Distribution, Purification, and Characterization of Thermostable Phenylalanine Dehydrogenase from Thermophilic Actinomycetes

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Phenylalanine dehydrogenase (l-phenylalanine:NAD oxidoreductase, deaminating; EC 1.4.1.1.-) isolated from Brevibacterium sp. isolated from soil (14) and purified by several mesophiles, Sporosarcina ureae (1, 3), Bacillus sphaericus (3), Bacillus badius (4), Rhodococcus sp. (5, 13), Rhodococcus maris (17), and Nocardia sp. (9), is important for the synthesis of L-phenylalanine and its related L amino acids from the corresponding oxo analogs and ammonia (2, 12, 13). We have shown that leucine dehydrogenase (EC 1.4.1.5) of a thermophile is much more stable than that of a mesophile not only at high temperatures but also under various other conditions (20). The thermostable leucine dehydrogenase is advantageous to substrate production in a membrane reactor (25) and for analysis of substrate amino acids and oxo acids (23).

In this report, we describe the distribution of phenylalanine dehydrogenase in thermophiles and the purification and characterization of the enzyme from T. intermedius IFO 14230.
Protein concentration was determined by the method of Lowry et al. (16), with bovine serum albumin as a standard protein.

Preparation of crude enzyme solution. Cells washed twice with 0.85% NaCl solution were suspended in 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.01% (vol/vol) 2-mercaptoethanol and were disrupted by ultrasonication. The cell debris was removed by centrifugation (20,000 × g, 20 min); the supernatant solution was used as the enzyme solution for screening of phenylalanine dehydrogenase and for purification.

Purification of phenylalanine dehydrogenase. Washed cells (160 g [wet weight]) of T. intermedium IFO 14230 were suspended in 300 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 1 mM EDTA. All procedures were carried out at room temperature (about 20°C) unless otherwise stated.

Step I. Heat treatment of crude enzyme solution. The cells were disrupted for 20 min in an ultrasonic oscillator. After centrifugation, solid NaCl was added to the supernatant solution (final concentration, 0.10 M), and then the enzyme solution was heated at 70°C for 1 h. The enzyme was centrifuged at 12,000 × g for 5 min, and the supernatant solution was dialyzed against 1,000 volumes of 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA and 0.01% 2-mercaptoethanol.

Step II. Red Sepharose 4B affinity chromatography. The enzyme solution (400 ml) was applied to five columns (3 by 22 cm) of Red Sepharose 4B equilibrated previously with dialysis buffer (pH 6.8). After each column was washed with the buffer (about 500 ml), the enzyme was eluted with buffer (about 500 ml) supplemented with 0.5 M NaCl. The active fractions were pooled, and then the enzyme (70 ml) was concentrated and deionized by ultrafiltration (Toyos Roshi ultrafilter UP 20 membrane). In this procedure, 10 mM Tris-HCl buffer (pH 8.0) containing 0.01% 2-mercaptoethanol and 1 mM EDTA was substituted for the elution buffer.

Step III. DEAE-Toyopearl column chromatography. The enzyme was applied to a DEAE-Toyopearl column (2.5 by 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 40 mM NaCl, 0.01% 2-mercaptoethanol, and 1 mM EDTA. The enzyme passed through the column under these conditions. The enzyme collected was concentrated by ultrafiltration.

Step IV. Sepharose CL-4B column chromatography. The enzyme solution (about 10 ml) was applied to a Sepharose CL-4B column (1.2 by 100 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 0.50 M NaCl, 0.01% 2-mercaptoethanol, and 1 mM EDTA. The enzyme was eluted in the fractions corresponding to the void volume of the column and was concentrated.

Step V. Sephadex G-100 column chromatography. The enzyme was further purified by Sephadex G-100 chromatography (column, 1.2 by 100 cm) by the procedure described for step IV.

Analytical gel electrophoresis. Disc gel electrophoresis (7.5% gel) was carried out as described by Davis (8), and sodium dodecyl sulfate (SDS) slab gel electrophoresis (12% gel, 2 mm thick) was performed by the procedure of Laemmli (15).

Molecular weight determination. Molecular weight was determined by gel filtration on a Toyopearl HW-55 column (1.6 by 62 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The following standard proteins were used to make a calibration curve: ferritin (molecular weight, 443,000), B. sphaericus alanine dehydrogenase (231,000), lactate dehydrogenase (145,000), bovine serum albumin (67,000), and ovalbumin (43,000). The subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis with the following marker proteins: bovine serum albumin (molecular weight, 67,000), catalase (57,500), ovalbumin (43,000), B- lactoglobulin (18,400), and cytochrome c (13,000).

Determination of stereospecificity of hydrogen transfer. The stereospecificity of hydrogen transfer of [4R-2H]NADH or [4S-2H]NADH catalyzed by phenylalanine dehydrogenase was studied by the 1H nuclear magnetic resonance (NMR) method (10). [4R-2H]NADH or [4S-2H]NADH (about 5 mM) was incubated with phenylalanine dehydrogenase (0.11 U) in the presence of 5 mM phenylpyruvate and 0.1 M NaNH in 0.6 ml of H2O. After incubation at 37°C for 3 h, the 1H NMR spectra of the aromatic region of the nicotinamide ring of NAD produced were measured on a 200-MHz NMR apparatus (Varian VXR-200).

RESULTS AND DISCUSSION

Distribution of phenylalanine dehydrogenase in thermophiles. To find organisms that produce thermostable phenylalanine dehydrogenase, we screened for enzyme activity in various thermophiles from culture collections. As shown in Table 1, high specific activity was found mainly in Thermotoga species; in particular, T. intermedium IFO 14230 and T. vulgaris DSM 43354 and DSM 43184 produced the enzyme abundantly. Enzyme activity was detected in almost all strains of thermophilic actinomycetes tested. The enzyme has been demonstrated in a few strains of mesophiles. Thus,

<table>
<thead>
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<th>TABLE 1. Screening of phenylalanine dehydrogenase in thermophiles</th>
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<tr>
<td>Strain</td>
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<tr>
<td>--------</td>
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<tr>
<td>Thermoactinomyces intermedius IFO 14230</td>
</tr>
<tr>
<td>T. vulgaris DSM 43354</td>
</tr>
<tr>
<td>T. vulgaris DSM 43184</td>
</tr>
<tr>
<td>T. vulgaris DSM 43062</td>
</tr>
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<td>T. vulgaris IFO 12516</td>
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<td>Pseudonocardia thermophila IFO 12133</td>
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<td>Microbispora bispora DSM 43038</td>
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<td>Thermosphaerobacter albus DSM 43310</td>
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</table>

*The following microorganisms have specific activities of 0.005 to 0.010 U/mg: Thermotoga species, for screening of thermophiles. To find organisms that produce thermostable phenylalanine dehydrogenase, we screened for enzyme activity in various thermophiles from culture collections. As shown in Table 1, high specific activity was found mainly in Thermotoga species; in particular, T. intermedium IFO 14230 and T. vulgaris DSM 43354 and DSM 43184 produced the enzyme abundantly. Enzyme activity was detected in almost all strains of thermophilic actinomycetes tested. The enzyme has been demonstrated in a few strains of mesophiles. Thus,
this is the first example showing the wide distribution of the enzyme in actinomycetes and suggests that the enzyme plays a role in the metabolism of these organisms. *Bacillus* sp. strain DSM 466 is only the thermophilic *Bacillus* strain producing phenylalanine dehydrogenase, although only poorly (0.0052 U/mg).

*T. intermedius* IFO 14230 exhibited the highest specific activity of phenylalanine dehydrogenase and produced the enzyme most abundantly. This strain was used for enzyme purification. Addition of L-phenylalanine (5 g/liter), L-tyrosine (0.4 g/liter), or phenylpyruvate (1 g/liter) to the basal medium did not affect enzyme production. The absence of enhancement with l-phenylalanine in enzyme production is peculiar to the *T. intermedius* IFO 14230 enzyme. Enzyme production in other microorganisms is significantly enhanced by addition of L-phenylalanine to basal medium containing peptone or corn steep liquor (1, 3, 13, 19).

**Purification of the enzyme.** Table 2 summarizes the result of a typical purification of phenylalanine dehydrogenase from the *T. intermedius* extract. The enzyme was purified about 125-fold with a 13% yield. The purified enzyme was found to be homogeneous by polyacrylamide disc gel electrophoresis (Fig. 1). The activity of this enzyme preparation is higher than that of the preparation obtained in a preliminary study, which probably was partially inactivated during purification (24).

**Molecular weight and subunit structure.** The molecular weight of the phenylalanine dehydrogenase was estimated to be about 270,000 by gel filtration on a Toyopearl HW-55 column (Fig. 2A). The subunit structure was studied by disc gel electrophoresis in the presence of 0.1% SDS. The electrophoresis indicated the presence of only one band, showing a molecular weight of about 41,000 (Fig. 2B). This value is good agreement with that (40,488) calculated from the amino acid sequence of the enzyme (27). Thus, the native enzyme probably has a hexamer structure composed of six identical or similar subunits. The *B. sphaericus*, *S. ureae* (3), and *B. badius* (4) enzymes are octamers, the *R. maris* enzyme (19) is a dimer, and the *Nocardia* sp. enzyme (9) is a monomer. This is the first example of the occurrence of a hexameric phenylalanine dehydrogenase.

**Stability.** The thermostability of the enzyme was examined. As shown in Fig. 3A, the enzyme retained its full

<table>
<thead>
<tr>
<th>Step</th>
<th>Total U</th>
<th>Total protein (mg)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
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<td>19,500</td>
<td>0.144</td>
<td>100</td>
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<td>362</td>
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<td>86.2</td>
<td>13</td>
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</table>

**FIG. 2.** Determination of molecular weights of the native phenylalanine dehydrogenase (A; ○) by gel filtration and of the subunit (B; ●) by SDS electrophoresis. Standard proteins (●) used are described in Materials and Methods.

**FIG. 3.** Effects of temperature and pH on enzyme stability. (A) Thermostability. After heat treatment of the pure enzyme in 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 1 mM EDTA, an aliquot of the enzyme solution was withdrawn and the remaining activity was assayed for the oxidative deamination of L-phenylalanine at 37°C. Heat treatment was carried out at 70°C (○) and 75°C (●). (B) Effect of pH on stability. After the enzyme in 50 mM buffer of various pHs was incubated at 50°C (○) and 75°C (●) for 10 min, an aliquot of the enzyme solution was withdrawn and the remaining activity was assayed for the oxidative deamination of L-phenylalanine at 37°C. The buffers (50 mM) used were acetate buffer (pH 4.5 to 6.5), potassium phosphate buffer (pH 6.8 to 8.5 and 10.8 to 11.5), and glycine-KCl-KOH buffer (pH 9 to 11).

**FIG. 1.** Disc gel electrophoresis of the purified enzyme (about 20 μg) in the absence of SDS. (A) Activity staining was done at 37°C in a mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM NAD, 10 mM L-phenylalanine, 0.4 mM phenazine methosulfate, and 0.5 mM p-iodonitrotetrazolium chloride until a band of sufficient intensity was visible. (B) The protein band was stained with Coomassie brilliant blue R-250. The position of the tracking dye was at the bottom of the gel.
activity upon heating at 70°C for 60 min, but the activity was lost rapidly by heating above 75°C. This feature of thermostability is similar to that of other dehydrogenases from moderate thermophiles such as *B. steaerothermophilus* leucine dehydrogenase (20). The stability of the enzyme at various pHs is shown in Fig. 3B. Activity was not lost upon incubation between pH 5.0 and 10.8 at 50°C for 10 min. The enzyme was stable between pH 5.5 and 9.5 upon heating at 70°C for 10 min. The *S. ureae* and *B. sphaericus* enzymes were completely inactivated by incubation at 50 and 60°C, respectively, for 10 min (3). Therefore, the *T. intermedius* enzyme is much more thermostable than the enzymes from mesophiles. This finding suggests the possibility that the thermostable enzyme is more useful than the enzymes from mesophiles as a biocatalyst for L-phenylalanine production (12, 14) and analysis (24).

**Effect of pH.** The enzyme showed maximum reactivity at pH 11.0 for the oxidative deamination of L-phenylalanine. The optimum pH of phenylpyruvate amination was around 9.2 (Fig. 4). The high reactivity of the enzyme at rather high pHs is similar to those of other amino acid dehydrogenases such as leucine dehydrogenase (19, 20) and alanine dehydrogenase (22).

**Substrate specificity.** The enzyme shows high specificity for L-phenylalanine in oxidative deamination, and L-tyrosine is inert; the *T. intermedius* enzyme differs from the enzymes of mesophiles (3, 5, 9) in this respect. As shown in Table 3, only L-4-aminophenylalanine and L-leucine are slightly oxidized by the enzyme. For reductive amination, phenylpyruvate is the preferred substrate. In addition, the enzyme catalyzes the amination of several 2-oxo acids such as 2-oxo-4-methylthiohdrbutyrate, 2-oxo-4-methylpentanoate, and 2-oxo-3-methylbutyrate, although slowly (Table 3). The enzyme does not act on 4-hydroxphenylpyruvate, unlike phenylalanine dehydrogenase from mesophiles (3, 4, 9, 13, 17). The high substrate specificity is favorable for the selective determination of L-phenylalanine and phenylpyruvate (24). Ammonia is an exclusive substrate as the amino donor for reductive amination. Hydroxylamine, methyamine, and ethylamine (0.15 M) were inert as the amino donor.

The enzyme required NAD (NADH) as a coenzyme, as does the mesophile enzyme, and NADP (NADPH) was inert.

**Steady-state kinetic mechanism.** A series of steady-state kinetic analyses was carried out to identify the reaction mechanism (6, 7, 19). First, initial velocity studies were performed. The double-reciprocal plots of initial velocity (v) against L-phenylalanine concentrations in the presence of several fixed concentrations of NAD gave intersecting straight lines (Fig. 5A). This pattern shows that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD and L-phenylalanine, as with other amino acid dehydrogenases (19, 22). The *Km* values for NAD and L-phenylalanine were calculated to be 0.078 and 0.22 mM, respectively. From the secondary plots of intercepts versus reciprocal concentrations of the other substrate, double-reciprocal plots of v against phenylpyruvate concentrations at several fixed concentrations of ammonia and a high and constant concentration of NADH gave straight intersecting lines (Fig. 5B). At a high concentration of phenylpyruvate, the double-reciprocal plots of v against NADH concentrations at several fixed concentrations of ammonia gave also intersecting lines (Fig. 5D). On the other hand, similar plots of v against NADH concentrations at several different concentrations of phenylpyruvate and a high concentration of ammonia gave parallel lines (Fig. 5C). These patterns may rule out the possibility of random addition of the substrates and represent a sequential ordered mechanism in which ammonia binds to the enzyme between other two substrates. The *Km* values for NADH, phenylpyruvate, and ammonia were determined to be 0.025, 0.045, and 106 mM, respectively.

Product inhibition studies of oxidative deamination were performed to determine the order of substrate addition and product release. With NADH as an inhibitor, the double-reciprocal plots of v versus NAD concentrations at a constant concentration of L-phenylalanine showed a typical competitive inhibition pattern (Fig. 6). This finding indicates that NAD and NADH can bind to the free form of the enzyme. NADH showed noncompetitive inhibition against L-phenylalanine. Phenylpyruvate showed noncompetitive inhibition.
and uncompetitive inhibitions with respect to phenylalanine and NAD, respectively. Ammonia showed uncompetitive inhibition with respect to both L-phenylalanine and NAD (data not shown). Noncompetitive inhibition by phenylpyruvate with respect to phenylalanine rules out the Thorell-Chance mechanism (28). These initial velocity and product inhibition patterns suggest a sequential ordered binary-tertiary mechanism, in which the sequence of substrate binding to the enzyme in the oxidative deamination is NAD and L-phenylalanine and then the sequence of product release is phenylpyruvate, ammonia, and NAD, but the inhibition by NADH against L-phenylalanine was noncompetitive. Only this feature does not fit with the suggested mechanism. Some unknown factor, now under investigation, is probably involved in the reaction.

**Stereo specificity of hydrogen transfer.** NAD- and NADP-dependent dehydrogenases show pro-R or pro-S stereo specificity for hydrogen removal from the C-4 position of the nicotinamide moiety of the reduced coenzymes. We studied the stereospecificity of hydrogen transfer of NADH catalyzed by phenylalanine dehydrogenase by using [4R-\textsuperscript{2H}]NADH and [4S-\textsuperscript{2H}]NADH. When [4R-\textsuperscript{2H}]NADH was incubated with phenylalanine dehydrogenase in the presence of ammonia and phenylpyruvate, a resonance doublet around 88.8 for the C-4 proton of NAD was not observed in the \textsuperscript{1}H NMR spectrum (Fig. 7A). This finding suggests that \textsuperscript{2}H is present at the C-4 position of NAD. In contrast, the doublet signal for the C-4 proton of NAD was shown in the NMR spectrum after incubation of a similar reaction mixture containing [4S-\textsuperscript{2H}]NADH (Fig. 7B). This result shows that the 4S-\textsuperscript{2}H of NADH was removed and \textsuperscript{1}H remained at the C-4 position of NAD. Thus, the thermostable phenylalanine dehydrogenase of *T. intermedia* is pro-S stereospecific, like leucine dehydrogenase (19), glutamate dehydrogenase (29), and *B. sphaericus* phenylalanine dehydrogenase (3) and unlike alanine dehydrogenase (20) and ε-lysine dehydrogenase (11).

**FIG. 5.** Initial velocity analysis. (A) Double-reciprocal plots of v (initial velocity) versus NAD concentration at a series of fixed concentrations of L-phenylalanine: 1.0 (1), 0.4 (2), 0.2 (3), and 0.1 (4) mM. (B) Double-reciprocal plots of v versus phenylpyruvate (Phepyr) concentration at several fixed concentrations of ammonia at a constant NADH concentration (0.10 mM). Ammonia was used at concentrations of 120 (1), 80 (2), 40 (3), and 20 (4) mM. Double-reciprocal plots of v versus NADH concentration at several fixed concentrations of phenylpyruvate at a constant ammonia concentration (200 mM). Phenylpyruvate was used at concentrations of 0.040 (1), 0.020 (2), 0.015 (3), and 0.010 (4) mM. (D) Double-reciprocal plots of v versus ammonia concentration at several fixed concentrations of NADH at a constant phenylpyruvate concentration (10 mM). NADH was used at concentrations of 0.040 (1), 0.020 (2), 0.014 (3), and 0.010 (4) mM.

**FIG. 6.** Product inhibition by NADH with respect to NAD. Inhibition by NADH was analyzed with NAD as the varied substrate in the presence of L-phenylalanine (5 mM). The NADH concentrations were 0 (1), 0.050 (2), 0.070 (3), and 0.100 (4) mM.

**FIG. 7.** Aromatic region of the \textsuperscript{1}H NMR spectra of NAD after incubation of [4R-\textsuperscript{2H}]NADH (A) or [4S-\textsuperscript{2H}]NADH (B) with phenylalanine dehydrogenase, ammonia, and phenylpyruvate (Phepyr). Other conditions are given in the text.
The results presented here show that phenylalanine dehydrogenase of *T. intermedii* IFO 14230 has a few characteristics distinct from those of mesophiles: it has a hexameric structure, high thermostability, and high substrate specificity. Its high stability and substrate specificity favor its use for amino acid production and analysis (24).

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