Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 338 (2005) 277–284
BBRC
www.elsevier.com/locate/ybbrc

# Distinctive iron requirement of tryptophan 5-monooxygenase: TPH1 requires dissociable ferrous iron

Hiroyuki Hasegawa [a,*], Arata Ichiyama [b]

[a] Department of Biosciences, Teikyo University of Science and Technology, Uenohara, Yamanashi 409-0193, Japan
[b] Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

Received 16 July 2005
Available online 16 September 2005

**Abstract**

A peripheral type of tryptophan 5-monooxygenase (EC 1.14.16.4), TPH1, is very unstable in vitro, but the inactivation was reversible and full reactivation occurs upon anaerobic incubation with a high concentration of dithiothreitol (DTT, 15 mM). In this study, distinctive iron requirement of TPH1 was revealed through analysis of the enzyme's inactivation and activation by DTT. For this purpose, all the glasswares, plastics, Sephadex G-25 gels, and reagents including protein solutions had been treated with metal chelators, and apo-TPH was prepared by treatment with EDTA. Apo-TPH thus prepared exclusively required free $Fe^{2+}$ for its catalytic activity; $10^{-8}$ M was enough under the strict absence of $Fe^{3+}$ but $10^{-12}$ M was too low. No other metal ions including $Fe^{3+}$ were effective. It appeared that $Fe^{3+}$ bound to the enzyme with a higher affinity than $Fe^{2+}$, resulting in the inactivation. Ascorbate, a non-thiol reducing agent, did not substitute DTT in the activation of TPH1, but enhanced the $Fe^{2+}$-dependent activity of apo-TPH as effectively as DTT. Thus, the DTT-activation was essentially substituted by preparation of apo-TPH by the EDTA treatment and the assay of apo-TPH in the presence of $Fe^{2+}$ and ascorbate. The activation of TPH1 by incubation with DTT was accompanied by exposure of 9 sulfhydryls out of the total 10 cysteine residues, but the cleavage of disulfide bonds seemed not to be crucial, even if it occurred. The effect of DTT was substituted by some other sulfhydryls whose structure was analogous to that of commonly used metal chelators. Based on these observations, the following dual roles of DTT are proposed: (1) in the activation of TPH, DTT removes inappropriate bound iron ($Fe^{3+}$) as a chelator, keeping $Fe^{3+}$ away from the enzyme's binding site which needs to bind $Fe^{2+}$ for the catalytic activity, and (2) in both the activation and reaction processes, DTT prevents oxidation of $Fe^{2+}$ to $Fe^{3+}$ as a reducing agent.


*Keywords:* Tryptophan hydroxylase; Serotonin biosynthesis; Tetrahydrobiopterin; Iron determination; Sulfhydryl determination; Disulfide cleavage

Tryptophan 5-monooxygenase (EC 1.14.16.4), which is commonly called as tryptophan hydroxylase, catalyzes conversion of L-tryptophan to 5-hydroxy-L-tryptophan (5HTP) using tetrahydrobiopterin as an essential cofactor [1–3].

L-Tryptophan + tetrahydrobiopterin + $O_2$
= 5-hydroxy-L-tryptophan + 4a-hydroxytetrahydrobiopterin

In the biological system, this enzyme catalyzes a rate-limiting step in the biosynthesis of serotonin (5-hydroxytryptamine). 5HTP is converted to serotonin by aromatic-L-amino acid decarboxylase. 4a-Hydroxytetrahydrobiopterin is recycled by conversion to quinononid-dihydrobiopterin, and subsequently to tetrahydrobiopterin by sequential actions of pterin 4a-carbinolamine dehydratase and dihydropteridine reductase. Tryptophan hydroxylase requires iron and has an iron binding site composed of neighboring His, His, and Glu on the 3D-structure, which was illustrated using recombinant human enzyme crystallized in the presence of $Fe^{3+}$ and 7,8-dihydrobiopterin [4]. Two homologous isoforms of tryptophan hydroxylase are known in mammals, and called TPH1 and TPH2 [5–7]. They are genetically coded on different chromosomes. TPH1 is located in both serotonin-producing cells in peripheral organs

* Corresponding author. Fax: +81 554 63 4431.
*E-mail address:* hasegawa@ntu.ac.jp (H. Hasegawa).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.045

and neurons in the brain, while distribution of TPH2 is limited to the brain. Before finding genetic identity of TPH2 in 2003, presence of the two types of TPH has been recognized empirically based on prominent differences in the properties of the enzyme preparations from respective sources [8–10]. The different identities of two tryptophan 5-hydroxylases were, however, not widely recognized, and description on characteristics of one isoform was occasionally taken as those of both isoforms. Moreover, it became more confusing when cDNA of the enzyme was cloned from a mRNA library of brain tissues and the recombinant enzyme was dealt with as the "brain enzyme" even though the code of the open reading frame was of TPH1 based on current knowledge. TPH1 was very unstable under laboratory conditions and lost virtually all the enzyme activity through purification procedures. Much earlier, Ichiyama and his coworkers found that the activity could be recovered by anaerobic treatment of the inactivated enzyme with a relatively high concentration of dithiothreitol (DTT) and a trace of $Fe^{2+}$ in the presence of catalase [11]. Based on this observation, they partially purified a typical peripheral TPH from bovine pineal gland and characterized it [12]. Similar activation with mercaptans was also observed with brain TPH [13–15]. Oxygen-sensitive inactivation of brain TPH and activation by DTT was reported by Kuhn et al. [16]. The empirical activation of the inactivated enzyme by incubation with DTT was further optimized as an unavoidable procedure for the assay of TPH of peripheral sources [17–19]. Effort to understand the mechanism of the reversible inactivation of the enzyme has been continued [20,21]. In the present study, we attempted to investigate the role of DTT in the activity of the peripheral TPH1 with reference to ferrous and ferric irons.

## Materials and methods

*Materials.* Bovine liver dihydropteridine reductase was purified as previously described [22]. Bovine liver catalase was purchased from Boehringer–Mannheim–Yamanouchi (Tokyo, Japan). Metal chelators, EDTA, 1:10-phenanthroline, EGTA, and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfate (PDTS), were purchased from Dojin Laboratories (Kumamoto, Japan). Affigel 202 and Chelex 100, from Bio-Rad (CA, USA); a fluorescent thiol reagent *N*-(7-dimethylamino-4-methyl coumarinyl) maleimide (DACM) and sulfhydryl compounds, from Wako Pure Chemical (Osaka, Japan); $HNO_3$ and $H_2SO_4$, Ultrex grade, from J.T. Baker (NJ, USA); HCl, $HClO_4$, formic acid, and acetic acid (Ultra Fine grade), from Nakarai Chemicals (Kyoto, Japan). All other reagents were of highest grade available from commercial sources.

*Preparation of solutions.* In the preparation of solutions special precautions were given to avoid contamination with iron as described in "Supplement #1." When $Fe^{2+}$ was added to experimental solutions which contained both oxidant ($O_2$) and reductants (tetrahydropterin and DTT), the redox state of iron might be uncontrollable even if it was added last. Nonetheless, the term "$Fe^{2+}$" is used in the present paper whenever $FeSO_4(NH_4)_2SO_4$ was added. Similarly, "$Fe^{3+}$" is used when solutions of $FeNH_4(SO_4)_2$ were added.

*Purification of tryptophan hydroxylase.* Mouse mastocytoma cells (P-815) were grown in the peritoneal cavity of BDF1 mice (F1-hybrid of female C57BL and male DBA/2) [23], and the enzyme was purified from packed cells by affinity chromatography with $DMPH_4$-bound agarose gels according to Nakata et al. [9] with modifications as described in "Supplement #2." The modified recipe attained a great yield of TPH1, 1.6–2.4 mg from 150 g of packed cells (50–70% recovery of the initial activity). The specific activity was about 5 μmol/min/mg with a purity of greater than 98% as estimated by SDS–PAGE.

*Preparation of apo-tryptophan hydroxylase.* Sephadex G-25 (superfine) used for preparation of apo-TPH1 must have been cleaned as follows. Dry gel was swollen in a 10 mM EDTA and 5 mM 1:10-phenanthroline solution (pH 6.8), and then autoclaved for 30 min at 120 °C. This resulted in the appearance of reddish color of 1:10-phenanthroline-$Fe^{2+}$. The gel was then stored in the 10 mM EDTA–5 mM 1:10-phenanthroline solution for at least 1 week and washed extensively with Chelex-treated buffer prior to use. Purified TPH (360 μg) kept in the stabilizing buffer (300 μl of 50 mM Tris–HCl (pH 7.5) containing 0.1 M KCl, 10% glycerol, and 0.05% Tween 20 [9]) was mixed with EDTA (final 10 mM) and left for 10 min at room temperature. The mixture was passed through a Sephadex G-25 column ($0.9 \times 12$ cm) equilibrated with the stabilizing buffer, and the enzyme fractions were collected (200 μl each). This preparation did not show any significant activity when the assay system was kept free of iron, but exhibited close to the full specific activity when measured under the standard assay conditions after the DTT activation.

*Anaerobic incubation of tryptophan hydroxylase with DTT.* Anaerobic conditions were established on ice by three cycles of evacuation and $N_2$-flushing in a Thunberg tube connected to a vacuum line which was switchable to $N_2$-gas [17]. The standard procedure of anaerobic preincubation for activation of TPH with DTT (usually 15 mM) was essentially the same as that described previously [11,18,19]. When the role of iron in the activation was to be investigated, $FeSO_4(NH_4)_2SO_4$ and catalase were omitted, and the minimum component of the mixture to allow the enzyme activation was DTT (15 mM in 50 mM Tris–acetate, pH 8.1).

*Tryptophan hydroxylase assay.* The standard reaction mixture (150 μl) contained 0.1 M potassium phosphate (pH 6.9), 0.33 mM $6MPH_4$, 0.67 mM L-tryptophan, 0.33 mM NADH, 13.3 μM $FeSO_4(NH_4)_2SO_4$, 33.3 μg/ml of bovine liver dihydropteridine reductase, 1 mg/ml of catalase, and the enzyme solution (including the components carried over from the preincubation step with DTT). When the $Fe^{2+}$ requirement was to be analyzed, $FeSO_4(NH_4)_2SO_4$ and catalase was omitted. Usually, 10–50 μl of the preincubation mixtures containing about 0.2 μg of the enzyme protein were assayed at 30 °C for 5 min. Reactions were terminated by addition of $HClO_4$ (final concentration, 0.84 M) and 5HTP formed was determined by high-performance liquid chromatography, as described previously [19].

*Determination of protein sulfhydryl groups.* A water-soluble fluorescent thiol reagent, *N*-(7-dimethylamino-4-methyl coumarinyl) maleimide (DACM), was employed for labeling protein sulfhydryl groups [24]. The assay procedure below was designed to analyze protein sulfhydryl content using about 100 μg protein. Prior to the reaction with DACM, reduction of protein samples was performed with 15 mM DTT in 0.1 M Tris–acetate (pH 8.1) under $N_2$. Enzyme protein denatured with 8 M guanidine–HCl was also subjected to the DTT reduction. The details of the procedure are described in "Supplement #3." The DACM–protein adduct was measured by fluorescence at excitation and emission wavelengths of 385 and 476 nm, respectively. The fluorescence intensity was expressed relative to that of TPH reduced under denaturing conditions in the presence of 8 M guanidine–HCl. The sulfhydryl content of BSA was determined both with 5,5′-dithio(2-nitrobenzoate) (DTNB) and DACM as sulfhydryl reactive reagent as described in "Supplement #4."

*Determination of iron.* Purified enzyme in the stabilizing buffer containing 10% glycerol was directly subjected to wet ashing before iron determination. In case of DTT/$N_2$-activated TPH, purified enzyme (157 μg) was subjected to the DTT/$N_2$ (-catalase, +$Fe^{2+}$) treatment and then adsorbed to concentrate the enzyme onto hydroxyapatite (0.4 ml) layered over a Sephadex G-25 gel filtration column (2.0 ml bed volume equilibrated with the stabilizing buffer). The enzyme was then eluted with 0.5 M phosphate buffer (pH 6.8) and 180-μl fractions of eluate containing more than 95% of applied enzyme were collected. Aliquot (170 μl) of all fractions was subjected to iron determinations after wet ashing. Iron was measured colorimetrically using 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfate (PDTS) as a chromogenic chelator ($\varepsilon_{562} = 27.9\ mM^{-1}\ cm^{-1}$) . Iron