodococcus* sp. M4 [17]

(B) PheDH from *Thermoactinomyces intermedius* [14]

and *Rhodococcus* sp. M4 (17) enzymes. In all cases, the pro-S hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine placing the PheDHs among the majority of amino acid dehydrogenases.

A series of steady-state kinetic analyzes provides information about the reaction mechanism. The oxidative deamination catalyzed by amino acid dehydrogenases proceed via the formation of a ternary complex with sequential or random substrate-binding mechanisms. However, diversity is found in the manner of substrate binding and product release. For PheDHs, two steady-state kinetic mechanisms have been described. The *Rhodococcus* sp. M4 (17) and *R. maris* (12) enzymes proceed through a sequential ordered mechanism in which NAD^{+} and L-phenylalanine bind to the enzyme in that order and three products, ammonia, phenylpyruvate and NADH, are released from the enzyme in that order after dehydrogenation. In case of the *R. maris* PheDH, Misono and coworkers noted that L-phenylalanine showed a noncompetitive inhibition pattern against phenylpyruvate, rather than the expected uncompetitive inhibition pattern. They suggested that L-phenylalanine might form dead-end complexes with the E⊠NADH form of the enzyme. This same discrepancy has been observed in the ValDH and DAPDH kinetic mechanisms (4).

The second kinetic mechanism determined for PheDHs is from the thermophile, *T. intermedius* (14). Its initial velocity and product inhibition patterns suggest a sequential ordered binary-ternary mechanism which is slightly different from the other mechanism. In this case, the order of substrate binding is the same as the first one but the order of release was observed to be phenylpyruvate, ammonia and NADH. This conclusion was drawn from both initial velocity and product inhibition experiments, but the authors did not comment on the somewhat unusual mechanism. However, it is interesting that inhibition pattern of NADH against L-phenylalanine showed a noncompetitive inhibition pattern which did not fit with the suggested mechanism. They comment that some unknown factor was probably involved in the reaction. The kinetic mechanisms of PheDHs are summarized in Figure 1.3.

1.3.2 Structure

Extensive developments of the techniques in gene cloning have enabled rapid determination of the primary structures of amino acid dehydrogenases. In addition, X-ray crystallographic analyses of several amino acid dehydrogenases have been undertaken and revealed their ternary and quaternary structures in detail [6].

Among amino acid dehydrogenases, primary structures of GluDHs, AlaDH, LeuDH, ValDH, DAPDH and PheDH have so far been determined by peptide and DNA sequencing methods [6]. The genes for five PheDHs, which were from *B. sphaericus* [20-21], *S. ureae* [9], *B. badius* [22], *T. intermidius* [23] and *Rhodococcus* sp. M4 [24], have been cloned and sequenced. Like other NAD(P)-dependent dehydrogenases, PheDH is composed of two domains based on sequence comparison; the N-terminal domain responsible for catalysis and amino acid binding and the C-terminal domain responsible for pyrimidine dinucleotide binding. Figure 1.4 shows an alignment of several amino acid dehydrogenases in the amino acid-binding and catalytic domain with secondary structure predictions based on the determined three-dimentional structure of the *Clostridium symbiosum* GluDH [24]. Although a computer-aided search of a protein sequence database revealed somewhat low similarities of overall sequenes among amino acid dehydrogenases, a common partial sequence of about 30 residues in NAD(P) binding domain was observed in all of the enzymes. The coenzyme binding domain which binds the adenine nucleotide moiety shows a high degree of conservation of tertiary structures; it consists of a two-stranded parallel β-sheet and one α-helix with virtually identical arrangement ($\beta\alpha\beta$-nucleotide-binding fold). Figure 1.5 shows a comparison of amino acid dehydrogenases in the carboxyl-terminal nucleotide-binding domain. From the x-ray crystallographically determined structure of the *C. symbiosum* GluDH, this region can be identified with residues in the βG-α10-βH region [24].

While the structures and functions of the coenzyme binding domains have been extensive studied, little is known about the substrate-binding domains of amino acid dehydrogenases. Accordingly, much attentions in the past few years have been paid for the investigation of substrate recognition mechanism

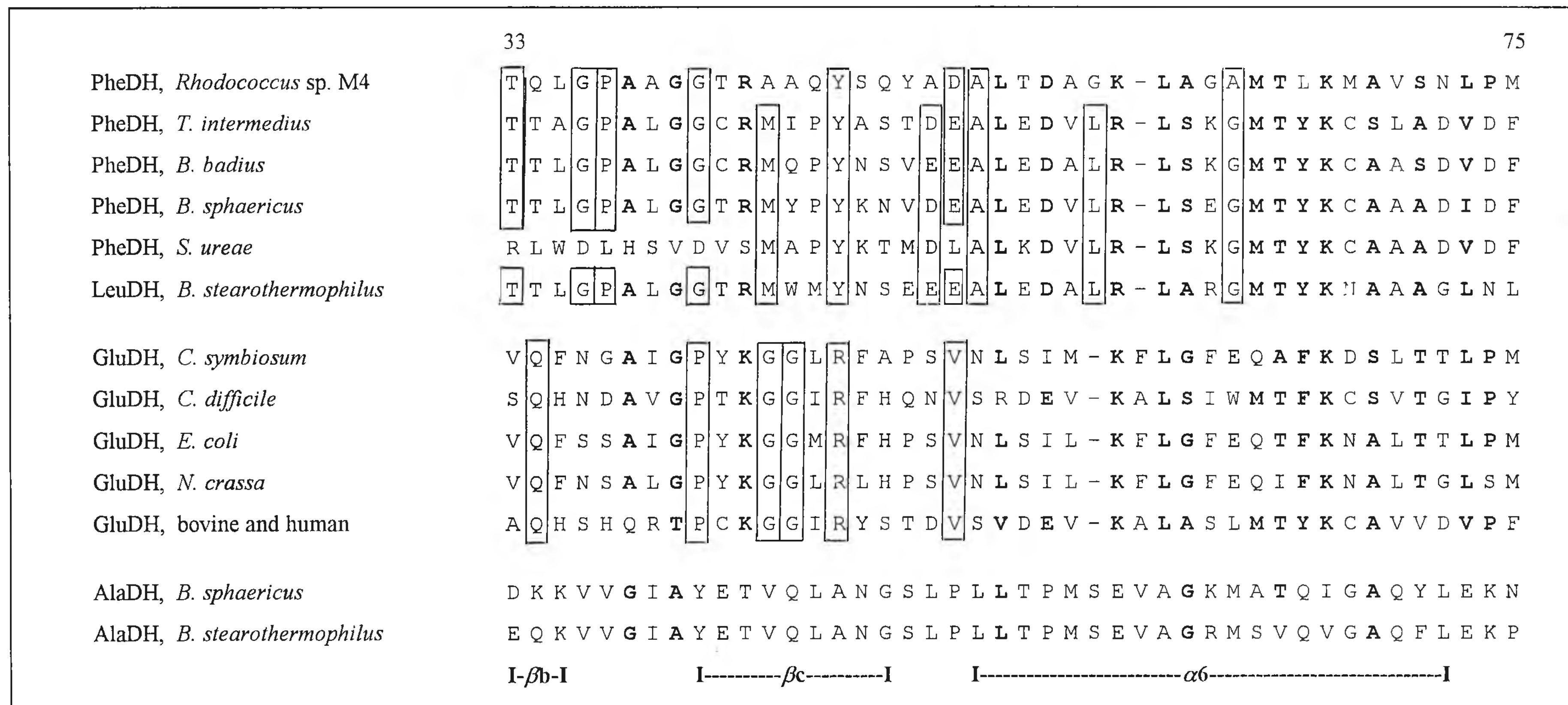

Figure 1.4 Sequence comparison of conserved residues in putative catalytic domains of several NAD(P)$^+$-dependent amino acid dehydrogenase [22,24]

Numbering is for the *Rhodococcus* enzyme. Secondary structure descriptions are based on the three-dimensional structure of *C. symbiosum* GluDH. Conserved residues in all dehydrogenases are shown in **boldface**. Residues conserved in either the glutamate (GluDH) or phenylalanine (PheDH) /leucine (LeuDH) dehydrogenases are boxed. Specific references are as follows: *T.*; *Thermoactinomyces*, *B.*; *Bacillus*, *S.*; *Sporosarcina*, *C.*; *Clostridium*, *E.*; *Escherichia*, *N.*; *Neurospora*.

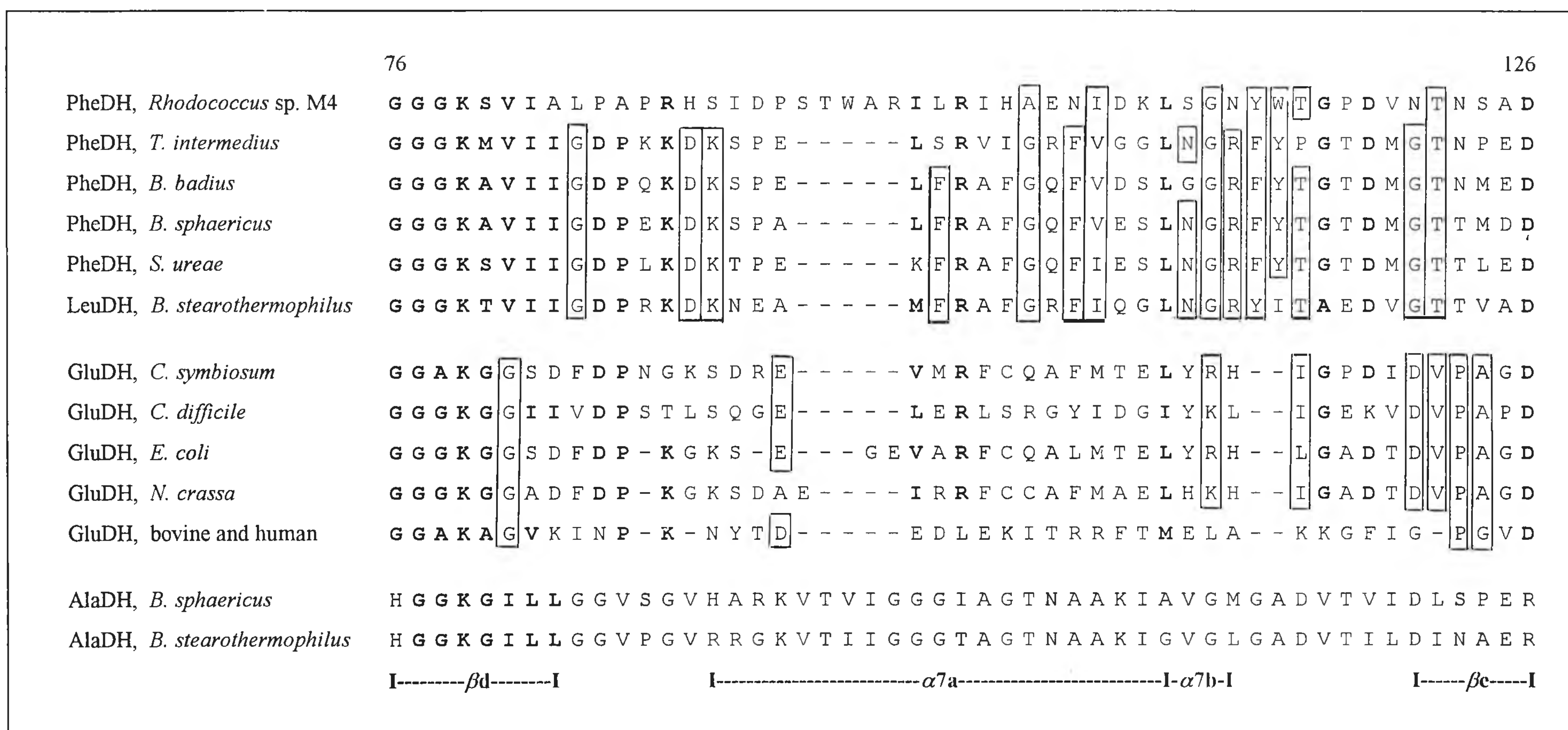

Figure 1.4 Sequence comparison of conserved residues in putative catalytic domains of several NAD(P)$^+$-dependent amino acid dehydrogenase [22,24] (Continue)

Numbering is for the *Rhodococcus* enzyme. Secondary structure descriptions are based on the three-dimensional structure of *C. symbiosum* GluDH. Conserved residues in all dehydrogenases are shown in *boldface*. Residues conserved in either the glutamate (GluDH) or phenylalanine (PheDH) /leucine (LeuDH) dehydrogenases are boxed. Specific references are described previously .

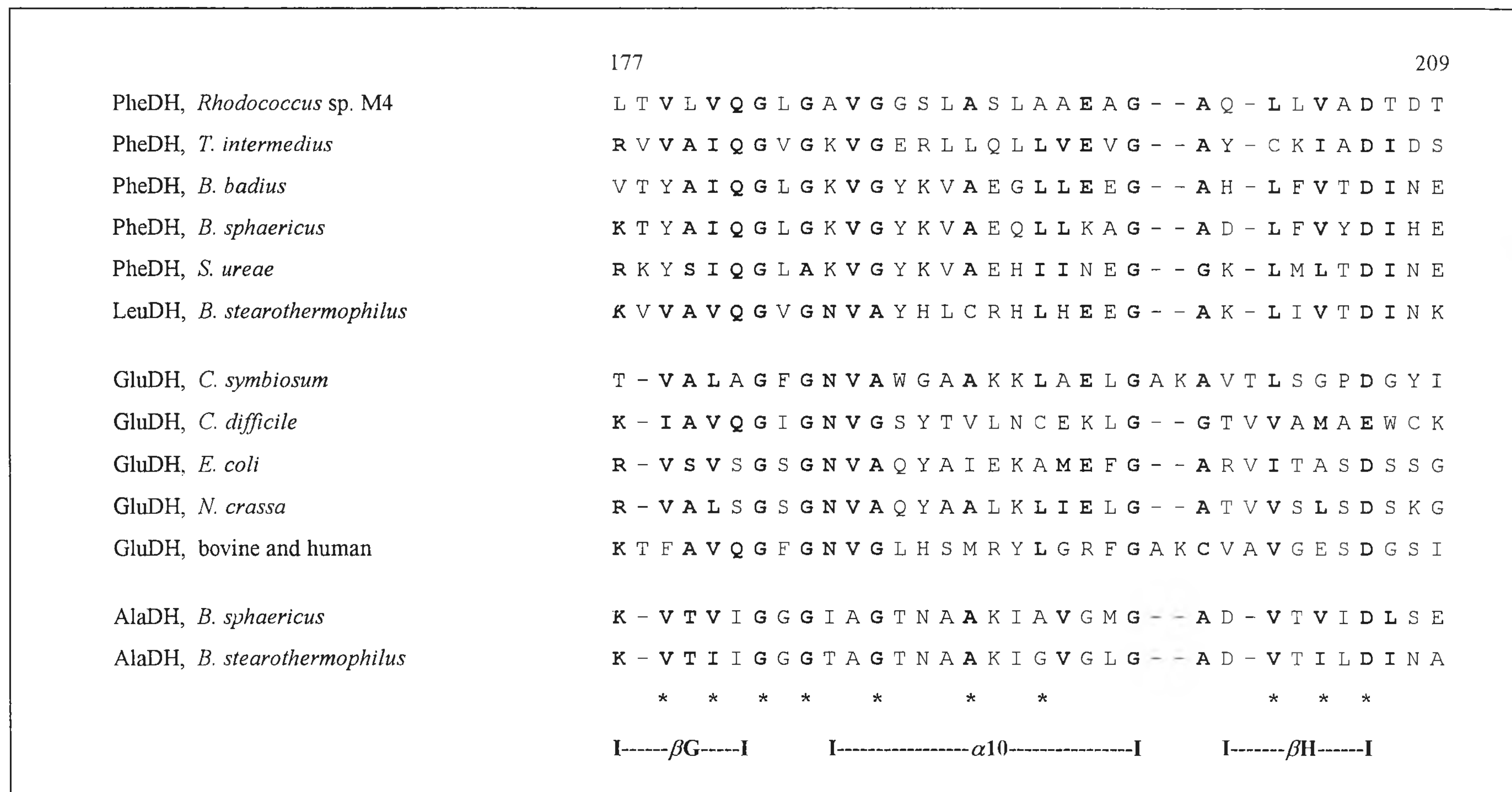

Figure 1.5 Sequence comparison of pyridine nucleotide-binding regions of several NAD(P)$^{+}$-dependent amino acid dehydrogenase [22,24]

Numbering is for the *Rhodococcus* enzyme. Secondary structure descriptions are based on the three-dimensional structure of *C. symbiosum* GluDH. Conserved residues are indicated in *boldface*. Residues important to the formation of the Rossmann fold are indicated by asterisks. Specific references are as listed in the legend to Figure 1.4.

and residues participating in substrate specificity of amino acid dehydrogenases [25-26] including PheDH, in particular with the *T. intermedius* enzyme studied by Kataoka *et al.* [27-28]. On the basis of the comparison of sequence homology between the *T. intermedius* PheDH and the *B. sterothermophilus* LeuDH, a 59 % sequence similarity in their substrate binding domains is found between the two enzymes (overall identities, 47 %). However, their substrate specificities are different; PheDH acts preferentially on L-phenylalanine and L-tyrosine whereas LeuDH acts on L-leucine and some other aliphatic L-amino acids. Thus, the substrate recognition mechanism of these two enzymes was investigated by means of site-directed mutagenesis. The hexapeptide segment (^{124}F-V-H-A-A-^{129}R) in the substrate binding domain of PheDH was replaced by the corresponding part of LeuDH (^{123}M-D-I-I-Y-^{128}Q) and the results suggested that the hexapeptide segment plays an important role in the substrate recognition by PheDH. In 1994, the same research group reported the genetic construction of a chimeric enzyme from two functionally related proteins sharing extensive sequence similarity and assessment of its catalytic properties, which was expected to provide valuable information on the structure-function relationship of the parent proteins [29]. 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 by genetic engineering and characterized in order to elucidate the substrate recognition mechanism of the two enzymes. Although the catalytic efficiency of the chimeric enzyme on L-phenylalanine is 6 % of that of the parental PheDH, the chimeric enzyme showed a similar K_m value for L-phenylalanine, pH optimum and the same stereospecificity for hydrogen transfer at the C-4 position of the NADH. In contrast, the substrate specificity of the chimeric enzyme differs from PheDH; the chimeric enzyme showed a lower substrate specificity than the parental PheDH (Figure 1.6). In addition to phenylalanine and its derivatives, it acts on poor substrates of both parent enzymes such as L-methionine, L-tryptophan and L-phenylglycine in the oxidative deamination. Furthermore, the chimeric enzyme acts on L-branched chain amino acids such as L-valine and L-isoleucine. The specificity of the chimeric enzyme in the reductive amination is an admixture of the specificities of the two parent enzymes. This suggests that a amino acid dehydrogenase that exhibits new substrate specificity was created. Indeed, the same group also reported the combination of chemical

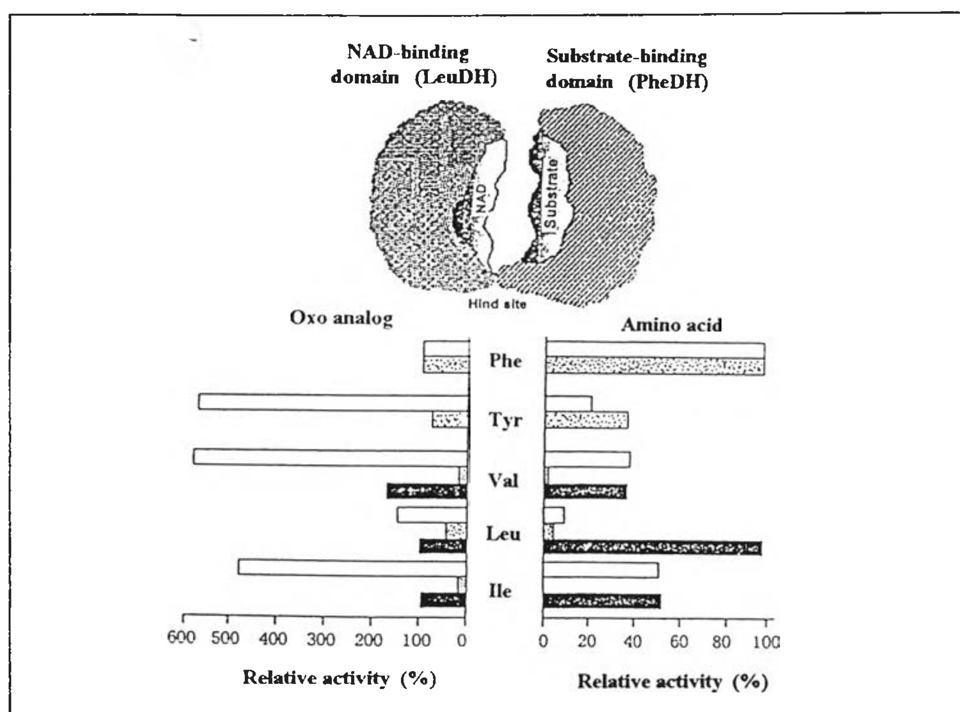

Figure 1.6 Scheme of the chimeric enzyme consisting of an amino terminal domain of phenylalanine dehydrogenase and a carboxy terminal domain of leucine dehydrogenase [(29)]

Comparison of substrate specificity of PheDH (□), chimeric enzyme (▩) and LeuDH (■) on both amination and deamination

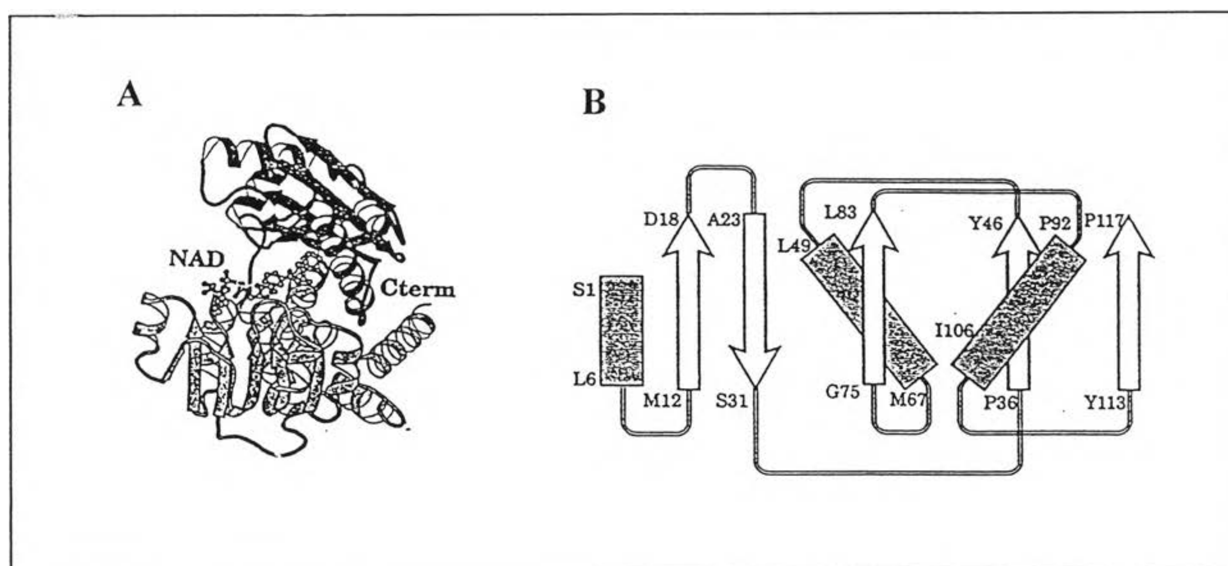

Figure 1.7 Structure of the *Rhodococcus* sp. M4 phenylalanine dehydrogenase [(16)]

(A) Ribbon representation of one subunit of PheDH • NAD^+ • phenylpyruvate ternary complex (B) The topology corresponding to the substrate binding domain. Rectangles and arrows represent α-helices and β-strands, respectively.

modification with a monoanionic acetylation reagent, methyl acetyl phosphate (MAP) and the site-directed mutagenesis of the *T. intermedius* PheDH which is useful not only for identification of active site lysyl residues (Lys-69, Lys-81) but also to elucidate the electrostatic environment around the active site.

In addition, PheDH from *B sphaericus* also has been studied on substrate recognition mechanism by Seah *et al.* [30]. Gly-124 and Leu-307, important residues in substrate specificity of the *B. sphaericus* PheDH were altered by site-specific mutagenesis to the corresponding residues in LeuDH; alanine and valine, respectively. The mutants showed decreased activity towards L-phenylalanine and increased catalytic activity towards most tested aliphatic amino acid substrates compared to the wild type.

By high-resolution x-ray analysis method, the structures of the dimeric PheDH from *Rhodococus* sp. M4 in the two ternary complexes with enzyme-NAD^+-phenylpyruvate and enzyme-NAD^+-β-phenylpropionate were recently reported by Vanhook *et al.* [16]. This is the first model of structure of the amino acid dehydrogenase with a ternary complex. Studies of the ternary complexes probably give more useful information for understanding the catalytic mechanism of enzymes. The PheDH is a homodimeric and each monomer is composed of distinct globular N- and C-terminal domains seperated by a deep cleft containing the active site. A ribbon representation of one subunit of the enzyme • NAD^+ • phenylpyruvate abortive complex is displayed in Figure 1.7 A. The N-terminal domain (Ser-1 to Gly-145) binds the amino acid substrate and plays an important role in the reaction on the subunit-subunit interface. Its motif contains five β-strands that form a mixed β-sheet with the overall topology shown in Figure 1.7 B. The C-terminal domain (Ala-146 to Ser-349) contains a typical Rossmann fold responsible for NAD binding as found for GluDH and LeuDH. This shows that amino acid dehydrogenases are composed of structurally independent coenzyme and substrate binding domains. The initial structural analyses of these two ternary complexes established that Lys-78 and Asp-118 function as the catalytic residues in active site. The studies have been continued by the same research group on the ionization behavior of these residues in steady-state turnover and also on the kinetic behavior of enzyme [17]. By analysis of the active-

site interactions in these models, structural data, along with the kinetic data, a chemical mechanism has been reformulated and proposed as in Figure 1.8.

1.4 Applications of phenylalanine dehydrogenase

Extensive research on characteristic and structure of PheDHs reflects their usefulness for applications catagorized to two major fields: industrial and medical fields.

1.4.1 Industrial applications

The commercial values of essential amino acids come from their wide applicability in both pharmaceutical and food industries. The worldwide market value of amino acids is approximately 2 billion dollars annually and the synthesis of optically active amino acids has been extensively studied [31]. Phenylalanine is an essential amino acid for human nutrition and is used as a component of amino acid infusions for medical purposes. It is a neuro-transmitter used to promote alertness. Because of its relationship to the action of the central nervous system, this amino acid can elevate mood, decrease pain, aid in memory and learning, suppress the appetite and treat Parkinson's disease [32-33]. In food industry, phenylalanine is used as a main intermediate of the diet artificial sweetener, aspartame [34-35]. Moreover, it is a precursor of benzaldehyde, an important aromatic compound participating in flavor, in the manufacture of cheese [36]. A variety methods may be used for the production of phenylalanine such as chemical synthesis [37], extraction from protein hydrolysates, fermentative or enzymatic methods. Direct fermentation leading to L-phenylalanine is known from alkanes with *Corynebacterium* sp. or from molasses [38-39]. In addition, other fermentative and enzymatic processes in L-phenylalanine production have been proposed and developed over the last two decades in several ways [40-41]. These include production from trans-cinnamic acid by *Rhodotorula glutinis* containing phenylalanine ammonia-lyase with 70 % conversion yield [42] ; from acetamidocinnamic acid by using

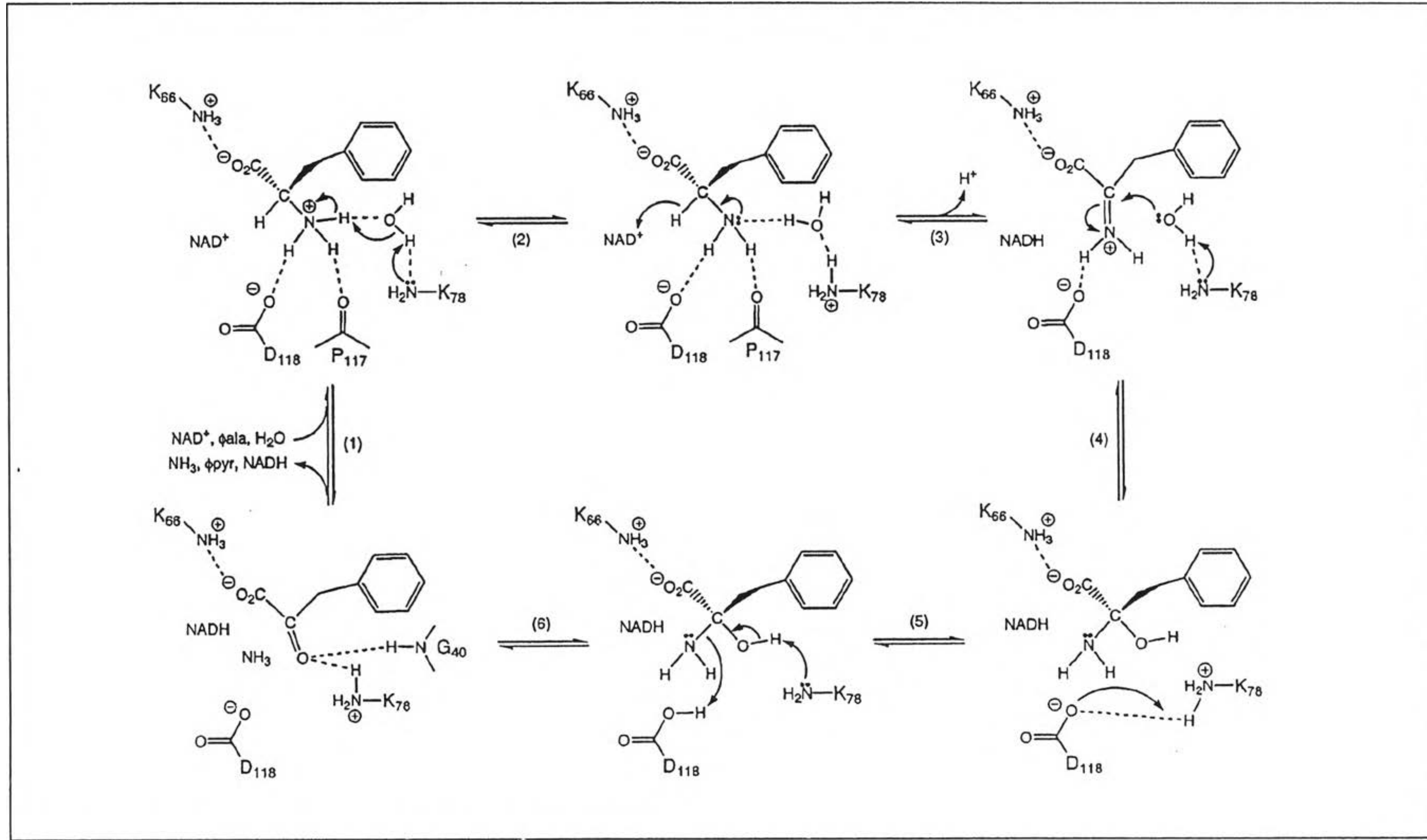

Figure 1.8 Chemical mechanism for phenylalanine dehydrogenase derived from kinetic and structural analyses [17]

a specific acylase and a transaminase found in *Alcaligenus faecalis* S-7 and *B. sphaericus* N-7 [(43)], respectively, or by using coimmobilized cells of *Corynebacterium* sp. and *Paracoccus denitrificans* [(44)]; from phenylpyruvate in combination with L-aspartate by aromatic amino acid aminotransferase [(45)]; from phenylpyruvate by *P. denitrificans* containing aminotransferase activity using intact cells (conversion yield 92.5 %) and using immobilized cells with κ-carragenan (conversion yield 90 %) [(46)]. Moreover, Chao *et al.* succesfully used the coupling reactions of aspartase and aminotransferase in *E.coli* for the selective production of L-aspartate (conversion yield 78 %) and L-phenylalanine (conversion yield 85 %), which are the essential raw materials utilized in the manufacture of aspartame [(47)].

Because the high stereospecificity enzymes are useful for the synthesis of chiral compounds such as L-amino acids. One of the enzymatic methods leading to L-amino acids is the reductive amination of corresponding α-keto acids with a specific NAD(H) amino acid dehydrogenase such as AlaDH, LeuDH or PheDH.

A method for enzymatic synthesis of L-phenylalanine with NAD^+-dependent PheDH has been investigated. However, the application of this enzyme to industrial production of L-phenylalanine has been hampered by the cost of coenzyme because it is complex and rather labile organic chemical. A multienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem. Thus, the enzymatic routes for phenylalanine production by PheDH, since has been reported, were summarized and catagorized in generous three ways. First, both the PheDH from *Brevibacterium* sp. [(48)] and *Rhodococcus* sp. M4 [(49)] have been used successfully for the continuous production of L-phenylalanine in a stirred tank membrane reactor. In a first step, phenylpyruvate was reductively aminated to L-phenylalanine by PheDH. The simultaneously oxidized NADH was regenerated by formate and formate dehydrogenase (FDH, E.C. 1.2.1.2) and therefore was required in catalytical amounts only (Figure 1.9A). In order to retain the coenzyme in analogy to the enzyme behind the ultrafiltration membrane, it was covalently bound to a water soluble polymer, polyethylene glycol 20,000. This way, the retention of the coenzyme by an ultrafiltration membrane could be accomplished

together with separation of the coenzyme from the product stream. With phenylpyruvate as substrate, nearly complete conversion can be reached. The activity of both enzymes was examined over a period of 12 days and showed a mean productivity as indicated in Table 1.3. As the similar enzymatic method, Asano *et al.* synthesized L-phenylalanine, tyrosine and some other amino aicds using a dialysis tube containing the *S. ureae* PheDH and *Candida boidinii* FDH [(50)]. The same group also reported that optically pure L-phenylalanine was synthesized with a yield of 99 % with a mixture of acetone-dried cells of *B. sphaericus* and *C. boidinii* as sources of PheDH and FDH, respectively [(51)].

Because of the instability of phenylpyruvate in aqueous solutions and its relatively high cost, two alternative routes have been studied. One starts from the racemic mixture of phenyllactate [(52)] while the other from acetamidocinnamic acid [(3)]. In both routes, phenylpyruvate is formed *in situ* and converted simultaneously by the action of PheDH to L-phenylalanine. The conversion of D,L-phenyllactate into the keto acid can be achieved utilizing the side reaction of two enzymes, D- and L-2-hydroxy-4-methyl-pentanoate dehydrogenase (2-hydroxy caproate dehydrogenase). NADH is regenerated continuously by the substrate oxidation (Figure 1.9 B). The kinetic properties of the enzyme involved in the cyclic reaction make this approach unfavourable. Acetamidocinnamic acid is another stable precursor of phenylpyruvate. Deacetylation results in an unstable enamine-imine derivative, which hydrolysis spontaneously to yield phenylpyruvate. The deacetylation can be accomplished enzymatically by an acylase isolated from a strain of *Brevibacterium* sp. In this route, FDH is necessary for coenzyme regeneration (Figure 1.9 C). A similar system also has been developed by Cho *et.al.* that an aminoacylase, inducibly formed in *B. thermoglucosidius* grown with a synthetic compound, acetamidocinnamic acid, and the *T. intermidius* PheDH were used for enzymatic synthesis of L-phenylalanine from chloroacetamidocinnamic acid [(53)]. The reaction system consisted of the hydrolysis of chloroacetamidocinnamic acid to phenylpyruvate by aminocyclase and the reductive amination of phenylpyruvate to L-phenylalanine by PheDH. The coenzyme NADH consumed was regenerated by a couple reaction with FDH. Under optimum conditions for L-phenylalanine production, more than 98 % conversion was obtained without

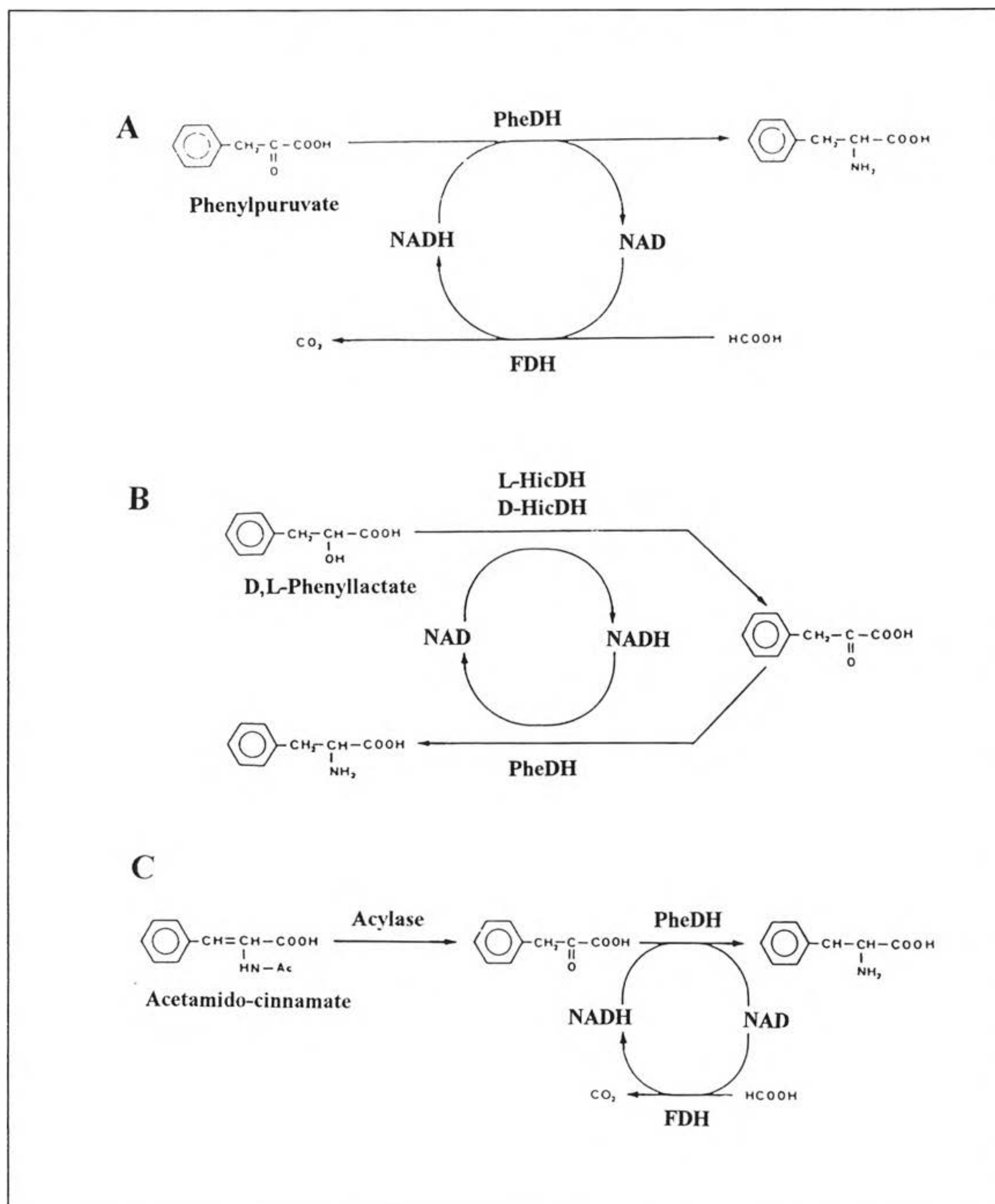

Figure 1.9 Enzymatic routes for the preparation of L-phenylalanine [(3)]

(A) Reductive amination of phenylpyruvate by phenylalanine dehydrogenase (PheDH) with simultaneously NADH regeneration using formate dehydrogenase (FDH) (B) Oxidation of DL-phenyllactate with D- and L-2-hydroxy-4-methylpentanoate dehydrogenase (HicDH) and simultaneous reductive amination of the *in situ* formed phenylpyruvate with PheDH. NADH is ' substrate-coupled ' regenerated from phenyllactate (C) *In situ* formation of phenylpyruvate by enzymatic deacetylation of acetamidocinnamic acid (acylase) followed by simultaneous reductive amination with PheDH

Table 1.3 Continuous production of L-phenylalanine with the aid of phenylalanine dehydrogenases and other dehydrogenases in an enzyme-membrane reactor

Enzyme mixture	Precursor	Conversion (%)	Space-time yield (g .l^{-1}.d^{-1})	Enzyme consumption (U)	Type of reaction routes (refered to Fig. 1.9)	Ref.
PheDH (*Brevibacterium* sp.) and FDH	phenylpyruvate	93.5	37.4	6102 (PheDH) 5969 (FDH)	A	48
PheDH (*Rhodococcus* sp. M4) and FDH	phenylpyruvate	95	456	1500 (PheDH) 150 (FDH)	A	49
PheDH , D-HmpDH and L-HmpDH [a]	DL-phenyllactate	43	28	-	B	52
PheDH , ACA acylase [b] and FDH	acetamido-cinnamate	88	277	1170 (acylase) 1770 (PheDH) 400 (FDH)	C	3

[a] HmpDH = 2-hydroxy-4-methylpentanoate dehydrogenase (or HicDH = 2-hydroxyisocaprate dehydrogenase)

[b] ACA acylase = Acetamidocinnamic acid acylase

- = No data

decomposition or racemization. This method may be promising as a commercial process, because chloroacetamidocinnamic acid and ammonium formate are both obtained easily at low prices. The comparison of continuous L-phenylalanine production with the aid of PheDHs and other enzymes in enzyme-membrane reactor is summarized in Table 1.3.

However, the instability of PheDH used in these processes has hampered the efficient operation of the systems and the industrial use of the systems depends chiefly on the cost of enzymes, although intact cells of microorganisms containing the enzymes can be used as catalyst in order to decrease costs [(54)]. In addition, genetic improvements through metabolic engineering are investigated. Galkin *et al.* recently reported a simple method for enzymatic synthesis of D and L-amino acids from α-keto acids with the recombinant *E. coli* TG1 cells which contained plasmids with heterologous genes necessary for biotransformation. L-amino acids were produced by thermostable L-amino acid dehydrogenase and FDH from α-keto acids and ammonium formate with only an intracellular pool of NAD^+ for the regeneration of NADH. By this method, plasmid containing FDH and PheDH genes was constructed (pFDHPheDH) for the synthesis of L-phenylalanine and L-tyrosine from phenylpyruvate and *p*-hydroxyphenylpyruvate with high yield of 95 % and 92 %, respectively. Moreover, the optical purity (enatiomeric excess) of them was 100 % [(54)].

1.4.1 Medical and pharmaceutical applications

PheDH has considerable commercial potential both for the chiral synthesis of novel nonprotogenic amino acids for use in the pharmaceutical industry and also for use as diagnostic reagents to monitor the serum levels of amino acids which accumulate in a range of metabolic diseases.

Phenylketonuria (PKU) and hyperphenylalaninemia, a related form of less harmful, are diseases mainly resulting from the deficiency of phenylalanine hydroxylase (EC. 1.14.16.1). Without this enzyme, phenylalanine and

it's breakdown intermediates from other enzyme routes accumulate in the blood and body tissues. PKU causes severe mental retardation if it is not early discovered in infancy (i.e., at 2-3 weeks of age) and treated immediately. Nowadays, it is mandatory in the United states and several other countries to have all newborns tested for PKU [55-57]. Much research is being directed towards the production of a simple sensitive test for the detection of elevated levels of phenylalanine in physiological fluids, a key parameter in PKU diagnosis, with enough speed and accuracy to allow the clinical control and the monitoring of dietary within a few hours. This has several advantages: (1) the clinician and the dietitian can compare the clinical and biochemical status of every patient and immediately make appropriate nutritional changes and (2) the general organization of the laboratory (in terms of time, reagents and labor cost) at the reference center, which usually is a children's hospital, is facilitated.

Phenylalanine can be measured in serum or plasma or in dried blood spots collected on filter paper. Microbiological method (Guthrie test), which is used worldwide as a semiquantitative test, is of low precision and cannot be applied in infants on antibiotic treatment [58]. Fluorimetric method can be automated ,but is susceptible to interference, especially for blood spots [59]. HPLC and amino acid analysis methods are accurate and precise but require expensive equipment, special expertise and are time comsuming [60-61]. Spectrophotometric methods using phenylalanine ammonia lyase, L-phenylalanine oxidase or derivative spectrophotometry are not routinely applied because of their insufficient specificity for phenylalanine [62]. None of these methods offers the combination of rapid, accurate and technically straightforward analysis.

Since the discovery of PheDH by Hummel *et al.*, PheDH seem to be the possible new and simpler ways to determined phenylalanine concentrations without interferences from other amino acids and phenylalanine derivatives. Hummel *et al.* were the first to suggest that PheDH from *Brevibacterium* sp. could be used to measure phenylalanine in biological samples [63]. However, the enzyme was not entirely specific for phenylalanine, which potential for misleading positive results arising from the activity of PheDH's towards other amino acids, especially for tyrosine. In 1989, Wendel *et al.* developed a colorimetric method to determine L-

phenylalanine in plasma or serum by coupling simultaneously the reaction of the *Rhodococcus* sp. M4 PheDH and a second reaction in which initially formed NADH and diaphorase converted iodonitro tetrazolium chloride (INT) to a formazan. This product was measured in the visible range at 492 nm [(64)]. The catalyzed reactions were summarized in Figure 1.10. This simple, rapid, accurate and precise photometric method can be applied for clinical routine and gave results identical to those of automated amino acid analysis. Later, the same research group also improved the sensitivity by devising a microplate assay [(65)] and a spectrophotometric end point assay [(66)] for routine hyperphenylalaninemia newborn screening. In addition, PheDH activity linked to a tetrazolium/intermediate electron accepter detector system has been produced in kit form as the Quantase[TM] phenylalanine assay for dietary monitoring using plasma or blood spots in manual and microtitre plate formats [(67)]. During 1989-1994, Cooper and colleagues developed and proposed the new method in determination of L-phenylalanine and phenylpyruvate in deproteinized tissue extracts based on enzymatic cycling assays [(68-69)]. The procedure involved the coupling of bacterial PheDH with rat kidney cytosolic glutamine transaminase K (Assay I). They suggested that glutamine transaminase K had a relatively broad specificity toward α-keto acids, but had a high affinity for phenylpyruvate ($K_m \leq 20$ μM) and PheDH exhibited some activity with tyrosine and other amino acid, but had the highest activity with (and affinity for) L-phenylalanine. Therefore, neither enzyme was completely specific for the L-phenylalanine/ phenylpyruvate pair. By combining the two enzymes in a recycling assay, the specificity toward L-phenylalanine is greatly improved. The coenzyme regeneration in system was provided by the combination of PheDH, L-amino acid oxidase and catalase (Assay II). This method provided a means of amplification so that small quantitives of a metabolite may be easily measured. The reactions in this cycling assay were shown in Figure 1.11.

However, these enzymatic assays are spectrophotometric or colormetric detection schemes which can be problematic when working with blood samples due to interferences from absorbance by various compounds and the turbidity of the sample. In addition, the enzyme cannot be reused after each assay and is only stable in solution for a few hours. This is economically unfeasible for clinical

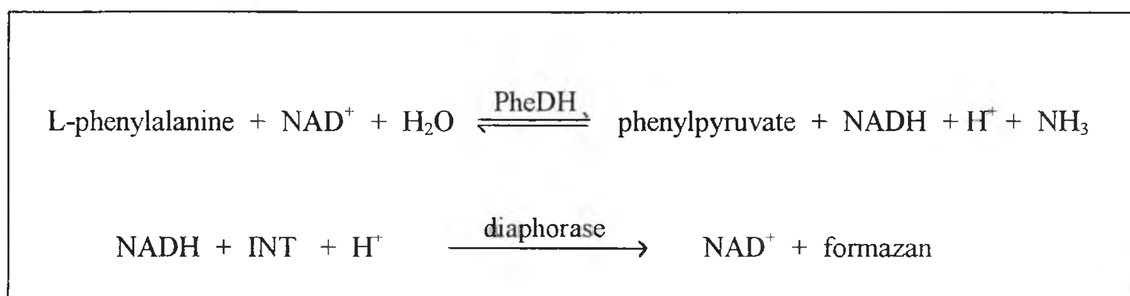

Figure 1.10 Coupling reactions of phenylalanine dehydrogenase and diaphorase for the determination of phenylalanine in plasma or serum [64]

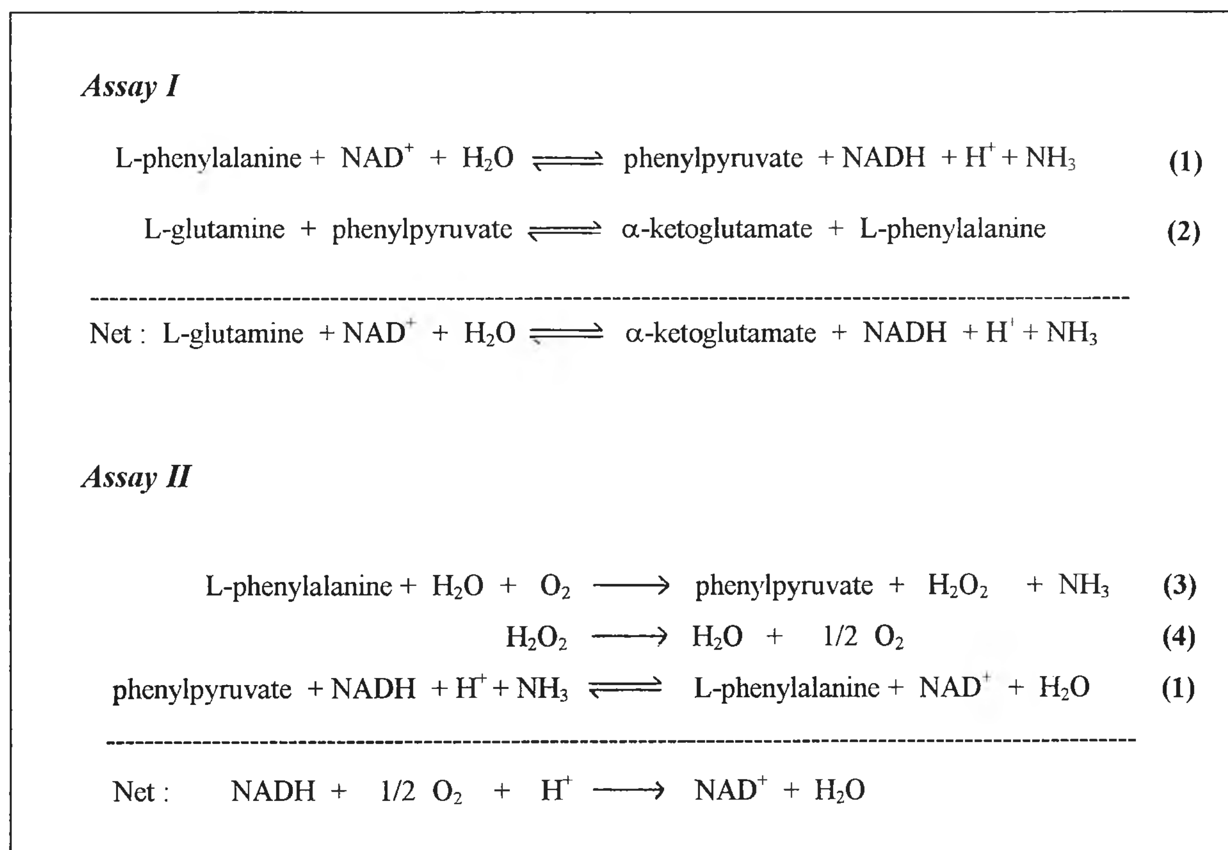

Figure 1.11 Recycling assay reactions for the determination of L-phenylalanine and phenylpyruvate in human blood [68-69]

Enzyme system in assay I contains PheDH (eq.1) and glutamine transaminase K (eq.2) and in assay II contains PheDH (eq.1), L-amino acid oxidase (eq.3) and catalase (eq.4).

analysis (where there are large number of samples) due to the high cost of obtaining enzymes. A less expensive alternative that can provide a fast and simple quantitative measurement of phenylalanine is recently described by Huang *et al.* by using an enzyme carbon paste electrode containing three different enzymes [70]. This sensor was based on the enzymatic/electrochemical recycling of tyrosinase in combination with salicylate hydroxylase and the *Rhodococcus* sp. M4 PheDH. As shown in Figure 1.12, salicylate hydroxylase and tyrosinase were coimmobilized first in a carbon paste electrode for the sensitive detection of NADH and this salicylate hydroxylase / tyrosinase bienzyme system was then coupled with PheDH for L-phenylalanine determination.

In the pharmaceutical purpose, PheDH also have been used in antihypertensive drug synthesis processes as described by Hanson *et al.* and Patel [71-72]. (*S*)-2-Amino-5 (1,3-dioxolan-2yl)-pentanoic acid (acetal amino acid or allysine ethylene acetal) is one of three building blocks used in an alternative synthesis of omapatrilat (VANLEV), an antihypertensive drug or vasopeptidase inhibitor now in clinical trials. Racemic allysine ethylene acetal has previously been prepared in an 8-step synthesis from 3,4-dihydro-2H-pyran for conversion into 1-piperideind-6-carboxylic acid, an intermediate for biosynthesis of β-lactam antibiotics. Their goal was to prepare allysine ethylene acetal compound by a simpler and more convenient route for synthesis of omapatrilat. The reductive amination of keto acids using amino acid dehydrogenases has been shown to be a useful method. Reductive amination of keto acid acetal to acetal amino acid was demonstrated using the *T. intermedius* PheDH. The reaction required ammonia and NADH. NAD^+ produced during the reaction was recycled to NADH by the oxidation of formate to CO_2 using FDH. An initial process was developed using heat-dried cells of *T. intermedius* as a source of PheDH and heat-dried cells of methanol-grown *C. boidinii* as a source of FDH (Figure 1.13). An improved process using PheDH from *T. intermedius* expressed in *E. coli* in combination with *C. boidinii* as a source of FDH and third generation process using methanol-grown *Pichia pastoris* containing endogenous FDH and recombinant protein expressing *T. intermedius* PheDH were also developed.

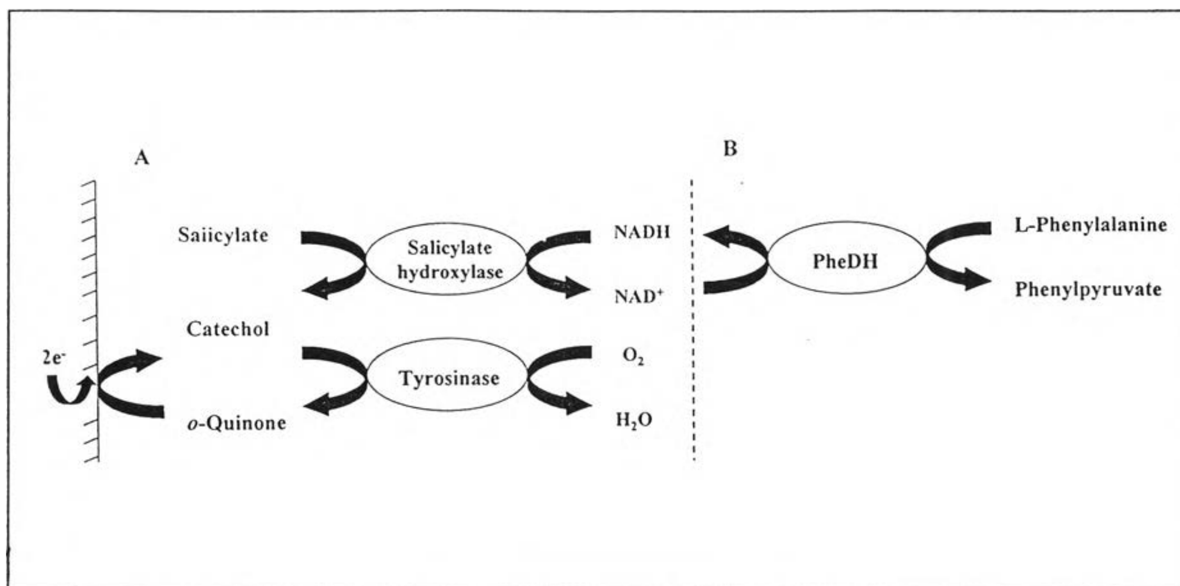

Figure 1.12 Schematic representation of an NADH-detecting biosensor [70]

(A) the salicylate/tyrosinase bioelectrocatalytic recycling system

(B) the coupling of PheDH to the bienzyme system

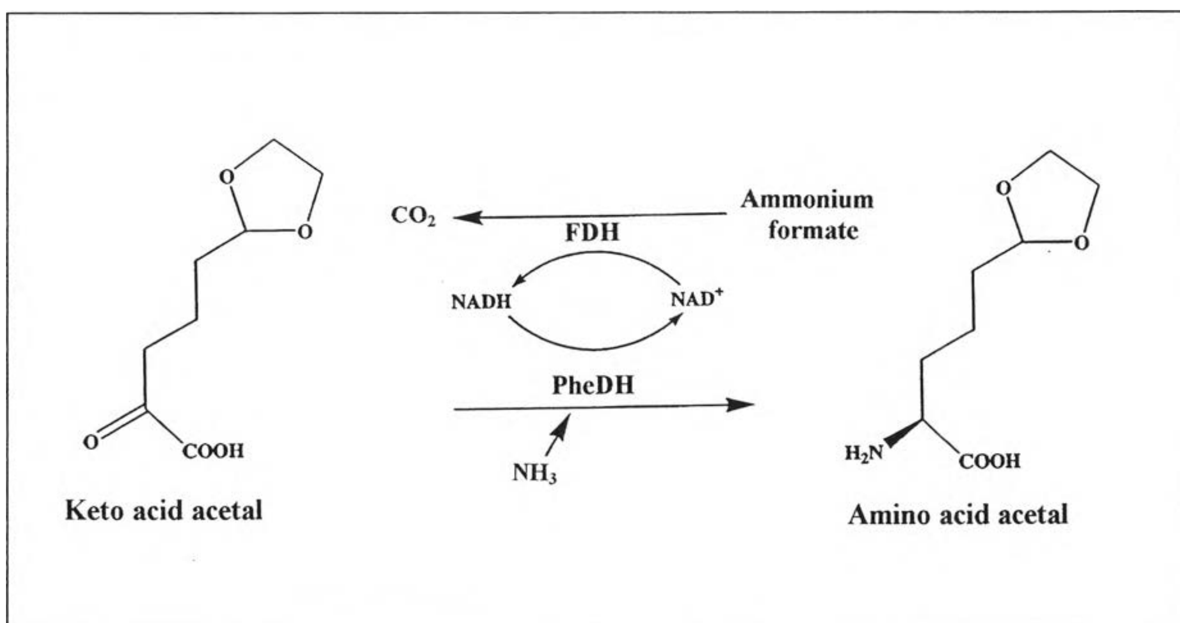

Figure 1.13 The synthesis of allysine ethylene acetal by phenylalanine dehydrogenase in pharmaceutical industry [71-72]

Reductive amination of keto acid acetal to amino acid acetal (allysine ethylene acetal) was catalyzed by PheDH and regeneration of NADH was carried out using FDH.

1.5 Objectives of this research

Since most amino acid dehydrogenases are from mesophilic bacteria, which have limited property in thermostability, much attention is being paid to the thermophilic bacteria producing thermostable enzyme. However, the application of these thermophiles to industrial processes has hampered by the cost of operating control systems because high temperature system was used for increasing bacterial growth. Therefore, the enzyme from mesophilic bacteria, which has thermotolerant property, has been proposed to be an interesting alternative because these bacteria can grow and produce the enzyme well at room temperature (25-35°C). The greatest attraction of using these bacteria are not only the ability to grow and produce enzyme well even at room temperature, but also still stable for high temperature (45-50°C) during industrial operations. In the previous study, thermotolerant bacteria producing amino acid dehydrogenase were screened by formazan forming and spectrophotometric method from various areas in Thailand and Japan by Suriyapanpong *et al.* [(73)]. Two, 4 and 6 isolates with NAD-dependent L-phenylalanine, L-leucine and L-serine dehydrogenase were obtained. Interestingly, high activity of PheDH from those two isolates was observed and the bacteria were named BC1 and BC2. Later, the isolate BC1 was identified as *Bacillus badius* by Chareonpanich *et al.* [(74)]. As an initial step in understanding the relationship between the structure and function of PheDH, the purification and characterization of enzyme are necessary to investigate.

The objective of this thesis :

1. To determine the optimal conditions for PheDH production bacterial strain BC1 and BC2
2. To purify PheDH
3. To characterize the biochemical properties of PheDH
4. To determine the effect of various group-specific reagents on PheDH activity.
5. To study kinetic mechanism of PheDH