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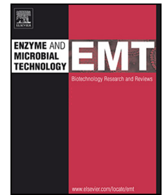

Biochemical characterization of an L-tryptophan dehydrogenase from the photoautotrophic cyanobacterium *Nostoc punctiforme*

Ryutaro Ogura [1,2], Taisuke Wakamatsu [*], Yuta Mutaguchi [4], Katsumi Doi, Toshihisa Ohshima [1,3]

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

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ABSTRACT

An NAD^+-dependent L-tryptophan dehydrogenase from *Nostoc punctiforme* NIES-2108 (NpTrpDH) was cloned and overexpressed in *Escherichia coli*. The recombinant NpTrpDH with a C-terminal His_6-tag was purified to homogeneity using a Ni-NTA agarose column, and was found to be a homodimer with a molecular mass of 76.1 kDa. The enzyme required NAD^+ and NADH as cofactors for oxidative deamination and reductive amination, respectively, but not $NADP^+$ or NADPH. L-Trp was the preferred substrate for deamination, though L-Phe was deaminated at a much lower rate. The enzyme exclusively aminated 3-indolepyruvate; phenylpyruvate was inert. The pH optima for the deamination of L-Trp and amination of 3-indolpyruvate were 11.0 and 7.5, respectively. For deamination of L-Trp, maximum enzymatic activity was observed at 45 °C. NpTrpDH retained more than 80% of its activity after incubation for 30 min at pHs ranging from 5.0 to 11.5 or incubation for 10 min at temperatures up to 40 °C. Unlike L-Trp dehydrogenases from higher plants, NpTrpDH activity was not activated by metal ions. Typical Michaelis–Menten kinetics were observed for NAD^+ and L-Trp for oxidative deamination, but with reductive amination there was marked substrate inhibition by 3-indolepyruvate. NMR analysis of the hydrogen transfer from the C4 position of the nicotinamide moiety of NADH showed that NpTrpDH has a pro-*S* (B-type) stereospecificity similar to the Glu/Leu/Phe/Val dehydrogenase family.

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1. Introduction

Amino acid dehydrogenases (EC 1.4.1.X) catalyze the reversible $NAD(P)^+$-dependent oxidative deamination of amino acids to their corresponding 2-oxoacids and ammonia [1–3]. More than fifteen kinds of amino acid dehydrogenases, including those acting on L-Glu, L-Ala, L-Ser, L-Val, L-Leu, L-Gly, L-Lys, L-Phe and L-Asp, have been identified in various organisms [1,4,5], and detailed analyses of the structures and functions of L-Glu dehydrogenase [5], L-Leu dehydrogenase [6,7] and L-Phe dehydrogenase [8] have been reported. In addition, several amino acid dehydrogenases have been applied for use in biosensors for L-amino acid, 2-oxoacid and ammonia assays [1,9], disease diagnosis [10,11], and amino acids synthesis [9,12].

As compared to other amino acid dehydrogenases, L-Trp dehydrogenase (EC 1.4.1.19, TrpDH), which catalyzes the reversible oxidative deamination of L-Trp to 3-indolepyruvate in the presence of $NAD(P)^+$, has not been extensively investigated from the viewpoint of it biochemical or biotechnological potential due in large part to its extremely limited distribution. The enzyme was first identified in several higher plants (e.g., *Pisum sativum*, *Spinacia oleracea* and *Zea mays*, etc.) in the mid 1980s [13], and was partially characterized at that time [14–17]. There was then no further investigation of the enzyme until the product of the Npun_R1275 gene (*npun_r1275*) from the cyanobacterium *Nostoc punctiforme* ATCC 29133 was found to exhibit NAD^+-dependent oxidative deamination activity toward L-Trp [18]. This was the first microbial TrpDH known, and its gene (*npun_r1275*) was located

Abbreviations: TrpDH, L-Trp dehydrogenase; NpTrpDH, NAD^+-dependent L-tryptophan dehydrogenase from *Nostoc punctiforme* NIES-2108.

* Corresponding author. Present address: Agricultural Science, Graduate School of Integrated Arts and Sciences, Kochi University, 200 Otsu, Monobe, Nankoku, Kochi 783-8502, Japan. Tel.: +81 88 864 5191; fax: +81 88 864 5191.
E-mail address: t-wakamatsu@kochi-u.ac.jp (T. Wakamatsu).
[1] These authors contributed equally to this work.
[2] Present address: Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.
[3] Present address: Department of Biomedical Engineering, Osaka Institute of Technology, 5-16-1 Ohmiya, Asahi-ku, Osaka 535-8585, Japan.
[4] Present address: Department of Biotechnology, Faculty of Bioresource Sciences, Akita Prefectural University, 241-438 Shimoshinjo-Nakano, Akita 010-0195, Japan.

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in the gene cluster for biosynthesis of scytonemin, a cyanobacterial radiation-absorbing pigment. The authors suggested that Npun_R1275 plays an important role in scytonemin biosynthesis by catalyzing the oxidative deamination of L-Trp, but no detailed analysis of the enzyme's biochemical properties were reported.

In the present study, we cloned a TrpDH homologous gene from *N. punctiforme* NIES-2108 (*nptrpdh*) in *Escherichia coli* and examined in detail the biochemical properties of the expressed product (NpTrpDH) from the viewpoint of its practical application.

2. Materials and methods

2.1. Materials

A pellet of *N. punctiforme* NIES-2108 was obtained from the Japan Society for Culture Collections. Enzymes that act on DNA, including restriction enzymes and KOD Plus Neo polymerase, were obtained from Takara Bio Inc. (Otsu, Japan) and TOYOBO (Osaka, Japan). DNA oligomers were synthesized by Hokkaido System Science (Sapporo, Japan). All other reagents used were of the highest commercially available grade.

2.2. Purification of NpTrpDH

N. punctiforme NIES-2108 cells were collected from the pellet and initially treated with lysozyme and proteinase K for 1 h at 37 °C. Thereafter, acetyl trimethyl ammonium bromide was added to the mixture (final concentration: 1% (w/v)), which was then incubated for 10 min at 65 °C. After removing the cellular debris by centrifugation, the genomic DNA was isolated from the solution using the phenol/chloroform extraction method, and the *nptrpdh* coding sequence was amplified by PCR using genomic DNA as the template with primers 5′-TATACATATGCTGCTATTTGAAACTGTTAGAGAAATGGGTCACGAACAAGTTCTTTTTTG-3′ (forward) and 5′-TATACTCGAGAGCTGCGATCGCTTTAGA CTTGCTGCGCTTACTATTG-3′ (reverse). These two primers were constructed based on the sequence of *npun_r1275*, with the forward primer containing a *Nde*I recognition site and the reverse primer containing a *Xho*I recognition site (underlined). The amplified gene fragments were ligated into plasmid pET-29b (Merck, Darmstadt, Germany) at the *Nde*I and *Xho*I sites, yielding an expression vector encoding NpTrpDH with a C-terminal His_6-tag, after which the sequence of the vector was confirmed. *E. coli* BL21(DE3) (Merck) cells were then transformed with the plasmid and cultured in 2.5 l of LB medium containing 20 μg kanamycin/ml at 37 °C until the $OD_{600} = 0.8$. Expression was then induced by addition of 0.6 mM IPTG, and the incubation was continued for an additional 3 h at 37 °C. The cells (wet weight: ∼10 g) were then harvested by centrifugation and stored at −20 °C.

The following procedures were carried out at 4 °C unless stated otherwise. Frozen cells (wet weight: ∼10 g) were thawed, suspended in 50 ml of buffer I (pH 8.0) [50 mM Tris–HCl, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole, 20% glycerol and a protease inhibitor cocktail tablet (EDTA-free; Roche, Basel, Switzerland)], disrupted by sonication on ice, and centrifuged (24,000 × g) for 30 min. The resultant supernatant was loaded onto a Ni-NTA agarose column (bed volume, 4 ml; QIAGEN, Hilden, Germany) equilibrated with buffer II (pH 8.0) [50 mM Tris–HCl, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole and 20% glycerol], and the proteins were eluted with a linear gradient of 0.06–1 M imidazole (total volume, 60 ml). Fractions containing NpTrpDH were dialyzed against buffer III (pH 8.0) [50 mM Tris–HCl, 300 mM NaCl, 1 mM DTT, and 20% glycerol]. The C-terminal Leu-Glu-His-His-His-His-His-His tag was retained.

2.3. Size exclusion chromatography

NpTrpDH was applied to a Superdex 200 10/300 GL column (1.0 cm × 30 cm, GE Healthcare, Little Chalfont, UK) and eluted with buffer IV (pH 7.5) [50 mM Tris–HCl, 100 mM KCl, and 1 mM DTT] at a flow rate of 0.5 ml/min using an ÄKTA Explorer System (GE Healthcare). The apparent molecular mass was estimated by comparing the protein's retention time with those of molecular mass markers (MWGF1000-1KT; Sigma–Aldrich, St. Louis, MO, USA).

2.4. Enzyme assays

NpTrpDH-catalyzed reduction of $NAD(P)^+$ or oxidation of NAD(P)H was followed at 340 nm using a UV-1600 UV–visible Spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature-controlled cuvette holder. Three independent experiments were performed for all enzyme assays. The standard assay mixture (pH 11.0, 1 ml) for oxidative deamination contained 200 mM Gly-NaOH, 5 mM L-Trp, 1.25 mM NAD^+ and 100 nM NpTrpDH. The mixture (900 μl) without NpTrpDH was first incubated for 3 min at 25 °C before the enzyme reaction was started by addition of NpTrpDH solution (100 μl, dissolved in buffer III) to the mixture. In each assay, the initial velocity was determined by measuring the linear increase in the absorbance at 340 nm. Because of the measurable absorbance of 3-indolepyruvate at 340 nm (extinction coefficient at 340 nm for 3-indolepyruvate: 680 M^{-1} cm^{-1} in 200 mM Gly-NaOH and 1% (v/v) DMSO, pH 11.0), we considered the effect of absorbance by 3-indolepyruvate on absorbance by NADH to calculate the initial velocity. One unit (U) of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADH from NAD^+ per minute under the standard conditions for oxidative deamination.

We measured the reductive amination activity of NpTrpDH using a reaction mixture (pH 7.5, 1 ml) containing 200 mM Tris–HCl, 6.25 μM 3-indolepyruvate (dissolved in 1% (v/v) DMSO), 400 mM NH_4Cl, 0.2 mM NADH and 15 nM NpTrpDH as the standard assay mixture. Just before the assay, 3-indolepyruvate was dissolved in DMSO, and added to the reaction mixture lacking NADH and NpTrpDH and incubated for 30 min at 25 °C. It was then assumed that the majority of 3-indolepyruvate was in its keto form [19]. The assay for reductive amination was then performed using assay procedures similar to those for oxidative deamination.

2.5. Kinetic analyses

The initial velocity for oxidative deamination was analyzed using the standard assay condition and the reaction mixture (pH 7.5, 1 ml) containing 200 mM Tris–HCl instead of 200 mM Gly-NaOH. To determine the kinetic constants for L-Trp and NAD^+, several different concentrations of L-Trp (0.0100–5.00 mM) or NAD^+ (0.0125–2.50 mM) were used. The initial velocity was then plotted against the substrate concentration, and the K_m and k_{cat} values were determined by curve fitting using Igor Pro ver. 3.14 software (WaveMetrics, Tigard, OR, USA).

The K_m and k_{cat} values for 3-indolepyruvate in the reductive amination were determined using various 3-indolepyruvate concentrations (0.00313–1.25 mM) in the presence of 400 mM NH_4Cl and 0.2 mM NADH. Because marked substrate inhibition by 3-indolepyruvate was observed, even when the concentration was relatively low, the reciprocal initial velocities were plotted against the reciprocals of the 3-indolepyruvate concentration, and the plots were fitted to the Haldane equation (Eq. (1)) using Igor Pro ver. 3.14 to obtain the K_m and k_{cat} values and the inhibition constant for 3-indolepyruvate (K_{iI}) [8,20].

$$v = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_{iI}} \quad (1)$$

Kinetic constants for ammonia were determined using different NH_4Cl concentrations (50.0–400 mM) in the presence of 4.17, 5.00 or 6.25 μM 3-indolepyruvate and 0.2 mM NADH. Determination of the K_m and k_{cat} values for ammonia was carried out using a relatively low concentration of 3-indolepyruvate to avoid substrate inhibition. The reciprocal initial velocities ($1/v$) were then plotted against the reciprocal NH_4Cl concentrations ($1/[NH_4Cl]$) at each 3-indolepyruvate concentration and analyzed using linear regression. These data were then fitted to the following equation (Eq. (2)) to calculate the K_m and k_{cat} values for ammonia [8].

$$v = \frac{V_{max}[NH_4Cl][3-\text{indolepyruvate}]}{K_{mA}[3-\text{indolepyruvate}] + K_{mI}[NH_4Cl] + [NH_4Cl][3-\text{indolepyruvate}] + K_{iA}K_{mI}} \quad (2)$$

where K_{mA} and K_{mI} are the K_ms for ammonia and 3-indolepyruvate, respectively. K_{iA} then indicates the dissociation constant for the NpTrpDH–ammonia complex.

Kinetic constants for NADH were calculated by varying the NADH concentration (0.00267–0.200 mM) in the presence of 4.17, 5.00 or 6.25 μM 3-indolepyruvate and 400 mM NH_4Cl. The reciprocal initial velocities ($1/v$) were then plotted against the reciprocal NADH concentrations (1/[NADH]) at each 3-indolepyruvate concentration and analyzed using linear regression. These data were then fitted to the following equation (Eq. (3)) to calculate the K_m and k_{cat} values for NADH [8].

$$v = \frac{V_{max}[NADH][3-\text{indolepyruvate}]}{K_{mN}[3-\text{indolepyruvate}] + K_{mI}[NADH] + [NADH][3-\text{indolepyruvate}]} \quad (3)$$

where K_{mN} and K_{mI} are K_ms for NADH and 3-indolepyruvate, respectively.

2.6. Effects of pH and temperature on enzyme activity

The pH dependences for oxidative deamination and reductive amination catalyzed by NpTrpDH were determined at 25 °C using 200 mM concentrations of the following buffers: MES-NaOH (pH 6.5 and 7.0), HEPES-NaOH (pH 7.0–8.0), TAPS-NaOH (pH 8.0–9.0), Gly-NaOH (pH 9.0–11.0), and Na_2HPO_4-Na_3PO_4 (pH 11.0–12.0).

The temperature dependence was evaluated by measuring the oxidative deamination activity at temperatures ranging from 20 °C to 60 °C.

These assays were monitored for 1 min.

2.7. Effects of pH and temperature on enzyme stability

The effect of pH on enzyme stability was evaluated by incubating 0.332 μM NpTrpDH for 30 min at 4 °C with 100 mM concentrations of the following buffers: Na-citrate (pH 3.0–6.0), MES-NaOH (pH 6.0–7.0), Tris–HCl (pH 7.0–8.0), TAPS-NaOH (pH 8.0–9.0), Gly-NaOH (pH 9.0–11.0) and Na_2HPO_4-Na_3PO_4 (pH 11.0–12.0). The enzyme solution was then rapidly cooled on ice, and the remaining activity was determined using the standard oxidative deamination assay. The thermal stability was determined by incubating 1.66 μM NpTrpDH in 50 mM HEPES-NaOH (pH 7.5) for 10 min at various temperatures (20–50 °C). The enzyme solution was then

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