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Purification and Characterization of a Dimeric Phenylalanine Dehydrogenase from *Rhodococcus maris* K-18

HARUO MISONO,* JUNICHI YONEZAWA, SHINJI NAGATA, AND SUSUMU NAGASAKI

Department of Agricultural Chemistry, Kochi University, Nankoku, Kochi 783, Japan

Received 20 June 1988/Accepted 30 September 1988

NAD$^+$-dependent phenylalanine dehydrogenase (EC 1.4.1.) was purified to homogeneity from a crude extract of *Rhodococcus maris* K-18 isolated from soil. The enzyme had a molecular mass of about 70,000 daltons and consisted of two identical subunits. The enzyme catalyzed the oxidative deamination of L-phenylalanine and several other L-amino acids and the reductive amination of phenylpyruvate and *p*-hydroxyphenylpyruvate. The enzyme required NAD$^+$ as a natural coenzyme. The NAD$^+$ analog 3-acetylpyridine-NAD$^+$ showed much greater coenzyme activity than did NAD$^+$. D-Phenylalanine, D-tyrosine, and phenylethylamine inhibited the oxidative deamination of L-phenylalanine. The enzyme reaction was inhibited by *p*-chloromercuribenzoate and $HgCl_2$. Initial-velocity and product inhibition studies showed that the reductive amination proceeded through a sequential ordered ternary-binary mechanism. NADH bound first to the enzyme, followed by phenylpyruvate and then ammonia, and the products were released in the order L-phenylalanine and NAD$^+$. The Michaelis constants were as follows: L-phenylalanine, 3.8 mM; NAD$^+$, 0.25 mM; NADH, 43 μM; phenylpyruvate, 0.50 mM; and ammonia, 70 mM.

Since NAD$^+$-dependent phenylalanine dehydrogenase (L-phenylalanine:NAD$^+$ oxidoreductase, EC 1.4.1.) was discovered by Hummel et al. (14), phenylalanine dehydrogenase has received much attention as a catalyst for the asymmetric synthesis of L-phenylalanine (1, 3, 12, 14). The enzyme occurs in various bacteria (2, 4, 5, 13, 14) and has been purified to homogeneity from *Sporosarcina ureae* (2), *Bacillus sphaericus* (4), and *Bacillus badius* (5). The enzymes from these bacteria are composed of eight identical subunits with molecular masses of 39,000 to 42,000 daltons (Da) (2, 4, 5). Although their enzymological properties have been characterized, the kinetic mechanism of the enzyme reaction has not been studied. During the course of a study on microbial degradation of L-phenylalanine, we found a dimeric NAD$^+$-dependent phenylalanine dehydrogenase in a soil bacterium identified as *Rhodococcus maris* K-18 and purified the enzyme to homogeneity to compare its properties with those of the octameric enzymes. We describe here the characterization of phenylalanine dehydrogenase purified from *R. maris* K-18, with emphasis on the kinetic mechanism of the enzyme reaction.

MATERIALS AND METHODS

Materials. NAD$^+$, NADP$^+$, and NADH were obtained from Kojin Biochemicals, Tokyo, Japan; NAD$^+$ analogs, α-keto acids (sodium salt), and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo.; DEAE-cellulose was obtained from Serva, Heidelberg, Federal Republic of Germany; Sephadex G-150, Phenyl-Sepharose CL-4B, Red-Sepharose CL-6B, and Mono Q HR5/5 anion-exchange column (5 by 50 mm) were obtained from Pharmacia, Uppsala, Sweden; TSK gel G3000SW was obtained from Toyo Soda, Tokyo, Japan; and marker proteins for molecular weight determinations were obtained from Oriental Yeast, Osaka, Japan. Hydroxyapatite was prepared according to the method of Tiselius et al. (29). Other chemicals used were analytical-grade reagents.

Medium and culture conditions. L-Phenylalanine-assimilating bacteria were isolated from soil by using a medium containing 1% L-phenylalanine, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract. The pH was adjusted to 7.2 with 4 M NaOH. Cultures were grown in test tubes containing 5 ml of medium at 30°C for 2 to 3 days on a reciprocal shaker. The isolated bacteria were maintained on medium containing 1% L-phenylalanine supplemented with 2% agar. Large-scale cultivation was carried out in 2-liter flasks containing 750 ml of medium supplemented with 0.5% peptone at 30°C for 21 h on a reciprocal shaker. The cells harvested by centrifugation were washed twice with 0.85% NaCl and stored at −20°C until used.

Enzyme assay. The standard reaction mixture for oxidative deamination contained 20 μmol of L-phenylalanine (pH 10.8), 2 μmol of NAD$^+$, 250 μmol of glycine-KCl-KOH buffer (pH 10.8), and enzyme in a final volume of 1.0 ml. The assay system for reductive amination consisted of 10 μmol of sodium phenylpyruvate, 0.2 μmol of NADH, 800 μmol of NH_4Cl, 200 μmol of glycine-KCl-KOH buffer (pH 9.9), and enzyme in a final volume of 1.0 ml. Substrate was replaced by water in a blank. Incubation was carried out at 30°C in a cuvette with a 1-cm light path. The reaction was started by addition of NAD$^+$ (or NADH) and monitored by measuring the initial change in A_{340} with a Shimadzu UV-140-02 double-beam spectrophotometer. One unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of NADH per min in the oxidative deamination. Specific activity was expressed as units per milligram of protein. Protein was measured by the method of Lowry et al. (18), with crystalline bovine serum albumin as the standard. Enzyme concentrations were derived from A_{280}. The absorption coefficient ($A^{1\%}_{1\,cm}$ at 280 nm = 15.3) was estimated by ultracentrifugal analysis with a specific refractive increment for solute protein of 1.874×10^{-3} (23).

Electrophoresis. Disc gel electrophoresis was performed by the method of Davis (9). Protein was stained with 0.04% Coomassie brilliant blue G-250 in 3.5% $HClO_4$. The enzyme was stained for activity with a solution (4.0 ml) containing 10 mM L-phenylalanine, 1 mM NAD$^+$, 0.125 M glycine-KCl-

* Corresponding author.

KOH buffer (pH 9.0), 40 μg of phenazine methosulfate, and 400 μg of nitroblue tetrazolium salt. Sodium dodecyl sulfate (SDS)-disc gel electrophoresis was carried out according to the method of Weber and Osborn (31).

Determination of molecular mass. Molecular mass was determined at room temperature by high-pressure liquid chromatography in a TSK gel G3000SW column (0.75 by 60 cm) (Toyo Soda) at a flow rate of 1.0 ml/min and an elution buffer consisting of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl. A calibration curve was made with the following proteins: yeast glutamate dehydrogenase (290,000 Da), pig heart lactate dehydrogenase (140,000 Da), yeast enolase (67,000 Da), yeast adenylate kinase (32,000 Da), and horse cytochrome *c* (12,400 Da). The molecular mass of the subunit was estimated by SDS-disc gel electrophoresis (31), using the following standard proteins: catalase (60,000 Da), ovalbumin (43,000 Da), yeast alcohol dehydrogenase (37,000 Da), α-chymotrypsinogen A (25,700 Da), and myoglobin (17,200 Da).

Purification of phenylalanine dehydrogenase. All procedures were performed at 0 to 5°C, and potassium phosphate buffer containing 0.01% 2-mercaptoethanol was used in the purification procedures unless otherwise stated.

(i) Step 1. Washed cells (about 1.5 kg [wet weight]) were suspended in 1 liter of 0.1 M buffer (pH 7.2) and disrupted by sonication. The intact cells and cell debris were removed by centrifugation.

(ii) Step 2. To the cell extract was added 1.0 ml of 1.0% protamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. After 10 min, the precipitate was removed by centrifugation.

(iii) Step 3. The supernatant was brought to 55% saturation with solid ammonium sulfate. The precipitate collected by centrifugation was dissolved in 10 mM buffer (pH 7.4) containing 10% glycerol and dialyzed against the same buffer.

(iv) Step 4. The enzyme solution was applied to a DEAE-cellulose column (4.8 by 42 cm) equilibrated with 10 mM buffer (pH 7.4) containing 10% glycerol. After the column was washed thoroughly with the buffer and then with the buffer supplemented with 0.1 M KCl, the enzyme was eluted with the buffer containing 0.15 M KCl. The active fractions were pooled and concentrated by ultrafiltration with a Pellicon Labocasette (Nihon Millipore Ltd., Tokyo, Japan) equipped with PT filters.

(v) Step 5. The enzyme was dialyzed against 1 mM buffer (pH 7.4) containing 0.1 M KCl and 20% glycerol and was then placed on a column (3.0 by 24 cm) of hydroxyapatite equilibrated with 1 mM buffer (pH 7.4) containing 0.1 M KCl and 20% glycerol. The enzyme was eluted with 10 mM buffer (pH 7.4) containing 0.1 M KCl and 20% glycerol. The active fractions were concentrated with a Pellicon Labocasette.

(vi) Step 6. To the enzyme solution was added the same volume of 0.9 M buffer (pH 7.4) containing 10% glycerol, and the preparation was applied to a Phenyl-Sepharose CL-4B column (1.5 by 16 cm) equilibrated with 0.5 M buffer containing 10% glycerol. After the column was washed with 0.4, 0.3, and 0.25 M buffer (pH 7.4) containing 10% glycerol, the enzyme was eluted with 0.2 M buffer (pH 7.4) containing 10% glycerol. The active fractions were concentrated with an ultrafiltration unit (model 200; Amicon Corp., Lexington, Mass.) and dialyzed against 10 mM buffer (pH 7.4) containing 20% glycerol.

(vii) Step 7. The enzyme solution was placed on a column (1.2 by 10 cm) of Red-Sepharose CL-6B equilibrated with 10 mM buffer (pH 7.4) containing 20% glycerol. After the column was washed with the same buffer and 0.1 M buffer (pH 7.4), the enzyme was eluted with 0.15 M buffer containing 20% glycerol. The active fractions were concentrated with an Amicon 200 ultrafiltration unit and dialyzed against 10 mM Tris hydrochloride buffer (pH 7.4) containing 10% glycerol.

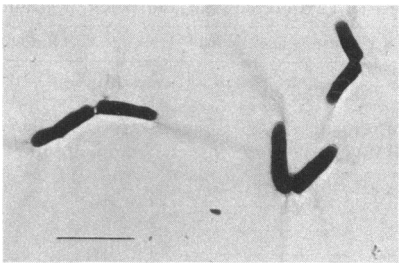

FIG. 1. Electron micrograph of *R. maris* strain K-18. Bar, 1.6 μm.

(viii) Step 8. The enzyme solution was applied to a Mono Q HR5/5 anion-exchange column (5 by 50 mm) equilibrated with 10 mM Tris hydrochloride buffer (pH 7.4) containing 10% glycerol. The column was equipped with a Pharmacia fast-protein liquid chromatography system and developed at a flow rate of 1.0 ml/min, with a 40-min linear gradient of KCl (0 to 0.3 M) in the same buffer. The active fractions were combined, concentrated with an Amicon 200 ultrafiltration unit, and stored at −20°C in the presence of 0.25 M sodium malonate.

RESULTS

Isolation of a bacterium having phenylalanine dehydrogenase. We isolated from soil 24 strains of bacteria that could utilize L-phenylalanine as the sole carbon source. Strain K-18 showed the highest L-phenylalanine dehydrogenase activity. Strain K-18 was a gram-negative, aerobic, coryneform bacterium that formed round, smooth colonies on the nutrient agar plate. No mycelium was formed. Cell division of strain K-18 was of the snapping type (32), and cells showed V forms (Fig. 1). The cell wall preparation contained *meso*-α,ε-diaminopimelate, arabinose, and galactose. Strain K-18 was positive in the glycolate test (30) and was not acid fast. From these morphological characteristics and the physiological characteristics listed in Table 1, strain K-18 seemed very similar to *R. maris* as described in *Bergey's Manual of Systematic Bacteriology* (11). Therefore, we named this strain *R. maris* K-18.

Optimal conditions for phenylalanine dehydrogenase production. Formation of phenylalanine dehydrogenase could be induced. Addition of 1.0% phenylalanine and 0.5% peptone to the medium was necessary to obtain maximal enzyme formation. The highest activity was obtained by cultivating the cells for 21 h at 30°C in medium containing 1% phenylalanine and 0.5% peptone.

Purification of phenylalanine dehydrogenase. Purification of the enzyme resulted in an approximately 85-fold enhancement of specific activity. Typical results of the purification procedure are shown in Table 2. The purified enzyme showed a single band on disc gel and SDS-disc gel electrophoresis (Fig. 2). The band stained for activity coincided with the protein band obtained by electrophoresis of the native enzyme.

Molecular mass and subunit structure. The molecular mass of the enzyme was estimated to be approximately 70,000 Da

TABLE 1. Taxonomic characteristics of strain K-18

Characteristic	Finding
Shape	Rod (0.4–0.5 by 1–2.5 μm)
Spores	None
Oxygen requirement	Aerobic
Mode of cell division	Snapping type
Growth at:	
37°C	Positive
42°C	Negative
Gram stain	Positive
Motility	Negative
Test for:	
Glycolate	Positive
Acid fastness	Negative
Catalase	Positive
Urease	Positive
Nitrate reduction	Positive
Hydrolysis of:	
Gelatin	Negative
Starch	Positive
Cellulose	Negative
Production of:	
Hydrogen sulfide	Negative
Indole	Negative
Voges-Proskauer test	Negative
Methyl red test	Negative
Assimilation of citrate	Positive
Production of acid from:	
Glucose	Positive
Sucrose, maltose, lactose, salicine, and sorbitol	Negative
Sensitivity to penicillin	Positive

by gel filtration on a TSK gel G3000SW column. The molecular mass of the subunit was calculated to be 36,000 Da from a similogarithmic plot of molecular mass versus mobility as determined by SDS-disc gel electrophoresis. These results suggested that the enzyme is a dimer composed of identical subunits.

Absorption spectrum. The absorption spectrum of the enzyme in 10 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol and 0.1 M KCl showed maximal absorbance at 278 nm, with a small shoulder at A_{283}. No absorption peak was detected in the region from 300 to 500 nm.

Stability. The purified enzyme was unstable, especially in the absence of glycerol and salt; 50% of activity was lost after the preparation was allowed to stand for 2 days at 4°C. Addition of glycerol (20%) and a high concentration of

TABLE 2. Summary of purification of phenylalanine dehydrogenase from *R. maris* K-18

Step no. (prepn)	Total protein[a] (mg)	Sp act (U/mg)	Total activity (U)	Yield (%)
1 (crude extract)	17,300	0.500	8,650	100
2 (protamine sulfate)	17,500	0.555	9,750	112
3 (ammonium sulfate)	15,700	0.618	9,700	112
4 (DEAE-cellulose)	3,500	1.37	4,810	55.6
5 (hydroxyapatite)	227	6.81	1,530	17.7
6 (Phenyl-Sepharose CL-4B)	24.6	30.1	740	8.55
7 (Red-Sepharose CL-6B)	3.81	66.4	253	2.92
8 (FPLC,[b] Mono Q HR5/5)	2.48	65.2	162	1.87

[a] Concentration of purified enzyme after the Red-Sepharose CL-6B step was determined from A_{280}, using the extinction coefficient ($A_1^{1\%}{}_{cm}$ = 15.7).
[b] FPLC, Pharmacia fast-protein liquid chromatography system.

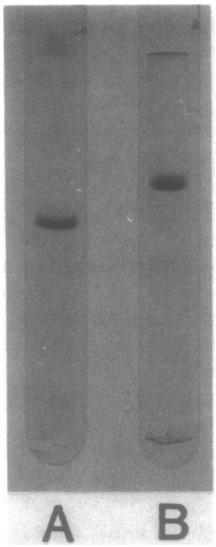

FIG. 2. Polyacrylamide disc gel electrophoresis of the purified enzyme. (Gel A) Purified enzyme (13 μg) electrophoresed at a current of 2.5 mA according to the method of Davis (9); (gel B) purified enzyme treated with 1% SDS and 0.1% 2-mercaptoethanol according to the method of Weber and Osborn (31). The SDS-treated enzyme (12 μg) was electrophoresed in the presence of 0.1% SDS at a current of 6 mA.

sodium malonate (0.25 M) or sodium glutarate (0.5 M) was found to stabilize the enzyme. In the presence of 0.25 M sodium malonate, the enzyme could be stored at 4°C for 1 month without loss of activity. When heated for 10 min in 10 mM potassium phosphate buffer (pH 7.4), the enzyme was stable at up to 35°C. Upon incubation at 35°C for 10 min, the enzyme was most stable over the pH range 6.7 to 10.

Effect of pH on enzyme activity. The enzyme showed maximal activity at pH 10.8 for the oxidative deamination of L-phenylalanine and at pH 9.8 for the reductive animation of phenylpyruvate. The rate of reductive amination at pH 9.8 (650 μmol/min per mg) was much higher than the rate of oxidative deamination at pH 10.8 (65.2 μmol/min per mg).

Substrate specificity. The ability of the enzyme to catalyze the oxidative deamination of various amino acids was examined at a concentration of 10 mM. In addition to L-phenylalanine (relative activity, 100), which was the preferred substrate, various L-amino acids served as substrates (relative activities given in parentheses): L-norleucine (15.6), L-ethionine (13.0), *p*-fluoro-DL-phenylalanine (8.3), *m*-fluoro-DL-phenylalanine (7.7), L-tryptophan (7.5), L-α-amino-β-phenylbutyrate (7.0), L-methionine (5.4), L-isoleucine (2.7), *o*-fluoro-DL-phenylalanine (2.3), L-tyrosine (2.0), L-leucine (2.0), DL-allylglycine (1.6), L-α-amino-*n*-butyrate (1.3), and *S*-methyl-L-cysteine (0.8). Phenylethylamine, L-phenylglycine, L-dopa, DL-phenylserine, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, DL-phenyllactate, L-*tert*-leucine, γ-aza-DL-leucine, L-norvaline, L-valine, L-alanine, L-lysine, L-arginine, L-histidine, L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-proline, L-serine, L-threonine, D-phenylalanine, D-tyrosine, D-tryptophan, D-methionine, D-leucine, D-isoleucine, D-norleucine, D-ethionine, D-*allo*-isoleucine, D-α-amino-*n*-butyrate, and *S*-methyl-D-cysteine were not substrates. Substrate specificities of the enzyme for reductive amination are given in Table 3. In addition to phenylpyruvate, *p*-hydroxyphenylpyruvate was a good substrate.

TABLE 3. Substrate specificities of phenylalanine dehydrogenase in reductive amination

Keto acid	Relative activity	K_m (mM)
Phenylpyruvate	100	0.5
p-Hydroxyphenylpyruvate	91	1.3
α-Ketocaproate	9.2	
α-Keto-γ-methiol-*n*-butyrate	9.0	
Indole-β-pyruvate	5.0	
β-Hydroxypyruvate	2.0	
α-Ketoisocaproate	1.2	
α-Ketovalerate	0	
α-Ketoisovalerate	0	
DL-α-Keto-β-methyl-*n*-valerate	0	
α-Ketobutyrate	0	
Oxaloacetate	0	
α-Ketomalonate	0	
α-Ketoglutarate	0	

[a] Keto acids were used at a concentration of 10 mM except for *p*-hydroxyphenylpyruvate (5 mM) and indole-β-pyruvate (2 mM).

Coenzyme specificity. The enzyme required NAD^+ as a natural coenzyme for oxidative deamination, and $NADP^+$ showed only slight activity (6.6% of the reactivity of NAD^+). In addition, some analogs of NAD^+ served as a coenzyme (Table 4). 3-Acetylpyridine-NAD^+ was a much better coenzyme than NAD^+. Thionicotinamide-NAD^+ and deamino-NAD^+ were similar to NAD^+ in cofactor activity.

Inhibitors. Among various compounds examined for inhibitory effects on the oxidative deamination of L-phenylalanine (Table 5), D-phenylalanine, D-tyrosine, phenylethylamine, 3-phenylpropionate, *trans*-cinnamate, L-phenylglycine, and *p*-hydroxyphenylethylamine inhibited the reaction competitively against L-phenylalanine.

The enzyme was inhibited completely by *p*-chloromercuribenzoate (1 μM) and $HgCl_2$ (10 μM) but was not inhibited by 1 mM monoiodoacetate or *N*-ethylmaleimide. Cu^{2+} was slightly inhibitory. Other metal ions (1 mM), such as Mg^{2+}, Co^{2+}, and Mn^{2+}, were not inhibitory. EDTA, α,α′-dipyridyl, NaF, NaN_3, Na_2SO_4, and pyridoxal 5′-phosphate had no effect on the oxidative deamination of L-phenylalanine. None of the following purine bases, nucleosides, and nucleotides affected activity: adenine, adenosine, AMP, ADP, ATP, guanine, GMP, GDP, and GTP.

TABLE 4. Coenzyme specificity[a]

Coenzyme[b]	Relative activity	K_m (mM)
NAD^+	100	0.25[c]
$NADP^+$	6.6	
$NAD^+ + NADP^+$	92	
3-Acetylpyridine-NAD^+	241	0.25[d]
Thionicotinamide-NAD^+	101	0.18[d]
Deamino-NAD^+	86	0.18[d]
3-Pyridinealdehyde-NAD^+	9.2	

[a] The reaction was carried out at pH 9.5 to avoid degradation of NAD^+ analogs at a more alkaline pH.

[b] Assays with NAD^+ analogs (2.5 mM) were conducted by measuring the increase in absorbance at the following wavelengths: 3-acetylpyridine-NAD^+, 363 nm (molar absorption coefficient [ε] = 9.1×10^3); thionicotinamide-NAD^+, 395 nm (ε = 11.3×10^3); deamino-NAD^+, 338 nm (ε = 6.2×10^3); and 3-pyridinealdehyde-NAD^+, 358 nm (ε = 9.3×10^3) (24).

[c] Obtained from the secondary plots of intercepts versus reciprocal concentrations of the substrate.

[d] The apparent K_m was determined by Lineweaver-Burk plots with the reaction system containing 20 mM L-phenylalanine.

TABLE 5. Inhibitory effects of various amino acids on oxidative deamination of L-phenylalanine[a]

Amino acid	Relative activity	K_i (mM)
None	100	
D-Phenylalanine	7	0.14
D-Tryptophan	90	
D-Tyrosine	36	1.58
D-Norleucine	90	
D-Methionine	90	
D-Ethionine	26	1.76
L-Phenylglycine	78	8.32
L-3-Phenyllactate	93	
Phenylethylamine	30	1.52
p-Hydroxyphenylethylamine	86	13.7
trans-Cinnamic acid	80	12.7
3-Phenylpropionate	57	2.6
α-Methyl-DL-phenylalanine	84	20.8
Phenylenediamine	50	4.36

[a] Amino acid concentration was 10 mM. D-Leucine, D-isoleucine, D-*allo*-isoleucine, D-α-amino-*n*-butyrate, L-dopa, DL-phenylserine, *N*-methyl-DL-phenylalanine, L-lysine, L-arginine, and L-histidine did not inhibit the reaction.

Kinetic mechanism. A series of steady-state kinetic analyses was carried out to investigate the reaction mechanism. First, initial-velocity studies for oxidative deamination were performed. Double-reciprocal plots of initial velocity against L-phenylalanine concentrations in the presence of various fixed concentrations of NAD^+ gave intersecting straight lines (Fig. 3). This result shows that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD^+ and L-phenylalanine (8). The Michaelis constants for L-phenylalanine and NAD^+ were calculated to be 3.8 and 0.25 mM, respectively, from the secondary plots of intercept versus reciprocal concentrations of the other substrate.

A kinetic analysis of reductive amination was performed to investigate several possible reaction mechanisms (8). Figure 4A shows double-reciprocal plots of velocities versus phenylpyruvate concentration at several concentrations of NADH and a constant concentration of ammonia. The

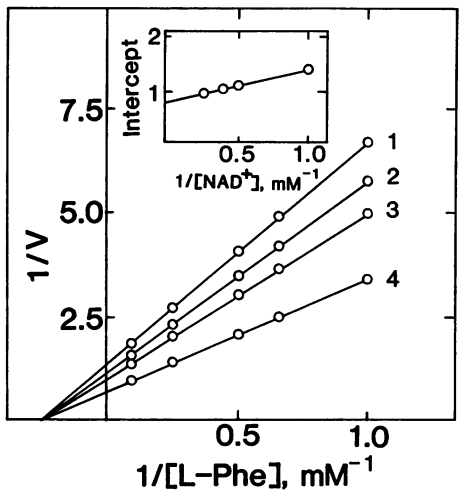

FIG. 3. Double-reciprocal plots of initial velocity versus L-phenylalanine concentration at a series of fixed concentrations of NAD^+. Concentrations of NAD^+ were: 1, 0.1 mM; 2, 0.15 mM; 3, 0.2 mM; and 4, 0.5 mM. The inset shows secondary plots of the intercepts versus the fixed NAD^+ concentrations.

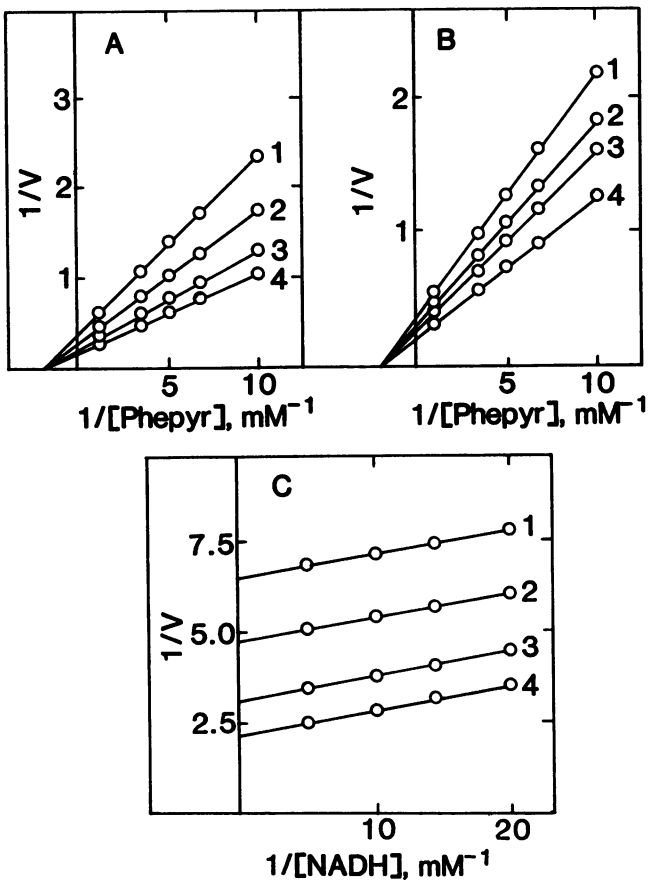

FIG. 4. Initial-velocity patterns for reductive amination. (A) Double-reciprocal plots of velocity versus phenylpyruvate (Phepyr) concentration at several fixed concentrations of NADH in the presence of a high concentration (0.6 M) of ammonia. Concentrations of NADH were: 1, 28 μM; 2, 46 μM; 3, 65 μM; and 4, 92 μM. (B) Double-reciprocal plots of velocity versus phenylpyruvate concentration at several fixed concentrations of ammonia in the presence of a high concentration (0.2 mM) of NADH. Concentrations of ammonia were: 1, 60 mM; 2, 80 mM; 3, 100 mM; and 4, 150 mM. (C) Double-reciprocal plots of velocity versus NADH concentration at several fixed concentrations of ammonia in the presence of a high concentration (10 mM) of phenylpyruvate. Concentrations of ammonia were: 1, 70 mM; 2, 100 mM; 3, 170 mM; and 4, 400 mM.

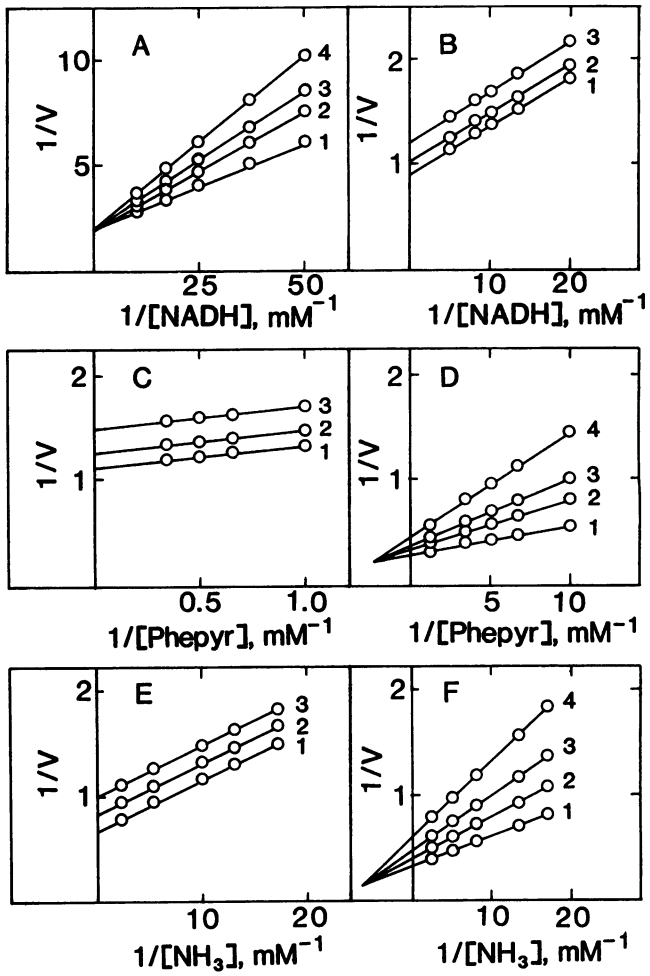

FIG. 5. Product inhibition of reductive amination by L-phenylalanine and NAD$^+$. (A) Product inhibition by NAD$^+$ with NADH as the varied substrate. Phenylpyruvate (10 mM) and ammonia (0.6 M) were held at constant concentrations. Concentrations of NAD$^+$ were: 1, 0 mM; 2, 0.3 mM; 3, 0.5 mM; and 4, 1.0 mM. (B) Product inhibition by L-phenylalanine with NADH as the varied substrate. Phenylpyruvate (10 mM) and ammonia (0.6 M) were held at constant concentrations. Concentrations of L-phenylalanine were: 1, 0 mM; 2, 3 mM; and 3, 10 mM. (C) Product inhibition by NAD$^+$ with phenylpyruvate (Phepyr) as the varied substrate. NADH (0.2 mM) and ammonia (0.6 M) were held at constant concentrations. Concentrations of NAD$^+$ were: 1, 0 mM; 2, 0.5 mM; and 3, 1.0 mM. (D) Product inhibition by L-phenylalanine with phenylpyruvate as the varied substrate. NADH (0.2 mM) and ammonia (0.6 M) were held at constant concentrations. Concentrations of L-phenylalanine were: 1, 0 mM; 2, 5 mM; 3, 10 mM; and 4, 20 mM. (E) Product inhibition by NAD$^+$ with ammonia as the varied substrate. NADH (0.2 mM) and phenylpyruvate (10 mM) were held at constant concentrations. Concentrations of NAD$^+$ were: 1, 0 mM; 2, 0.5 mM; and 3, 1.0 mM. (F) Product inhibition by L-phenylalanine with ammonia as the varied substrate. Phenylpyruvate (10 mM) and NADH (0.2 mM) were held at constant concentrations. Concentrations of L-phenylalanine were: 1, 0 mM; 2, 5 mM; 3, 10 mM; and 4, 20 mM.

double-reciprocal plots gave straight lines intersecting on the abscissa. At a high concentration of NADH, the double-reciprocal plots of velocities versus phenylpyruvate concentration at several fixed concentrations of ammonia also gave straight intersecting lines (Fig. 4B). However, with phenylpyruvate at a saturating concentration, the double-reciprocal plots of velocities versus NADH concentration at several different concentrations of ammonia were quite different from those shown in Fig. 4A and B and gave parallel lines (Fig. 4C). These observed kinetic patterns rule out the possibility of random addition of substrates and indicate a sequential ordered mechanism in which phenylpyruvate binds to the enzyme between NADH and ammonia (8). The K_m values for NADH, phenylpyruvate, and ammonia were calculated to be 43 μM, 0.50 mM, and 70 mM, respectively.

Product inhibition studies of the reductive amination reaction to determine the order of substrate addition and product release were performed according to the method of Cleland (8). With NAD$^+$ as an inhibitor, the double-reciprocal plots of velocities versus NADH concentration at high and constant concentrations of phenylpyruvate and ammonia showed competitive inhibition (Fig. 5A). L-Phenylalanine showed uncompetitive inhibition with respect to NADH (Fig. 5B). These results suggest that NAD$^+$ and NADH can bind to the free form of the enzyme. The other product

inhibition patterns observed with NAD^+ and L-phenylalanine as inhibitors were identical to the predicted patterns for the sequential ordered ternary-binary mechanism except for the noncompetitive inhibition by L-phenylalanine with respect to phenylpyruvate (Fig. 5D). This finding suggests that L-phenylalanine may also bind to the NADH-enzyme complex. Noncompetitive inhibition by phenylalanine with respect to ammonia rules out the Theorell-Chance mechanism (28). The results obtained from these initial-velocity and product inhibition studies show that the sequence of addition of substrates in reductive amination is NADH, phenylpyruvate, and ammonia and that the sequence of release of products is L-phenylalanine and NAD^+.

DISCUSSION

The results presented above show that the phenylalanine dehydrogenase of *R. maris* K-18 is a dimer, whereas phenylalanine dehydrogenases from *S. ureae* (4), *B. sphaericus* (4) and *B. badius* (5) are octamers. This is the first example of occurrence of a dimeric phenylalanine dehydrogenase. A similar dimeric structure has been reported for *meso*-diaminopimelate dehydrogenase (19), 2,4-diaminopentanoate dehydrogenase (26), and L-*erythro*-3,5-diaminohexanoate dehydrogenase (6), whereas glutamate dehydrogenase (25), leucine dehydrogenase (20), and alanine dehydrogenase (21) are composed of six subunits. In general, the dimeric amino acid dehydrogenases are unstable, whereas the octameric or hexameric enzymes are stable. Phenylalanine dehydrogenase from *R. maris* K-18 was also unstable. We examined the conditions favorable for stability and found that the presence of a high concentration of sodium malonate (0.25 M) or sodium glutarate (0.5 M) stabilizes the enzyme. Stabilization of amino acid dehydrogenases by dicarboxylates has not been reported previously. Stabilization is very important for use of an unstable enzyme.

The phenylalanine dehydrogenase of *R. maris* K-18 is inducible, which indicates that, as has been found for enzymes from other bacteria (2, 4, 5, 13, 14), this enzyme functions in phenylalanine degradation.

Although phenylalanine dehydrogenases from various sources have broad substrate specificities, the enzyme from *R. maris* K-18 was different in substrate specificity from the enzymes of *S. ureae* (4), *B. sphaericus* (4), *B. badius* (5), *Rhodococcus* sp. strain M4 (13), and *Brevibacterium* sp. (14). *p*-Hydroxyphenylpyruvate was a good substrate for reductive amination of the enzyme from *R. maris* K-18, but L-tyrosine was a poor substrate for oxidative deamination.

NAD^+ is replaced by some of the NAD^+ analogs as a coenzyme for phenylalanine dehydrogenase from *R. maris* K-18. 3-Acetylpyridine-NAD^+ is reduced by the enzyme more rapidly than is NAD^+, as has been reported for other dehydrogenases (15, 20, 22). Deamino-NAD^+ and NAD^+ have closely similar reactivities. Thus, the amino groups of the nicotinamide and adenine moieties of NAD^+ are not of crucial importance for coenzyme activity.

Asano et al. (4) reported that phenylalanine dehydrogenases from *S. ureae* and *B. sphaericus* are inhibited by D-amino acids, although the inhibition pattern and inhibition constants were not described. The enzyme from *R. maris* K-18 was also inhibited by D-phenylalanine and D-tyrosine but not by D-leucine and D-isoleucine. *p*-phenylethylamine and 3-phenylpropionate also strongly inhibited the deamination of L-phenylalanine. These compounds behave as competitive inhibitors with respect to L-phenylalanine. Inhibition by the D-enantiomer of the substrates has been reported for leucine dehydrogenase (20). The degree of inhibition, however, was stronger for phenylalanine dehydrogenase than for leucine dehydrogenase.

The amino acid dehydrogenases studied so far catalyze the reaction by a sequential ordered mechanism (16, 17, 20, 21, 27), with the exception of bovine liver glutamate dehydrogenase, which shows a random mechanism (7, 10). The sequence of substrate binding, however, varies. Kinetic studies on the phenylalanine dehydrogenase from *R. maris* K-18 showed that reductive amination proceeds through the sequential ordered ternary-binary mechanism, similar to the mechanisms found for NAD^+-specific glutamate dehydrogenase (16, 17, 27), leucine dehydrogenase (20), and *meso*-diaminopimelate dehydrogenase (19) but different from those found for $NADP^+$-specific glutamate dehydrogenase (16) and alanine dehydrogenase (21).

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