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

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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

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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 (0.9×12 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

in wet-ashed protein samples was reduced with ascorbic acid, mixed with PDTS, and the absorbance was determined essentially by the method of Gottschall et al. [25]. Wet ashing with $HClO_4$ was performed essentially as described previously [26]. Details of the combustion of sample proteins with HNO_3 in a buffer containing glycerol and following colorimetry are described in "Supplement #5." The limit of the iron determination by the PDTS-method was about 0.3 nano-atoms. For each sample, five different volumes were separately wet ashed, and the amount of Fe vs. amount of applied protein was calculated by linear regression. Determinations of the same sample with correlation coefficients >0.9 were taken as positive measurement.

Others. Calculation of the free Fe^{2+} concentration in the aqueous Fe^{2+}–EGTA or Fe^{2+}–EDTA mixtures was performed based on the method of Eriksson [27] using EQCAL software [28].

Results

Exposed sulfhydryl groups on tryptophan hydroxylase

Since DTT is a reducing reagent which effectively cleaves disulfide bond of proteins, the activation of TPH by incubation with DTT under anaerobic conditions could be explained, at least in part, by the disulfide bond cleavage. Therefore, sulfhydryl groups of TPH were determined before and after the DTT treatment. For this purpose, a method was developed in which sulfhydryl groups of the activated enzyme was labeled with DACM, a water-soluble fluorogenic reagent, when the enzyme was still fully active. In order to minimize oxidative inactivation before the labeling, the DTT-activated enzyme was passed through a Sephadex G-25 column equilibrated with DACM immediately after the activation procedure, so that the labeling of protein sulfhydryl groups occurred just after separation from DTT with minimal exposure of the protein mixture to air [20], as described in detail in "Supplement #3." In a preliminary experiment using crystalline BSA, only one SH out of a total of 35 cysteine residues per molecule was labeled with DACM after the DTT treatment, while all the 35 sulfhydryls were labeled when the protein was denatured in 8 M guanidine–HCl and subjected to DTT treatment ("Supplement #4"). These results clearly indicated that the DTT treatment did not reach buried cysteine or half cystine residues (cf. [29]) of non-denatured BSA, while it reduced virtually all 34 buried sulfhydryls after denaturation.

Four preparations of purified TPH were subjected to the DACM labeling, and the sulfhydryl content expressed in terms of the relative fluorescence was compared on the protein molar basis. The enzyme from mouse mastocytoma P-815 contains 10 cysteine residues out of a total of 447 amino acids [30]. The sulfhydryl content measured after the DTT treatment in the presence of 8 M guanidine–HCl was assumed to represent the total 10 cysteines (100%). The SH of the non-reduced native protein (virtually no enzyme activity) was calculated to be 2 residues (21.5%), suggesting that these SH groups were exposed to DACM and the remaining 8 sulfhydryls were somehow "masked" or buried. With DTT-treated native enzyme (fully active), 9 residues (91.9%) were exposed to DACM, suggesting that the newly exposed 7 sulfhydryls were not buried but had been somehow "masked." The remaining one sulfhydryl out of total 10 (100 − 91.9%) was likely to be buried, which was exposed only by denaturation in 8 M guanidine–HCl.

Comparison of the ability of various sulfhydryl compounds to activate tryptophan hydroxylase

The ability of various sulfhydryl compounds to activate TPH is summarized in Table 1. In the absence of any reducing reagent, the preincubation under anaerobic conditions caused no activation, and even residual enzyme activity was lost during the preincubation. DTT was the most effective among the reagents tested, and 2-mercaptoethanol, 2-mercaptoethylamine, and cysteine were comparably effective (group 1). *N*-Acetylcysteine, glutathione, 2-mercaptopropionic acid, 3-mercaptopropionic acid, and thioglycerol (data not shown) were less effective, and prolonged incubation did not improve the results (group 2). 8-Mercaptoquinoline inactivated the enzyme, probably because it was a strong iron chelator. Non-sulfhydryl reducing reagents such as ascorbate and sodium hydrosulfite also failed to activate the enzyme, but prevented further loss of activity during the incubation. The concentration of the reagent required for effective activation of TPH was much higher than that expected for disulfide bond cleavage, suggesting that the role of DTT was unlikely to be a disulfide bond breaker for which a 30-fold molar excess was thought to be sufficient. It is worth emphasizing that the highly effective reductants (group 1) have a common structural feature, HS-C-C-OH or HS-C-C-NH [31], analogous to the structure of the most commonly used metal chelators such as EDTA.

Table 1
Activation of tryptophan hydroxylase by sulfhydryl compounds

Group	Reductant	Enzyme activity (μmol/min/mg)	Relative to DTT (%)
1	DTT	1.61	100
	2-Mercaptoethylamine	1.51	93.8
	2-Mercaptoethanol	1.28	79.3
	Cysteine	1.28	79.5
2	*N*-Acetylcysteine	0.502	31.2
	3-Mercaptopropionate	0.354	22.0
	2-Mercaptopropionate	0.189	11.7
	Glutathione	0.260	16.1
3	8-Mercaptoquinoline	—[a]	<1
	None	—[a]	<1

Purified TPH was preincubated with various sulfhydryl compounds in 50 mM Tris–acetate (pH 8.1) containing 10 μM $FeSO_4(NH_4)_2SO_4$ and 2 mg/ml of catalase at 30 °C for 10 min under air. The reductant concentration in the preincubation mixture was 30 mM, except for DTT whose concentration was 10 mM. Then a 50-μl aliquot of the preincubation mixture was subjected to the TPH assay, as described in Materials and methods. Sulfhydryl compounds were dissolved in acidic solution and neutralized with NaOH just prior to use. The reductants are arbitrarily classified into three groups based on their effectiveness relative to DTT.

[a] 5HTP formation was less than 0.02 μmol/min/mg.

The iron content of purified tryptophan hydroxylase

Since tetrahydropterin-dependent monooxygenases are generally thought to be iron containing enzymes, the iron content of our purified TPH preparations was determined. Wet ashing of TPH preparations maintained in an enzyme-stabilizing buffer containing 10% glycerol was carefully done, and iron in wet-ashed protein samples was measured colorimetrically as described in Materials and methods. In three independent determinations, the amounts of iron detected were <0.3, 0.6, and 0.4 nano-atoms per 1.31, 3.86, and 1.82 nmol of the enzyme subunit (51.3 kDa [30]), respectively; representing <0.23, 0.15, and 0.22 atom/subunit. Since these enzymes had virtually no activity unless measured after the DTT-activation, the values obtained could be of non-functional iron, presumably Fe^{3+}.

The iron content of TPH was then determined after the DTT/N_2 activation which was carried out in the presence of 10 μM Fe^{2+} but in the absence of catalase. The activated enzyme was applied to a small bed of hydroxyapatite (0.75 × 1 cm) which had been layered over a Sephadex G-25 column, and the elution was carried out with 0.5 M potassium phosphate (pH 6.8). Each protein fraction which was eluted ahead of phosphate was directly ashed and subjected to the iron determination. The sum of iron detected in all enzyme fractions was 0.73 nano-atoms and that of protein was 15.8 nmol of the enzyme subunit; representing 0.046 atom/subunit. In a control experiment carried out in parallel with a similar amount of catalase, a value of about 1.04 atoms of iron per subunit (M_r = 50,000) was observed. These results suggest that the fully activated TPH had lost the bound iron through the gel filtration procedure and thus Fe^{2+} bound to TPH is easily dissociable.

Iron as a dissociable ligand

We then carried out EDTA-treatment to prepare iron-free apo-TPH whose activity was absolutely dependent on supplemented Fe^{2+} in the reaction mixture [21]. After many unsuccessful trials, we finally obtained it by mixing the purified enzyme with EDTA (10 mM), followed by removal of the chelator by gel filtration. It was critical that all the glasswares, plastics, and Sephadex G-25 gels had been washed to make them iron-free, and all reagents used had been passed through a Chelex-100 column, as described in Materials and methods. The apo-TPH obtained by the EDTA procedure was inactive under the strict absence of Fe^{2+}, but was fully active when measured after the DTT activation under the standard assay conditions. Without the DTT-treatment, the apo-enzyme expressed a definite activity (10.2%) in the presence of Fe^{2+} (50 μM) alone, and further addition of catalase (1 mg/ml) or ascorbate (10 mM) enhanced the activity (40% and 60% of the full activity, respectively) as shown in Table 2.

It was possible that DTT and other highly effective sulfhydryl compounds, which have structural resemblance to common chelators such as EDTA, acted as both chelator and reductant of Fe^{3+}, thereby causing full activation. The effect on the activity of the EDTA-washed apo-TPH was compared in the presence of Fe^{2+} and DTT or in the presence of Fe^{2+} and a non-thiol reducing reagent, ascorbate. Where indicated, catalase was added to protect the enzyme from inactivation by H_2O_2. As shown in Table 2, the enzyme exhibited about 66% of the full activity in the presence of ascorbate (10 mM), Fe^{2+}, and catalase. Since the activity observed with DTT (10 mM) was 64% under the same conditions, ascorbate was judged to be able to completely replace DTT in supporting the activity of apo-TPH. It was worth noting that ascorbate did not replace DTT in activating TPH before the EDTA-treatment, suggesting that EDTA exposed a site for accepting Fe^{2+}. It was, therefore, conceivable that "EDTA-washing" substituted for an essential part of the "DTT-activation."

Table 2
Activation of apo-tryptophan hydroxylase by Fe^{2+} and ascorbate

Additions	Activity[a] (5HTP formed in 5 min)	
	μmol/mg	Percentage[b]
50 μM Fe^{2+} + 10 mM Asc + 1 mg/ml catalase	15.0	66.4
50 μM Fe^{2+} + 10 mM Asc	13.5	59.7
50 μM Fe^{2+} + 1 mg/ml catalase	8.80	38.9
50 μM Fe^{2+} + 10 mM DTT + 1 mg/ml catalase	14.4	63.8
50 μM Fe^{2+}	2.30	10.2
1 mg/ml catalase	0.535	2.4
None	—[c]	<1

Apo-TPH was assayed without the DTT-activation. The reaction mixture (150 μl) contained the indicated additions in 0.1 M potassium phosphate (pH 6.9) containing 0.33 mM $6MPH_4$, 0.67 mM L-tryptophan, 0.33 mM NADH, 33.3 μg/ml of bovine liver dihydropteridine reductase, and the enzyme. Solutions of Fe^{2+} were kept in 0.1 M ascorbic acid (lines 1 and 2) or 10 mM HCl (lines 3–5) until use. The pH of the ascorbate was adjusted to neutrality with 0.1 M NaOH just before use. The reaction was initiated by addition of the enzyme, and carried out at 30 °C for 5 min, then 5HTP formation was determined. All procedures were conducted in air except for the DTT-activation which served as a positive control. Data are means of triplicate assay.

[a] Note that the enzyme reaction did not always proceed linearly with time, especially in the absence of catalase (cf. Fig. 1).

[b] Percent of the control activity (22.6 μmol/mg) determined after the standard DTT-activation.

[c] 5HTP formation was less than 0.1 μmol/mg.

Requirement of apo-TPH for Fe^{2+} and the protective effect of catalase were further demonstrated in an experiment shown in Fig. 1 in which apo-TPH had been preincubated anaerobically with DTT in the absence of both Fe^{2+} and catalase. No significant activity was observed without addition of Fe^{2+}, even when catalase was present. 5HTP formation commenced upon addition of Fe^{2+} without a significant lag period regardless of the presence or absence of catalase. Fe^{2+} alone was sufficient for activity but the enzyme seemed to be unstable under this conditions. However, the absence of Fe^{2+} did not lead to irreversible loss of enzyme activity, since the enzyme reaction commenced upon subsequent addition of Fe^{2+} with no detectable lag period (right panel). Catalase alone did not support the enzyme activity but stabilized the enzyme in the presence of Fe^{2+}.

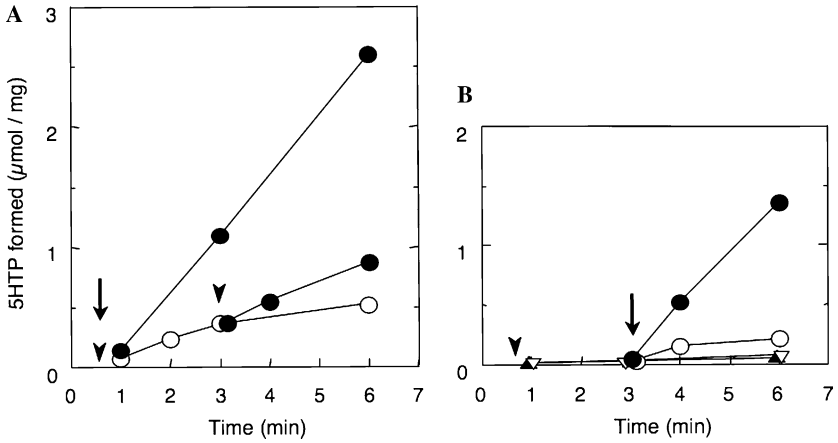

Fig. 1. Time course of 5HTP formation by apo-tryptophan hydroxylase in the presence or absence of Fe^{2+} and catalase. Apo-TPH prepared as described in the text was preincubated anaerobically with DTT in the absence of both Fe^{2+} and catalase. The reaction mixture contained all of the components of the standard assay system, except that Fe^{2+} (A) or both Fe^{2+} and catalase (B) were initially omitted. The preincubated enzyme was added at 40 s of incubation. Fe^{2+} was added at the times indicated by arrows and the reactions were carried out in the presence (closed circles) or absence (open circles) of catalase which was added as indicated by arrowheads. Open triangles represent 5HTP formation in the absence of both Fe^{2+} and catalase, and closed triangles represent 5HTP formation in the absence of Fe^{2+} but in the presence of catalase. Final concentrations of Fe^{2+} and catalase were 50 μM and 2 mg/ml, respectively.

Specific requirement of tryptophan hydroxylase for Fe^{2+}

Apo-TPH did not catalyze detectable 5HTP formation with any of the following metals added (100 μM) to the preincubation mixture in place of Fe^{2+}: $CuSO_4$, $Cd(CH_3COO)_2$, $Co(CH_3COO)_2$, $NiCl_2$, $Pb(CH_3COO)_2$, $Zn(CH_3COO)_2$, $MnCl_2$, $MgCl_2$, or $CaCl_2$. Iron containing compounds such as cytochrome *c*, hemin, and ferricyanide, at 50 μM each, also failed to activate the enzyme. Thus, free iron is specifically required for the TPH activity. In order to see if these metal ions could compete with iron, apoenzyme was preincubated with DTT in the presence of various metal ions at 10 μM each, but without Fe^{2+}, and the activity was then determined in the presence of both 50 μM Fe^{2+} and 3.3 μM each of the metal ions which were carried over with the enzyme. The enzyme activity in the presence of Fe^{2+} alone was 4.13 μmol/min/mg and activities (μmol/min/mg) in the presence of other metal ions were: Mn^{2+}, 1.15 (28%); Zn^{2+}, 0.66 (16%); Cu^{2+}, 3.94 (95%); Ca^{2+}, 4.00 (97%); and Mg^{2+}, 3.88 (94%). These results suggest that Mn^{2+} and Zn^{2+} competed with Fe^{2+}.

The specificity of iron requirement (Fe^{2+} or Fe^{3+}) of apo-TPH was then examined. In order to ensure that free iron in the reaction mixture was maintained solely in the Fe^{2+} or Fe^{3+} form, Fe^{2+} and limited concentrations of EDTA or 1:10-phenanthroline were added to the apo-enzyme. EDTA chelates Fe^{3+} more effectively than Fe^{2+} as evidenced by its stability constant, $\log(K_{ML})$ of 25.1 and 14.3 for Fe^{3+} and Fe^{2+}, respectively. On the other hand, 1:10-phenanthroline forms a 1:3 complex with iron with greater affinity for Fe^{2+} ($\log(K_{ML3}) = 21.2$). Fig. 2 shows the results of an experiment in this line. Addition of Fe^{3+} together with 1:10-phenanthroline might be a most strict condition for the enzyme to be isolated from Fe^{2+}; even

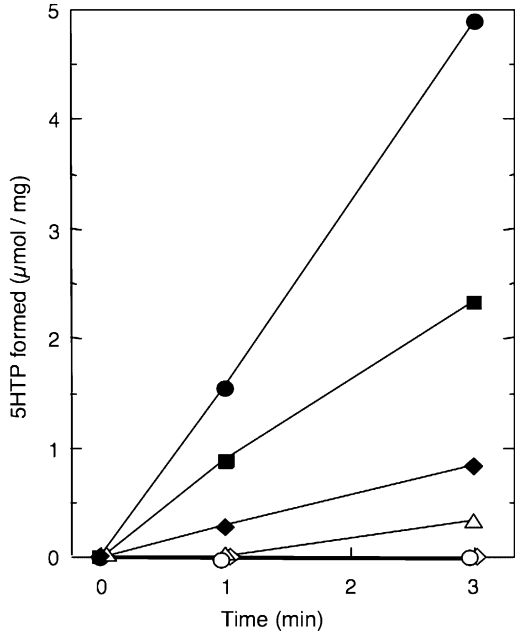

Fig. 2. Absolute requirement of apo-tryptophan hydroxylase for Fe^{2+}. Apoenzyme was mixed with 50 mM Tris–acetate (pH 8.1) containing 15 mM DTT in the main chamber of Thunberg tubes, the side arms of which contained $FeSO_4(NH_4)_2SO_4$ or $FeNH_4(SO_4)_2$ alone or the iron salts in combination with either EDTA or 1:10-phenanthroline. The tubes were incubated at 30 °C for 10 min under N_2 and the two solutions were then mixed while anaerobiosis was maintained. Final concentrations of Fe^{2+}, Fe^{3+}, EDTA, and 1:10-phenanthroline after mixing were 70, 70, 50, and 150 μM, respectively. Immediately after mixing (within 15 s), 50 μl aliquots of the mixtures were dispensed into reaction mixtures which contained the standard assay components, except for $FeSO_4(NH_4)_2SO_4$. Reactions were carried out at 30 °C for the indicated periods. Closed circles, Fe^{2+} alone; closed squares, Fe^{2+} plus EDTA; closed diamonds, Fe^{2+} plus 1:10-phenanthroline; open triangles, Fe^{3+} plus EDTA; open diamonds, Fe^{3+} plus 1:10-phenanthroline; open circles, no iron or chelator.

if Fe^{2+} appeared in the mixture by reduction with DTT; the formed Fe^{2+} must have been selectively masked, because 1:10-phenanthroline preferentially bound to Fe^{2+}, leaving free Fe^{3+} at a calculated concentration of 20 μM or greater due to its weak affinity for 1:10-phenanthroline. On the other hand, virtually no Fe^{3+} may have existed in its free form when Fe^{2+} was given in the presence of EDTA, even if Fe^{3+} was produced in situ by oxidation of Fe^{2+}. Following conditions containing 70 μM Fe^{2+} or Fe^{3+} plus 50 μM EDTA or 150 μM 1:10-phenanthroline were used. (a) Fe^{2+} alone, a trace of Fe^{+3} oxidized from Fe^{2+} might exist, (b) Fe^{2+} plus EDTA, a trace of Fe^{3+} oxidized from Fe^{2+} might be masked leaving Fe^{2+} near 20 μM, (c) Fe^{2+} plus 1:10-phenanthroline, 50 μM Fe^{2+} might be masked leaving a trace of Fe^{3+}, (d) Fe^{3+} plus EDTA, 50 μM Fe^{3+} might be masked leaving a trace of Fe^{2+} which was reduced from Fe^{3+} by DTT, (e) Fe^{3+} plus 1:10-phenanthroline, even a trace of Fe^{2+} might be masked leaving more than 20 μM Fe^{3+}, and (f) no iron. In condition (e), no activity was detected while considerable activity was observed in condition (b). This is a demonstration that the enzyme has an absolute requirement for Fe^{2+} for its activity.

Estimation of affinity of Fe^{2+} to tryptophan hydroxylase

In search of appropriate chelators which extensively masked trace of Fe^{3+} leaving Fe^{2+} to be available for the enzyme, we found that EGTA allowed the enzyme to be remarkably active. As shown in Table 3, the enzyme exhibited as much as 80% of the full activity, when 100 μM Fe^{2+} was added in the presence of 1 mM EGTA, 2 mg/ml of catalase, and 10 mM ascorbate at pH 6.7. The stability constant of EGTA $\log(K_{ML})$ for Fe^{2+} and Fe^{3+} at pH 6.7 is 11.9 and 20.5, respectively. Under this condition, Fe^{2+} was also bound to EGTA, but due to weaker affinity than Fe^{3+} a portion of it should remain as free Fe^{2+} whose concentration was suggested to be no higher than 8.70×10^{-9} M (calculated by a PC software EQCAL using 10^{-4} M for Fe^{2+}, 10^{-3} M for EGTA and pH 6.7). On the other hand, the enzyme was inactive when EGTA was replaced by 1 mM EDTA at the same pH (data not shown), indicating that the enzyme was unable to utilize free Fe^{2+} at 1.8×10^{-12} M (calculated based on $\log(K_{ML})$ of EDTA for Fe^{2+} of 14.3). Hence, the effective Fe^{2+} concentration for the enzyme was considered to be in a range between 10^{-12} and 10^{-8} M under the strict absence of Fe^{3+}.

Table 3
Fe^{2+} requirement of EDTA-washed apo-tryptophan hydroxylase

Additions		Enzyme activity		
Fe^{2+} (M)	EGTA (M)	A (μmol/min/mg)	B (μmol/min/mg)	A/B × 100 (%)
1×10^{-6}	0	2.67	3.93	68.0
1×10^{-5}	0	2.52	4.03	62.6
1×10^{-4}	0	3.60	5.06	71.3
1×10^{-4}	1×10^{-3}	4.06	5.08	79.8
5×10^{-4}	1×10^{-3}	2.48	3.30	75.2
1×10^{-3}	1×10^{-3}	1.40	2.18	64.5

The reaction of EDTA-washed apo-TPH was performed in the presence of various concentration of Fe^{2+}. (A) Apo-TPH was used directly, (B) the enzyme was treated with 10 mM DTT in 50 mM Tris–acetate (pH 8.1) at 30 °C for 10 min under N_2. The reaction mixture contained indicated concentrations of Fe^{2+} and/or EGTA, 10 mM ascorbic acid, and 2 mg/ml of catalase, in addition to the basal components (pH 6.7) described in the legend to Table 2. The enzyme reaction was initiated by addition of the enzyme and carried out at 30 °C for 5 min, then 5HTP formation was determined.

Discussion

In the present study, a distinctive feature of iron requirement of mastocytoma TPH, a representative TPH1, was revealed. We showed that the enzyme required loosely bound Fe^{2+} for the enzyme activity. (1) Purified mastocytoma TPH contained only 0.15–0.23 atoms per mole of enzyme subunit and this enzyme was virtually inactive unless it was subjected to the DTT activation. (2) The remaining iron could be easily removed just by mixing purified TPH preparations with EDTA, followed by gel filtration to remove small molecules. (3) Resulting apo-TPH exhibited absolute requirement for Fe^{2+} for its enzyme activity. No other metals including Fe^{3+} were effective, and the effective concentration of Fe^{2+} for the enzyme was calculated to be fairly low, between 10^{-12} and 10^{-8} M. (4) Although we do not yet have direct evidence, it appeared that the enzyme was inactivated by binding with Fe^{3+} even in the presence of Fe^{2+} due to a presumably higher affinity of Fe^{3+} to the enzyme than Fe^{2+}. For example, for the catalysis of apo-TPH to proceed efficiently and linearly inclusion into the reaction mixture of a reducing reagent such as DTT and ascorbate was essential, in addition to Fe^{2+}. The above results are in good agreement with the crystallographic studies on 3D-structure of TPH of Wang et al. [4] who showed that Fe^{3+} was bound to the bottom of active site by coordinations to two histidines and glutamic acid, and other apexes of Fe^{3+} octahedron were open. However, according to the information we got in the present study, the bound iron should be in the Fe^{2+} form for the enzyme to be catalytically active.

As to the role of DTT in the activation of TPH1, it was suggested that DTT removed bound Fe^{3+} as a chelator reducing it to Fe^{2+}, but cleavage of disulfide bond seemed not to be crucial, even if it occurred. (1) Many sulfhydryl compounds (group 2, Table 1) having a potential of breaking the disulfide bond could not or could only partially activate TPH, while those having structures common to metal chelators, HS-C-C-OH or HS-C-C-NH (group 1, Table 1), were effective. (2) The DTT-activation was replaced by treating TPH with EDTA resulting in the formation of apo-TPH and by adding Fe^{2+} to the assay mixture together with a reductant such as DTT or ascorbate and catalase. In this case, the reductants were thought to prevent oxidation of Fe^{2+} to Fe^{3+} and to eliminate Fe^{3+} by its reduction to Fe^{2+}. Catalase has been thought to protect inactivation

of the enzyme by scavenging H_2O_2 formed from Fe^{2+} in the presence of O_2.

Thus, the present work has revealed a unique feature of TPH1 requirement for Fe^{2+} that the activity is affected by concentration of available Fe^{2+}. In vitro and in the absence of adequate reducing reagents, rapid oxidation of free Fe^{2+} and preferable association of Fe^{3+} with the enzyme may occur resulting in its rapid but reversible inactivation. In the living cells, intracellular environment seems to be more reductive than in vitro conditions, but intracellular concentration of Fe^{2+} may be below saturation of TPH [32,33]. Considering wide distribution of TPH1 both in the brain and peripheral serotonergic tissues, the above feature of TPH1 in iron acquisition requires further elucidation for understanding serotonin-related physiology, pathology, and development of neural, vascular, and immune systems of human body.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.09.045.

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