Critical Reviews in Biochemistry and Molecular Biology, 29(6):415–467 (1994)

The Biochemistry and Enzymology of Amino Acid Dehydrogenases

*Norbert M. W. Brunhuber and John S. Blanchard**

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

* Author to whom correspondence should be addressed.

Referee: Dr. Harvey F, Fisher, Dept. of Veterans Affairs, Medical Center, 4801 Linwood Boulevard, Kansas City, MO 64128

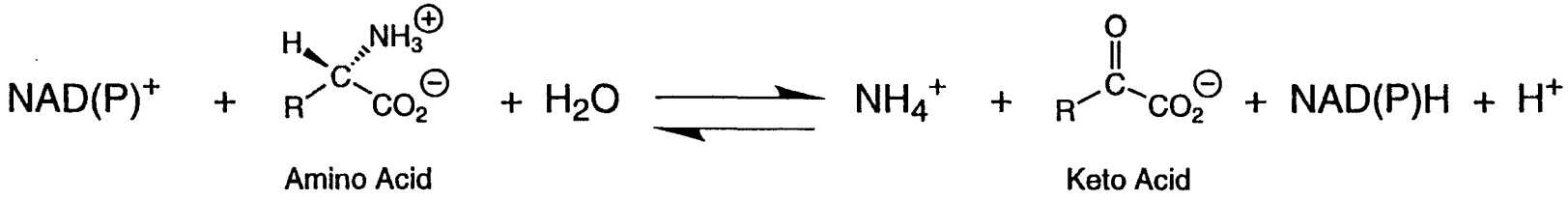
ABSTRACT: This review is an exhaustive description of the biochemistry and enzymology of all 17 known NAD(P)$^+$-amino acid dehydrogenases. These enzymes catalyze the oxidative deamination of an amino acid to its keto acid and ammonia, with the concomitant reduction of either NAD^+ or $NADP^+$. These enzymes have many important applications in industrial and medical settings and have been the object of prodigious enzymological research. This article describes all that is known about the poorly characterized members of the family and contains detailed information on the better characterized enzymes, including valine, phenylalanine, leucine, alanine, and glutamate dehydrogenases. The latter three enzymes have been the subject of extensive enzymological experimentation, and, consequently, their chemical mechanisms are discussed. The three-dimensional structure of the *Clostridium symbiosum* glutamate dehydrogenase has been determined recently and remains the only structure known of any amino acid dehydrogenase. The three-dimensional structure and its implications to the chemical mechanisms and rate-limiting steps of the amino acid dehydrogenase family are discussed.

KEY WORDS: amino acid, amino acid dehydrogenase, catalytic mechanism, chemical mechanism, isotope effects, kinetic mechanism, metabolism, nucleotide specificity, pH profiles, pyridine nucleotide, substrate specificity.

I. GENERAL DESCRIPTION OF AMINO ACID DEHYDROGENASES

The amino acid dehydrogenases (EC 1.4.1.X) are a family of enzymes that are part of the oxidoreductase superfamily. They catalyze the removal of the amino group, generally from an L-amino acid, with the formation of the corresponding keto acid and with the concomitant reduction of NAD(P)$^+$. The general formula for this reaction can be written as shown in Scheme 1. Any molecule can be considered an amino acid if it has an amino group and a carboxylic acid. Most amino acid dehydrogenases are specific for the α-amino acids

1040-9238/94/$.50



SCHEME 1. General reaction of amino acid dehydrogenases.

TABLE 1
List of NAD(P)$^+$-Dependent Amino Acid Dehydrogenases Listed in the 1992 Enzyme Nomenclature Catalog

Substrate	EC Number
Alanine	1.4.1.1
L-Amino Acid (general)	1.4.1.5
L-*erythro*-3,5-Diaminohexanoate	1.4.1.11
2,4-Diaminopentanoate	1.4.1.12
meso-Diaminopimelate	1.4.1.16
Glutamate	1.4.1.2-4
Glycine	1.4.1.10
Leucine	1.4.1.9
Lysine (α-deaminating)	1.4.1.15
Lysine (ε-deaminating)	1.4.1.18
N-Methylalanine	1.4.1.17
Phenylalanine	1.4.1.20
Serine	1.4.1.7
Tryptophan	1.4.1.19
Valine	1.4.1.8

incorporated into proteins, but others are known that operate on β- and ε-amino acids, for example. These enzymes are found in an extensive number of diverse prokaryotic and eukaryotic organisms. In this review, the oxidative deamination of the amino acid substrate will be defined as the forward direction, and the reductive amination of the keto acid will be referred to as the reverse direction.

Amino acid dehydrogenases are categorized based on the specificity they display toward their amino acid substrate. Currently there are 17 different enzymes listed in the 1992 *Enzyme Nomenclature* catalog (see Table 1). This review will present what is known about each of these 17 different enzymes. The sections discussing the less well-characterized enzymes will often present all that is known about them. The sections discussing the best characterized enzymes; leucine, alanine, and glutamate dehydrogenases are nearly complete summaries, but are not strictly exhaustive. The individual section on glutamate dehydrogenase in particular focuses mainly on mechanism since there are two reviews available that describe this amino acid dehydrogenase fairly comprehensively (Smith et al., 1975; Hudson and Daniel, 1993). There are two short, general reviews on amino acid dehydrogenases that have recently appeared (Hummel and Kula, 1989; Ohshima and Soda, 1990).

II. FUNCTIONS OF AMINO ACID DEHYDROGENASES

Amino acid dehydrogenases are important enzymes that exist at the interface of nitrogen and carbon metabolism. Before

the carbon skeleton of an amino acid can be metabolized for energy through the glycolytic or TCA cycle reactions, the amino group must first be removed. This can be accomplished by pyridoxyl phosphate-dependent transaminases, which transfer the amino group from one amino acid to another, usually glutamate, a predominant nitrogen storage molecule of the cell (see Scheme 2). A second method is to employ a deaminases such as phenylalanine-ammonia lyase, or aspartase, which do not change the oxidation level of the substrate (see Scheme 3). The third option is to use an amino acid dehydrogenase. These enzymes have the advantage of removing the amino group as free ammonia, which can then be used by the cell in diverse ways. In addition, the amino acid dehydrogenases couple deamination to the production of a reduced nucleotide, which can be subsequently used in a variety of energy requiring processes. Amino acid dehydrogenases, like the transaminases and hydrolases, operate reversibly, and are capable of sequestering ammonia by incorporation into an amino acid. This occurs in certain nitrogen fixing organisms, as well as others when their levels of free ammonia are high.

The amino acid dehydrogenases have been studied intensively because of their ubiquitous distribution and also because of a number of potential industrial applications. They have been used in the design of biosensors, where they can monitor the levels of free amino acids in solution, especially in screening blood serum for elevated levels of amino acids associated with certain diseases. The amino acid dehydrogenases have also been used in the industrial synthesis of amino acids. Since these enzymes act stereospecifically, they produce the pure, natural L-isomer of the amino acids, which are important for pharmaceutical and dietary consumption purposes.

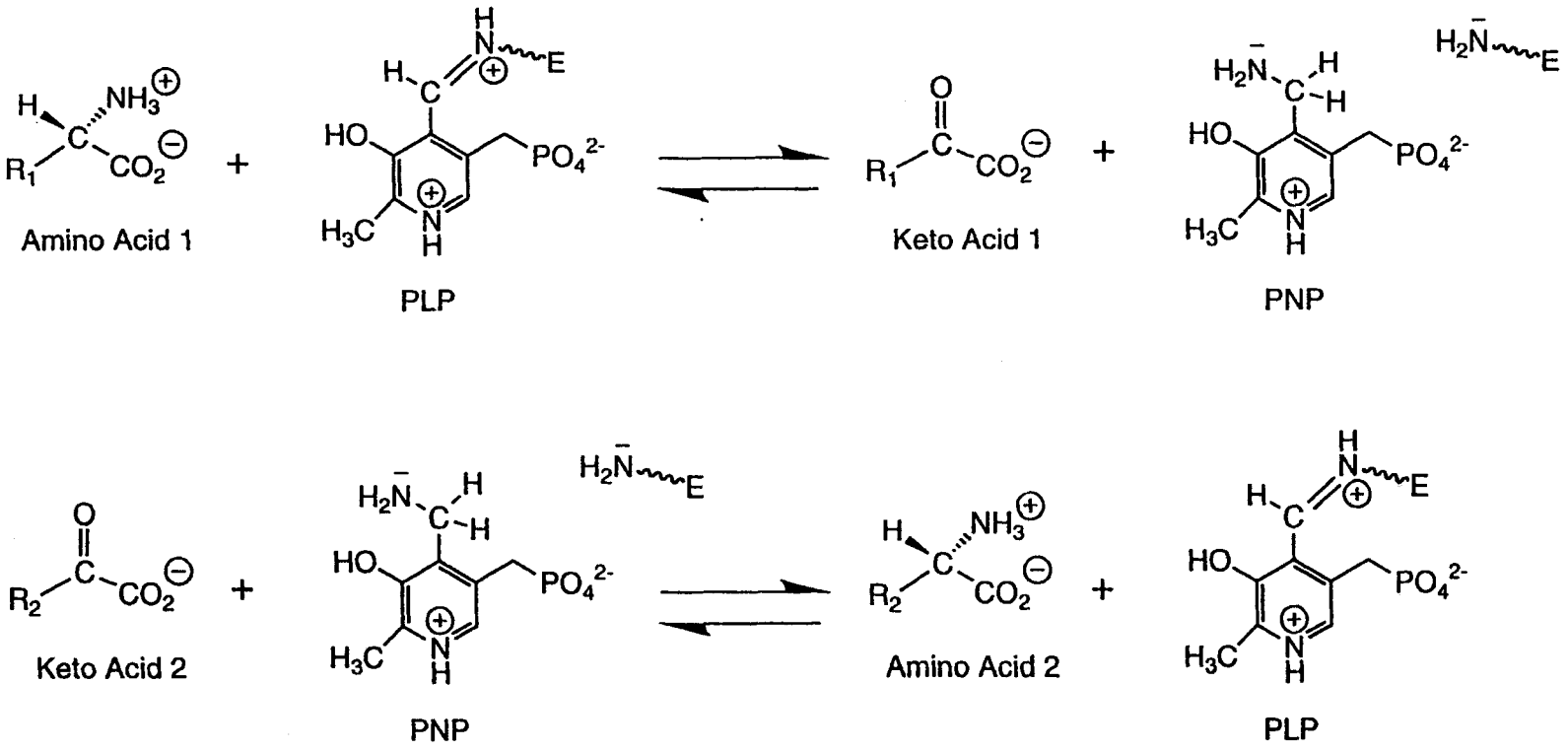
III. SPECIFIC EXAMPLES OF AMINO ACID DEHYDROGENASES

A. Poorly Characterized Amino Acid Dehydrogenases

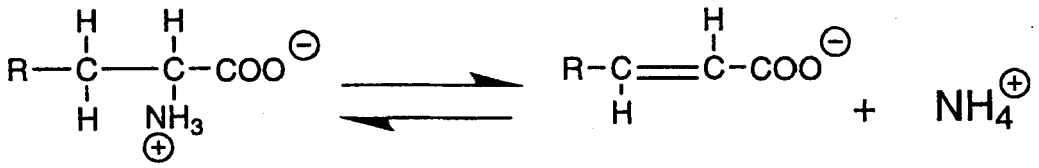
There are a number of amino acid dehydrogenases listed in *Enzyme Nomenclature* for which there is very little published literature. These ten enzymes are summarized here, in most cases with all available pertinent information known about them. This first section will cover the amino acid dehydrogenases that use serine, tryptophan, L-amino, glycine, *N*-methylalanine, L-*erythro*-3,5-diaminohexanoate, 2,4-diaminopentanoate, lysine, lysine ε-, and *meso*-α,ε-diaminopimelate as their substrates.

1. Serine Dehydrogenase

A single Russian report on serine dehydrogenase (1.4.1.7) demonstrated the NAD^+-specific deamination of serine to 3-hydroxypyruvate (Kretovich and Stepanovick, 1966). The protein, isolated from parsley leaves, was reported to be very labile. The enzyme has an optimum pH of 10 in the direction of serine synthesis, and the activity could be inhibited by 5 μ*M* *p*-chloromercuribenzoate, a sulfhydryl alkylating agent. Two K_m values were reported; that of hydroxypyruvate (0.5 μ*M*) and of am-



SCHEME 2. General reaction of amino acid transaminases.



SCHEME 3. General reaction of amino acid lyases.

monia (15 μ*M*). No other reports of this activity from any source have appeared since this report.

2. Tryptophan Dehydrogenase

Tryptophan dehydrogenase (1.4.1.19) has also been poorly characterized, with the only example of this enzyme being isolated from spinach leaves (Vackova et al., 1985). It catalyzes the NAD^+-or $NADP^+$-specific conversion of tryptophan into (indol-3-yl)pyruvate. The researchers noted that the activity was higher in chloroplasts than in mitochondria, and that it could be increased by the addition of calcium ions.

3. L-Amino Acid Dehydrogenase

L-amino acid dehydrogenase (1.4.1.5) was isolated from *Clostridium sporogenes* and is specific for $NADP^+$ as a substrate (Nisman and Mager, 1952). Although no quantitation was provided, the authors report a broad specificity for amino acid substrates. The amino acid substrates, listed in order of descending activity, are L-alanine > L-norleucine > L-leucine > L-isoleucine > L-valine. The lack of a serious characterization of this enzyme leads one to suspect that this may simply be an alanine dehydrogenase.

4. Glycine Dehydrogenase

Another poorly characterized enzyme is glycine dehydrogenase (1.4.1.10) isolated from *Mycobacterium tuberculosis* ($H_{37}R_a$) (Goldman, 1959). This enzyme is NAD^+-specific and catalyzes the oxidative deamination of glycine to form glyoxylate. The enzyme displays noncompetitive inhibition by serine vs. glyoxylate with steady-state kinetic constants of K_{is} = 4.4 m*M* and K_{ii} = 3.6 m*M*, exhibits a K_m for glyoxylate = 0.22 m*M* and a K_m for ammonia = 2.9 M. The enzyme is optimally active for glycine synthesis at pH = 6.4, and a $K_{eq} = 2.3 \times 10^{-11}$ M^2 was reported (Goldman, 1959).

5. N-Methyl-L-Alanine Dehydrogenase

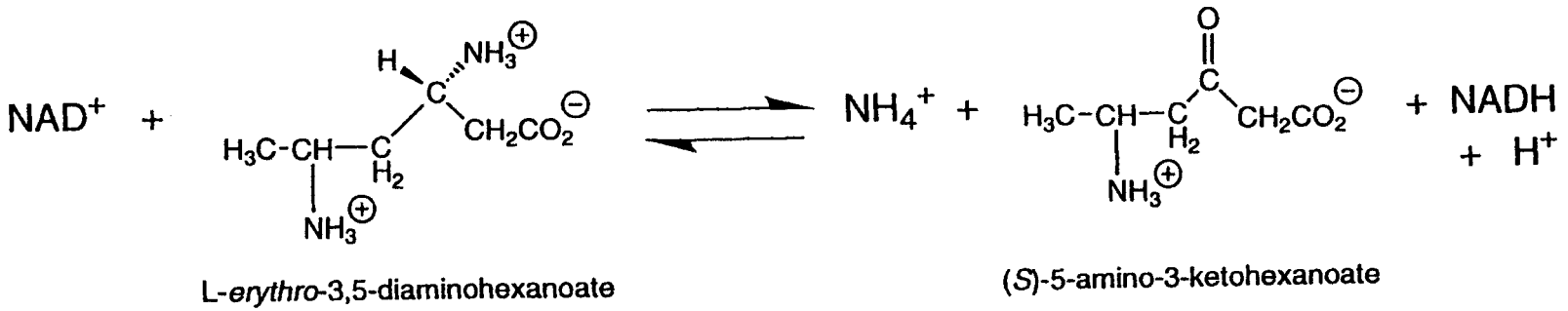
An unusual amino acid dehydrogenase has been isolated from *Pseudomonas* MS (Lin and Wagner, 1975) that catalyzes the formation of *N*-methyl-L-alanine from pyruvate and methylamine, rather than the usual amino acid dehydrogenase substrate, ammonia. The authors speculated that the organism uses this enzyme to sequester

potentially toxic methylamine, and subsequently catabolize it by a 1-carbon folate-dependent process. The enzyme was purified from authentic alanine dehydrogenase, and did not exhibit activity using ammonia as a substrate. The protein has a native molecular weight of 77 kDa, and a subunit molecular weight of 36.5 kDa, suggesting it exists in solution as a homodimer. It is $NADP^+$-specific, and can use alternate keto acid substrates such as oxaloacetate (64% of the rate of pyruvate) and α-ketobutyrate (14%). Its pH optimum is 8.4, in the direction of *N*-methyl-L-alanine oxidation, and is unstable unless DTT is present in solution. The steady-state K_m's at pH 8.5 for substrates are NADPH = 0.035 m*M*, pyruvate = 15 m*M*, and methylamine = 75 m*M*.

6. Diaminohexanoate and Diaminopentanoate Dehydrogenases

The following two amino acid dehydrogenases have similar substrates, but are poorly characterized. These enzymes have been reviewed by Stadtman (1973). The first is L-*erythro*-3,5-diaminohexanoate dehydrogenase (1.4.1.11), which converts this substrate into (*S*)-5-amino-3-ketohexanoate, illustrated in Scheme 4. It is the only amino acid dehydrogenase known to react with a β-amino acid. Two forms of this enzyme have been studied. One was isolated from *Clostridium* SB4, and is used by the organism in lysine catabolism (Baker et al., 1972). The protein is a tetramer of 140 kDa, composed of 37 kDa subunits, however active dimers are formed at high pH, or low ionic strength, and are about one third as active as the tetramer. The enzyme has an optimum pH of 7.0 for amino acid synthesis, and 8.9 for amino acid oxidation. The K_{eq} is 4×10^{-10} M^2 favoring amino acid formation, a value higher than that of other general amino acid dehydrogenases. The kinetic parameters exhibited by substrates are NAD^+ = 0.28 m*M*, diaminohexanoate = 0.18 m*M*, NADH = 0.074 m*M*, 5-amino-3-ketohexanoate = 0.26 m*M*, and ammonia = 140 m*M*. The inhibition constants of two noncompetitive inhibitors were measured, D-*erythro*-3,5-diaminohexanoate (K_i = 0.5 m*M*) and D,L-*erythro*-3,5-diaminohexanoate (K_i = 1.2 m*M*). The authors noted the enzyme's discrimination between amino acid substrate analogs. The enzyme is NAD^+-specific, but does use the analog 3-acetylpyridine NAD^+ (70%). The kinetic mechanism of the enzyme was partially determined, with NAD^+ binding first, followed by diaminohexanoate. After catalysis, the order of product release was not determined, except that NADH release was confirmed to occur last.

The other example of a diaminohexanote dehydrogenase was isolated from *C. sticklandii* (Baker and van der Drift, 1974). This protein was quite different from the *C.* SB4 example. The protein exhibits a native molecule weight of 79 kDa, and consists of two 39 kDa subunits. It also had a number of regulatory properties, not seen with the *C.* SB4 enzyme. These include inhibition by ATP and GTP, which supports the enzyme's role in lysine fermentation. Inhibition by the end products of lysine fermentation, 5-amino-3-ketohexanoate and ATP, suggests that regulation of this enzyme is crucial to balancing the use of lysine for production of NAD(P)H and other cellular needs. In addition, each subunit has two nucleotide binding sites, one being



SCHEME 4. Reaction of L-*erythro*-3,5-diaminohexanoate dehydrogenase.

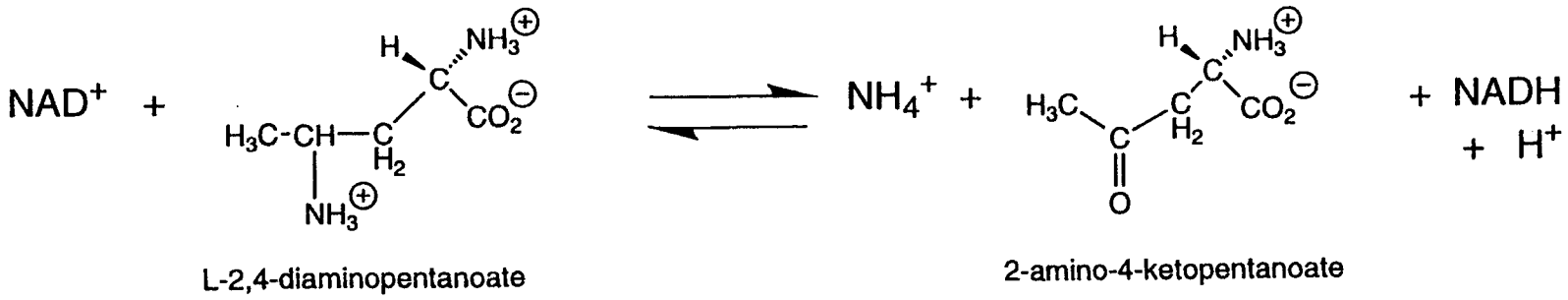
regulatory in nature, and the other in the catalytic site. Divalent cations were shown to activate the enzyme at high pH, and the enzyme has a pH optimum of 8.9 for amino acid oxidation, similar to the *C.* SB4 enzyme. The nucleotide specificity was broad, with NAD^+, $NADP^+$, and alternate nucleotides being used including, 3-acetylpyridineNAD^+ (10% of V of NAD^+) and deaminoNAD^+ (25% of V of NAD^+). The K_m's for the reaction are NAD^+ = 0.3 m*M* (V = 15.9 units/mg), $NADP^+$ = 0.03 m*M* (V = 17.1 units/mg), diaminohexanoate = 0.16 m*M*. The authors studied a number of reaction inhibitors, such as D-*erythro*-3,5-diaminohexanoate, K_i = 0.1 m*M*. They found that good inhibitors had a terminal $CH_3CH(NH_2)CH_2$ configuration, which was more important than chain length.

The second type of enzyme of this pair is known as 2,4-diaminopentanoate dehydrogenase (1.4.1.12), which is one of the few amino acid dehydrogenases that catalyzes the deamination of an α-amino acid at other than the α-position. This enzyme is involved in ornithine degradation, but may also play a role in lysine fermentation because of its ability to use 2,5-diaminohexanoate as an alternate substrate. The activity is known as C_4 activity when 2,4-diaminopentanoate is used, with the subscript denoting the position of the deamination. In this case, the product is 2-amino-4-ketopentanoate, as shown in Scheme 5. If 2,5-diaminohexanoate is used, the enzyme is known as a C_5 enzyme, since the product is 2-amino-5-ketohexanoate. This product cyclizes non-enzymatically to Δ^1-pyrroline-2-methyl-5-carboxylic acid, illustrated in Scheme 6. In comparison, the previously discussed L-*erythro*-3,5-diaminohexanoate dehydrogenase is a C_3 enzyme since it deaminates at the 3 position to form 5-amino-3-ketohexanoate.

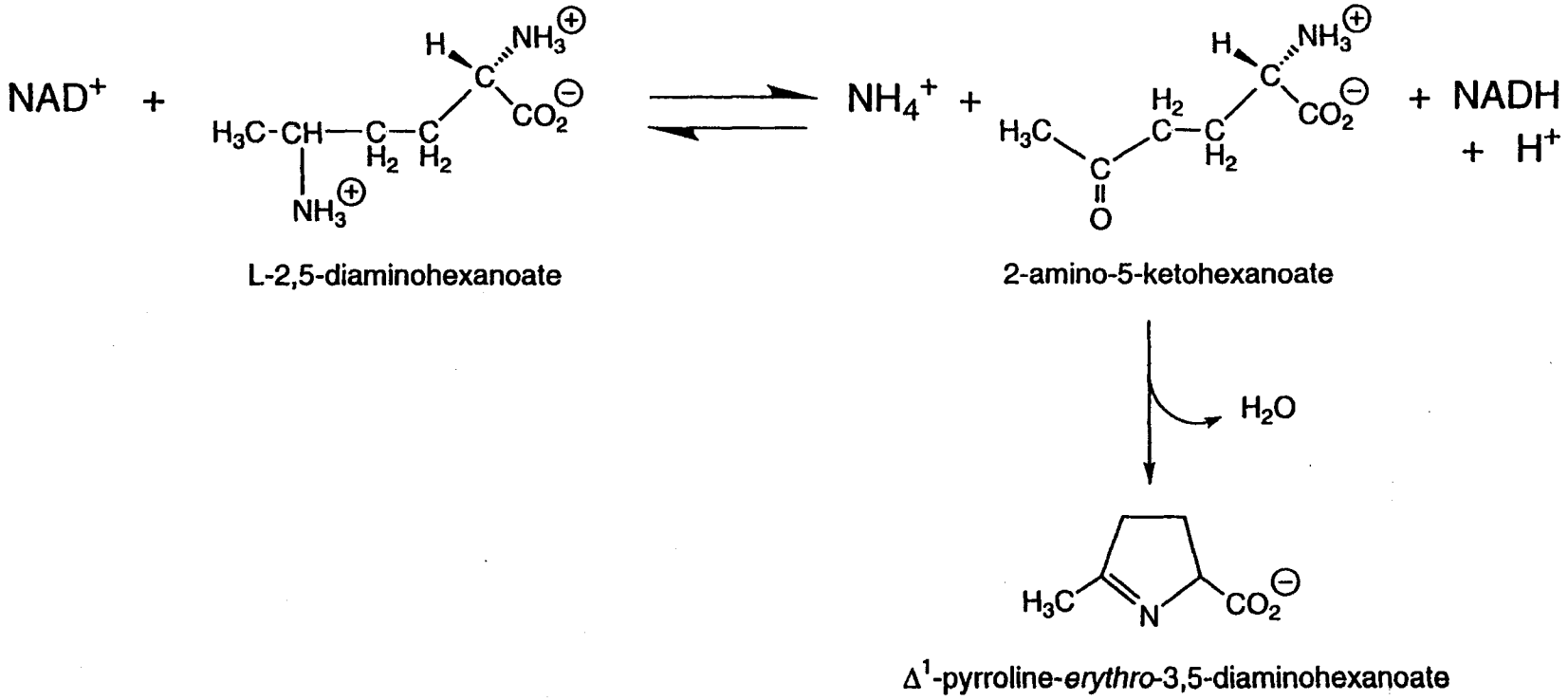
Two examples of diaminopentanoate dehydrogenase are known. The first was isolated from *C. sticklandii* (Tsuda and Friedmann, 1970). The reaction has a K_{eq} = 1.04×10^{-14} M^2, favoring amino acid synthesis, and the pH optimum for amino acid oxidation is 8.8. The enzyme displays only slight preferences for $NADP^+$, and exhibits Michaelis constants for NAD^+ = 1.8 m*M*, $NADP^+$ = 0.15 m*M*, and diaminopentanoate = 1.8 m*M*. If the slow substrate 2,5-diaminohexanoate is used the K_m's are: NAD^+ = 3.3 m*M*, $NADP^+$ = 0.28 m*M*, and diaminohexanoate = 2.5 m*M*. Although the Michaelis constants for these two substrates are similar, the velocity for the diaminohexanoate reaction is only 0.1% that of diaminopentanoate (Stadtman, 1973). The quaternary structure was reported by Somack and Costilow (1973) as being a 72 kDa dimer of 40 kDa subunits. They also showed that the enzyme had catalytically important sulfhydryl groups through alkylation-inactivation experiments. Other examples of this enzyme have been demonstrated from *C.* SB4 and ME, but these have not yet been characterized (Stadtman, 1973).

7. Lysine Dehydrogenase (α Deaminating)

Two examples of amino acid dehydrogenases that use L-lysine as an oxidizable substrate have been reported. The most poorly characterized is lysine dehydrogenase (1.4.1.15), which catalyzes the oxidative deamination of the α-amino group. The single report on this enzyme (Sivaram and Sharma, 1966) reported its isolation from human liver, and showed that it uses NAD^+, however, $NADP^+$ was not tested as



SCHEME 5. Reaction of 2,4-diaminopentanoate dehydrogenase using 2,4-diaminopentanoate as a substrate.



SCHEME 6. Reaction of 2,4-diaminopentanoate dehydrogenase using 2,5-diaminohexanoate.

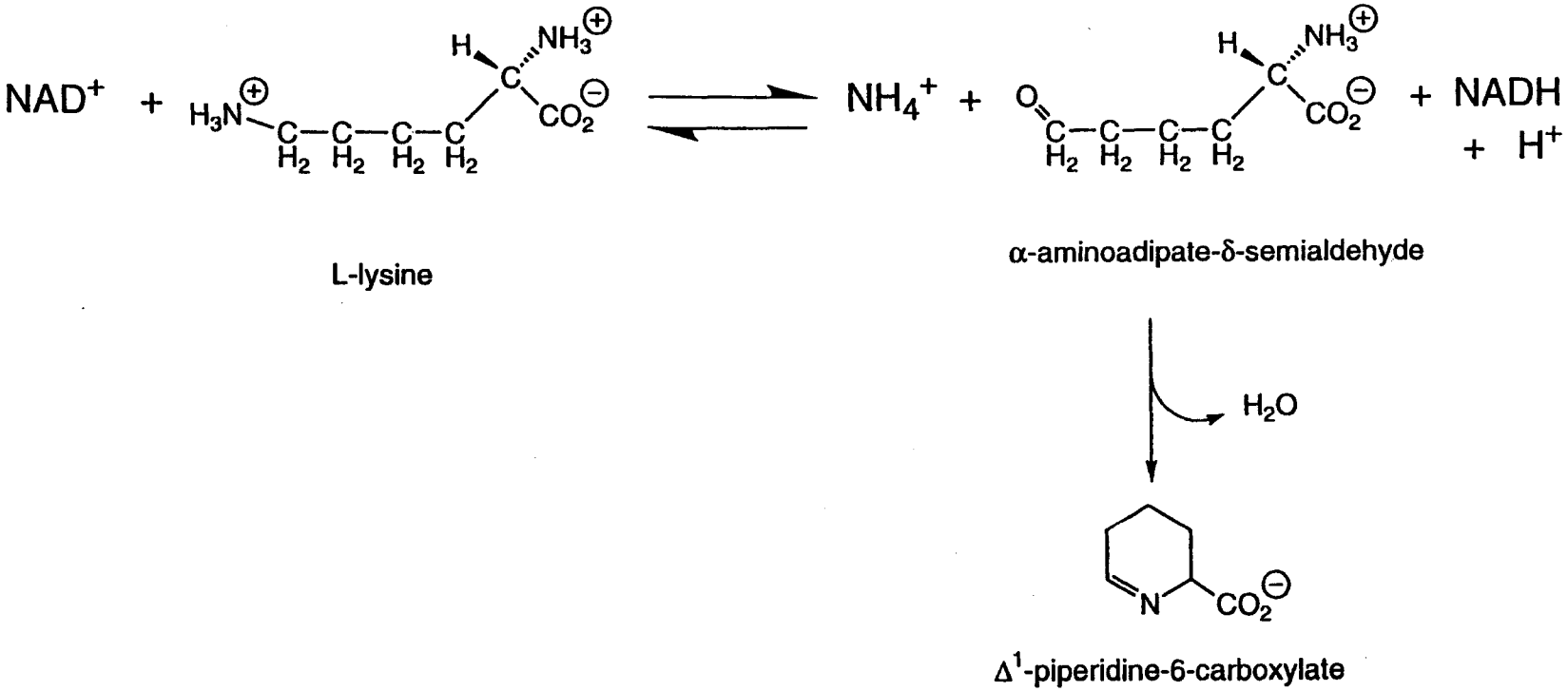
a substrate. In addition, the product was not characterized, which raises the possibility that the enzyme is operating on the ε-amino group, an activity which has been positively demonstrated by a number of authors (see below).

8. Lysine Dehydrogenase (ε Deaminating)

A number of reports have been published on lysine ε-dehydrogenase (1.4.1.18), which deaminates the side chain 6-amino group of L-lysine to form α-aminoadipate δ-semialdehyde, which non-enzymatically cyclizes to form Δ^1-piperidine-6-carboxylate, detailed in Scheme 7. Most work has been done on the enzyme isolated from *Agrobacterium tumefaciens*, where it is important in lysine catabolism, and can be induced by adding lysine to the growth media (Misono and Nagasaki, 1983). It has been shown to be a 70 kDa dimer of 39 kDa subunits (Misono et al., 1985). However, exogenously added lysine can induce the reversible formation of higher activity tetramers (Misono et al., 1989a). This interconversion may be due to a second binding site for lysine. Modification of the protein with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate), prevents this interconversion from occurring (Misono et al., 1989a). More evidence for an activator binding site comes from studies on compounds that were activators but neither substrates nor inhibitors. Molecules such as 6-amino-caproate, 7-aminoheptanoate, 8-aminooctanoate, D,L-α-hydroxy-*n*-caproate, and L-phenyllactate could activate the enzyme, which led the authors to conclude that the activator molecule must be monocarboxylic with a hydrophobic side chain. Monoamines or *N*-acetyl derivatives of L-lysine did not activate the enzyme (Hashimoto et al., 1989a). Binding studies were used to show that there were two activator binding sites per dimer, but only one catalytic site (Hashimoto et al., 1989a). Thus, the tetrameric form of the enzyme would have two active sites and four activator binding sites (Hashimoto et al., 1989a).

In the direction of amino acid oxidation, the pH optimum was 9.7 (Misono and Nagasaki, 1982). The enzyme is NAD^+-specific, but alternate nucleotide substrates are tolerated, including deaminoNAD$^+$ (42%), 3-acetylpyridineNAD$^+$ (16%), pyridine aldehydeNAD$^+$ (0.26%), and thioNAD$^+$ (0.19%) (Misono et al., 1989a). The stereospecificity of hydride transfer was shown to be *pro*-R specific (Hashimoto et al., 1989b), which is very unusual as most of the amino acid dehydrogenases have been shown to be *pro*-S specific, with the notable exception being alanine dehydrogenase. L-Lysine was the preferred substrate, with only a single lysine analog, S-(β-aminoethyl)-L-cysteine, used to any degree (2.9%) (Misono et al., 1985). The Michaelis constants for the natural substrates are NAD^+ = 0.059 m*M* and L-lysine = 1.5 m*M* (Misono et al., 1985).

The only additional report of lysine ε-dehydrogenase was from the yeast *Candida albicans* (Hammer et al., 1991). The authors noted the enzyme has a native molecular weight of 87 kDa, and a pH optimum of 9.5 for amino acid oxidation, but could be completely inhibited by the mercuricals, $HgCl_2$ and *p*-chloromercuribenzoate. In terms of its substrate specificity, the yeast enzyme is radically different from the bacterial form of the enzyme. The



SCHEME 7. Reaction of L-lysine ε-dehydrogenase.

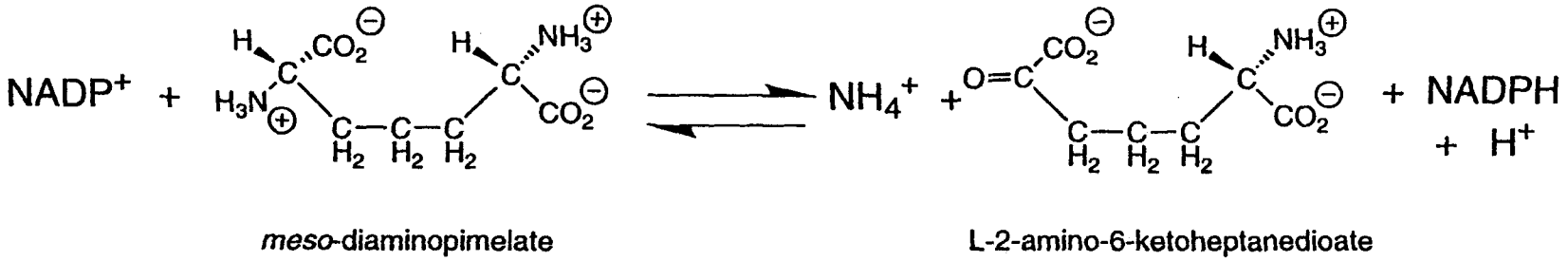
yeast enzyme is specific for $NADP^+$, exhibiting a K_m of 0.071 m*M*. The K_m for L-lysine is 0.87 m*M*, but this enzyme exhibits a broad specificity for amino acid substrate. The maximum rates of substrate analog oxidation compared to L-lysine's activity are: *S*-(β-aminoethyl)L-cysteine (42%), L-leucine (21%), δ-hydroxylysine (21%), L-ornithine (14%), and L-isoleucine (5%).

9. Diaminopimelate Dehydrogenase

Probably the most unusual amino acid dehydrogenase is diaminopimelate D-dehydrogenase (1.4.1.16), which is specific for a D-stereocenter, converting *meso*-2,6-diaminopimelate into L-2-amino-6-ketoheptanedioate (Misono et al., 1979), see Scheme 8. The first report of this enzyme activity was from *Bacillus sphaericus* (Misono et al., 1976), but extensive work has also been done on the enzyme from *Corynebacterium glutamicum*, including sequencing the gene encoding the enzyme (Ishino et al., 1987). The enzyme is used in this organism as an alternate pathway for lysine synthesis. Quantitative analysis showed that 30% of synthesized lysine came from this anabolic route, while the rest came from succinylated intermediates (Sonntag et al., 1993).

The *Bacillus* enzyme is an 80 kDa dimer of 41 kDa subunits (Misono and Soda, 1980a), similar to the *Corynebacterium* enzyme, which is a 70 kDa dimer of 39 kDa subunits (Misono et al., 1986a). Both enzymes have similar pH optima for amino acid oxidation of 10.5 and 9.8, respectively (Misono and Soda, 1980b; Misono et al., 1986a). The *Corynebacterium* enzyme has an optimum pH of 7.9 for the amino acid synthetic reaction. The *Bacillus* enzyme could be inactived by mercurials, including $HgCl_2$ and *p*-chloromercuribenzoate (Misono and Soda, 1980a). Additional experimentation showed that each subunit of the enzyme had a single reactive cysteine residue and an intrasubunit disulfide (Misono et al., 1981). The authors suggested that the reactive cysteine is not involved in catalysis, but rather is positioned near the active site allowing bulky modification molecules, like *p*-CMB or DTNB, to prevent substrate binding at the active site and causing the loss of activity (Misono et al., 1981). The K_{eq} of this enzyme was determined to be 4.46×10^{-14} M^2 (Misono and Soda, 1980a), making amino acid synthesis extremely favorable. This enzyme is one of the few amino acid dehydrogenases that clearly function *in vivo* in the direction of the favorable thermodynamic equilibrium (Sonntag et al., 1993). The unusually low K_m for ammonia (36 m*M*) when compared with most amino acid dehydrogenases may be a reflection of this cellular function (Sonntag et al., 1993).

The *Bacillus* enzyme exhibits Michaelis constants for diaminopimelate = 2.5 m*M* and $NADP^+$ = 0.00083 m*M* (Misono et al., 1980b). The enzyme was shown to be specific for $NADP^+$, and for the *meso* form of diaminopimelate; neither L,L- nor D,D-isomers were substrates (Misono et al., 1976). The kinetic mechanism of the enzyme is ordered sequential with $NADP^+$ and diaminopimelate binding in that order, and ammonia, ketopimelate, and NADPH being released in that order (Misono and Soda, 1980a). One product inhibition pattern (keto acid as inhibitor vs. diaminopimelate) was noncompetitive, instead of the expected



SCHEME 8. Reaction of diaminopimelate D-dehydrogenase.

uncompetitive pattern. This result may be due to the keto acid binding to the E · $NADP^+$ complex. These authors also determined that the enzyme was *pro*-S specific and exhibited a large but unquantitated primary deuterium isotope effect on hydride transfer (Misono and Soda, 1980a). Detailed enzymology was not performed on the *Corynebacterium* enzyme, but its Michaelis constants were reported as $NADP^+$ = 0.12 m*M*, diaminopimelate = 3.1 m*M*, ammonia = 36 m*M*, ketopimelate = 0.28 m*M*, and NADPH = 0.13 m*M* (Misono et al., 1986a).

Two brief reports appeared on the diaminopimelate dehydrogenases isolated from soybeans (Wenko et al., 1985) and a *Brevibacterium* sp. (Misono et al., 1986b). Little is known about the soybean enzyme, but it has an unusual monomeric quaternary structure of 67 kDa molecular weight. The pH optimum (8.0) for amino acid oxidation is also different. The *Brevibacterium* enzyme has been more extensively characterized. It has a dimeric structure, with a native molecular weight of 70 kDa and a subunit molecular weight of 39 kDa. The pH optimum for amino acid oxidation is 10.5 and for the reverse reaction it is 8.5. The enzyme was shown to be $NADP^+$ specific having a K_m = 0.14 m*M*. Other kinetic constants are diaminopimelate = 6.25 m*M*, ammonia = 62.5 m*M*, keto acid = 0.21 m*M*, and NADPH = 0.23 m*M*.

B. Valine Dehydrogenase

Valine dehydrogenase (1.4.1.8) has drawn considerable enzymological attention because many of the purified enzymes have come from *Streptomyces* species, where it is important in the synthetic pathway of the oligoketide antibiotic tylosin (Figure 1) (Õmura et al., 1983; Priestley and Robinson, 1989). Valine is deaminated and decarboxylated to form *iso*-butyrate, and then isomerized to *n*-butyrate, which is a direct precursor for protylonolide biosynthesis (Õmura et al., 1983). In *S. fradiae*, valine dehydrogenase activity, and hence protylosin levels, are controlled by the amount of ammonia in the medium (Õmura et al., 1983).

Valine dehydrogenase, along with leucine dehydrogenase, belongs to a group of amino acid dehydrogenases that has specificity for branched-chain amino acid substrates. The differences between these two enzymes is in their order of preference for the substrates valine and leucine. They are unique enzymes in *S. aureofaciens*, since valine and isoleucine were effective inducers of the enzyme, but leucine was not (Vancurová et al., 1988a). Valine dehydrogenase is crucial in *Streptomyces'* ability to use valine as a sole nitrogen source, since disrupting the *vdh* encoding the valine dehydrogenase gene caused a loss of this ability (Tang and Hutchinson, 1993). One gene encoding valine dehydrogenase from *Streptomyces coelicolor* has been sequenced (Tang and Hutchinson, 1993), and has a 55 to 60% identity with the protein sequences of *Bacillus* leucine and phenylalanine dehydrogenases, including the characteristic consensus sequence involved in dinucleotide substrate binding.

The characteristics of each of the enzymes isolated from the four *Streptomyces* sources, and the *Alcaligenes faecalis* enzyme, are presented in Table 2. The quaternary structure of each of these enzymes is diverse, with most being dimers, but

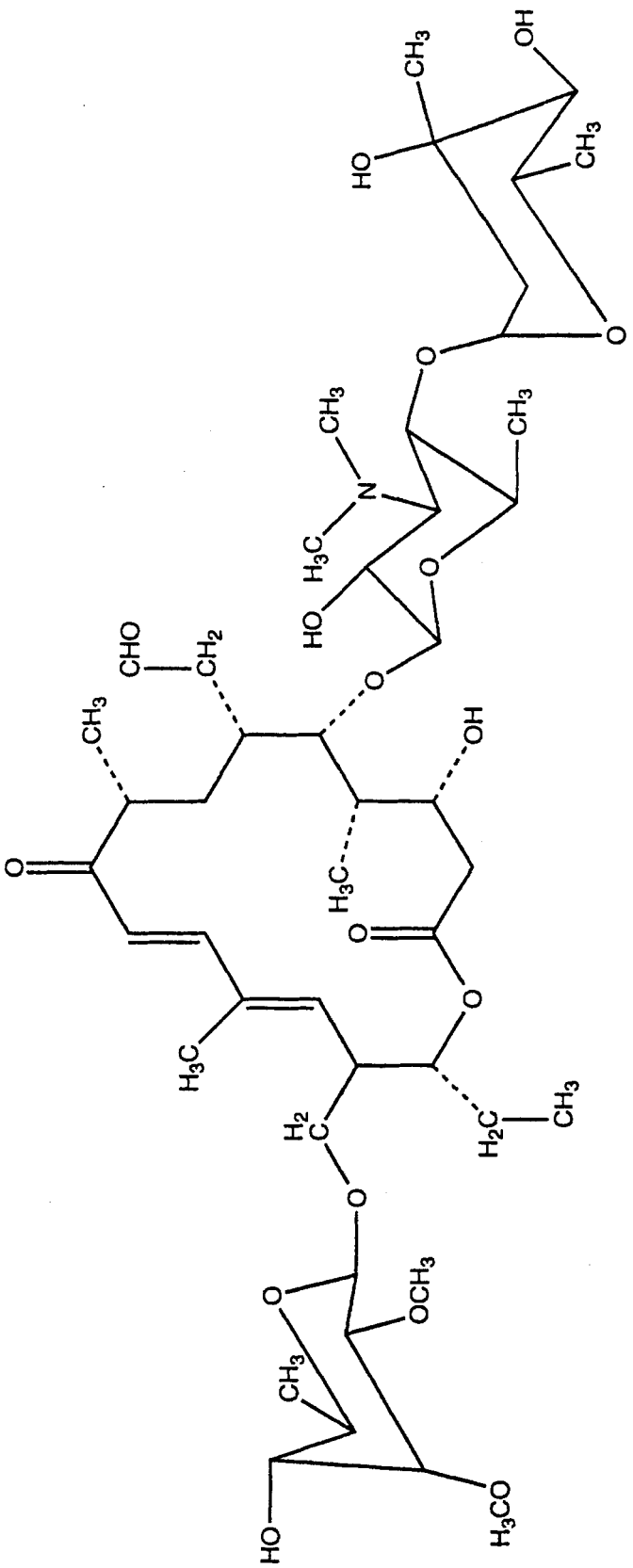


FIGURE 1. Structure of the antiobiotic tylosin.

TABLE 2
Properties of Valine Dehydrogenases

Parameter	*S. aureofaciens*	*S. coelicolor*	*S. fradiae*	*S. cinnamon.*	*A. faecalis*
Native MW	118 kDa	70	215	88	72
Subunit MW	29 kDa	41	18	41	40
4° Structure	Tetramer	Dimer	Dodecamer	Dimer	Dimer
Nucleotide specificity	NAD^+	NAD^+	$NAD(P)^+$	NAD^+	NAD^+
pH optimum for amino acid:					
Oxidation	10.7	10.5	10.6	10.5	10.8
Reduction	9.0	9.0	8.8	9.5	8.8
K_m NAD^+ m*M*	0.10	0.17	0.03	0.18	0.05
K_m valine m*M*	2.5	10.0	1.0	1.3	2.3
K_m amm. m*M*	18	—	22	55	250
K_m keto m*M*	1.25	0.6	0.8	0.8	0.8
K_m NADH	0.023	0.093	0.050	0.074	0.087
Inhibited by:	Mercurials	—	Mercurials	Mercurials	Mercurials
Stereospecificity of hydride transfer	—	—	—	*pro*-S	*pro*-S
% Rate of:					
NAD^+	100	100	100	100	100
AcetylNAD^+	100	—	65	—	714
ThioNAD^+	0	—	0	—	—
DeaminoNAD^+	84	—	94	—	440
AldehydeNAD^+	37	—	0	—	173
NGD^+	72	—	93	—	—
DeamidoNAD^+	0	—	—	—	—
Acetyldeamido-NAD^+	—	—	—	—	150
% Rate of:					
Valine	100	100	100	100	100
Isoleucine	47	28	29	2	72
Norvaline	43	—	98	26	44
Leucine	36	8	25	14	73
L-α-aminobutyrate	17	130	69	24	33
Norleucine	11	—	52	3	16
Alanine	2	5	15	—	—
Cysteine	—	3	—	—	—
Methionine	—	—	—	—	2
tert-D,L-leucine	—	—	—	—	22
% Rate of:					
Ketoisvalerate	100	—	100	82	100
Ketobutyrate	48	—	78	218	22
Ketoisocaproate	38	—	20	42	58
Keto-β-methyl-*n*-valerate	22	—	23	—	68
Ketovalerate	—	—	80	100	5
Ketocaproate	—	—	44	39	6
Pyruvate	—	—	32	3	2

Note: References are *S. aureofaciens* (Vancurová et al., 1988a); *S. coelicolor* (Navarrete et al., 1990); *S. fradiae* (Ōmura et al., 1983; Vancura et al., 1988); *S. cinnamonensis* (Priestley and Robinson, 1989); *A. faecalis* (Ohshima and Soda, 1993).

tetramers and dodecamers have been detected. The nucleotide specificities and pH optima of all valine dehydrogenases are generally the same. The *Streptomyces* enzymes, however, share an unusually low K_m value for ammonia, which may reflect valine dehydrogenases' special requirement in antibiotic biosynthesis (Vancura et al., 1988). The *A. faecalis* enzyme has a K_m value for ammonia much higher and closer to that observed for other amino acid dehydrogenases. An important listing in Table 2 is the amino acid preferences for each of these enzymes. These data show that each of these enzymes have distinct preferences for branched-chain substrates; however, all exhibit the highest preference for valine.

Steady-state kinetic mechanisms have been determined for the valine dehydrogenases of *S. cinnamonensis* (Priestley and Robinson, 1989) and *A. faecalis* (Ohshima and Soda, 1993). Both exhibit ordered sequential addition of NAD^+ and valine. Following catalysis, the *Streptomyces* enzymes continue with the sequential release of products; ammonia, keto acid, and NADH. The *Alcaligenes* enzyme, however, exhibits a random order of release of ammonia and keto acid, followed by the release of NADH.

C. Phenylalanine Dehydrogenase

Phenylalanine dehydrogenase (1.4.1.20), discovered in 1984, is the most recently discovered amino acid dehydrogenase; however, since its discovery the enzyme has been the focus of numerous studies. This is largely due to its potential importance as a commercial method of producing L-phenylalanine, a component of the artificial sweetener aspartame (L-aspartate-L-phenylalanine-1-methyl ester, or NutraSweet). The enyzme is rarely detected, except in Gram-positive nocardioforms, where it is used in the catabolism of L-phenylalanine. The enzyme has been partially purified from a *Brevibacterium* species (Hummel et al., 1984), where it was discovered first, and subsequently from *Sporosarcina ureae* (Asano and Nakazawa, 1985), *Bacillus sphaericus* (Asano et al., 1987a) *B. badius* (Asano et al., 1987b) *Rhodococcus maris* (Misono et al., 1989b), *Rh. sp. M4* (Hummel et al., 1987), *Nocardia sp. 239* (de Boer et al., 1989), and *Thermoactinomyces intermedius* (Ohshima et al., 1991), a thermophilic organism. In some cases, enzyme activity is inducible by addition of L-phenylalanine to the medium and can also be induced by other amino acids, such as D-phenylalanine and L-histidine (Hummel et al., 1984, 1987).

The genes for four phenylalanine dehydrogenases have been cloned and sequenced. Two reports, for the *B. sphaericus* (Okazaki et al., 1988) and the *S. ureae* (Hibino et al., 1990) phenylalanine dehydrogenase gene recently appeared. The paper describing the *T. intermedius* sequence (Takada et al., 1991), additionally compared the gene and protein sequences of the *T. intermedius* enzyme to the *Bacillus* and *Sporosarcina* sequences with 56% and 42% homology at the amino acid level, respectively (Takada et al., 1991). The authors suggested that the enzyme is composed of two domains, the N-terminal domain responsible for amino acid binding and the C-terminal domain responsible for nucleotide binding. The fourth phenylalanine dehydrogenase sequence reported, from *Rh. sp. M4*, presents a detailed comparative analysis of a number of different

amino acid dehydrogenases (Brunhuber et al., 1994). In this report, the protein sequences of the phenylalanine dehydrogenases from *B. sphaericus*, *T. intermedius*, *Rh. M4*, and the sequence of leucine dehydrogenase from *B. stearothermophilus* were shown to be between 32 and 53% identical (Brunhuber et al., 1994). The same comparisons with a number of glutamate and alanine dehydrogenases showed significantly lower identity (less than 29%). The conserved residues in the amino acid substrate binding domain of phenylalanine, leucine, glutamate, and alanine dehydrogenases were compared in detail, and suggestions were made concerning residues that may be involved in general amino acid binding, and in amino acid discrimination (Brunhuber et al., 1994). This kind of sequence alignment information has been used in attempts to change the substrate specificity of *T. intermedius* phenylalanine dehydrogenase from L-phenylalanine to L-leucine (Kataoka et al., 1993).

Phenylalanine dehydrogenases do not share a common quaternary structure. The first enzymes described from *S. ureae*, *B. sphaericus*, and *B. badius* were shown to exhibit native molecular weights of 305, 340, and 335 kDa, respectively, and were shown to be octomers (Asano and Nakazawa, 1985; Asano et al., 1987a, b). Since then, nearly every other possible quaternary structure has been found. The *Thermoactinomyces* enzyme is a 270 kDa hexamer (Ohshima, et al., 1991), the *Rh. sp M4* enzyme is a 150 kDa tetramer (Brunhuber et al., 1994), the *Rh. maris* enzyme is a 70 kDa dimer (Misono et al., 1989b), and the *Nocardia* enzyme is a 42 kDa monomer (Boer et al., 1989). None of the phenylalanine dehydrogenases have yet been structurally characterized by X-ray crystallography.

All phenylalanine dehydrogenases are NAD^+-specific. Alternate nucleotide substrates have been tested with the *Rh. maris* enzyme and the relative rates are similar to those seen for other amino acid dehydrogenases. The relative rates were: NAD^+, 100%; 3-acetylpyridineNAD^+, 241%; thioNAD^+, 101%; deaminoNAD^+, 86%; and pyridine aldehydeNAD^+, 9% (Misono et al., 1989b). Phenylalanine dehydrogenases are very selective for phenylalanine, and occasionally tolerate tyrosine and its corresponding keto acid, *p*-hydroxyphenylpyruvate, as a substrate. The relative rates for a variety of amino acid substrates are listed in Table 3. Also listed in this table are the reported Michaelis constants for the phenylalanine dehydrogenases. In addition to these, the constants for the *Brevibacterium* enzyme are, NAD^+ = 0.125 m*M*, L-phenylalanine = 0.385 m*M*, NADH = 0.047 m*M*, phenylpyruvate = 0.177 m*M*, and ammonia = 431 m*M* (Hummel et al., 1986).

The optimal reaction conditions for the phenylalanine dehydrogenases mirror those for the amino acid dehydrogenase family as a whole. Where it was tested, sulfhydryl-modifying agents have been shown to inhibit the enzymatic reaction (Asano et al., 1987a, b; Misono et al., 1989b). The pH optima for both forward and reverse reactions are high, as listed in Table 3. The pH optima for the *Brevibacterium* enzyme were reported to be 8.5 for reductive amination and 10.5 for oxidative deamination (Hummel et al., 1984). As with other amino acid dehydrogenases, phenylalanine dehydrogenases have equilibrium constants that lie far toward amino acid synthesis, despite their predominantly catabolic roles. The equilibrium constant reported for the *B. sphaericus* enzyme was reported to be 1.4×10^{-15} M^2 (Asano et al., 1987a), while

TABLE 3
Properties of Phenylalanine Dehydrogenases

Parameter	*Rh. M4*	*S. ureae*	*B. sphaer.*	*B. badius*	*Rh. maris*	*N. 239*	*T. inter.*
% Rate of:							
Phenylalanine	100	100	100	100	100	100	100
Tyrosine	12	5	72	9	2	—	—
Tryptophan	2	5	1	4	8	—	—
Methionine	4	4	3	8	5	—	—
Norvaline	—	6	1	—	—	—	—
Norleucine	—	15	4	—	16	—	—
p-Fluoro-phenylalanine%	62	—	—	34	8	—	—
Rate of:							
Phenylpyruvate	100	100	100	100	100	100	100
p-Hydroxy phenylpyruvate	5	24	136	53	91	28	—
Indolepyruvate	3	1	0	—	5	54	—
2-Keto-4-methyl mercaptobutyrate	33	27	11	16	9	—	14
2-Keto-isovalerate	—	2	6	13	—	—	6
2-Keto-isocaproate	—	13	8	—	—	240	—
2-Ketovalerate	—	9	6	12	—	—	—
2-Ketocaproate	—	32	0	31	9	—	—
K_m NAD^+ m*M*	0.27	0.14	0.17	0.15	0.25	0.23	0.078
K_m L-phe	0.87	0.096	0.22	0.088	3.8	0.75	0.22
K_m amm.	387	85	78	127	70	96	106
K_m phenylpyruvate	0.13	0.16	0.4	0.106	0.5	0.06	0.045
K_m NADH	0.13	0.072	0.025	0.21	0.043	—	0.025
pH optimum for amino acid:							
Oxidation	10.1	10.5	11.3	10.4	10.8	—	11.0
Reduction	9.25	9.0	10.3	9.4	9.8	10.0	9.2

Note: References are: *Rh. M4* (Hummel et al., 1987; Bradshaw et al., 1991); *S. ureae* and *B. sphaericus* (Asano et al., 1987a); *B. badius* (Asano et al., 1987b); *Rh. maris* (Misono et al., 1989b); *N. 239* (Boer et al., 1989); *T. intermedius* (Ohshima et al., 1991).

the *Nocardia* enzyme has been reported to have a K_{eq} of 3.2×10^{-18} M^2 (Boer et al., 1989).

The stereochemistry of hydride transfer has been determined for the *B. sphaericus* and *Thermoactinomyces* enzymes (Asano et al., 1987a; Ohshima et al., 1991). In both cases, the *pro*-S hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine, placing the phenylalanine dehydrogenases among the majority of amino acid dehydrogenases. Two steady-state kinetic mechanisms have been described for phenylalanine dehydrogenases. The *Rh. maris* enzyme exhibits an ordered sequential mechanism with NAD^+ and L-phenylalanine binding in that order, and with ammonia, phenylpyruvate, and NADH released in that order (Misono et al., 1989b). This mechanism is consistent with those reported for valine dehydrogenase from *S. cinnamonensis* and diaminopimelate dehydrogenase from *B. sphaericus*. Misono and coworkers note that one product inhibition pattern (L-phenylalanine was used as an inhibitor against phenylpyruvate) showed a noncompetitive inhibition pattern, rather than the expected uncompetitive inhibition pattern. They suggest that L-phenylalanine may form dead-end complexes with the E · NADH form of the enzyme. This same discrepancy has been observed in the valine and diaminopimelate dehydrogenase kinetic mechanisms.

The second kinetic mechanism determined for phenylalanine dehydrogenases is from the thermophile, *Thermoactinomyces*, and is slightly different from the other mechanisms. In this case the order of substrate binding is the same, but the order of release was observed to be phenylpyruvate, ammonia, and NADH (Ohshima et al., 1991). This conclusion was drawn from both initial velocity and product inhibition experiments, but the authors did not comment on the somewhat unusual mechanism.

D. Leucine Dehydrogenase

The leucine dehydrogenases (1.4.1.9) have been relatively well characterized from an enzymological perspective. All of the purified examples have come from *Bacillus* species, namely, *B. cereus*, *B. subtilis*, *B. sphaericus*, and *B. stearothermophilus*, the latter being a thermophilic species. These enzymes are likely to be physiologically important in the catabolism of branched chain amino acids. In *B. subtilis*, the enzyme can be induced when isoleucine or valine are present in the growth medium (Obermeier and Poralla, 1976). The enzyme also plays an important role in bacterial spore germination, allowing leucine to be used as a nitrogen and carbon source. Only the *B. stearothermophilus* gene has been sequenced (Nagata et al., 1988). Sequence comparisons between this enzyme and other amino acid dehydrogenases revealed the typical residues of a Rossmann fold in the C-terminal domain. The N-terminal domain, responsible for amino acid binding, has many residues in common with other amino acid dehydrogenases (Brunhuber et al., 1994).

The quaternary structures of leucine dehydrogenase are not simple. The *B. sphaericus* and *B. stearothermophilus* enzymes are both hexamers of 245 and 300 kDa, respectively (Ohshima et al., 1978, 1985), while the *B. cereus* enzyme is an octomer, based on electron microscopy (Lünsdorf and Tsai, 1985). The latter au-

thors suggest that the *B. cereus* quaternary structure consists of two staggered rings of four subunits each. The *B. sphaericus* enzyme has been crystallized recently, and preliminary X-ray crystallographic investigations suggest an octomeric structure (Turnbull et al., 1994) in conflict with the hexameric structure suggested by Ohshima et al. (1982).

All of the leucine dehydrogenases studied are NAD^+-specific. Alternate nucleotides were tested with the *B. sphaericus* enzyme and the relative rates of reaction are: NAD^+ (100%), acetylpyridineNAD^+ (166%), deaminoNAD^+ (81%), acetylpyridinedeaminoNAD^+ (100%), pyridine aldehydeNAD^+ (19%), and thioNAD^+ (21%) (Ohshima et al., 1978). The preferred amino acid substrate is always leucine, but other branched chained amino acids are accepted as substrates. For example, the *B. subtilis* enzyme shows a substrate preference for leucine (100%), valine (81%), isoleucine (64%), norleucine (24%), and norvaline (21%) (Zink and Sanwal, 1962). Other enzyme sources display similar preferences. The relative rates for the *B. sphaericus* enzyme were leucine (100%), valine (74%), isoleucine (58%), norvaline (41%), L-α-aminobutyrate (14%), norleucine (10%), γ-methylallylglycine (8%), and *tert*-D,L-leucine (2%) (Ohshima et al., 1978). The Michaelis constants for each of these enzymes' substrates are shown in Table 4.

The thermodynamic equilibrium for the reaction favors amino acid synthesis. The K_{eq} for the *B. cereus* enzyme was determined to be 11.1×10^{-14} M^2 (Sanwal and Zink, 1961). The optimal pHs for amino acid oxidation are generally high (11.3 for *B. cereus*, 10.7 for *B. sphaericus*, and 11.0 for *B. stearothermophilus*) and slightly lower for amino acid synthesis (9.3 for *B. sphaericus*, and 9.7 for *B. stearothermophilus*). Interestingly, when the keto analog of valine, ketoisovalerate, was used with the *B. stearothermophilus* enzyme, the pH optimum for amino acid synthesis was lowered to 8.8 (Ohshima et al., 1985). Sulfhydryl-modifying agents were shown to inactivate both the *B. cereus* and *B. sphaericus* dehydrogenases (Sanwal and Zink, 1961; Ohshima et al., 1978), and it was also shown that the *B. sphaericus* enzyme could be inactivated by pyridoxal phosphate (Ohshima et al., 1978).

Detailed enzymological characterization has been performed on the *B. sphaer-*

TABLE 4
Michaelis Constants for Leucine Dehydrogenases

Parameter	*B. cereus*	*B. subtilis*	*B. sphaericus*	*B. stearo.*
K_m NAD^+	0.16 m*M*	0.16	0.39	0.49
K_m leucine	6.2	6.2	1.0	4.4
K_m ammonia	13	13	200	—
K_m keto acid	2.2	3.3	0.31	—
K_m NADH	0.10	0.12	0.04	—

Note: References are *B. cereus* (Sanwal and Zink, 1961); *B. subtilis* (Zink and Sanwal, 1962); *B. sphaericus* (Ohshima et al., 1978); *B. stearothermophilus* (Ohshima et al., 1985).

icus and *B. stearothermophilus* enzymes. It was determined that the *B. sphaericus* enzyme exhibits *pro*-S stereospecificity for hydride transfer, and that its kinetic mechanism is sequential ordered Bi-Ter with NAD^+ and leucine binding in that order, followed by ammonia, keto acid, and NADH release (Ohshima et al., 1978). The *B. stearothermophilus* kinetic mechanism was shown to be identical to the *B. sphaericus* enzyme (Sekimoto et al., 1993). The authors noted that when leucine was used as a product inhibitor vs. the corresponding keto acid, the inhibition pattern was noncompetitive, instead of the expected uncompetitive pattern, indicating that leucine could bind to the E · NADH complex and form the dead-end complex.

Pyridoxal phosphate was used in modification experiments with the *B. stearothermophilus* enzyme, where the reagent can form Schiff bases with reactive lysine residues. The enzyme was shown to be inactivated by modification of K80, the lysine conserved in amino acid dehydrogenases in the "GGGK" region (Matsuyama et al., 1992; Brunhuber et al., 1994). The optimal pH for inactivation was 8.5 and the enzyme could be protected from inactivation by either NAD^+ and leucine or NADH alone. NAD^+ alone did not afford significant protection (Matsuyama et al., 1992), and modification did not inhibit NADH from binding. To confirm these findings, a K80A mutant was prepared; it exhibited lower levels of activity, which were not sensitive to PLP modification. The function of this lysine residue in catalysis could either involve the formation of a Schiff base with the keto acid, or it could participate in acid/base chemistry. Matsuyama and coworkers (1992) suggested the latter as more likely based on their observations that at either alkaline pH values or in the presence of added primary amines some of the activity of the K80A mutant was restored.

To understand the chemical mechanism of this enzyme, the pH dependence of the kinetic constants, V and V/K, were measured (Sekimoto et al., 1993) for the wild type enzyme and K80A, K80N, and K80R mutants. The wild type enzyme exhibits $V/K_{leucine}$ pK values of 8.9 and 10.7, while the V profile depends on one group exhibiting pK values of 8.6. The group at 8.9/8.6 was assigned to K80, due to its inactivation pK of 8.5, which is very similar to the pH profile values. This pK was shifted higher in the $V/K_{leucine}$ profile of the K80A and K80N mutants (to 9.4 and 9.3, respectively). Curiously, the arginine mutant has a lower pK = 8.1. The authors concluded that lysine80 is acting as a general base to raise the nucleophilicity of an attacking water molecule. This conclusion was compatible with solvent kinetic isotope effect experiments (Sekimoto et al., 1993), where the wild-type enzyme had a small solvent kinetic isotope effect of 1.32, whereas the K80A mutant had a solvent kinetic isotope effect of 3.18, suggesting that without lysine 80 enhancing the nucleophilicity of a water molecule, its attack on the imino acid intermediate became a rate-limiting step.

The second pK value of 10.7 seen in the V/K profile for the wild-type enzyme was not seen in the K80A and K80N mutants, and was shifted to 11.1 in the K80R mutant. The residue responsible for this pK was suggested to be involved in binding of the keto acid substrate based on other data from a pK_i profile performed with the competitive inhibitor isocaproate, which had a pK at 10.1 for the wild type enzyme, and 10.5 for the K80A mutant.

The authors assign the V/K pK to the conserved lysine residue found at position 68. This suggestion was confirmed by Sekimoto and co-workers (1994) by generating K68A and K68R mutants. These mutants lost the high pK value seen in the wild type enzyme pH profiles. The authors also observed an unexpected loss of activity in the direction of oxidative deamination, which they explained was due to an altered active site environment.

Based on these observations, Sekimoto et al. (1993) propose a model for the chemical mechanism of leucine dehydrogenase (Figure 2). In the forward direction after NAD^+ binds, leucine is bound with its carboxyl group hydrogen-bonded to K68. Hydride transfer from the Cα position of the L-amino acid to NAD^+ forms the imino acid intermediate and NADH. An active site water molecule is hydrogen bonded to K80, activating it, and enhancing its attack at the α-carbon of the leucine amino acid forming a carbinolamine. This intermediate decomposes to the keto acid and ammonia. The authors note that their model and supporting data are very similar to the mechanism proposed for glutamate dehydrogenase (see below).

E. Alanine Dehydrogenase

This enzyme (1.4.1.1) has been one of the most extensively studied amino acid dehydrogenases since its discovery in *Bacillus subtilis* in 1955 (Wiame and Piérard, 1955). The enzyme has subsequently been purified from many different species of *Bacillus* where it has a role in spore germination (Yoshida and Freese, 1964; O'Connor and Halvorson, 1961; McCormick and Halvorson, 1964; McCowen and Phibbs, 1974). The cells could be rescued, and caused to germinate, by the addition of pyruvate, which suggests that the function of alanine dehydrogenase in germinating spores is the production of energy, both directly via formation of NADH, and indirectly since pyruvate directly enters the TCA cycle and can be oxidized with the production of NADH (Siranosian et al., 1993), and at the same time free ammonia is generated. It was also shown genetically that sporulation was defective when the alanine dehydrogenase gene was disrupted (Siranosian et al., 1993). Alanine dehydrogenase levels are controlled both by the amount of alanine in the medium and by sporulation (Siranosian et al., 1993).

Alanine dehydrogenase has been isolated from other organisms that have different metabolic uses for the enzyme, including nitrogen assimilation. Nitrogen fixing organisms, after converting N_2 gas into ammonia, need to store ammonia. The most common way is through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, however at high ammonia concentrations, many organisms employ a second pathway that uses glutamate dehydrogenase to store nitrogen. Organisms lacking glutamate dehydrogenase, however, will use alanine dehydrogenase for the same function (Murrell and Dalton, 1983). The nitrogen-fixing bacteria in soybean nodules, *Bradyrhizobium japonicum*, and the nitrogen-fixing, blue-green alga, *Anabaena cylindrica*, are examples of organisms that use alanine dehydrogenase in this way; the enzyme has been purified from both of these sources (Smith and Emerich, 1993a; Rowell and Stewart, 1976). The

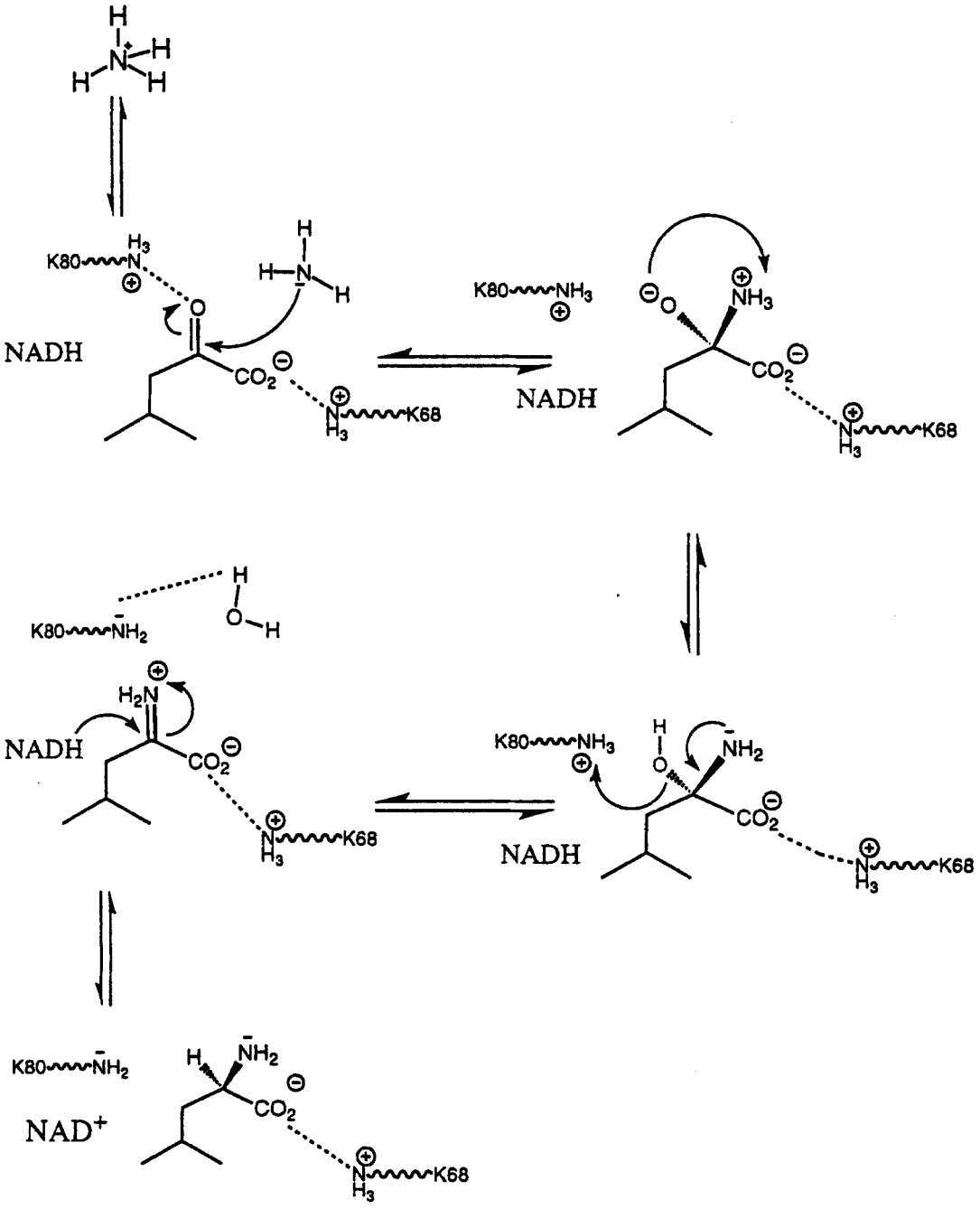


FIGURE 2. Chemical mechanism of leucine dehydrogenase (adapted from Sekimoto et al., 1993).

role of this enzyme in *Pseudomonas sp. MA* is similar to its role in these nitrogen-fixing organisms. This obligate methylotroph uses the enzyme to store ammonia generated from the degradation of methylamine (Bellion and Tan, 1987).

Alanine dehydrogenase has been found in numerous other organisms where its function is less clear, and may simply be involved in the interconversion of alanine and pyruvate. An interesting source of the enzyme is *Mycobacterium tuberculosis*, where the enzyme is expressed at high levels, compared to *M. bovis* BCG, which contains no detectable enzyme (Goldman, 1959; Andersen et al., 1992). Its function in *M. tuberculosis* may be in cell wall synthesis, where D-alanine is a component of the peptidoglycan layer (Andersen et al., 1992). The enzyme has also been purified from the halophiles, *Halobacterium cutirubrum* (Kim and Fitt, 1977) and *H. salinarium*, (Keradjopoulos and Holldorf, 1979), and from thermophiles *Thermus thermophilus* (Váli et al., 1980). The enzyme has also been purified from *Propionibacterium freudenreichii* (Crow, 1987) and *Streptomyces aureofaciens* (Vancurová et al., 1988b).

Four alanine dehydrogenase gene sequences have been determined; one from *M. tuberculosis* (Andersen et al., 1992), and three others from *Bacillus* species. The amino acid sequence of the enzyme from *B. subtilis* was reported to be 65% and 66% identical to the predicted amino acid sequences of the *B. sphaericus* (strain IFO 3525) and *B. stearothermophilus* genes (Siranosian et al., 1993). The latter two amino acid sequences were 73% homologous to each other, but showed low levels of homology to other enzymes in the database (Kuroda et al., 1990). The authors of this report describe the C-terminal domain to be the nucleotide-binding domain, and the N-terminal domain to be the catalytic domain, as has been suggested for other amino acid dehydrogenases.

There have been no reports of crystal structures for alanine dehydrogenases, limiting our information about their tertiary structures. Crystallization may be complicated by the quaternary structures of these dehydrogenases. The enzymes from *B. subtilis*, *B. cereus*, *B. sphaericus*, *B. stearothermophilus*, as well as *Mycobacterium*, *Thermus*, and *Anabaena* species are all hexamers. (Yoshida and Freese, 1970; Porumb et al., 1987; Ohshima et al., 1990; Kuroda et al., 1990; Andersen et al., 1992; Váli et al., 1980; Rowell and Stewart, 1976). Other enzymes exhibit a variety of structures; those from *Bradyrhizobium* and *Pseudomonas* are tetrameric structures, while both *Halobacterium* enzymes are monomers (Smith and Emerich, 1993a; Bellion and Tan, 1987; Kim and Fitt, 1977; Keradjopoulos and Holldorf, 1979). The enzyme purified from *S. aureofaciens* is an octomer (Vancurová et al., 1988b).

All of the purified alanine dehydrogenases are NAD^+-specific, but there are no reports on the nucleotide specificity of these enzymes. The K_{eq} of the reaction was measured with the *M. tuberculosis* enzyme, and a value of 5.5×10^{-11} M^2 was reported (Goldman, 1959). The two *Halobacterium* enzymes are unusual in their requirements for monovalent cations for full activity. The *H. salinarium* enzyme is activated nonspecifically by cations (Keradjopoulos and Holldorf, 1979), while the *H. cutirubrum* enzyme is nonspecifically activated by monovalent cations in the reverse reaction, but has an absolute requirement for potassium in the amino acid oxidative reaction (Kim and Fitt, 1977).

Alanine dehydrogenase is well known for its unusual *pro*-R hydride transfer stereochemistry, first reported for the *B. subtilis* enzyme (Alizade et al., 1975)

TABLE 5
Properties of Alanine Dehydrogenases

Parameter	*B. japonicum*	*A. cylind.*	*Pseudomonas*	*H. cutirub.*	*H. salinar.*	*S. aureo.*	*M. tuber.*	*T. thermo.*
K_m NAD^+ m*M*	0.2	0.014	—	0.5	1.5	0.11	—	0.12
K_m ala	1.0	0.4	—	7.0	5.3	5.0	—	0.18
K_m amm.	8.9	8.0	26	0.82	500	6.67	—	59
K_m pyr.	0.49	0.11	4.3	0.8	0.95	0.56	—	0.75
K_m NADH	0.086	—	0.059	0.2	0.031	0.029	—	0.035
pH optimum for amino acid:								
Oxidation	10.0	9.6	9.0	9.0	9.0	10.0	9.8	10.5
Reduction	8.5	8.0	9.0	9.0	9.0	8.5	9.3	8.0
Inhibition by mercurials?	—	yes	—	—	—	—	yes	—
Highly alanine specific?	—	yes	—	—	—	yes	yes	yes
Native MW, kDa	190	270	217	72.5	60	395	240	290

Note: References are *B. japonicum* (Smith and Emerich, 1993a); *A. cylindrica* (Rowell and Stewart, 1976); *Pseudomonas* (Bellion and Tan 1987); *H. cutirubrum* (Kim and Fitt, 1977); *H. salinarium* (Keradjopoulos and Holldorf, 1979); *S. aureofaciens* (Vancurová et al., 1988b) *M. tuberculosis* (Goldman, 1959, Andersen et al., 1992); *T. thermophilus* (Váli et al., 1980).

TABLE 6
Properties of *Bacillus* Alanine Dehydrogenases

Parameter	*B. subtilis*	*B. cereus*	*B. sphaericus DSM 462*	*B. sphaericus IFO 3525*	*B. licheni-formis*	*B. stearo.*
K_m NAD^+, m*M*	0.18	0.18	0.26	0.23	0.045	—
K_m alanine	1.73	12.5	10.5	18.9	5.0	—
K_m ammonia	38	30	38	28.2	12	—
K_m pyruvate	0.54	0.48	0.5	1.7	0.37	—
K_m NADH	0.023	0.037	0.1	0.01	0.025	—
pH optimum for amino acid						
Oxidation	10.25	10.75	10.5	10.5	—	—
Reduction	8.9	8.75	8.2	9.0	—	—
K_{eq} (M^2)	3.1×10^{-14}	1.31×10^{-14}	4.3×10^{-17}	—	—	—
Inhibition by mercuricals?	Yes	—	—	Yes	—	—
Native MW, kDa	230	248	230	230	—	235

Note: References are *B. subtilis* (Yoshida and Freese, 1965); *B. cereus* (McCormick and Halvorson, 1964, Porumb et al., 1987); *B. sphaericus* DSM 462 (Ohshima et al., 1990); *B. sphaericus* IFO 3525 (Ohashima and Soda, 1979); *B. licheniformis* (McCowen and Phibbs, 1974); *B. stearothermophilus* (Kuroda et al., 1990).

and later reported for the *B. sphaericus* IFO 3525 enzyme (Ohshima and Soda, 1979). This feature makes alanine dehydrogenase and lysine ε-dehydrogenase the only amino acid dehydrogenases that exhibit the *pro*-R stereochemistry.

The kinetic mechanisms of alanine dehydrogenase are not all alike. A preliminary analysis was performed on the enzyme from *Propionibacterium* that suggested the very unusual order of binding of alanine and NAD^+, followed by the random release of pyruvate and NADH, followed by the ordered release of ammonia (Crow, 1987). A preliminary analysis was also performed on the *Mycobacterium* enzyme, and the order suggested was again alanine and NAD^+ binding in that order, followed by the ordered release of NADH, pyruvate, and ammonia (Goldman, 1959). The enzyme from *Bradyrhizobium* has undergone a much more rigorous treatment (Smith and Emerich, 1993b). The authors show that the kinetic mechanism for the enzyme from this nitrogen fixing organism is a rapid-equilibrium ordered one, with the binding of the substrate ammonia being at thermodynamic equilibrium. The order of addition follows a Ter-Bi Theorell-Chance mechanism, with NADH binding first, followed by the equilibrium binding of ammonia. Pyruvate binds and alanine is released as the first product, followed by the release of NAD^+. The authors suggest this type of mechanism is ideal for the local environment of the enzyme, where a high concentration of ammonia exists (estimated to be 12 m*M*). The authors also noted that pyruvate could bind to the $E \cdot NAD^+$ complex, forming a dead-end complex (Smith and Emerich, 1993b).

The *B. subtilis* enzyme has had a full enzymological characterization (Grimshaw and Cleland, 1981; Grimshaw et al., 1981). At low pH, the kinetic mechanism was proposed to be ordered sequential with NAD^+ and alanine binding in that order, followed by ammonia, pyruvate, and NADH release. It was shown however, that the $E \cdot NAD^+$ complex must first isomerize to $E^* \cdot NAD^+$ before it can bind alanine. Combination of alanine with $E \cdot NAD^+$ leads to substrate inhibition due to the inhibition of subsequent isomerization. This inhibition occurs with alanine in its zwitterionic state and with an enzymic group of pK = 7.5 in its deprotonated state. The possibility of an isomerization phenomenon was suggested previously for the *B. cereus* enzyme, where it was noted that a kinetically significant isomerization could account for the observed enhanced rate of enzyme reactivation when NADH was bound (Porumb et al., 1987). The kinetic mechanism of the *B. subtilis* enzyme is complicated at pH values above 10.9, where NAD^+ binding becomes equilibrium ordered with the enzyme. Additionally, at pH values above 9.35, alanine is nonenzymatically converted into iminopyruvate, complicating the measure of reaction rates (Grimshaw and Cleland, 1981). These authors also suggested that ammonia does not bind to the active site but rather reacts with the enzyme-bound keto acid based on the observation that all of the bimolecular rate constants approach diffusion limited values at optimal states of enzyme and substrate ionization, except for the V/K of ammonia (Grimshaw and Cleland, 1981).

The chemical mechanism was determined for the *B. subtilis* enzyme using a combination of pH analysis and isotope effects (Grimshaw et al., 1981). Alanine was shown to be a "sticky" substrate,

whereas serine, a slow substrate, was not. Ammonia, not ammonium ion, and the monoanionic form of the amino acid are the true substrates. A lysine on the enzyme with a pK = 9.0-9.6 in the E · NAD^+ complex and pK >10.0 in the E · NADH complex binds the carboxyl group of the amino acid/keto acid substrate. A histidine of pK ~7.0 in both the E · NAD^+ and E · NADH complexes participates in acid/base chemistry, being unprotonated in the forward reaction and protonated in the reverse reaction.

The model in Figure 3 has alanine bound as a monoanion with its carboxyl group interacting with an active site lysine. NAD^+ oxidizes alanine to form iminopyruvate, after it is brought into proximity to alanine by a conformational change. Water, activated by an adjacent histidine functioning as a base, now attacks the iminopyruvate from the same side. The protonated histidine donates a proton to the neutral amino group of the newly formed carbinolamine. The deprotonated histidine then removes a proton from the hydroxyl group of the carbinolamine, initiating the expulsion of ammonia, forming pyruvate. The identity of the active site lysine and histidine residues have been tentatively assigned based on the *B. sphaericus* gene sequence (Kuroda et al., 1990). These authors suggest the lysine in question is Lys156 which is part of the "GGGK" motif, a fingerprint region conserved in all amino acid dehydrogenase substrate-binding domains. They propose that the conserved His153 is the histidine performing acid/base chemistry. No other studies, such as site-directed mutagenesis, have been performed to confirm these predictions. This assignment to lysine156 contradicts that suggested for leucine dehydrogenase, where the "GGGK" lysine is given a catalytic, rather than a binding role (Sekimoto et al., 1993).

The relative rates of chemical steps within the proposed mechanism discussed above were extended using detailed isotope effect methodologies (Weiss et al., 1988). In the reverse reaction, it was shown that the chemical steps involved in carbinolamine formation, imine formation, and reduction of imine to amino acid were equally rate-limiting. In the overall reaction, the release of oxidized nucleotide was suggested to be the rate-limiting step; when alternate nucleotides or substrates were used, hydride transfer began to limit V_{max} for these slower substrates.

F. Glutamate Dehydrogenase

The discussion of glutamate dehydrogenases will not be as inclusive or historically exhaustive as the discussions in previous sections, due to the wealth of information present in the literature. This section will only summarize some of the general aspects of the enzyme and then discuss the mechanistic and structural studies of the bovine liver and *Clostridium symbiosum* enzymes. The reader is referred to two general and more complete reviews (Smith et al., 1975; Hudson and Daniel, 1993).

Glutamate dehydrogenase has been demonstrated in nearly every organism, because of its critical role at the crossroads between nitrogen and carbon metabolism. The enzyme can be used to assimilate free ammonia into the cell's nitrogen-storage molecule, glutamate. This system is used when free ammonia levels are high. When ammonia levels are low, cells use a

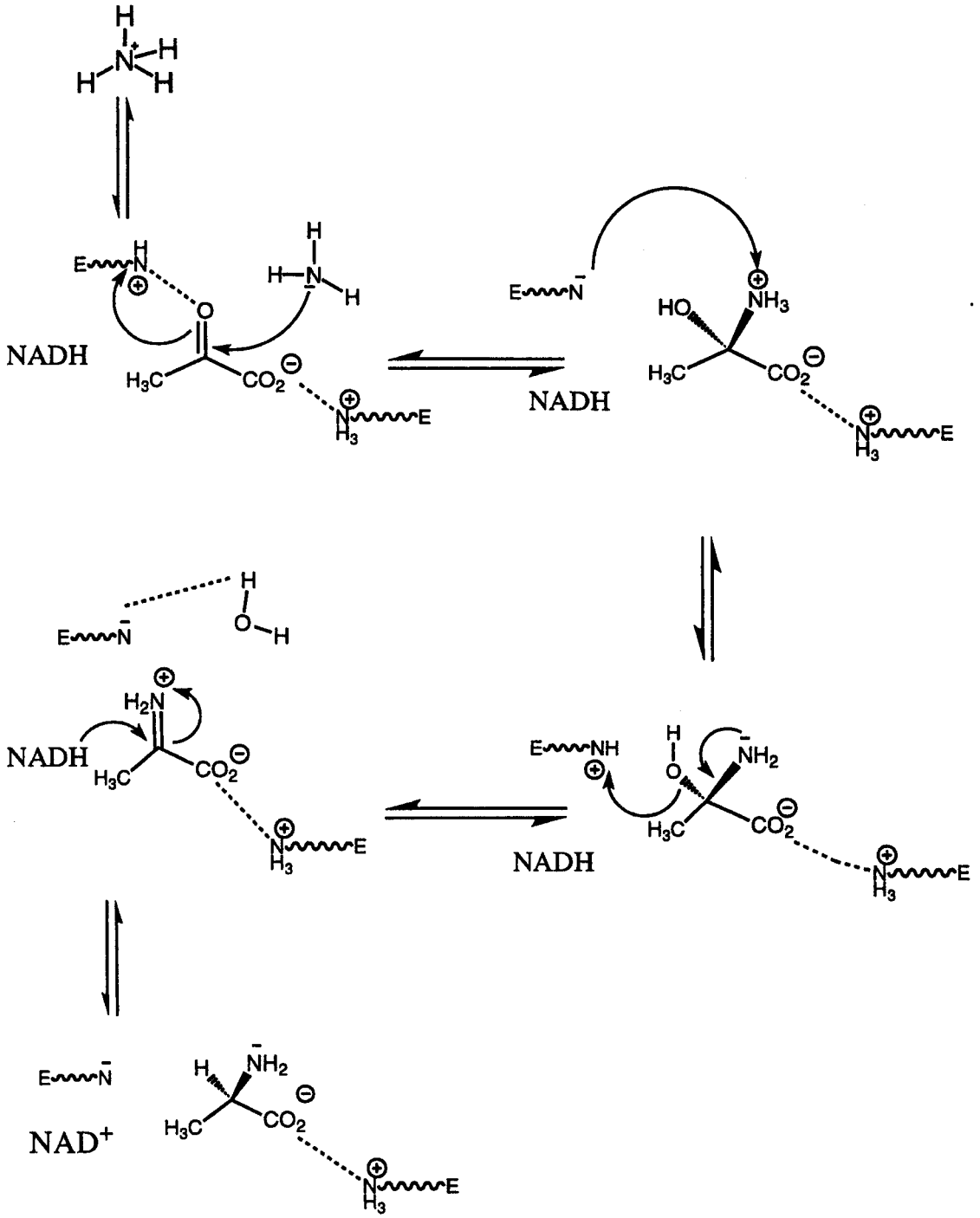


FIGURE 3. Chemical mechanism of alanine dehydrogenase (Adapted from Grimshaw et al., 1981).

glutamine synthetase/glutamate synthase system, since glutamine synthetase has a lower K_m for ammonia than glutamate dehydrogenase, and so is able to operate more efficiently at low concentrations of ammonia. Alternatively, glutamate dehydrogen-

ase may also be used in a catabolic fashion, degrading excess glutamate to the TCA cycle intermediate, α-ketoglutarate, which becomes available for energy generation.

There are three types of glutamate dehydrogenases; those which are NAD^+-specific (1.4.1.2), $NADP^+$-specific (1.4.1.4), or have mixed specificity (1.4.1.3). Usually, $NADP^+$-specific enzymes are used for anabolic functions, that is, the formation of glutamate, and NAD^+-specific enzymes are used for glutamate degradation. This strategy accords with the usual biosynthetic and catabolic function which $NADP^+$ and NAD^+, respectively, are conscripted for. The mixed type of enzyme is found in the mitochondrial matrix of all animal tissue types. There are little, if any, differences in the properties of the enzyme isolated from various tissue sources. There is also remarkable similarity between various animal sources (Smith et al., 1975). Higher fungi express separate NAD^+- and $NADP^+$-specific forms of the enzyme, while lower fungi have only the NAD^+-specific enzyme. Bacteria generally express a single enzyme, and they can be either NAD^+- or $NADP^+$-specific. Plants express separate NAD^+- and $NADP^+$-specific enzymes which are compartmentalized into their mitochondria and chloroplasts (Smith et al., 1975).

The mixed-specificity enzymes from animals are subject to extensive allosteric control, and a recent review suggests at least three different sites for effector binding in addition to the active site (Hudson and Daniel, 1993). The most well described effectors are ADP, which enhances activity, and GTP, which decreases it. NAD^+, NADH, and NADPH can also alter the enzyme's activity, as can the hormones diethylstilbestrol, estradiol, progesterone, Δ^4-androstene-3,17-dione, testosterone, and thyroxine (Hudson and Daniel, 1993). Amino acids such as leucine, isoleucine, and methionine are known to stimulate the enzyme (Hudson and Daniel, 1993). A different method of enzymatic control is the enzyme's tendency to aggregate into linear rods when protein concentrations or ionic strength levels are high. The concentrations of glutamate dehydrogenases in most cells exceed the critical polymerization concentration; however, not all cells have a high enough level of enzyme, and some species of mammals have glutamate dehydrogenases that do not polymerize at all (Smith et al., 1975). The allosteric effectors that modify activity, also modulate the degree of aggregation. Added to these various layers of control are potential allosteric interactions between subunits of the hexameric oligomer, reinforcing the critical importance of this enzyme in the cell. In lower organisms, the level of activity of glutamate dehydrogenase is controlled through expression of its gene. This type of control is especially evident if an organism has both NAD^+- and $NADP^+$-specific forms of the enzyme, where the ratio of gene expression oscillates depending on cellular conditions (Smith et al., 1975).

Glutamate dehydrogenases have a native molecular weight of about 330 kDa and are usually hexamers. Notable exceptions are some fungal NAD^+-specific enzymes, which are tetramers of approximately 100 kDa subunit native molecular weights. For the majority of glutamate dehydrogenases, the pH optima for reductive amination lies between pH 7.6 and 8.0, while the corresponding optima for oxidative deamination are between 8.5 and 9.0. All glutamate dehydrogenases exhibit *pro*-S hydride specificity, and those studied have a random kinetic mechanism of

substrate addition and product release. The K_m values for NAD^+ or $NADP^+$, α-ketoglutarate, and NADPH are remarkably similar regardless of source, whereas the steady-state values for glutamate, ammonia, and NADH differ widely (Hudson and Daniel, 1993).

The remainder of this section will discuss the mechanistic and structural aspects of two specific glutamate dehydrogenases, bovine liver and *C. symbiosum*. The former has historically been the focus of enzymological studies particularly since its amino acid sequence was determined (Moon and Smith, 1973), whereas the latter has been the first glutamate dehydrogenase to have its three-dimensional structure solved by X-ray crystallography (Baker et al., 1992).

The kinetic mechanism of glutamate dehydrogenase has been the subject of substantial debate. To minimize ambiguity, most well-characterized enzymes have not had kinetic studies determined in the presence of added allosteric effectors, and assays are performed with NADP(H) rather than NAD(H), since the latter acts as an effector. Steady-state enzymological studies use dilute solution of protein, avoiding aggregation. Despite these precautions, the enzyme did not reveal its kinetic mechanism easily. Initially it was proposed that the kinetic mechanism was random (Hudson and Daniel, 1993); however, a rigorous study of the kinetic mechanism using product and dead-end inhibitors (Rife and Cleland, 1980a) suggested that while $NADP^+$ and glutamate binding were random, the preferred mechanism binds $NADP^+$ first to the free enzyme. The order of release of products was ordered with ammonia, α-ketoglutarate, and NADPH released in that order. The possibility of a random release of α-ketoglutarate and NADPH, with the favored pathway releasing keto acid first, could not be ruled out, however, because of evidence for an E · α-ketoglutarate complex (Rife and Cleland, 1980a). The use of monocarboxylic alternate substrates changed the kinetic mechanism to a rapid equilibrium ordered one, where the keto acid was in equilibrium between its bound and free states. This observation was confirmed in an independent study of the steady-state reaction that focused strictly on the alternate substrate norvaline (LiMuti and Bell, 1983).

Rife and Cleland (1980a) also describe some of the reasons for the substrate inhibition seen with glutamate dehydrogenase. Substrate inhibition by α-ketoglutarate is due to the formation of an E · $NADP^+$ · α-ketoglutarate dead-end complex. A pre-steady-state study suggested the formation of the same complex could explain the substrate inhibition by α-ketoglutarate, but also suggested the formation of an abortive E · α-ketoglutarate complex (Colen, 1978). It was claimed that most of the substrate inhibition comes from the ternary dead-end complex, which exhibits the characteristics of a slow, tight binding inhibitor.

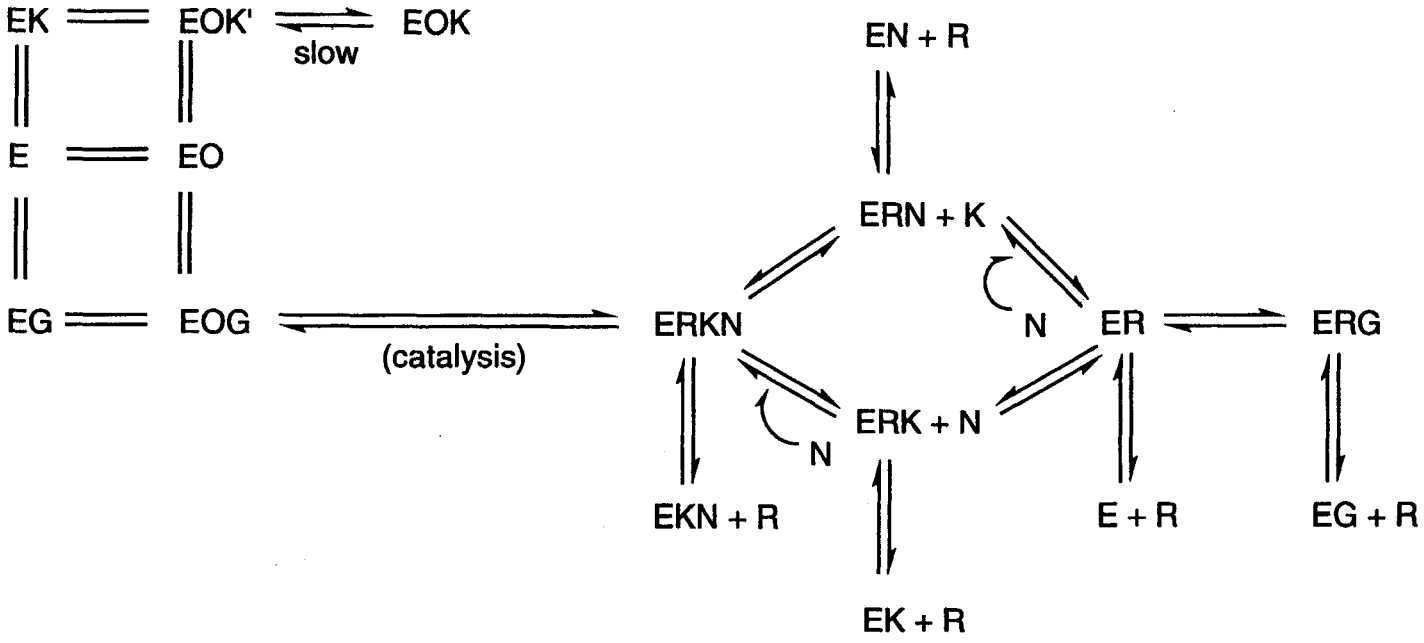
The steady-state reaction is also inhibited by ammonia. The nature of this inhibition was investigated by Brown et al. (1979). These authors noted that before steady-state turnover is achieved there is a pre-steady-state burst of product, a phenomenon that had been reported earlier through pioneering work by Iwatsubo and others (Jallon et al., 1975; di Franco and Iwatsubo, 1971). The pre-steady state burst reflects the slow release of reduced nucleotide before catalysis can continue. The strong binding of NADPH is well known (Rife and Cleland, 1980a), and its release is probably the rate-limiting step in the

overall reaction. When the reaction was studied at inhibitory concentrations of ammonia, the size of product burst was decreased (Brown et al., 1979). Ammonia was also shown to be a noncompetitive inhibitor vs. glutamate when concentrations of glutamate and ammonia were high (Brown et al., 1979). These results prompted these authors to add steps to the product release section of the kinetic mechanism, which permits ammonia binding to the enzyme and inhibits the reaction.

The findings of the Colen and Brown studies can be summarized in Scheme 9 where E = enzyme; O = oxidized nucleotide; G = glutamate; N = ammonia; K = α-ketoglutarate; R = reduced nucleotide. The double bars without arrowheads indicate complexes that are in equilibrium with each other. This mechanism does not correspond precisely to that proposed by Rife and Cleland (1980a), who commented that ammonia displayed greater affinity for the enzyme when dicarboxylic substrates were used, compared to monocarboxylic substrates.

Rife and Cleland (1980b) also investigated the chemical mechanism of bovine liver glutamate dehydrogenase. They studied the pH dependence of the steady-state kinetic parameters, V and V/K, with all of the natural substrates of the reaction, plus some alternate, monocarboxylic substrates, and dead-end inhibitors. Their observations can be summarized as follows. An enzymic carboxylic group of pK = 5.2 must be protonated for efficient binding of both mono- and dicarboxylic substrates in the reverse reaction, and unprotonated in the forward reaction. A lysine residue of pK = 7.8 in the reverse reaction and pK = 8.7 – 9.3 in the forward reaction must be protonated for dicarboxylic substrates and unprotonated for monocarboxylic substrates. This group likely binds the γ-carboxyl group of the α-ketoglutarate substrate. The amino acid substrate must bind with its amino group protonated, and ammonia, not ammonium ion, is the substrate for the reaction. Finally, a lysine of pK> 10.5 must be protonated for catalysis in the reverse direction, while in the forward direction it must be unprotonated and exhibits a pK value of 7.6 to 8.0. This catalytic lysine has since been assigned in an X-ray crystallography model (see below) to Lys126, that is, the lysine that is part of the "GGGK" motif widely conserved within the amino acid dehydrogenases (Brunhuber et al., 1994). The assignment of this abnormally low pK positively confirms the suspicion of its catalytic importance put forth since its initial observation by E. L. Smith (Smith et al., 1970).

These data were compiled to generate a model for the chemical mechanism that has been generally accepted (Figure 4). In the direction of amino acid synthesis, α-ketoglutarate binds to E · NADPH with its γ-carboxyl to a protonated lysine. An active site carboxyl must be protonated to maintain proper active site conformations. Ammonia now enters the active site and reacts with the α-keto group. The α-carbonyl oxygen accepts a proton from an active site lysine, forming the carbinolamine. The carbonyl oxygen then accepts a second proton from the enzymic carboxyl, which concurrently abstracts a proton from the substrate's amine. The carbinolamine collapses to the imine, with the concommitant release of a water molecule. The imine is reduced by NADPH to form an unprotonated amino acid, which is protonated by the active site carboxyl, and is released as the protonated amino acid product.



SCHEME 9. Proposed kinetic mechanism of glutamate dehydrogenase.

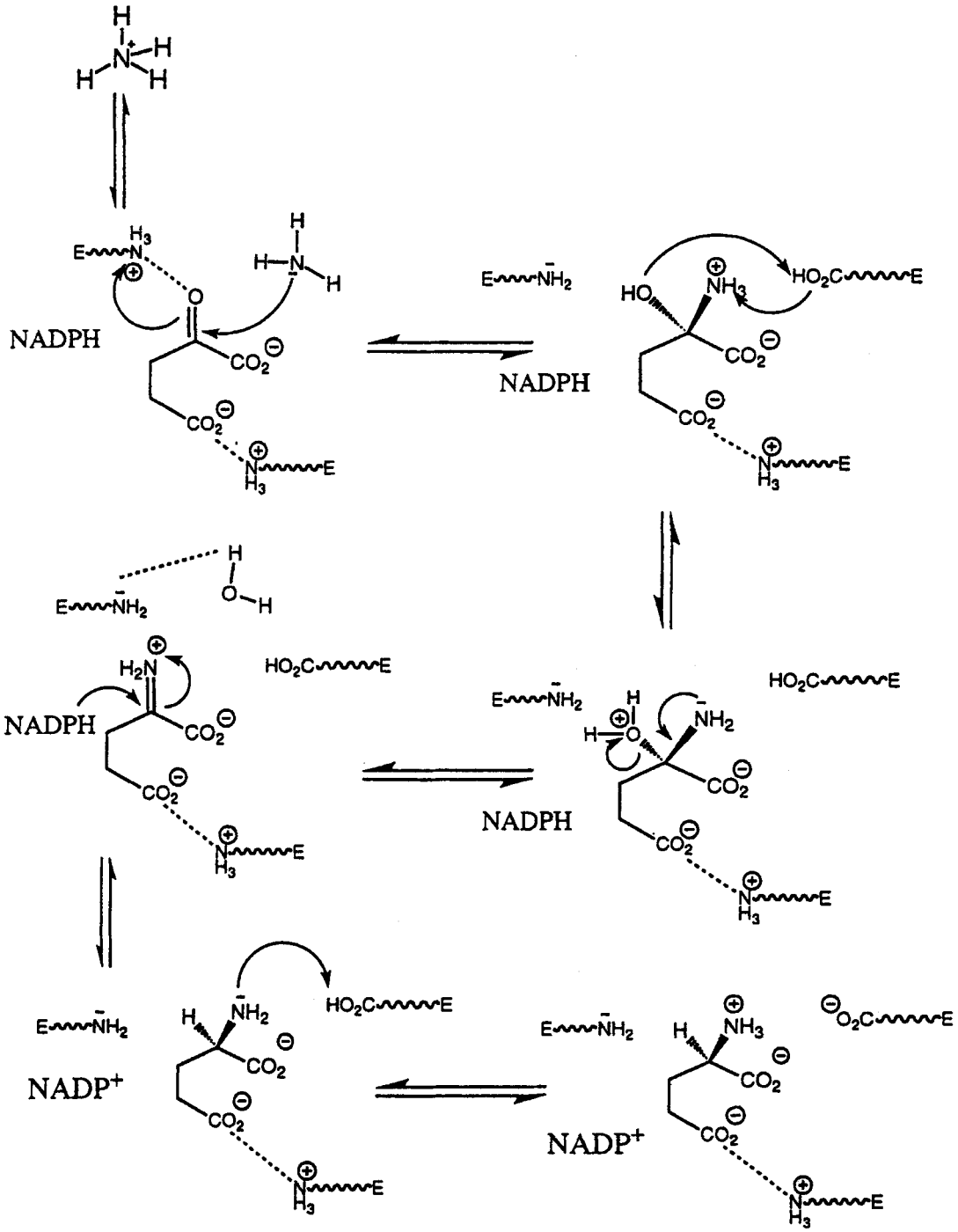


FIGURE 4. Chemical mechanism of glutamate dehydrogenase.

This model was evaluated using proline and its keto acid. This alternate substrate tests only the redox portion of the model, since it exists as an imine. Srinivasan and Fisher (1985) determined pH profiles and primary deuterium isotope effects for this substrate. They saw a larger isotope effect on the reduction of this substrate (3.9, reverse reaction; >3, forward reaction) than Rife and Cleland (1980a) saw with glutamate (about 1.2), indicating that hydride transfer chemistry is more rate-limiting with this alternate substrate. Srinivasan and Fisher also observed that the keto acid must be protonated for activity, proline must be deprotonated for activity, and that an enzymic group of pK = 8.6 enhanced the rate of the reaction when unprotonated.

The authors used their results to comment on the model of the chemical mechanism of glutamate dehydrogenase using glutamate. Once the intermediate, α-iminoglutarate is formed in the active site it must be protonated to undergo oxidation by bound nucleotide. This conclusion agrees with the model postulated by Rife and Cleland (1980b). Conversely, glutamate must bind and react in its deprotonated form, which is opposite to the earlier suggestion. The authors concluded that neither the γ-carboxyl of the substrate, nor the other enzymic active site residues, are necessary for redox chemistry.

Srinivasan and Fisher (1985) modified the Rife and Cleland model at a stage after the initial formation of the carbinolamine (Figure 5). In the next step, the active site lysine removes a proton from the amine, and the carbinolamine then collapses into the imine with the hydroxyl-α-carbon bond cleaved with the removal of a proton from the active site carboxyl. At this point the protonated imine is reduced by NADPH and the unprotonated amino acid is then released as product.

The revised model was clarified and extended by other investigations. In the forward direction, the model does not specify whether water must enter the active site before ammonia release as was suggested by Fisher et al. (1987). They concluded that if water is not available for direct attack, the intermediate would have to form a Schiff base with an enzymic residue. Carbonyl addition by an enzymic group was shown not to occur through experiments with α-ketoglutarate and its competitive inhibitor, oxalylglycine. Oxalylglycine forms the same hydrogen bonds as α-ketoglutarate, but cannot undergo any carbonyl additions because its carbonyl group is part of an amide group. In the kinetic experiments performed, oxalylglycine behaved the same as α-ketoglutarate, eliminating the possibility of any kinetically significant carbonyl addition reactions. In order to prove that water must bind to the active site before ammonia elimination, Srinivasan et al. (1988) measured the rate of ^{18}O carbonyl exchange on α-ketoglutarate. If a enzymic Schiff base did form, isotopic exchange should take place; however, insignificant exchange occurred unless ammonia was added to the reaction, ruling out enzymic Schiff base formation. The authors also noted that the γ-carboxyl of the substrate does not participate in catalysis (for example, in an intramolecular, cyclic manner), since no differences were observed in the rates of model nonenzymatic dehydration reactions with the hydrated forms of α-ketoglutarate or pyruvate, which lacks a second carboxyl group (Srinivasan et al., 1988).

The relative rates of the chemical steps in the model were compared using isotope effects (Weiss et al., 1988). In the reverse

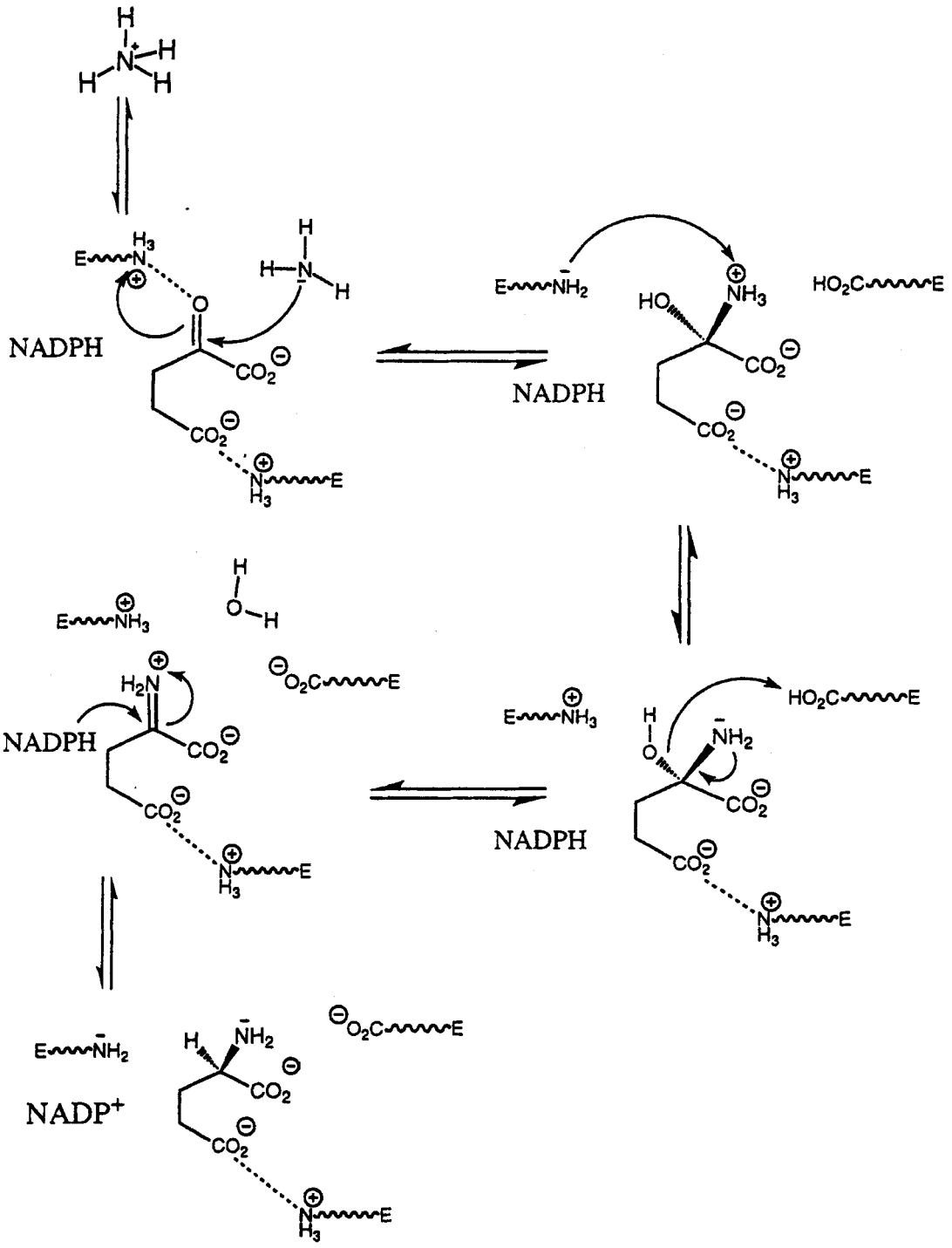


FIGURE 5. Revised chemical mechanism of glutamate dehydrogenase.

reaction, it was shown that hydride transfer was not as rate-limiting as the steps for carbinolamine formation, imine formation, and the release of glutamate product. The use of alternate keto acid substrates, such as α-ketovalerate, causes hydride transfer

to become more rate limiting, and can be made even more rate limiting if the alternate nucleotide acetylpyridineNAD$^+$ is used. However, the alternate nucleotide, thioNAD$^+$, causes carbinolamine formation to become largely rate limiting, independent of which keto acid substrate is used.

The non-rate-limiting nature of the hydride transfer step in the overall mechanism was previously noted (Rife and Cleland, 1980a). Slow steps occurring after the pre-steady-state burst of product and during the attainment of the steady state, mask the primary deuterium isotope effect measured in steady-state experiments. While the results from the study of Weiss et al. (1988) suggest that slow product release is the cause of the masking, Rife and Cleland (1980a) noted that the primary deuterium isotope effect remains low even during the initial burst of product formation, indicating that there are isotopically insensitive steps in this stage that are slow and could mask the isotope effect.

Pre-steady-state techniques were used by Fisher et al. (1992) to identify a slow proton release step occurring before hydride transfer in the ternary E · NADP$^+$ · glutamate complex. This slow step was seen before in an earlier study (Fisher et al., 1980), but its chemical description was not clear. This proton release could also be observed when competitive inhibitors, which do not undergo catalysis, were used. This observation ruled out the possibility of the released proton coming from the amino group of the substrate. The authors suggest that the proton release is triggered either by a conformation change upon ternary complex formation or a change in an enzymic pK upon ternary complex formation.

Further pre-steady-state experiments refined the enzyme's chemical mechanism (Figure 6). Singh et al. (1993) propose that glutamate binds to an open conformation of the E · NADP$^+$ complex by binding its carboxyls to protonated Lys114 (α-carboxyl) and Lys90 (γ-carboxyl). The next step is a conformational change, in which bulk solvent is expelled from the active site, and glutamate and the nicotinamide ring are brought into catalytic proximity. The slow loss of a proton also occurs in this step, released from Lys126, which then allows the residue to bind a water molecule. This water molecule will later nucleophilically attack the imine intermediate, and is bound before ammonia release, as verified by other experiments mentioned above (Fisher et al., 1987; Srinivasan et al., 1988). These steps all occur before hydride transfer occurs, even in the initial burst phase of product formation, and can account for the masking of the primary deuterium isotope effect on hydride transfer.

The mechanism of the *C. symbiosum* glutamate dehydrogenase has not been as thoroughly studied as that for the liver enzyme, but its kinetic mechanism has been investigated and some chemical modification studies have been performed. Although this strict anaerobe's glutamate dehydrogenase is not regulated by complex allosteric effectors, its kinetic mechanism has not been trivial to elucidate. Steady-state kinetics were used to characterize this enzyme, and it was found to be NAD$^+$ dependent, although it could use acetylpyridineNAD$^+$ and deaminoNAD$^+$ as well as the natural substrate. The enzyme is essentially L-glutamate specific (Syed et al., 1991), and experiments suggested that the kinetic mechanism was random for both substrate binding and product release. The authors concede, however, that their data are not unambiguous, and product release

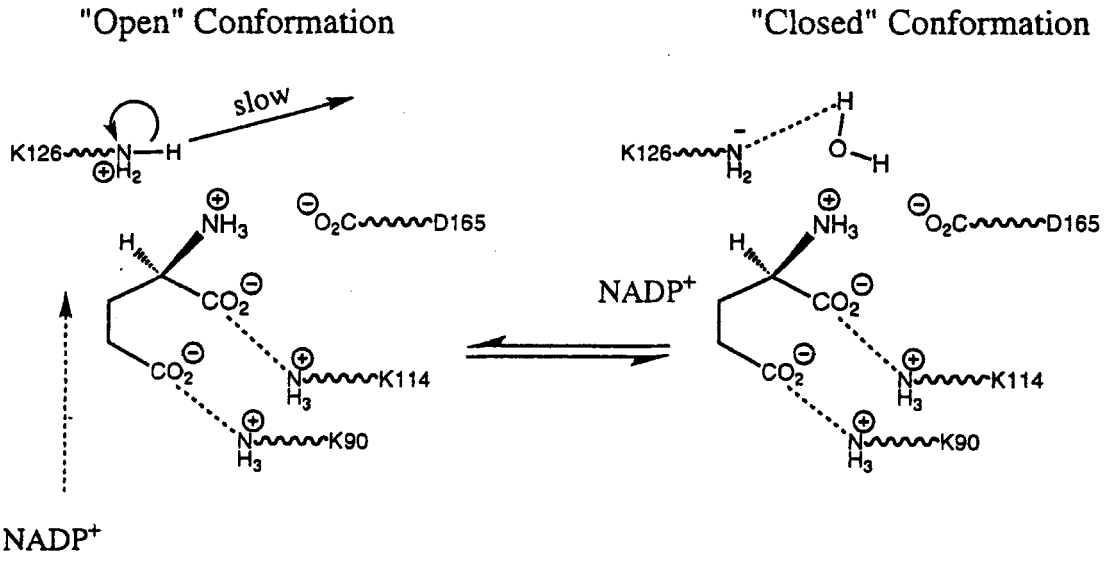


FIGURE 6. Model of pre-steady-state chemical mechanism.

could be rapid-equilibrium random (Syed et al., 1991). They also noticed a biphasic rate for glutamate dehydrogenase, which depended on the concentration of NAD^+, a phenomenon that had been observed for other glutamate dehydrogenase sources, but is unexplained (Syed et al., 1991).

The *C. symbiosum* enzyme was subjected to pyridoxal phosphate modification, and three lysines were found to react with this reagent (Lilley and Engel, 1988). These lysines (Lys89, Lys114, Lys125) are located in the amino acid binding pocket of the enzyme as observed in the X-ray crystal structure (see below). The modifications could be prevented by incubation with the substrates, and NAD^+, NADH, glutamate, succinate, and α-ketoglutarate could all partially protect the enzyme, but the highest level of protection was offered by NADH and α-ketoglutarate (Lilley and Engel, 1992). Although all three lysines could be modified, once a particular lysine was modified, the others became unreactive toward further modification, indicating the proximity of three residues to each other (Lilley and Engel, 1992). The enzyme was also reacted with sulfhydryl modifying agents (Syed et al., 1994), and only one of the two cysteines in each subunit could be modified with either *p*CMB or DTNB, while other modification agents were ineffective. The modified cysteine (Cys320) lies within the active site, but is not necessary for catalysis, since mutating the residue to serine, threonine, or alanine did not affect activity (Syed et al., 1994). The authors suggested the bulky modification agent inactivates the enzyme by sterically intruding on the active site. The enzyme could be protected against thiol modification by substrates, with the best protection observed in the presence of NADH and glutamate (Syed et al., 1994).

The mechanism of substrate binding was studied using stopped-flow techniques (Basso et al., 1993). The intrinsic enzyme fluorescence changed little upon formation

of either the E · NAD^+ or E · glutamate complexes, but when the ternary complex was generated there was a significant change in fluorescence. Measuring this change with different orders of substrate addition allowed the authors to suggest the kinetic mechanism was rapid equilibrium random. Furthermore, binding of the first substrate synergistically enhanced the binding efficiency of the second, with E · NAD^+ formation being the preferred pathway (Basso et al., 1993). Pre-steady state experiments showed that there was no burst of product formation before steady-state rates were achieved, unlike the bovine liver enzyme. This observation, coupled with their fluorescence studies, led the authors to suggest that there is a rate-limiting step present between ternary complex formation and hydride transfer, postulated to be a slow conformational change (Basso et al., 1993). This phenomenon may be related to another study that showed that the enzyme could have two states of activity (Syed and Engel, 1990). At low pH, the enzyme has a higher rate of activity than at higher pHs. The change in activity was not due to protein aggregation, but could be enhanced by ionic strength, temperature, and substrate binding, particularly α-ketoglutarate and nucleotide. The authors suggest the different states of protein activity may be due to a slow pH-dependent conformational change (Syed and Engel, 1990). This step could be similar to the one proposed for bovine liver enzyme (Singh et al., 1993).

The *Clostridium* enzyme has become of great significance to the amino acid dehydrogenase family because it was the first, and presently only, enzyme to be crystallized and have its three-dimensional structure solved. Bovine liver enzyme crystallization has been hampered by its tendency to aggregate under high protein conditions. The *Clostridium* enzyme also has the advantage that it is not subject to allosteric regulation. Extensive sequence comparisons, however, have shown that the hexameric glutamate dehydrogenases share significant homology with each other, implying that the information gleaned from the structural description of the *Clostridium* enzyme could be used as a paradigm for other glutamate dehydrogenases, and possibly other amino acid dehydrogenases (Teller et al., 1992).

In a preliminary note, Rice et al. (1985) reported their successful crystallization of native *Clostridium* glutamate dehydrogenase from 35 to 45% ammonium sulfate at pHs 6 to 8. Large rhombohedral crystals could be cut from crystal clusters. These crystals existed in a *C*2 space group, with unit cell dimensions of a = 147 Å, b = 151 Å, c = 108 Å, and β = 140°. This report was followed by a model of the structure at 6 Å resolution (Rice et al., 1987). This structure was based on the native data set, plus two mercury heavy atom derivatives that crystallized in the same monoclinic *C*2 space group. The authors found that three subunits of the hexamer were in the asymmetric unit, and the hexamer is a squat cylinder 108 Å in height and has a 44 Å radius. The authors also collected a data set of the crystals with NAD^+ soaked into them.

The authors' model showed that the protein was approximately 55% α-helix and consisted of two globular domains separated by a deep cleft, which presumably is the active site (Rice et al., 1987). The N-terminal domain (domain A or I) makes most of the intersubunit contacts within the hexamer. It was also implied that this domain binds the amino acid substrate. The

C-terminal domain (domain B or II) binds NAD^+ in the intrasubunit cleft, and NAD^+ is bound in a position similar to that seen for other NAD^+-dependent dehydrogenases. Binding of NAD^+ caused no large changes in the apoprotein structure (Rice et al., 1987).

The model was refined to 1.96 Å with a R factor = 0.227 (Baker et al., 1992). With this model, clear fitting of the main chain atoms into electron density was possible, including positioning of side chains. The hexamer is arranged in a cylindrical shape formed by a dimer of trimers with a center cavity 11 Å wide, which is lined by hydrophilic residues. The monomer consists of 17 α-helices and 13 β-strands. Domain I begins with five helices, followed by two antiparallel β-strands, then a β-sheet containing both parallel and antiparallel strands, flanked by α-helices. Domain II consists of a β-sheet of six parallel and one antiparallel β-strands, flanked by α-helices, similar to the NAD^+-binding domains of other dehydrogenases, the so-called Rossmann fold, with the exception that here one of the strands' direction is reversed. The connections between the two domains of each subunit are limited to just two helices and a peptide loop, potentially allowing freedom of movement between domains (Baker et al., 1992).

The bound NAD^+ molecule was clearly seen in this model. Both the adenine and nicotinamide ribose rings are bound in a C2′ *endo* position. The nicotinamide ring is bound in the *syn* position about the glycosidic bond because of specific contacts made between the enzyme and the carboxamide moiety. This mode of binding packs the *re* face on the ring against the enzyme surface with the pro-*R* hydrogen shielded by a methyl group. The *si* face of the ring is adjacent to the large cleft in the protein, but the C_4 carbon of the nicotinamide ring is at least 6.3 Å away from a putative glutamate-binding site, thus precluding catalysis from occurring in this configuration (Baker et al., 1992). $NADP^+$ is discriminated against, because the presence of a 2′ phosphate is not accommodated by an appropriate pocket on the enzyme surface.

New crystals were grown by using L-glutamate as the precipitant (Stillman et al., 1992). These crystals existed in a *R*32 space group, different from the one seen in the native and NAD^+ bound crystals. The cell dimensions are a = b = 162 Å, c = 103 Å, α = β = 90°, γ = 120°. In addition there was only 1 subunit per asymmetric unit. The authors believed that such a radical change in crystal space groups could only come from a conformational change in the protein. This report preceded a full three-dimensional structural determination with glutamate bound, at 1.9 Å resolution and a R factor of 0.171 (Stillman et al., 1993). The structure was solved using two heavy atom derivatives, pCMBS and $K_2Pt(CN)4$, which when soaked into the crystal could cause the space group change from *C*2 to *R*32, just as glutamate could (Stillman et al., 1993).

The model showed that with glutamate bound, the enzyme changes to a closed conformation that excludes bulk solvent from the cleft. This makes the active site more hydrophobic and better suited for hydride transfer. One ordered water molecule is left at the active site and is bound to the neutral side chain of Lys125. Glutamate lies in the cleft bound to domain I. Its γ-carboxyl group is bound to Lys89 as well as Ser380. In the NAD^+ bound model, Lys113 makes contact with Asn373, which is in domain II. With glutamate, the side

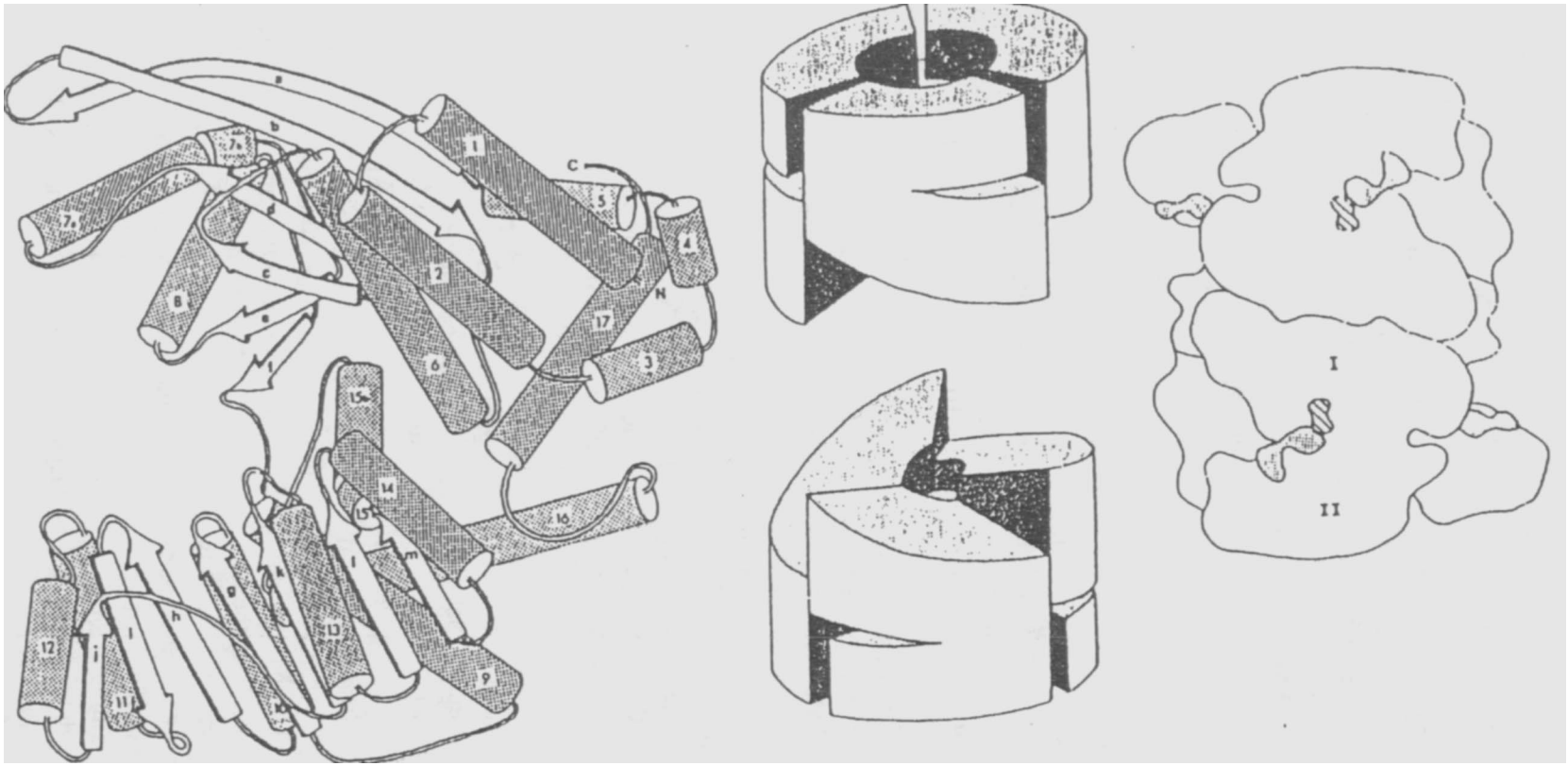


FIGURE 7. A schematic representation of the glutamate α-helices represented as cylinders and β-sheets as arrows, the positive interlocking of the subunits about the two-fold axis is illustrated, middle, and the assembly of six subunits into the active hexamer is portrayed, right, with the shaded segments representing the bound cofactor NAD^+ and hatched areas the model of bound glutamate. The two domains of each subunit, I and II, are clearly visible (from Baker et al., 1992).

chain of Lys113 moves significantly to bind the α-carboxyl group of glutamate, but the closed conformation of the protein brings Asn373 into a new position which allows it to contact Lys113. The C_3 and C_4 atoms of glutamate make van der Waals contacts with V377 and A163.

The model shows how the heavy atom derivatives can stabilize the closed conformation of the enzyme. $Pt(CN)_4$ molecules bind in the same pocket as glutamate, while mercury binds to Cys320, which is not directly in the active site, but can be modified by bulky reagents, inactivating the enzyme (Syed et al., 1994).

The principal interactions between the enzyme and NAD^+ are not significantly different in the closed conformation of the enzyme. Modeling NAD^+ into the active site of the closed conformation, however, shows that the C_4 atom is 4.0 Å away from the α-carbon of glutamate. While this is closer than the 7.4 Å distance in the open conformation, it is still too far for efficient hydride transfer. The authors speculate that when the ternary complex forms, there must be additional, slight conformational changes that will bring the C_4 atom of the nicotinamide ring within a catalytically appropriate, but unspecified, distance to the αC of glutamate (Stillman et al., 1993).

From their models of the open and closed conformations of the enzyme, plus the limited enzymological information for this enzyme, the authors propose a model for the chemical mechanism of the enzyme that is somewhat different from the revised models of Rife and Cleland (see above). When viewed from the reverse direction, this model begins with α-ketoglutarate bound with its side chain carboxyl bound to Lys89 and its α-carboxyl bound to Lys113. Free ammonia attacks the keto acid's α-carbon and the carbonyl oxygen is protonated by Asp165, a residue conserved among glutamate dehydrogenases and present in the proper position for this role in the crystal structure (Stillman et al., 1993). Asp165 triggers the carbinolamine collapse to the imine by removing a proton from the amine moiety and the substrate oxygen leaves as water by abstracting a second proton from Lys125. The imine is then reduced by NADH, and the nitrogen is protonated by Asp165 to form the zwitterionic form of L-glutamate.

A D165S mutant enzyme was generated and kinetic studies showed that it displayed less activity than the normal enzyme, but bound its substrates with the same affinity, except for ammonia, which had a substantially larger K_m (Dean et al., 1994). These data were used to confirm Asp165 catalytic role in the chemical mechanism.

The authors note that Lys125, which has a low pK value, is essential in activating the ordered water molecule in the active site, where it behaves as a nucleophile in the forward direction. When this occurs, Lys125 is protonated and must lose this proton in order to bind a second water molecule for the next round of catalysis. This step may actually be quite slow and may account for the proton burst seen in the pre-steady-state experiments with bovine liver glutamate dehydrogenase (Singh et al., 1993).

IV. CONCLUSIONS

Although the amino acid dehydrogenases occupy only a small corner of the pyridine nucleotide-dependent dehydrogenase world, they have many important industrial and medical applications, and have served as a fascinating landscape for enzymological research. The best charac-

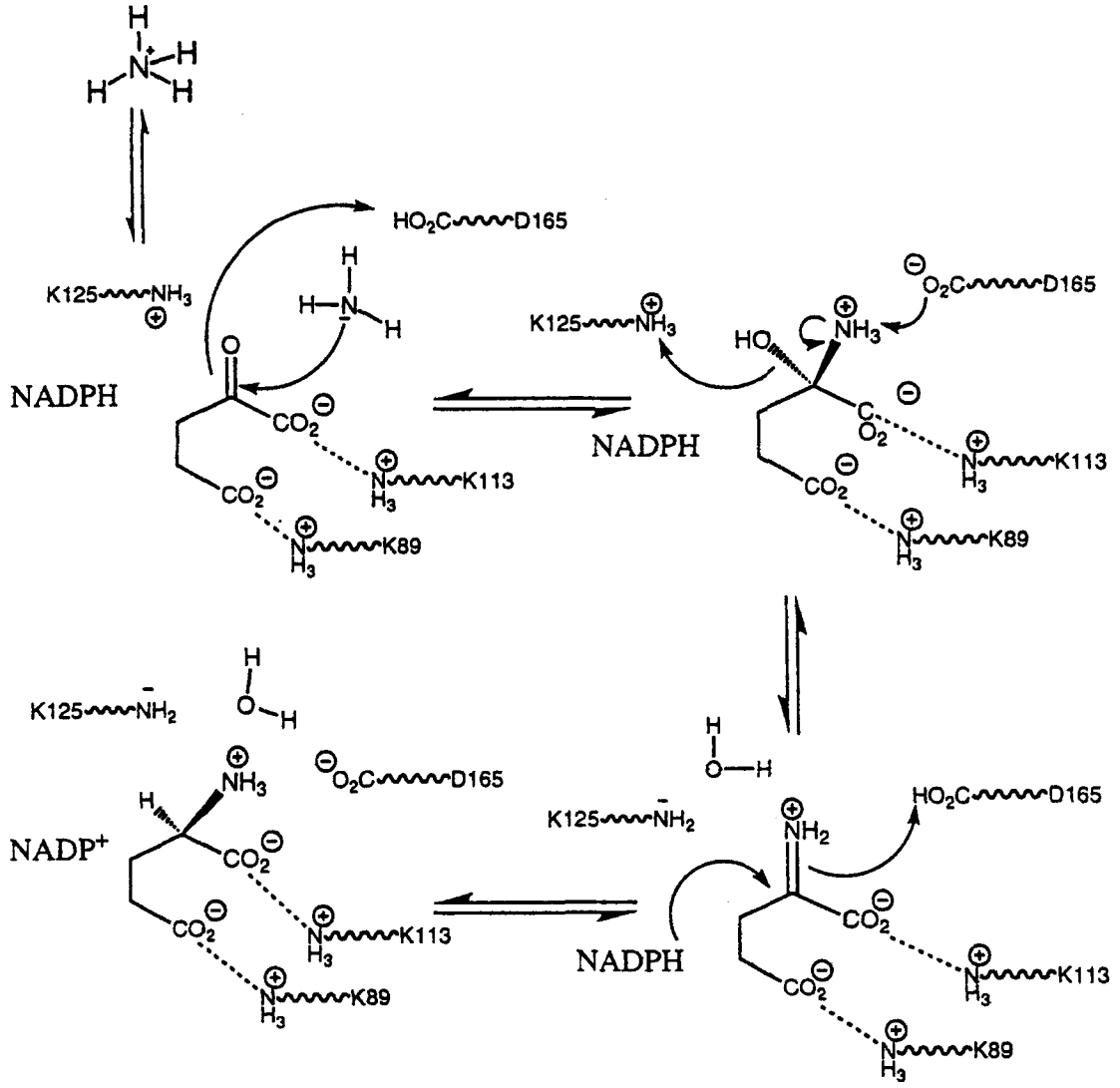


FIGURE 8. Chemical mechanism of glutamate dehydrogenase based on X-ray crystallographic evidence.

terized of these enzymes have revealed homologous amino acid sequences that allow for the identification of conserved residues involved in the catalysis of identical reactions, while also indicating how a progenitor enzyme can be adapted to use different types of substrates. Despite performing similar chemistry on their respective amino acid substrates, the details of leucine, alanine, and glutamate chemical mechanisms and their rate determining steps have been revealed as subtly different. Because a wealth of enzymological data are available on this class of enzymes, and a three dimensional structure has recently been determined, serious structure/function relationships can be undertaken. This enzyme family provides

ACKNOWLEDGMENTS

The authors would like to thank Dr. Julius Marmur for his helpful comments. This work was supported by National Institutes of Health grant GM 33449 (to J. S. B.) and National Institutes of Health predoctoral training grant 5T32 GM 07260 (to N. M. W. B.). The information in this paper is part of a thesis to be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine.

Alizade, M. A., Bressler, R., and Brendel, K. (1975). Stereochemistry of the hydrogen transfer to NAD^+ Catalyzed by (S)alanine dehydrogenase from *Bacillus subtilis*. *Biochim. Biophys. Acta* **397**, 5–8.

Andersen, Å. B., Andersen, P., and Ljungqvist, L. (1992). Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. *Infect. Immun.* **60**, 2317–2323.

Asano, Y. and Nakazawa, A. (1985). Crystallization of phenylalanine dehydrogenase from *Sporosarcina ureae*. *Agric. Biol. Chem.* **49**,

Asano, Y., Nakazawa, A., and Endo, K. (1987a). Novel phenylalanine dehydrogenases from *Sporosarcina ureae* and *Bacillus sphaericus*. *J. Biol. Chem.* **262**, 10346–10354.

Baker, P. J., Britton, K. L., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W., and Stillman, T. J. (1992). Subunit assembly and active site location in the structure of glutamate dehydrogenase. *Proteins: Struct., Funct., and Gen.* **12**, 75–86.

Baker, J. J. and van der Drift, C. (1974). Purification and properties of L-*erythro*-3,5-diaminohexanoate dehydrogenase from *Clostridium sticklandii*. *Biochemistry* **13**, 292–299.

Baker, J. J., Jeng, I., and Barker, H. A. (1972). Purification and properties of L-*erythro*-3,5-diaminohexanoate dehydrogenase from a lysine-fermenting *Clostridium*. *J. Biol. Chem.* **247**, 7724–7734.

Basso, L. A., Engel, P. C., and Walmsley, A. R. (1993). The Mechanism of substrate and coenzyme binding to clostridial glutamate dehydrogenase during oxidative deamination. *Eur. J. Biochem.* **213**, 935–945.

Bellion, E. and Tan, F. (1987). An NAD^+-dependent alanine dehydrogenase from a methylotrophic bacterium. *Biochem. J.* **244**, 565–570.

Boer, L. de, Rijssel, M. van, Euverink, G. J., and Dijkhuizen, L. (1989). Purification, characterization and regulation of a monomeric L-phenylalanine dehydrogenase from the facultative methyltroph *Nocardia* sp. *239*. *Arch. Microbiol.* **153**, 12–18.

Bradshaw, C. W., Wong, C.-H., Hummel, W., and Kula, M.-R. (1991). Enzyme-catalyzed asymmetric synthesis of (*S*)-2-amino-4-phenylbutanoic acid and (R)-2-hydroxyl-4-phenylbutanoic acid. *Bioorganic Chem.* **19**,

Brown, A., Colen, A. H., and Fisher, H. F. (1979). Effect of ammonia on the glutamate dehydrogenase catalyzed oxidative deamination of L-glutamate. The steady state. *Biochemistry* **26**, 5924–5928.

quencing, and expression of *Rhodococcus* L-phenylalanine dehydrogenase. *J. Biol. Chem.* **269**, 16203–16211.

Colen, A. H. (1978). Transient-state kinetics of L-glutamate dehydrogenase: mechanism of α-ketoglutarate inhibition in the burst phase. *Biochemistry* **17**, 528–533.

Crow, V. L. (1987). Properties of alanine dehydrogenase and aspartase from *Propionibacterium freudenreichii* subsp. *shermanii*. *Appl. Environ. Microbiol.* **53**, 1885–1892.

Dean, J. L. E., Wang, X.-G., Teller, J. K., Waugh, M. L., Britton, K. L., Baker, P. J., Stillman, T. J., Martin, S. R., Rice, D. W., and Engel, P. C. (1994). The catalytic role of aspartate in the active site of glutamate dehydrogenase. *Biochem. J.* **301**, 13–16.

Fisher, H. F., Maniscalco, S., Singh, N., Mehrotra, R. N., and Srinivasan, R. (1992). A slow obligatory proton release step precedes hydride transfer in the liver glutamate dehydrogenase catalytic mechanism. *Biochim. Biophys. Acta* **1119**, 52–56.

Fisher, H. F., Pazhanisamy, S., and Medary, R. T. (1987). The anomalous properties of the glutamate dehydrogenase-NADPH-α-ketoglutarate complex are not ascribable to a carbonyl addition reaction. *J. Biol. Chem.* **262**, 11684–11687.

Fisher, H. F., Stickel, D. C., and Colen, A. H. (1980). pH-dependent thermodynamic parameters of the glutamate dehydrogenase-α-ketoglutarate-NADPH complex. *Biochim. Biophys. Acta* **615**, 27-33.

di Franco, A. and Iwatsubo, M. (1971). Transient complexes in the catalysis of glutamate dehydrogenase. *Biochimie* **53**, 153–159.

Goldman, D. S. (1959). Enzyme systems in the *Mycobacteria*. *Biochim. Biophys. Acta* **34**, 527–539.

Grimshaw, C. E. and Cleland, W. W. (1981). Kinetic mechanism of *Bacillus subtilis* L-alanine dehydrogenase. *Biochemistry* **20**, 5650–5655.

Grimshaw, C. E., Cook, P. F., and Cleland, W. W. (1981). Use of isotope effects and pH studies to determine the chemical mechanism of *Bacillus subtilis* L-alanine dehydrogenase. *Biochemistry* **20**, 5655–5661.

Hammer, T., Bode, R., and Birnbaum, D. (1991). Occurrence of a novel yeast enzyme, L-lysine ε-dehydrogenase, which catalyses the first step of lysine catabolism in *Candida albicans*. *J. Gen. Microbiol.* **137**, 711–715.

Hashimoto, H., Misono, H., Nagata, S., and Nagasaki, S. (1989a). Activation of L-lysine ε-dehydrogenase from *Agrobacterium tumefaciens* by several amino acids and monocarboxylates. *J. Biochem.* **106**, 76–80.

Hashimoto, H., Misono, H., Nagata, S., and Nagasaki, S. (1989b). Stereospecificity of hydrogen transfer of the coenzyme catalyzed by L-lysine ε-dehydrogenase. *Agric. Biol. Chem.* **53**, 1175–1176.

Hibino, Y., Asano, Y., Okazaki, N., and Numao, N. (1990). Isolated Phenylalanine Dehydrogenase Gene and Process for Production of Phenylalanine Dehydrogenase. U.S. Patent 4,970,157.

Hudson, R. C. and Daniel, R. M. (1993). L-Glutamate dehydrogenases: distribution, properties and mechanism. *Comp. Biochem. Physiol.* B **106**, 767–792.

Hummel, W. and Kula, M.-R. (1989). Dehydrogenases for the synthesis of chiral compounds. *Eur. J. Biochem.* **184**, 1–13.

Hummel, W., Schütte, H., Schmidt, E., Wandrey, C., and Kula, M.-R. (1987). Isolation of L-phenylalanine dehydrogenase from *Rhodococcus* sp. *M4* and its application for the production of L-phenylalanine. *Appl. Microbiol. Biotechnol.* **26**, 409–416.

Hummel, W., Schmidt, E., Wandrey, C., and Kula, M.-R. (1986). L-Phenylalanine dehydrogenase from *Brevibacterium* sp. for production of L-phenylalanine by reductive amination of phenylpyruvate. *Appl. Microbiol. Biotechnol.* **25**, 175–185.

Hummel, W., Weiss, N., and Kula, M.-R. (1984). Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. *Arch. Microbiol.* **137**, 47–52.

Ishino, S., Mizukami, T., Yamaguchi, K., Katsumata, R., and Araki, K. (1987). Nucleotide sequence of the *meso*-α,ε-diaminopimelate D-dehydrogenase gene from *Corynebacterium glutamicum*. *Nucleic Acids Res.* **15**, 3917.

Jallon, J.-M., Risler, Y., and Iwatsubo, M. (1975). Beef liver L-glutamate dehydrogenase mechanism: presteady state study of the catalytic reduction of 2.oxoglutarate by NADPH. *Biochem. Biophys. Res. Commun.* **67**, 1527–1536.

Kataoka, K., Takada, H., Yoshimura, T., Furuyoshi, S., Esaki, N., Ohshima, T., and Soda, K. (1993). Site-directed mutagenesis of a hexapeptide segment involved in substrate recognition of phenylalanine dehydrogenase from *Thermoactinomyces intermedius*. *J. Biochem. 114*, 69–75.

Keradjopoulos, D. and Holldorf, A. W. (1979). Purification and properties of alanine dehydrogenase from *Halobacterium salinarium*. *Biochim. Biophys. Acta* **570**, 1–10.

Kim, E. K. and Fitt, P. S. (1977). Partial purification and properties of *Halobacterium cutirubrum* L-alanine dehydrogenase. *Biochem. J.* **161**, 313–320.

Kretovich, W. L. and Stepanovich, K. M. (1966). The enzyme catalyzing hydroxypyruvate reductive amination. *Izv. Akad. Nauk S.S.S.R. Ser. Biol.* **No. 2**, 295–301.

Kuroda, S., Tanizawa, K., Sakamoto, Y., Tanaka, H., and Soda, K. (1990). Alanine dehydrogenases from two *Bacillus* species with distinct thermostabilities: molecular cloning, DNA and protein sequence determination, and structural comparison with other NAD(P)$^+$-dependent dehydrogenases. *Biochemistry* **29**, 1009–1015.

Lilley, K. S. and Engel, P. C. (1988). Identification of three essential lysine residues probably involved in substrate binding of the NAD$^+$-dependent glutamate dehydrogenase of *Clostridium symbiosum*. *Biochem. Soc. Trans.* **16**, 875–876.

Lilley, K. S. and Engel, P. C. (1992). The essential active-site lysines of clostridal glutamate dehydrogenase. *Eur. J. Biochem.* **207**, 533–540.

LiMuti, C., and Bell, J. E. (1983). A steady-state random-order mechanism for the oxidative deamination of norvaline by glutamate dehydrogenase. *Biochem. J.* **211**, 99–107.

Lin, M. C. M. and Wagner, C. (1975). Purification and characterization of *N*-methylalanine dehydrogenase. *J. Biol. Chem.* **250**, 3746–3751.

Lünsdorf, H. and Tsai, H. (1985). Quaternary organization of subunits in the L-leucine dehydrogenase from *Bacillus cereus*. *FEBS Lett.* **193**, 261–266.

Matsuyama, T., Soda, K., Fukui, T., and Tanizawa, K. (1992). Leucine dehydrogenase form *Bacillus stearothermophilus*: identification of active-site lysine by modification with pyridoxal phosphate. *J. Biochem.* **112**, 258–265.

McCormick, N. G. and Halvorson, H. O. (1964). Purification and properties of L-alanine dehydrogenase from vegetative cells of *Bacillus cereus*. *J. Bacteriol.* **87**, 68–74.

McCowen, S. M. and Phibbs, P. V. Jr. (1974). Regulation of alanine dehydrogenase in *Bacillus licheniformis*. *J. Bacteriol.* **118**, 590–597.

Misono, H., Hashimoto, H., Uehigashi, H., Nagata, S., and Nagasaki, S. (1989a). Properties of L-lysine ε-dehydrogenase from *Agrobacterium tumefaciens*. *J. Biochem.* **105**, 1002–1008.

Misono, H., Ogasawara, M., and Nagasaki, S. (1986a). Characterization of *meso*-α,ε-diaminopimelate dehydrogenase from *Corynebacterium glutamicum* and its distribution in bacteria. *Agric. Biol. Chem.* **50**, 2729–2734.

Misono, H., Ogasawara, M., and Nagasaki, S. (1986b). Purification and properties of *meso*-

diaminopimelate dehydrogenase from *Brevibacterium* sp. *Agric. Biol. Chem.* **50**, 1329–1330.

Misono, H. and Nagasaki, S. (1982). Occurrence of L-lysine ε-dehydrogenase in *Agrobacterium tumefaciens*. *J. Bacteriol.* **150**, 398–401.

Misono, H. and Nagasaki, S. (1983). Distribution and physiological function of L-lysine ε-dehydrogenase. *Agric. Biol. Chem.* **47**, 631–633.

Misono, H., Nagasaki, S., and Soda, K. (1981). *meso*-α,ε-diaminopimelate D-dehydrogenase: sulfhydryl group modification. *Agric. Biol. Chem.* **45**, 1455–1460.

Misono, H. and Soda, K., (1980a). Properties of *meso*-α,ε-diaminopimelate D-dehydrogenase from *Bacillus sphaericus*. *J. Biol. Chem.* **255**, 10599–10605.

Misono, H. and Soda, K., (1980b). Purification and properties of *meso*-α,ε-diaminopimelate D-dehydrogenase from *Bacillus sphaericus*. *Agric. Biol. Chem.* **44**, 227–229.

Misono, H., Togawa, H., Yamamoto, T., and Soda, K. (1976). Occurrence of *meso*-α,ε-diaminopimelate dehydrogenase in Bacillus sphaericus. *Biochem. Biophys. Res. Commun.* **72**, 89–93.

Misono, H., Togawa, H., Yamamoto, T., and Soda, K. (1979). *meso*-α,ε-Diaminopimelate D-dehydrogenase: distribution and the reaction product. *J. Bacteriol.* **137**, 22–27.

Misono, H., Uehigashi, H., Morimoto, E., and Nagasaki, S. (1985). Purification and properties of L-lysine ε-dehydrogenase from *Agrobacterium tumefaciens*. *Agric. Biol. Chem.* **49**, 2253–2255.

Misono, H., Yonezawa, J., Nagata, S., and Nagasaki, S. (1989b). Purification and characterization of a dimeric phenylalanine dehydrogenase from *Rhodococcus maris* K-18. *J. Bacteriol.* **171**, 30–36.

Moon, K. and Smith, E. L. (1973). Sequence of bovine liver glutamate dehydrogenase. VIII. Peptides produced by specific chemical cleavages; the complete sequence of the protein. *J. Biol. Chem.* **248**, 3082–3088.

Murrell, J. C. and Dalton, H. (1983). Ammonia assimilation in *Methylcoccus capsulatus* (bath) and other obligate methanotrophs. *J. Gen. Microbiol.* **129**, 1197–1206.

Nagata, S., Tanizawa, K., Esaki, N., Sakamoto, Y., Ohshima, T., Tanaka, H., and Soda, K. (1988). Gene cloning and sequence determination of leucine dehydrogenase from *Bacillus stearothermophilus* and structural comparison with other NAD(P)$^+$-dependent dehydrogenases. *Biochem.* **27**, 9056–9062.

Navarrete, R. M., Vara, J. A., and Hutchinson, C. R. (1990). Purification of an inducible L-valine dehydrogenase of *Streptomyces coelicolor* A3(2). *J. Gen. Microbiol.* **136**, 273–281.

Nisman, B. and Mager, J. (1952). Diphosphopyridine nucleotide and phosphate requirement for oxidation of amino-acids by cell-free extracts of obligate anaerobes. *Nature* **169**, 243–244.

Obermeier, N. and Poralla, K. (1976) Some physiological functions of the L-leucine dehydrogenase in *Bacillus subtilis*. *Arch. Microbiol.* **109**, 59–63.

O'Connor, R. J., and Halvorson, H. O. (1961). L-alanine dehydrogenase: a mechanism controlling the specificity of amino acid-induced germination of *Bacillus cereus* Spores. *J. Bacteriol.* **82**, 706–713.

Ohashima, T. and Soda, K. (1979). Purification and properties of alanine dehydrogenase from *Bacillus sphaericus*. *Eur. J. Biochem.* **100**, 29–39.

Okazaki, N., Hibino, Y., Asano, Y., Ohmori, M., Numao, N., and Kondo, K. (1988). Cloning and nucleotide sequencing of phenylalanine dehydrogenase gene of *Bacillus sphaericus*. *Gene* **63**, 337–341.

Ohshima, T., Misono, H., and Soda, K. (1978). Properties of crystalline leucine dehydrogenase from *Bacillus sphaericus*. *J. Biol. Chem.* **253**, 5719–5725.

Ohshima, T., Nagata, S., and Soda, K. (1985). Purification and characterization of thermostable leucine dehydrogenase from *Bacillus stearothermophilus*. *Arch. Microbiol.* **141**, 407–411.

Ohshima, T., Sakane, M., Yamazaki, T., and Soda, K. (1990). Thermostable alanine dehydrogenase from thermophilic *Bacillus sphaericus* DSM 462. *Eur. J. Biochem.* **191**, 715–720.

Ohshima, T. and Soda, K. (1990). Biochemistry and biotechnology of amino acid dehydrogenases. *Adv. Biochem. Eng./Biotech.* **42**, 187–209.

Ohshima, T. and Soda, K. (1993). Valine dehydrogenase from a non-spore-forming bacterium, *Alcaligenes faecalis*: purification and characterization. *Biochim. Biophys. Acta* **1162**, 221–226.

Ohshima, T., Takada, H., Yoshimura, T., Esaki, N., and Soda, K. (1991). Distribution, purification, and characterization of thermostable phenylalanine dehydrogenase from *Thermophilic Actinomycetes*. *J. Bacteriol.* **173**, 3943–3948.

Ômura, S., Tanaka, Y., Mamada, H., and Masuma, R. (1983). Ammonium ion suppresses the biosynthesis of tylosin aglycone by interference with valine catabolism in *Streptomyces fradiae*. *J. Antibiotics* **36**, 1792–1794.

Porumb, H., Vancea, D., Muresan, L., Presecan, E., Lascu, I., Petrescu, I., Porumb, T., Pop, R., and Bârzu, O. (1987). Structural and catalytic properties of L-alanine dehydrogenase from *Bacillus cereus*. *J. Biol. Chem.* **262**, 4610–4615.

Priestley, N. D. and Robinson, J. A. (1989). Purification and catalytic properties of L-valine dehydrogenase from *Streptomyces cinnamonensis*. *Biochem. J.* **261**, 853–861.

Rice, D. W., Baker, P. J., Farrants, G. W., and Hornby, D. P. (1987). The crystal structure of glutamate dehydrogenase from *Clostridium symbiosum* at 0.6 nm Resolution. *Biochem. J.* **242**, 789–795.

Rice, D. W., Hornby, D. P., and Engel, P. C. (1985). Crystallization of an NAD^+-dependent glutamate dehydrogenase from *Clostridium symbiosum*. *J. Mol. Biol.* **181**, 147–149.

Rife, J. E. and Cleland, W. W. (1980a). Kinetic mechanism of glutamate dehydrogenase. *Biochemistry* **19**, 2321–2328.

Rife, J. E. and Cleland, W. W. (1980b). Determination of the chemical mechanism of glutamate dehydrogenase from pH studies. *Biochemistry* **19**, 2328–2333.

Rowell, P. and Stewart, W. D. P. (1976). Alanine dehydrogenase of the N_2-fixing blue-green alga, *Anabaena cylindrica*. *Arch. Microbiol.* **107**, 115–124.

Sanwal, B. D. and Zink, M. W. (1961). L-Leucine dehydrogenase of *Bacillus cereus*. *Arch. Biochem. Biophys.* **94**, 430–435.

Sekimoto, T., Fukui, T., and Tanizawa, K. (1994). Involvement of conserved lysine 68 of *Bacillus stearothermophilus* leucine dehydrogenase in substrate binding. *J. Biol. Chem.* **269**, 7262–7266.

Sekimoto, T., Matsuyama, T., Fukui, T., and Tanizawa, K. (1993). Evidence for lysine 80 as general base catalyst of leucine dehydrogenase. *J. Biol. Chem.* **268**, 27039–27045.

Singh, N., Maniscalco, S. J., and Fisher, H. F. (1993). The real-time resolution of proton-related transient-state steps in an enzymatic reaction. *J. Biol. Chem.* **268**, 21–28.

Siranosian, K. J., Ireton, K., and Grossman, A. D. (1993). Alanine dehydrogenase (*ald*) is required for normal sporulation in *Bacillus subtilis*. *J. Bacteriol.* **175**, 6789–6796.

Smith, E. L., Austen, B. M., Blumenthal, K. M., and Nyc, J. F. (1975). Glutamate dehydrogenases. in *The Enzymes* (Boyer, P. D., Ed.), Vol. 11, pp. 293–367. Academic Press, New York.

Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970). Bovine liver glutamate dehydrogenase: tentative amino acid sequence; identification of a reactive lysine; nitration of a specific tyrosine and loss of allosteric inhibition

by guanosine triphosphate. *Proced. Nat. Acad. Sci.* **67**, 724–730.

Smith, M. T. and Emerich, D. W. (1993a). Alanine dehydrogenase from soybean nodule bacteroids: purification and properties. *Arch. Biochem. Biophys.* **304**, 379–385.

Smith, M. T. and Emerich, D. W. (1993b). Alanine dehydrogenase from soybean nodule bacteroids. *J. Biol. Chem.* **268**, 10746–10753.

Somack, R. and Costilow, R. N. (1973). 2,4-Diaminopentanoic acid C_4 dehydrogenase. *J. Biol. Chem.* **247**, 385–388.

Sonntag, K., Eggeling, L., De Graaf, A. A., and Sahm, H. (1993). Flux partitioning in the split pathway of lysine synthesis in *Corynebacterium glutamicum*. *Eur. J. Biochem.* **213**, 1325–1331.

Srinivasan, R. and Fisher, H. F. (1985). Reversible reduction of an α-imino acid to an α-amino acid catalyzed by glutamate dehydrogenase: effect of ionizable functional groups. *Biochemistry.* **24**, 618–622.

Srinivasan, R., Viswanathan, T. S., and Fisher, H. F. (1988). Mechanism of formation of bound α-iminoglutarate from α-ketoglutarate in the glutamate dehydrogenase reaction. *J. Biol. Chem.* **263**, 2304–2308.

Stadtman, T. C. (1973). Lysine metabolism by *Clostridia*. *Adv. Enzymol.* **38**, 413–448.

Stillman, T. J., Baker, P. J., Britton, K. L., Rice, D. W., and Rodgers, H. F. (1992). Effect of additives on the crystallization of glutamate dehydrogenase from *Clostridium symbiosum*. *J. Mol. Biol.* **224**, 1181–1184.

Stillman, T. J., Baker, P. J., Britton, K. L., and Rice, D. W. (1993). Conformational flexibility in glutamate dehydrogenase. *J. Mol. Biol.* **234**, 1131–1139.

Syed, S. E.-H. and Engel, P. C. (1990). A pH-dependent activation-inactivation equilibrium in glutamate dehydrogenase of *Clostridium symbiosum*. *Biochem. J.* **271**, 351–355.

Syed, S. E.-H., Engel, P. C., and Parker, D. M. (1991). Function studies of a glutamate dehydrogenase with known three-dimensional structure: steady-state kinetics of the forward and reverse reactions catalyzed by the NAD^+-dependent glutamate dehydrogenase of *Clostridium symbiosum*. *Biochim. Biophys. Acta* **1115**, 123–130.

Syed, S. E.-H., Hornby, D. P., Brown, P. E., Fitton, J. E., and Engel, P. C. (1994). Site and significance of chemically modifiable cysteine residues in glutamate dehydrogenase of *Clostridium symbiosum* and the use of protection studies to measure coenzyme binding. *Biochem. J.* **298**, 107–113.

Takada, H., Yoshimura, T., Ohshima, T., Esaki, N., and Soda, K. (1991). Thermostable phenylalanine dehydrogenase of *Thermoactinomyces intermedius*: cloning, expression, and sequencing of its gene. *J. Biochem.* **109**, 371–376.

Tang, L. and Hutchinson, C. R. (1993). Sequence, transcriptional, and functional analyses of the (branched-chain amino acid) dehydrogenase gene of *Streptomyces coelicolor*. *J. Bacteriol.* **175**, 4176–4185.

Teller, J. K., Smith, R. J., McPherson, M. J., Engel, P. C., and Guest, J. R. (1992). The glutamate dehydrogenase gene of *Clostridium symbiosum*. *Eur. J. Biochem.* **206**, 151–159.

Tsuda, Y. and Friedmann, H. C. (1970). Ornithine metabolism by *Clostridium sticklandii*. *J. Biol. Chem.* **245**, 5914–5926.

Turnbull, A. P., Ashford, S. R., Baker, P. J., Rice, D. W., Rodgers, F. H., Stillman, T. J., and Hanson, R. L. (1994). Crystallization and quaternary structure analysis of the NAD^+-dependent leucine dehydrogenase from *Bacillus sphaericus*. *J. Mol. Biol.* **236**, 663–665.

Vackova, K., Mehta, A., and Kutacek, M. (1985). Tryptophan aminotransferase and tryptophan dehydrogenase activities in some cell compartments of spinach leaves: the effect of

calcium ions on tryptophan dehydrogenase. *Biol. Plant.* **27**, 154–158.

Váli, Z., Kilár, F., Lakatos, S., Venyaminov, S. A., and Závodszky, P. (1980). L-Alanine dehydrogenase from *Thermus thermophilus*. *Biochim. Biophys. Acta* **615**, 34–47.

Vancura, A., Vancurová, I., Volc, J., Fussey, S. P. M., Flieger, M., Neuzil, J., Marsálec, J., and Bêhal, V. (1988). Valine dehydrogenase from *Streptomyces fradiae*: purification and properties. *J. Gen. Microbiol.* **134**, 3213–3219.

Vancurová, I., Vancura, A., Volc, J., Neuzil, J., Flieger, M., Basarová, G., and Bêhal, V. (1988a). Isolation and characterization of valine dehydrogenase from *Streptomyces aureofaciens*. *J. Bacteriol.* **170**, 5192–5196.

Vancurová, I., Vancura, A., Volc, J., Neuzil, J., Flieger, M., Basarová, G., and Bêhal, V. (1988b). Purification and partial characterization of alanine dehydrogenase from *Streptomyces aureofaciens*. *Arch. Microbiol.* **150**, 438–440.

Weiss, P. M., Chen, C.-Y., Cleland, W. W., and Cook, P. F. (1988). Use of primary deuterium and ^{15}N isotope effects to deduce the relative rates of steps in the mechanisms of alanine and glutamate dehydrogenases. *Biochemistry* **27**, 4814–4822.

Wenko, L. K., Treick, R. W., and Wilson, K. G. (1985). Isolation and characterization of a gene encoding *meso*-diaminopimelate dehydrogenase from *Glycine max*. *Plant Mol. Biol.* **4**, 197–204.

Wiame, J. M. and Piérard, A. (1955). Occurrence of an L(+)-alanine-dehydrogenase in *Bacillus subtilis*. *Nature (London)* **176**, 1073–1075.

Yoshida, A. and Freese, E. (1964). Purification and chemical characterization of alanine dehydrogenase of *Bacillus subtilis*. *Biochim. Biophys. Acta* **92**, 33–43.

Yoshida, A. and Freese, E. (1965). Enzymic properties of alanine dehydrogenase of *Bacillus subtilis*. *Biochim. Biophys. Acta* **96**, 248–262.

Yoshida, A. and Freese, E. (1970). L-Alanine dehydrogenase (*Bacillus subtilis*). *Methods Enzymol.* **17**, 176–181.

Zink, M. W. and Sanwal, B. D. (1962). The distribution and substrate specificity of L-leucine dehydrogenase. *Arch. Biochem. Biophys.* **99**, 72–77.