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Effect of metals and phenylalanine on the activity of human tryptophan hydroxylase-2: Comparison with that on tyrosine hydroxylase activity

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Abstract

We cloned the human tryptophan hydroxylase-2 (hTPH2) gene by RT-PCR, and expressed and purified its product as a maltose-binding protein (MBP)-fusion protein. We investigated the effects of essential divalent cations and L-phenylalanine (L-Phe) on the hTPH2 activity for the first time, and compared them with those on human tyrosine hydroxylase (hTH1) activity. We found that cobaltous and manganous ions inhibited the activities of both enzymes but that hTH1 was affected at lower concentrations than hTPH2. From kinetic analyses, we found that phenylalanine acted as an inhibitor more strongly against hTPH2 than against hTH1. These data are important for elucidating the molecular mechanism underlying the alterations in the contents of serotonin and catecholamines in the brain under pathological and physiological conditions, such as hyperphenylalaninemia and chronic manganese toxicity.

Keywords: Tryptophan hydroxylase-2; Tyrosine hydroxylase; Manganese; Phenylalanine; Hyperphenylalaninemia; Parkinsonism

Aromatic amino acid hydroxylases such as tyrosine hydroxylase (tyrosine 3-monooxygenase, TH; EC 1.14.16.2) [17], phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH; EC 1.14.16.1) [8], and tryptophan hydroxylase (tryptophan 5-monooxygenase, TPH; EC 1.14.16.4) [3], are widely expressed in higher organisms. These enzymes require an aromatic amino acid and molecular oxygen as substrates, as well as ferrous ion and (*6R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH4) as cofactors [2,9,11,17]. TPH catalyzes the initial and rate-limiting step of the biosynthesis of serotonin (5-hydroxytryptamine, 5-HT). This enzyme hydroxylates L-tryptophan (L-Trp) to form 5-hydroxytryptophan (5-HTP), and the product 5-HTP is then decarboxylated to serotonin by aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28). AADC also catalyzes the decarboxylation of L-dihydroxyphenylalanine (L-DOPA), produced from L-tyrosine (L-Tyr) by the action of TH, to dopamine.

Until recently, it had been thought that TPH was translated from mRNA transcribed from a single gene and that the differences in the properties of neuronal and peripheral enzymes were due to post-translational modification [10]. From their study on $Tph^{-/-}$ mice, Walther and Bader [21] discovered that the amount of serotonin in the mutant brain was unchanged, although the amounts of serotonin in the peripheral tissues were almost depleted in the knockout mice. They discovered that brain serotonin in these animals was formed by a previously unknown brain-specific isoform of TPH, which they referred to as TPH2 [21,22]. These investigators demonstrated that the conventional TPH gene (now called TPH1) was expressed in the pineal gland and gut but that the TPH2 gene was expressed only in 5-HT-producing neurons in the brain.

The enzymatic properties of TPH2 have been poorly understood. Very recently, McKinney et al. [14] reported the characterization of human tryptophan hydroxylase-2 (hTPH2) and compared its properties with those of TPH1. In this present study, we cloned human *TPH2* cDNA and expressed its enzyme product in *Escherichia coli* (*E. coli*) as a fusion protein with maltose-binding protein (MBP), since it was earlier reported that TPH1 was difficult to purify [1] and that the MBP-fusion protein was the most effective to express and purify TPH1 [15]. In order to investigate the differences in the characteristics of TPH2 with those of the other aromatic amino acid hydroxylase in the brain, we also expressed and purified human TH type 1 (hTH1) as an MBP-fused form. As calcium, magnesium, cobalt, manganese, and iron ions are essential divalent cations in higher organisms, we examined their effects on hTPH2 and hTH1 activities, especially since metal ions may interfere with the reaction

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of the ferrous ion, an essential cofactor for catalysis by these enzymes.

Expression vector pMAL-c2e and amylose resin were from New England Biolabs Inc. (Beverly, MA, USA). Human fetal brain RNA was purchased from Clontech (Palo Alto, CA, USA). All reagents were of analytical grade and used without further purification.

Human *TPH2* cDNA was cloned by reverse transcription and PCR amplification. Briefly, human fetal brain mRNA was reverse-transcribed to cDNA with SuperScript™ II RNase H⁻ reverse transcriptase and random hexamers as primers. Full-length h*TPH2* was amplified by PCR using forward primer 5′-ggtaccgATGCAGCCAGCAATGATGATG-3′ and reverse primer 5′-tctAgATCCCCAGATATTGGTTCATTTTG-3′. The underlined nucleotides of the forward primer represent the *Kpn*I site at the upstream region of the initiation codon, and those of the reverse primer, the *Xba*I site; and the small letters represent the bases mutated from the original sequence (GENBANK: AY098914). The termination codon of hTPH2 is TAA; but in order to generate the *Xba*I site at the termination codon, the codon was changed to TAG. These restriction enzyme sites were introduced in order to facilitate subsequent cloning into the expression vector used. After the sequence had been verified, the full-length h*TPH2* gene was digested with restriction enzymes *Kpn*I and *Xba*I, and the resulting *Kpn*I–*Xba*I fragment was inserted into the pMAL-c2e vector, thus generating pMAL-c2e-hTPH2. pMAL-c2e is an *E. coli* expression vector used in protein fusion and purification systems, and was designed to create fusion between a desired cloned gene and the *E. coli mal E* gene. pMAL-c2e-hTPH2 was used to transform *E. coli* strain BL21(*DE3*).

Expression and purification of MBP-tagged hTPH2 by amylose resin affinity chromatography were performed according to the manufacturer's protocol with slight modification, i.e., ferrous ammonium sulfate at the concentration of 0.2 mM was added to the culture medium of the transformed *E. coli*. After having been harvested, the cells were sonicated in cell lysis buffer, which was composed of 20 mM Tris–HCl (pH 7.4) containing 10% glycerol, 200 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM EDTA. The cell lysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant was collected and fractionated by using ammonium sulfate from 41% to 60%; and these fractions were applied to an amylose resin column. Human TPH2 fraction was eluted with the same lysis buffer containing 10 mM maltose, the eluate was applied to a column of Superose 6 HR 10/30, and active fractions were collected. The purity of hTPH2 was checked by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie Brilliant Blue R-250 (CBB) staining at each step, and protein concentration was determined by the method of Bradford with γ-globulin used as a standard.

Human TH1 gene fused with a pMAL vector was kindly donated by Nakashima [18], and was used to transform *E. coli* BL21(*DE3*). Expression and purification of MBP-tagged hTH1 was the same as for hTPH2 except that hTH1 fractions were collected from 31% to 50% ammonium sulfate.

The enzyme activity of hTPH2 was measured by HPLC-fluorescence detection [5] with slight modifications. The standard reaction mixture (total volume 100 μl) contained 120 mM Na-HEPES (pH 7.2), 2.5 mM DTT, 0.1 mg/ml catalase, 20 μM ferrous ammonium sulfate, 100 μM L-tryptophan (otherwise indicated), and the enzyme. The reaction was started by the addition of BH4 solution to achieve a final concentration of 500 μM. The reaction mixture was incubated in air at 30 °C for 10 min, filtered, and injected into the HPLC-fluorescence detection apparatus, which was equipped with a reverse-phase column CAPCELL PAK C18 UG80 S-5 (4.6 mm i.d. × 250 mm, Shiseido, Tokyo, Japan); and fluorescence was detected at 350 nm with excitation at 302 nm. The mobile phase for the HPLC was a solution composed of 15 mM acetate–citric buffer (pH 3.5), 5% acetonitrile, and 7.5% methanol. For assessing the effects of divalent cations, we varied the concentrations of them from 0 to 1.0 mM. The divalent cations selected were cobalt, manganese, calcium, and magnesium. In the case of calcium ions, EGTA was added to the reaction mixture at the final concentration of 1.0 μM to remove any trace amount of contaminating calcium ions in the distilled water. For the kinetic assay of hTPH2, the concentration of L-Trp was varied from 0 to 800 μM, L-phenylalanine was added at the final concentration of 50, 100, and 200 μM to each set of reaction mixtures, and the mixtures were incubated for 5 min.

Enzyme activity of hTH1 was measured by an HPLC-electrochemical detection (ECD) method [19]. The standard reaction mixture (total volume 200 μl) contained 200 mM Na-HEPES (pH 7.0), 100 mM 2-mercaptoethanol, 0.2 mg/ml catalase, 200 μM L-tyrosine (otherwise indicated), and the enzyme. The reaction was started by the addition of BH4 at the final concentration of 1.0 mM. The reaction mixture was incubated in air at 37 °C for 10 min, and the L-DOPA produced was separated with a reverse-phase column of NUCLEOSIL 100-7C-18 (4.0 mm i.d. × 250 mm, GL Science, Tokyo, Japan), and detected electrochemically with an applied voltage of 600 mV. The mobile phase for the HPLC was 0.1 M sodium phosphate buffer (pH 3.5) containing 8 μM EDTA and 1% methanol. In the experiments on the effects of the divalent cations, the conditions were the same as those used for the TPH assay. In the kinetic assay for hTH1, the concentration of L-Tyr was varied from 0 to 400 μM, L-phenylalanine was added at the final concentrations of 50, 100, and 200 μM to each set of reaction mixtures, and the mixtures were incubated for 5 min.

First, we cloned human TPH2 cDNA from human fetal brain mRNA by RT-PCR. By sequencing the cloned cDNA, we found a single nucleotide polymorphism (SNP) at the base position of A936G (SNP ID: rs7305115), though it did not change the amino acid specified, i.e., proline^{312}. The digested DNA fragment was cloned into pMAL-c2e vector; and MBP-tagged hTPH2 was expressed in *E. coli*, purified by ammonium sulfate fractionation, amylose resin affinity chromatography, and gel filtration chromatography. MBP-tagged hTH1 was also expressed and purified in the same way. These two MBP-tagged enzymes were subjected to SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gels, and the homogeneity of the enzymes was confirmed (Fig. 1). Molecular weights of MBP-tagged hTPH2 and hTH1 determined from the SDS–PAGE were 97 and 113 kDa, respectively.