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Molecular Mechanism for Pterin-Mediated Inactivation of Tyrosine Hydroxylase: Formation of Insoluble Aggregates of Tyrosine Hydroxylase

Fumi Urano[1,6], Nobuhiro Hayashi[2], Fumio Arisaka[3], Hideki Kurita[4], Shizuaki Murata[5] and Hiroshi Ichinose[1,*]

[1]Department of Life Science and [3]Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501; [2]Institute for Comprehensive Medical Science and [4]Department of Hygiene, School of Medicine, Fujita Health University, Toyoake 470-1192; [5]Graduate School of Environmental Studies, Nagoya University, Nagoya 464-8601; and [6]Japan Association for the Advancement of Medical Equipment, Tokyo 113-0033

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Tyrosine hydroxylase (TH), an iron-containing enzyme, catalyzes the first and rate-limiting step of catecholamine biosynthesis, and requires tetrahydrobiopterin (BH4) as a cofactor. We found that preincubation of recombinant human TH with BH4 results in the irreversible inactivation of the enzyme at a concentration far less than the K_m value toward BH4 in spite of its cofactor role, whereas oxidized biopterin, which has no cofactor activity, does not affect the enzyme activity. We show that TH is inactivated by BH4 in competition with the binding of dopamine. The sequential addition of BH4 to TH results in a gradual decrease in the intensity of the fluorescence and CD spectra without changing their overall profiles. Sedimentation velocity analysis demonstrated an association of TH molecules with each other in the presence of BH4, and studies using gel-permeation chromatography, turbidity measurements, and transmission electron microscopy demonstrated the formation of amorphous aggregates with large molecular weights following the association of the TH proteins. These results suggest that BH4 not only acts as a cofactor, but also accelerates the aggregation of TH. We propose a novel mechanism for regulating the amount of TH protein, and discuss its physiological significance.

Key words: aggregation, tetrahydrobiopterin, tyrosine hydroxylase.

Abbreviations: BH4, tetrahydrobiopterin; CA, catecholamine; dopamine, DA; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; NOS, nitric oxide synthase; BH2, dihydrobiopterin; 6-methyl-PH4, 6-methyltetrahydropterin; TEM, transmission electron microscopy; PD, Parkinson's disease.

Tyrosine hydroxylase [TH; tyrosine mono-oxygenase (EC 1.14.16.2)] catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine, which is the rate-limiting step in the biosynthesis of catecholamines (*1*, *2*). TH requires (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin [(6*R*)BH4] as a cofactor, molecular oxygen, and iron for its reaction. The enzyme activity of TH is strictly regulated by many factors in order to control the biosynthesis of catecholamines in the neurons and adrenal medulla.

The phosphorylation of TH and the binding of catecholamines to it are two major post-translational mechanisms for regulating TH activity. Catecholamines, the end products of the TH reaction, bind to TH through a charge-transfer interaction with iron (*3*, *4*). Although catecholamine-bound TH is inactive, it has a higher thermo-stability than the unbound enzyme and is protected from proteolytic digestion (*5*, *6*). The bound catecholamine is released upon phosphorylation at Ser40 of TH by cAMP-dependent protein kinase (*7*).

We found that the protein content of TH is greatly decreased in BH4-deficient mice produced by disruption of a gene involved in BH4 synthesis (*8*). The TH protein level is recovered by repeated administration of BH4. Interestingly, the amount of tryptophan hydroxylase protein (EC 1.14.16.4, TPH), which, like TH, utilizes BH4 as a cofactor, is unaffected (*8*). Our findings suggested that the intracellular concentration of BH4 regulates the level of TH protein in the cell. The molecular mechanism, however, remained to be resolved.

Alterations in the level of TH protein, especially in the nerve terminals, are important for neural transmission. When the number of TH molecules decreases in a synapse, the rate of dopamine production is reduced. If the firing rate of the neuron is low, the decreased supply of newly synthesized dopamine is not a problem, because an adequate amount of dopamine can be stored in synaptic vesicles prior to the next excitation. If the firing rate is high, however, a serious problem arises, because the slow supply of dopamine cannot keep pace with its consumption. The efficiency for neurotransmission in the neurons is thus be reduced.

A group of cofactors that bind tightly to certain enzymes is known to have a stabilizing effect on the enzyme structure. BH4 is essential for the enzymatic production of nitric oxide by the action of nitric oxide synthase [NOS; (*9*, *10*)].

*To whom correspondence should be addressed. Tel: +81-45-924-5822, Fax: +81-45-924-5807, E-mail: hichinos@bio.titech.ac.jp

BH4 binds tightly to endothelial and neural NOSs with dissociation constants in the nanomolar range, and this binding is reported to stabilize the dimeric structure of NOS (*11–13*). The affinity toward BH4 is so strong that purified NOS typically contains 0.2–0.5 BH4 molecules per heme moiety (*14*). In the case of TH and other aromatic amino acid hydroxylases, however, the purified enzymes do not contain any BH4 as part of their molecular structure. This indicates that the affinity between BH4 and TH is weaker than those for NOSs. There has been no report of a stabilizing effect of BH4 on the TH molecule.

For the present study, we examined the effect of the preincubation of recombinant human TH with BH4, and found that TH is inactivated by incubation with a nearly stoichiometric amount of BH4, and that high-molecular-weight aggregates of the enzyme are formed. Furthermore, we explored the molecular mechanism for this inactivation mediated by BH4. Our results show that TH is first inactivated by a conformational change, followed by an association of the inactivated TH molecules, and that these associated TH proteins tend to form huge insoluble aggregates. Based on our findings, we discuss the physiological significance of this phenomenon and its possible implications in Parkinson's disease.

EXPERIMENTAL PROCEDURES

Materials—(6*R*)BH4 and 7,8-dihydro-L-biopterin (BH2) were purchased from Schircks Laboratories (Jona, Switzerland). 3,4-Dihydroxyphenylethylamine (dopamine) and 6-methyl-5,6,7,8-tetrahydropterin (6-methyl-PH4) were from Sigma Chemical Co. (St. Louis, USA). (6*S*)BH4 was a gift from Dr. S. Matsuura. All other reagents used were purchased from Sigma Chemical Co., Wako (Osaka, Japan), Boehringer-Mannheim (Mannheim, Germany), Nacalai (Kyoto, Japan), and Dojindo Laboratories (Kumamoto, Japan).

Expression and Purification of Recombinant hTH1—Recombinant human TH type 1 was expressed in a pET system of *Escherichia coli* and purified to homogeneity (>90%) as previously described (*15*, *16*). The purified protein was dialyzed against 20 mM Tris-HCl buffer (pH 7.3) containing 1 mM dithiothreitol and 8% sucrose, and concentrated with a membrane filter. The concentration of the purified TH protein was determined by calculation after measurement of its absorbance at 280 nm (A_{280} = 1.04 at 1 mg/ml/cm path-length, Ref. *17*). The purified TH contained 0.53 atoms of iron/subunit, as determined by atomic absorption spectrometry; and the specific activity was 156 nmol DOPA formed/min/mg protein at pH 7.0. The enzyme was divided into small aliquots, and stored at –80°C until use. Molar concentrations of TH used in each experiment were designated as the concentration of the subunit, not that of the tetrameric TH molecule.

Assay of Tyrosine Hydroxylase Activity—TH activity was assayed as previously described (*15*). The incubation mixture (200 μl) consisted of 0.2 mM 2-morpholinoethanesulfonic acid (MES)–Na buffer (pH 7.0), 200 μM L-tyrosine, 100 mM β-mercaptoethanol, 0.2 mg/ml catalase, 1 mM (6*R*)BH4, and the enzyme preparation. The reaction was started by the addition of (6*R*)BH4 and was carried out at 37°C for 10 min under normal atmospheric conditions. Unless otherwise noted, preincubation of the enzyme with the final specified concentration of the compounds tested was performed at 30°C for 10 min in 25 μl of 50 mM MES–Na buffer (pH 7.0) containing 1 mM dithiothreitol and 8% sucrose.

Iron Content Measurement—Iron contents in TH were determined by atomic absorption using a polarized Zeeman atomic absorption spectrophotometer type z-8100 (Hitachi, Tokyo, Japan).

Polyacrylamide Gel Electrophoresis (PAGE)—After preincubation with pterin analogs or dopamine at 30°C for 10 min, TH was subjected to native-PAGE and SDS-PAGE in 2–15% gradient gels, and visualized by silver staining.

Fluorescence Measurements—Fluorescence measurements were obtained in an FP-777 Spectrofluorometer (Jasco Co. Ltd., Tokyo) with a cuvette with a pathlength of 0.1 cm. Samples consisted of 60 μg/ml TH (1.0 μM) in 20 mM Tris-HCl buffer (pH 7.0) containing 8% sucrose. The sample chamber was maintained at 30°C, and samples were equilibrated at this temperature for 15 min before measurements. Concentrated BH4 solution was added drop-wise to the sample, with mixing by pipetting. The mixture was then left to stand for 5 min, and then measured for fluorescence. Fluorescence emission was measured between 300–450 nm with an excitation wavelength at 295 nm. Figures show corrected emission spectra obtained by eliminating the background spectra of the buffer and solvents.

Circular Dichroism Measurements—Circular dichroism spectra of TH were obtained in a J-720 spectropolarimeter (Jasco Co. Ltd., Tokyo). Samples consisted of 60 μg/ml TH (1.0 μM) in 20 mM Tris-HCl buffer (pH 7.3) containing 8% sucrose in a cell with a 2-mm pathlength, and measurements were made with continuous N_2 gas flow through the sample chamber. Samples were scanned 3 times from 200 to 260 nm at 20 nm/min. The concentrations and the secondary structures of soluble TH proteins were calculated using JASCO J-700 for Windows software (*18*).

Analytical Ultracentrifugation—Sedimentation velocity was measured in an Optima XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with An60Ti rotor at 20°C. The TH solution (0.95 mg/ml, 17 μM) was first incubated in the presence or absence of 85 μM BH4 or BH2 at 30°C for 10 min in 20 mM Tris-HCl buffer (pH 7.3) containing 8% sucrose, 1 mM dithiothreitol and then centrifuged (400 μl total volume). A buffer with the same composition as that used for the final gel filtration column chromatography was served as an optical reference. Before centrifugation, 0.1 M NaCl was added to the sample solution and control buffer to adjust the ionic strength. Samples were centrifuged at 30,000 rpm, and the moving boundaries were monitored at a wavelength of 280 nm in the continuous scanning mode. The sedimentation absorbance profiles were analyzed to obtain the distribution of sedimentation coefficients c(s) using SEDFIT software (*19*).

Gel-Permeation Chromatography—Samples were loaded onto a Superose 6HR 10/30 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl buffer (pH 7.3) containing 8% sucrose and 1 mM dithiothreitol, and eluted at a flow rate of 0.2 ml/min.

Transmission Electron Microscopy (TEM)—A droplet of TH solution that had been incubated with BH4 at 30°C for 10 min was placed on a carbon-film grid; 2 min later, the

excess liquid was blotted off with filter paper. After the grid had been partially dried, 1 drop of staining solution, 2% (w/v) aqueous uranyl acetate, was applied to the grid, which was then blotted with filter paper 2 min later and permitted to dry at room temperature. The samples were examined with a JEOL JEM-2000 EX (TEM) operated at 100 kV by the Hanaichi-Institute of Electron Microscopy (Aichi, Japan).

Turbidity Assay—TH solutions were incubated with or without BH4 derivatives and left to stand at 4°C for the desired period. The absorbance of the samples was monitored at 600 nm.

RESULTS

Pterin-Mediated Inactivation of Tyrosine Hydroxylase—First, we examined the effect of preincubation of the purified recombinant TH with BH4. The purified enzyme (0.17 μM) was incubated with (6*R*)BH4, (6*S*)BH4, 6-methyl-PH4, BH2, or biopterin at 30°C for 10 min in 25 μl of 50 mM MES–Na buffer (pH 7.0) containing 8% sucrose and 1 mM dithiothreitol. After the preincubation, we assayed TH activity by using the preincubated samples as the enzyme solution under saturating concentrations of both tyrosine (0.2 mM) and BH4 (1 mM) in air. Preincubation of TH with (6*R*)BH4 resulted in a dose-dependent loss of TH activity compared with the activity of the control samples, which were preincubated without test compounds (Fig. 1A). This inactivation of TH occurred even at concentrations of BH4 less than 1 μM. Experiments with a variety of other pterin analogues indicated that only the tetrahydro-forms of pterin analogues, *i.e.*, (6*S*)BH4 and 6-methyl-PH4, inactivated TH as well as (6*R*)BH4, whereas the oxidized forms of pterins, such as BH2 and biopterin, which do not have cofactor activity, had no inhibitory effect on the TH activity (Fig. 1, A and B).

Next we examined the kinetics of TH inactivated by preincubation with BH4 and compared it with that of TH preincubated without BH4. The double reciprocal plot against BH4 was biphasic for both enzymes, confirming our earlier report of 2 different apparent K_m values for BH4 under the conditions of 0.2 mM tyrosine (*15*, *20*). Two different V_{max} values were obtained for both enzymes. The V_{max} values of the inactivated TH were smaller than those of the intact enzyme, respectively, whereas the K_m values of the inactivated TH were similar to those of the control sample (Fig. 2).

Next, we preincubated TH (0.17 μM) with 1.7 μM BH4 in the presence of phenylalanine as a poor substrate in order to examine the effect of substrate on the pterin-mediated inactivation. We chose phenylalanine instead of tyrosine as the substrate, because dopa produced from tyrosine is known to form an inhibitory complex with TH through its catechol-structure. The binding site of phenylalanine in TH has been shown to be identical to that of tyrosine (*21*), whereas the K_m value for phenylalanine is 7 times higher than that for tyrosine (*22*). The presence of 1–200 μM phenylalanine did not affect the BH4-mediated inactivation of TH, and the TH activity of the sample preincubated with BH4 was decreased to 18% that of the sample preincubated without BH4 even in the presence of 200 μM phenylalanine. These results demonstrate that the pterin-mediated inactivation occurs in the presence of a substrate as well.

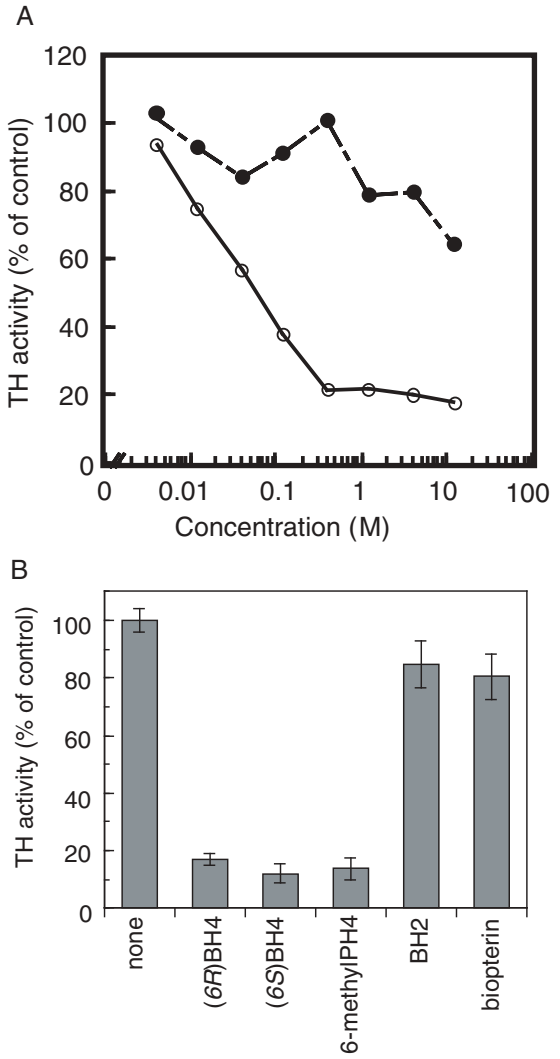
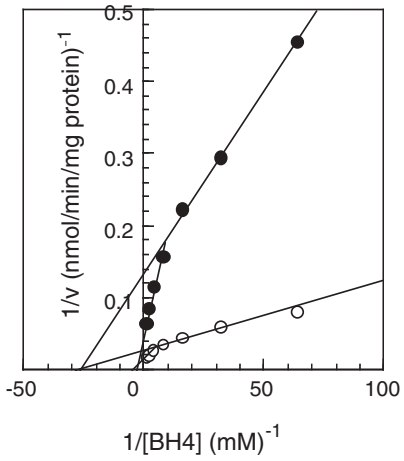


Fig. 1. **Effects of preincubation with pterin analogues on TH activity.** TH (0.01 mg/ml, 0.17 μM) was preincubated with pterin analogues at 30°C for 10 min in 25 μl of 50 mM MES–Na buffer (pH 7.0) containing 8% sucrose and 1 mM dithiothreitol, and immediately assayed for TH activity as described in "MATERIALS AND METHODS." Control activity of the preincubated sample without test compounds was 68.2 ± 6.3 nmol DOPA/min/mg of protein. (A) The enzyme was preincubated with (6*R*)BH4 (open circles) or dihydrobiopterin (closed circles). The data shown are the averages of 5 samples. (B) TH was preincubated with 1.7 μM pterin analogues. The data shown are the averages of the following number of samples: n = 5 for none, (6*R*)BH4, and BH2; and n = 4 for (6*S*)BH4, 6-methyl-PH4 and biopterin.

We examined the inactivated TH protein for bound BH4 by removing of low-molecular weight compounds by gel-permeation chromatography. We did not detect any substantial amounts of BH4 or oxidized biopterin in the protein fraction (data not shown). The activity of the TH protein preincubated with BH4 remained low, 15% that of the TH protein preincubated without BH4, after removal of low-molecular weight compounds. These results indicate that BH4 does not bind tightly to TH, and that the inactivation of TH by BH4 is irreversible.

A



B

	additive at preincubation	*K*m (mM)	TH activity (nmol/min/mg of protein)
1	vehicle	0.233	72.2
		0.026	32.7
2	BH4	0.232	17.9
		0.042	8.0

Fig. 2. **(A) Double-reciprocal plots and (B) kinetic parameters of TH preincubated with or without BH4.** TH (0.01 mg/ml, 0.17 μM) was preincubated without BH4 (open circles, control) or with 1.7 μM BH4 (closed circles) at 30°C, and then the enzyme activity was assayed at 37°C in the presence of BH4 as a cofactor for the hydroxylation reaction.

Table 1. **Protection by dopamine against BH4-mediated inactivation of TH.**

No.	dopamine	BH4	TH activity (% of control)	
			Assay at pH 7.0	Assay at pH 6.0
1	–	–	100	100
2	–	+	25	36
3	+	–	30	110
4	+	+	28	121

TH (0.01 mg/ml, 0.17 μM) was preincubated with or without 1.7 μM dopamine at 30°C for 5 min at pH 7.0, and then BH4 (final concentration of 1.7 μM) or vehicle alone (0.4 mM HCl) was added. Incubation was then continued for another 5 min at 30°C. Following the preincubation, the TH activities were assayed at pH 6.0 or 7.0. The data are the averages of 2 experiments. Control activity of the preincubated sample without test compounds was 50.3 nmol DOPA/min/mg of protein at pH 7.0, and 34.4 nmol DOPA/min/mg of protein at pH 6.0.

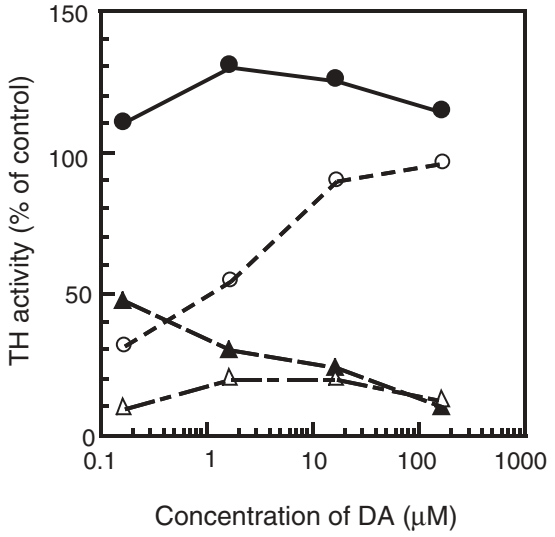


Fig. 3. **Competition between BH4 and dopamine.** TH (0.17 μM) was preincubated with various concentrations of dopamine and 1.7 μM BH4 (open symbols) or vehicle (filled symbols) for 10 min at pH 7.0. Following the preincubation, TH activity was assayed at pH 6.0 (circles) or 7.0 (triangles). Control activity for the preincubated sample without test compounds was 42.8 nmol DOPA/min/mg of protein at pH 7.0, and 19.5 nmol DOPA/min/mg of protein at pH 6.0. The data shown are the averages of 2 experiments.

Next we tested the possibility that the inactivation by BH4 might be due to the generation of free radicals generated during the TH reaction. The addition of catalase to the preincubation solution, however, failed to protect TH from inactivation. Next, we equilibrated all solutions with argon and performed preincubation under an argon atmosphere. The strength of inactivation by BH4 was the same under both aerobic and anaerobic conditions (data not shown).

Competition between Dopamine and BH4—Catecholamines are known to inhibit TH activity by forming a tight complex with ferric iron at the active site of the enzyme (*4*, *23*). *In vitro*, this inhibition by catecholamines is observed over pH 7.0, since dopamine is released from ferric ion under acidic conditions with recovery of TH activity (Table 1, No. 3). In contrast, TH inactivated by BH4 shows decreased activities at both pH 6.0 and pH 7.0 (Table 1, No. 2). A TH solution (0.17 μM) was first incubated with 1.7 μM dopamine for 5 min at pH 7.0 prior to preincubation with 1.7 μM BH4, and then the TH activity was assayed at both pH 6.0 and pH 7.0. When TH was preincubated with dopamine prior to the addition of BH4, the TH activity assayed at pH 6.0 was not decreased, indicating that BH4 did not react with the dopamine-bound enzyme during the preincubation at pH 7.0 (Table 1, No. 4). This result shows that the binding of dopamine to TH protects the enzyme from inactivation by BH4.

Next we examined the effects of the simultaneous addition of dopamine and BH4, and compared their affinity for TH. We added various amounts of dopamine and 1.7 μM BH4 to the enzyme solution (0.17 μM), and preincubated the solution at 30°C for 10 min. The simultaneous addition of dopamine and BH4 to the enzyme solution attenuated the BH4-mediated inactivation of TH in a concentration-dependent manner, as shown in Fig. 3 (open circles). About half of the activity was restored when the concentration of dopamine was nearly equimolar with that of

BH4, suggesting the affinity between TH and BH4 is as strong as that between TH and dopamine.

Iron Contents and Effect of General Reductants—The competition experiment suggests that an interaction of BH4 with the iron at the active site may be involved in the pterin-mediated inactivation, as catecholamines are known to bind to TH by chelating the iron with the 2 oxygen atoms of the catecholquinone (*23*, *24*).

We checked for a possible alteration in the iron content of the TH protein. Our preparation of recombinant TH originally contained 0.53 atom of iron/subunit. TH (2.8 mg/ml, 50 μM) was incubated at 30°C for 10 min with or without a 10-fold molar excess (500 μM) of BH4. Then, the low-molecular weight compounds were removed by gel-permeation chromatography through Sephadex G-25, and the iron content in the protein fraction was determined by atomic absorption spectrometry. The iron content of the sample incubated without BH4 was 0.40 atom/subunit, and that of the sample incubated with BH4 was decreased to 0.23 atom/subunit (average, $n = 2$). When ferrous iron was added to the TH solution after removal of the low-molecular weight compounds, however, noactivity was recovered (data not shown). These results suggest that the inactivated TH shows a tendency to release iron probably due to a conformational change, and that the TH activity can not be restored by the addition of iron.

It is known that BH4 acts bi-functionally in the reaction with TH. One function is the reduction of iron at the active site from the ferric to the ferrous state, and the other is hydroxylation of the substrate through its action as an electron donor (*25*, *26*) (see Fig. 11). In order to examine whether the reducing ability of tetrahydropterin is the cause of the inactivation, we preincubated TH (0.17 μM) with a general reductant, β-mercaptoethanol or dithiothreitol. Neither had any significant effect at concentrations of 1.7 μM or 170 μM. As shown in Fig. 1, tetrahydropterins attenuate TH activity to 20% of the control, even at 1.7 μM. These results suggest that the BH4-dependent inactivation of TH cannot be explained solely by the reducing properties of BH4.

Polyacrylamide Gel Electrophoresis—Next we performed native polyacrylamide gel electrophoresis (PAGE) to look for a possible conformational change in the enzyme. TH was incubated at 30°C for 10 min with BH4, biopterin, dopamine, or dithiothreitol as a general reductant. Half of the incubated sample was subjected to native-PAGE, and the other half was subjected to SDS-PAGE to confirm the presence of the TH protein. As shown in Fig. 4A, on the native PAGE gel the TH band was absent when the enzyme had been preincubated with BH4 (lanes 3 and 4), whereas preincubation with biopterin, dopamine or dithiothreitol did not affect the intensity of the band (lanes 2, 5, and 6). We did not detect any shifted band corresponding to the absent band even at the top of the gel. The presence of the TH protein in all samples was confirmed by SDS-PAGE (Fig. 4B). When TH was incubated with various amounts of BH4, the intensity of the TH band gradually weakened in a dose-dependent manner (data not shown). These data suggest that an alteration in the charge-distribution on the surface of the TH protein or insolubilization of the TH protein by incubation with BH4 causes the disappearance of the TH band in the native PAGE gel. In case of the SDS-PAGE, the insoluble fraction is supposedly solubilized by SDS.

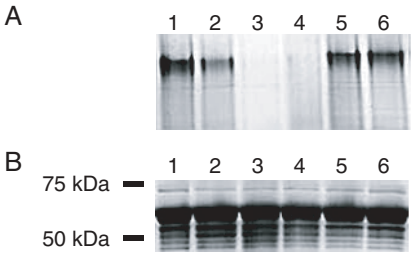


Fig. 4. **Polyacrylamide gel electrophoresis of TH after incubation with BH4.** Native-PAGE (A) and SDS-PAGE (B) of TH (0.95 μg). TH (4.3 μM) was incubated with buffer (lane 1) or with buffer containing 4.3 mM dithiothreitol (lane 2), 4.3 mM dithiothreitol and 0.43 mM (6*R*)BH4 (lane 3), 0.43 mM (6*R*)BH4 (lane 4), 0.16 mM biopterin (lane 5) or 0.43 mM dopamine (lane 6) at 30°C for 10 min, and then loaded onto a gel for electrophoresis under non-denaturing (A) or denaturing (B) conditions.

Quenching the Fluorescence and Circular Dichroism (CD) of TH—In order to examine for alterations in the higher-order structure of TH caused by pterin-mediated inactivation, we measured the tryptophan fluorescence and CD spectra of the inactivated TH. Using an excitation wave length of 295 nm, we measured the fluorescence intensity between 300–450 nm. The emission maximum was found to be around 340 nm for the human recombinant TH protein (Fig. 5A). This emission maximum is typical for partially solvent-exposed residues (type-II tryptophan fluorescence) (*27*). The fluorescence spectra of TH (1 μM) mixed with increasing amounts of BH4 are also shown in Fig. 5A. At first, the fluorescence intensity was slightly elevated after the addition of 1 to 2 μM BH4, and then it gradually decreased with increasing concentrations of BH4 (Fig. 5, A and B). The addition of a large excess of BH4 (850 μM) resulted in complete quenching of the fluorescence. In the process of quenching the fluorescence, conformational changes in TH accompanying some kind of energy transfer might have occurred. Although the details are hard to analyze, the findings strongly suggest that the content of the TH protein in solution is decreased in the process because the fluorescence was finally quenched completely.

CD spectra were collected in a similar way as those for fluorescence. The sequential addition of BH4 also attenuated the intensity of the CD spectrum as a whole without changing the profile of the entire spectrum, while CD signals longer than 210 nm could not be measured properly when 840 μM BH4 was added (Fig. 6A). The attenuation in the intensities of the fluorescence and CD spectra suggest that the number of TH molecules in solution decreases after the addition of BH4, and that the apparent concentration of TH is reduced. Interestingly, when we added biopterin instead of BH4, the CD profile changed, indicating that a conformational change in TH is induced by biopterin (Fig. 6B). We calculated the secondary structure of native TH and TH in the presence of biopterin. The calculation showed that the native TH protein contains 69% α-helix, 31% turn (profile "a" in Fig. 6B), while TH contains 58% α-helix, 42% turn in the presence of 100 μM biopterin (profile "d" in Fig. 6B),

Sedimentation Velocity Analysis—Sedimentation velocity analysis was performed to determine the size

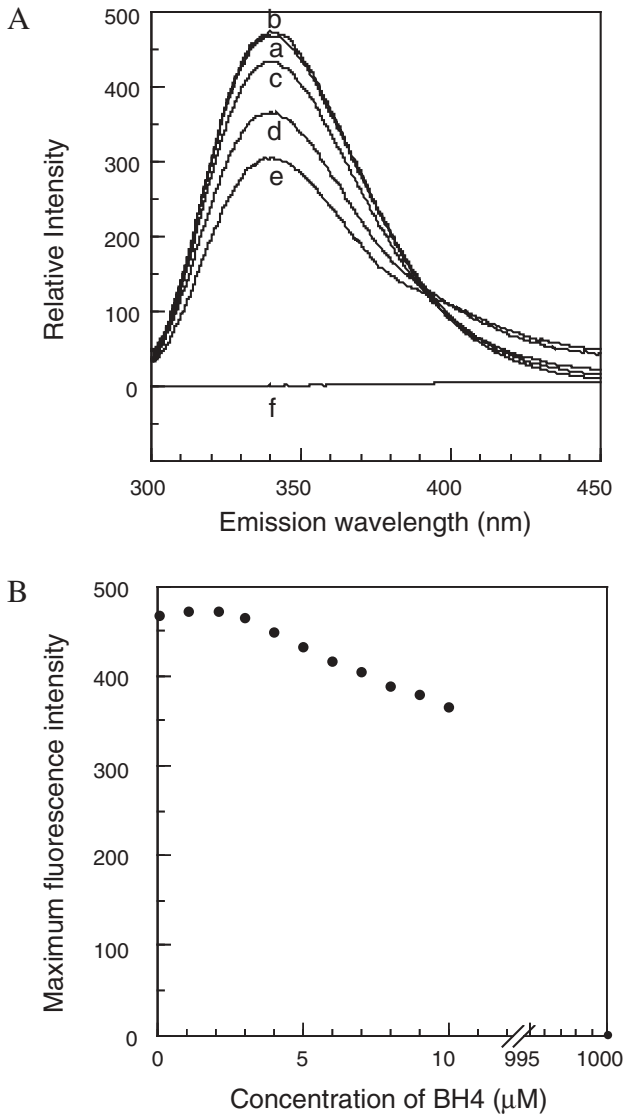


Fig. 5. **Fluorescence of TH.** (A) The fluorescence spectrum of TH (60 μg/ml, 1 μM) was obtained after equilibration at 30°C for 15 min (a). In the same way, the fluorescence spectra of samples were measured after BH4 in 0.01 M HCl had been added dropwise to the enzyme solution to a final concentration of 2 μM (b), 5 μM (c), 10 μM (d), 20 μM (e) or 840 μM (f) and mixed by pipetting. (B) The maximum fluorescence intensity was plotted against the BH4 concentration.

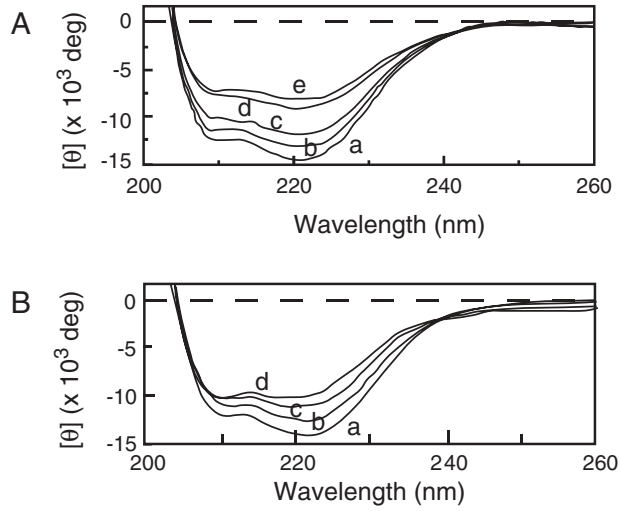


Fig. 6. **CD spectra of TH.** (A) The CD spectrum of TH (60 μg/ml, 1 μM) in Tris-HCl buffer (pH 7.3) was recorded as described in "MATERIALS AND METHODS" (trace "a"). BH4 was then added to a final concentration of 1 μM (b), 5 μM (c), 10 μM (d), or 20 μM (e). (B) Biopterin was added to the TH solution to a final concentration of 10 μM (b), 50 μM (c) or 100 μM (d).

distribution of TH in the presence of 5 equivalents of BH4 or BH2 per TH subunit. This concentration, which is less than the equivalent molar concentration used in the other analyses, was chosen because BH4 and BH2 exhibit absorption maxima at 292 nm, which interferes with the detection of protein at 280 nm. However, we confirmed that this concentration of BH4 or BH2 is sufficient to induce inactivation. Figure 7 shows sedimentation profiles (A–C) and c(s)-sedimentation coefficient distribution plots (D–F) of TH in the absence (A, D) and presence of 85 μM BH4 (B, E) or 85 μM BH2 (C, F) at 20°C. The sedimentation of intact TH reveals a homogeneous distribution (95.6% of total species) of native tetrameric TH species with a sedimentation coefficient of 6.18 S (Fig. 7D) and a molecular weight calculated by SEDFIT software of 218,600 ± 7,200. The calculated molecular weight is in a very good agreement with molecular weight deduced from the amino acid sequence (55,533 × 4 = 222,132). In the presence of BH4, the distribution of the sedimentation coefficients for TH shows heterogeneity, *i.e.*, 9.29 S (14.9%; M_r = 302,900 ± 22,300) as well as 6.45 S (75.7%; M_r = 176,200 ± 7,500) (Fig. 7E). Since the molecular weights of the inactivated TH were calculated using the same frictional coefficient as the native enzyme, the values do not necessarily correspond to the values calculated from the amino-acid sequence. This result suggests that the addition of BH4 induces a conformational change in TH and the association of two TH molecules, because the calculated molecular weight of the protein peak at 9.29 S is roughly twice that of the protein peak at 6.45 S. As compared with the effect of BH4 on the sedimentation of TH, BH2 had no effect on the sedimentation distribution of TH (Fig. 7F) or on its coefficient (6.19 S, 94.0%).

Furthermore, when we performed the sedimentation velocity analysis of TH at 30°C with BH4, the sedimentation profile of TH revealed a heterogeneous distribution and showed the formation of oligomeric species (Fig. 7G). This profile indicates a rapid exchange between association and dissociation states of the TH protein in the presence of BH4, since an increase in temperature would tend to promote the dissociation.

Gel-Permeation Chromatography of Inactivated TH— We also performed gel-permeation chromatography to examine for alterations in the molecular weight of the inactivated TH after incubation with BH4. After TH had been incubated with a 100-fold molar excess of BH4 at 30°C for 10 min, the mixture was left to stand at 4°C for 15 min, 1 day or 4 days. As shown in Fig. 8, profile "a," when TH was incubated without BH4, most of the protein was eluted

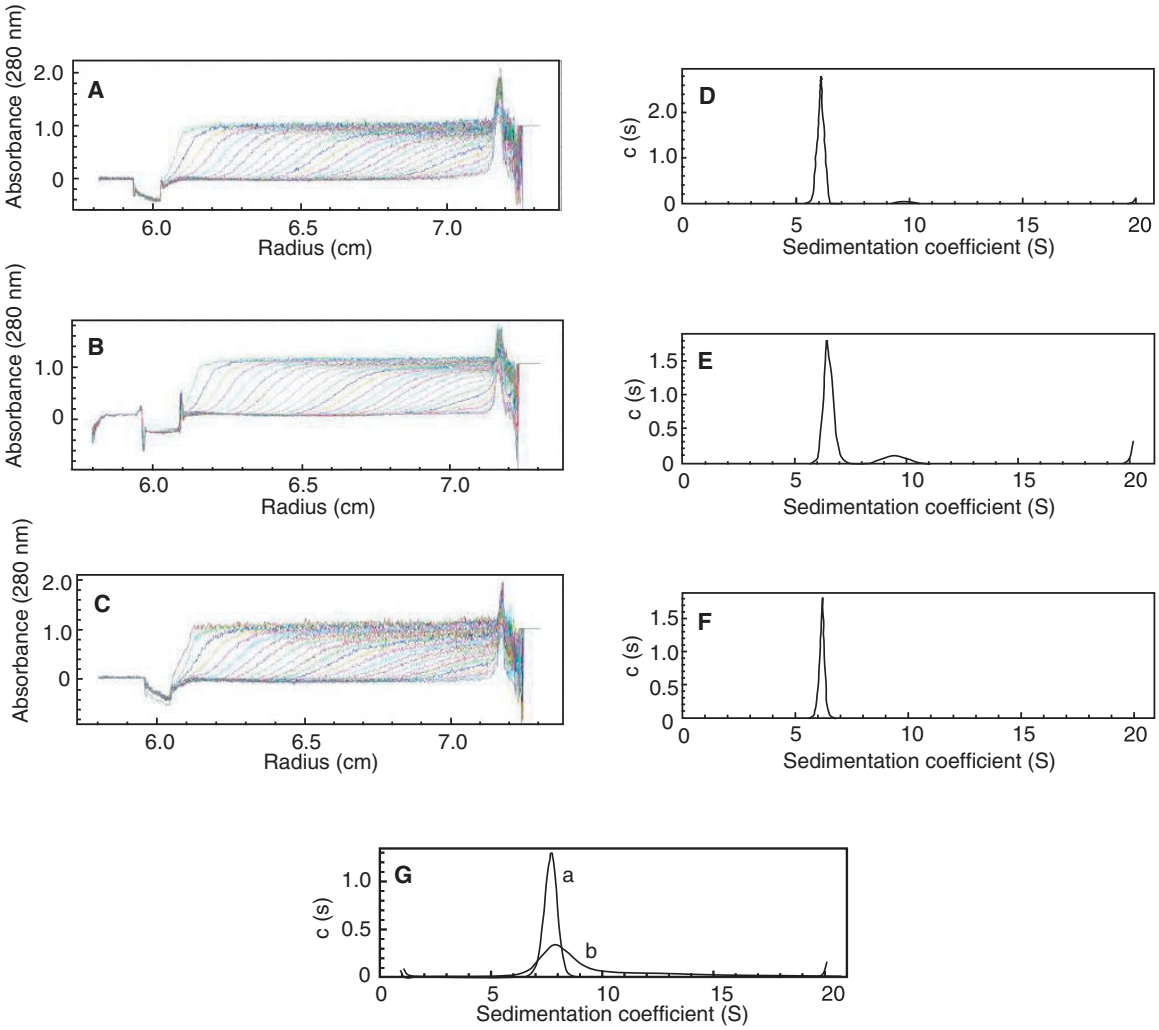


Fig. 7. **Analytical ultracentrifugation.** Sedimentation velocity profiles (A–C) and c(s)-sedimentation coefficient distribution plots (D–F) at 20°C were obtained. 17 μM TH solutions were incubated without compound (A, D) or with 85 μM BH4 (B, E) or 85 μM BH2 (C, F) at 30°C for 10 min before centrifugation. In Fig. 6G, the sedimentation coefficient distribution of TH, c(s), at 30°C for TH without (a) or with BH4 (b) is shown.

at fractions corresponding to a molecular weight of 220,000, *i.e.*, the molecular weight of the native tetrameric TH. However, when TH was preincubated with BH4, a shift of some of the protein peak around 220,000 to a position corresponding to the void volume was detectable from as early as 15 min (profile "b"); and this shift increased after 1 day (profile "c") and was almost complete after 4 days (profile "d").

Transmission Electron Microscopy (TEM)—We also obtained TEM images of the TH protein stored at 4°C for 4 days after incubation with or without BH4 at 30°C for 10 min. We observed many small particles (Fig. 9, A and B) suggested to be TH tetramers by comparison with the size of the truncated TH found in the X-ray studies: width, 5.5 nm; height, 4.0 nm; depth, 4.0 nm (*28*). Incubation of TH with BH4 resulted in the formation of very large amorphous aggregates (Fig. 9C), and the individual particles of TH were a little larger than tetrameric TH (Fig. 9D). Although TH incubated without BH4 also formed a few aggregates (Fig. 9B), they were rare and smaller in size than those formed by incubation with BH4. In the samples incubated with BH4, we also found fibrillar structures with a lengths of several micrometers (Fig. 9E).

Measurement of the Turbidity—After incubation of TH (0.9 mg/ml, 16 μM) with 810 μM BH4 or BH2, we measured the absorbance of the samples at 600 nm. The turbidity of the TH solution incubated with BH4 was slightly but rapidly elevated from just after the addition of BH4, and gradually increased over 2 days (Fig. 10). The increased turbidity was visible to the naked eye. The sample incubated with BH2 showed no change in absorption at 600 nm, nor was any precipitate formed. Following measurements for 4 days, we added 810 μM BH4 to the samples containing TH alone or TH and BH2. The turbidity of these solutions was elevated within 10 min after the addition of BH4, and the absorbance at 600 nm finally reached the same level as that of samples incubated with BH4 from the beginning. These results, together with those presented above, demonstrate that TH becomes inactive and partly insoluble soon after the reaction with BH4, and that the

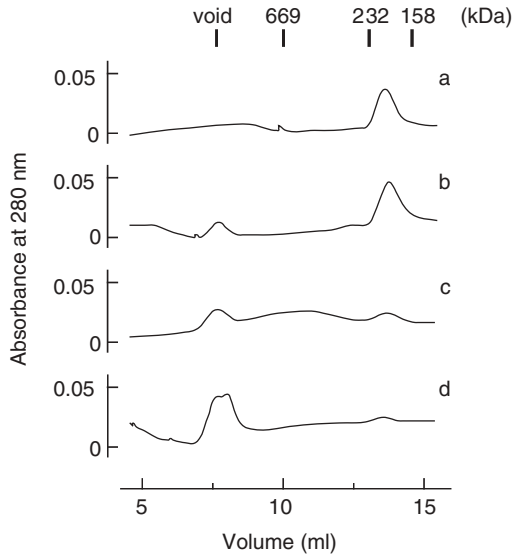


Fig. 8. **Gel-permeation chromatography.** After incubation without (a) or with (b–d) 600 μM BH4 (100 molar equivalents per TH subunit), 6 μM TH solution was stored at 4°C for 15 min (b), 1 day (c), 4 days (a, d). Thereafter, the solution was loaded onto a column and chromatography was performed at 4°C.

inactivated protein tends to form insoluble aggregates with large molecular weights.

DISCUSSION

The complex of TH and ferric iron, designated as TH-Fe(III) in Fig. 11, reacts with both catecholamines and BH4. Because the affinities for both compounds are comparable, their intracellular concentrations would be important determinants for the fate of TH-Fe(III). When TH-Fe(III) reacts with catecholamine, the complex is stable and protected from pterin-mediated inactivation until the catecholamine is liberated from the ferric iron by phosphorylation of the enzyme (*22*, *29*). When TH-Fe(III) reacts with BH4, the ferric iron is reduced to its ferrous form and is ready for the catalytic reaction. Because the K_m values of phosphorylated TH toward BH4 were shown to be around 10–30 μM (*15*, *26*), more than 10 μM BH4 would be necessary for efficient catalysis. Indeed, the intracellular concentration of BH4 in the striatum is assumed to be approximately 10 μM, *i.e.*, around the K_m value (*30*). When the concentration of catecholamine becomes enough high to bind to the iron at the active site, TH would form a stable complex with dopamine, thus halting the catalytic reaction. However, when the concentration of BH4 might be decreased to less than the K_m value, the hydroxylation reaction hardly proceeds, and the concentration of catecholamines in the cell would be decreased. Since BH4 can still react with TH even at concentrations below the K_m value (Fig. 1), the TH protein would be inactivated and aggregated.

We previously reported that the level of the TH protein is greatly decreased in the brains of BH4-deficient mice (*8*). In view of the decreased BH4 concentration in these mice, we speculate that the TH protein tends to form aggregates in the cell as was observed to occur in solution in the

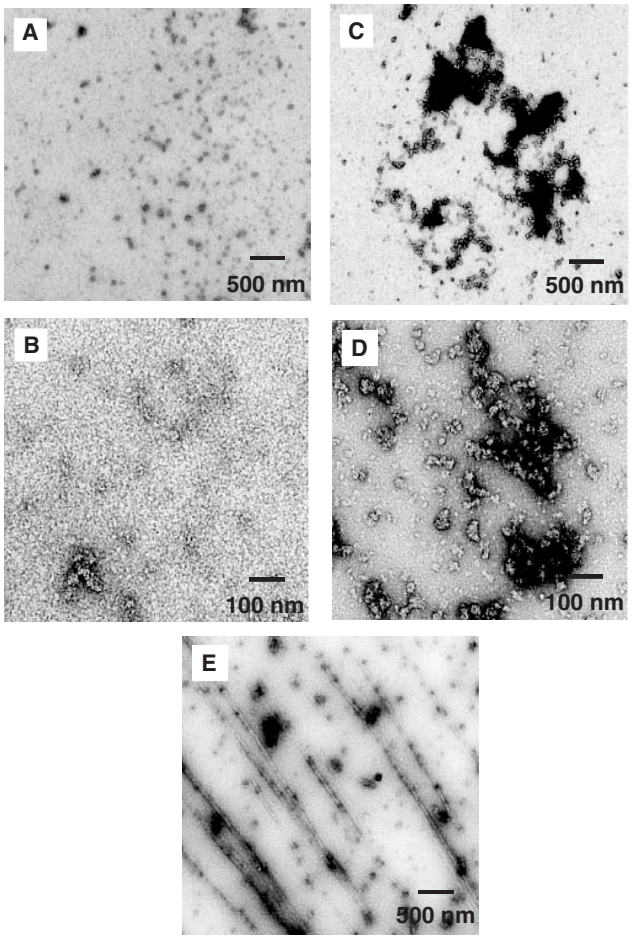


Fig. 9. **Transmission electron microscopic image of TH.** Images were obtained after storage at 4°C for 4 days of 0.4 mg/ml TH (7 μM) incubated without (A, B) or with (C–E) 700 μM BH4 at 30°C for 10 min. The scale bars represent (A, C, E) 500 nm and 100 nm (B, D).

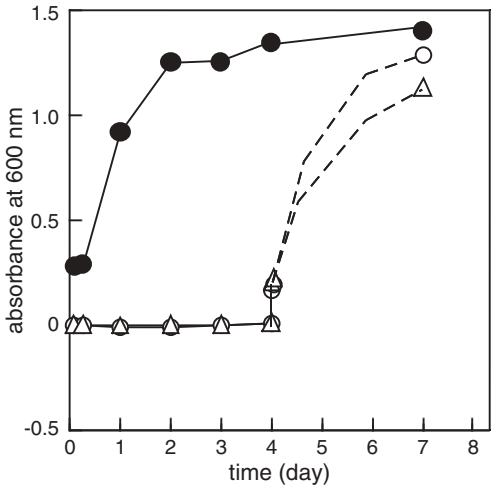


Fig. 10. **Turbidity analyses.** Visible absorbance at 600 nm was measured after storage at 4°C of 0.9 mg/ml TH (17 μM) incubated with 0.5 mM HCl (open circle) or 810 μM BH4 (closed circle) or 810 μM BH2 (open triangle) at 30°C for 10 min. Then, 810 μM BH4 was added to TH mixed with HCl or BH2 after 4 days' storage (broken lines).

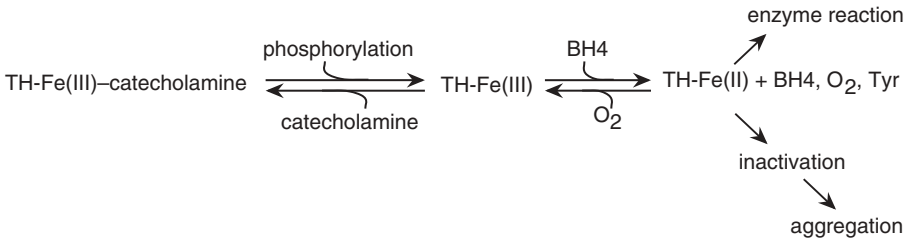


Fig. 11. **Proposed mechanism for regulating the amount of TH protein by BH4 and catecholamines.** Ferric iron in the enzyme is reduced to ferrous iron by BH4 and the catalytic cycle proceeds (*25*, *26*). Catecholamine, the end product of the TH reaction, reacts with the ferric iron, forming an inactive and stable complex (*4*, *7*). When the intracellular concentration of BH4 is lower than the K_m value, inactivation and subsequent aggregation of the TH protein would be prominent.

present *in vitro* study. This aggregated TH might be degraded rapidly in the cell, most likely by the ubiquitin-proteasome system (*31*), or may accumulate as insoluble aggregates. As a result, the amount of TH protein would be decreased when the intracellular concentration of BH4 is decreased.

The amount of TH protein in the striatum has also been reported to be decreased in mice homozygous for a mutant dopamine transporter gene (*32*). As a consequence of the defective dopamine transporter, the intracellular concentration of dopamine in the nerve terminals is reduced to only 5% of the wild-type level, and the TH protein level in knockout mice was only 10% of the wild-type level in the striatum (*32*). The balance between the intracellular concentrations of catecholamines and BH4 and the TH protein level in the cell is something to be emphasized.

The pterin-mediated inactivation of TH was previously described by Kuhn and Lovenberg (*33*) and Roskoski *et al.* (*34*) who used native TH purified from bovine adrenal medulla and PC12 cells, respectively; however, they did not elucidate the molecular mechanism of the inactivation. Roskoski *et al.* reported that TH phosphorylated by cAMP-dependent protein kinase is more susceptible to pterin-mediated inactivation, and that the EC50 value for phosphorylated TH is around 1 μM (*34*). When TH was isolated from nonrecombinant sources, the purified enzyme was reported to contain endogenous catecholamines bound to the iron at the active site of TH (*3*). Because phosphorylation of TH by cAMP-dependent protein kinase liberates the bound catechol from the TH protein (*29*), unphosphorylated recombinant TH, which we used in this study, and phosphorylated native TH should be in a similar state. Their results support our finding that catecholamine-bound TH is hardly inactivated by BH4 and that pterin-mediated inactivation of TH occurs at a concentration lower than the K_m value for BH4.

Tetrahydropterins, *i.e.*, (6*R*)BH4, (6*S*)BH4, and 6-methyl-PH4, inactivate TH, whereas the oxidized form of biopterin did not cause any inhibition (Fig. 1). Because oxidized biopterin does not inactivate TH, the reducing ability of BH4 must be essential for pterin-mediated inactivation. When general reductants such as β-mercaptoethanol and dithiothreitol were examined, however, they did not inactivate TH as strongly as BH4; nor did they alter the fluorescence spectrum (data not shown). On the other hand, the results of our CD measurements show that the oxidized form of biopterin alters the profile of the CD spectrum of the TH solution (Fig. 6B). In addition, the frictional ratio of TH was slightly decreased from 1.78 to 1.64 by the addition of BH2 in the ultracentrifugation analysis (Fig. 7F), indicating that a slight conformational change is induced by BH2. The addition of BH4 further decreases the frictional ratio. These results suggest that both the reducing ability of BH4 and a conformational change in the TH protein induced by the pterin backbone are necessary to inactivate TH efficiently.

From the results of the TH activity assay, we showed that BH4-mediated inactivation occurrs within a few minutes after incubation with BH4 at 30°C. The conformation of the inactivated TH is changed as shown by the alteration in the distribution of the sedimentation coefficients of tetrameric TH species from a sharp peak at 6.18 S to a slightly broader peak at 6.45 S. The species with the sedimentation coefficient of 6.45 S may be an inactivated form of tetrameric TH. Moreover, the addition of BH4 made the distribution of sedimentation coefficients for TH heterogeneous. We assume that the species with the larger sedimentation coefficient is a dimer of tetrameric TH molecules from the molecular weight of these species calculated using SEDFIT software. The association of TH molecules at 30°C is in rapid dynamic equilibrium, in contrast with TH at 20°C, where the kinetics of association and dissociation is slow. Turbidity analysis showed that the inactivated TH is partly insoluble. Examination by gel permeation chromatography also showed the amount of the protein in the void-volume fraction to increase gradually up to 4 days when the samples were stored at 4°C (Fig. 8). These analyses clarified that the association of TH caused by BH4 is followed by the formation of huge aggregates within a few days. These results are consistent with the result showing that the turbidity of TH in the presence of BH4 increases within 10 min, and shows a significant increase after standing for 6 h at 4°C, as shown in Fig. 10. We theorize that TH is first inactivated by the conformational change induced by BH4, and that the altered TH molecules then associate rapidly. These associated TH molecules would then form small globular intermediates, probably corresponding to the small particles surrounding the aggregates observed in the TEM analysis (Fig. 9). Finally, large amorphous aggregates or fibrils of huge molecular weight would gradually be formed.

Recent studies on neurodegenerative disorders associated with the denaturation of proteins suggest that protofibrillar or globular intermediate forms in aggregates are more toxic (*35*) than the mature fibers (*36*), and that the inclusions are protective rather than toxic toward the survival of neurons (*37*). In idiopathic PD, intracytoplasmic inclusions, called Lewy bodies, are found in the soma of nigral dopaminergic neurons. While α-synuclein and some other proteins have been identified as components of Lewy

bodies, immunohistochemical analysis suggested that TH is also a component of these bodies (*38*). Although α-synuclein is widely expressed in the brain, TH is distributed only in catecholaminergic neurons, those that are mainly affected in PD. Since it was recently reported that α-synuclein interacts with TH in rat brain (*39*), it is possible that the aggregation of TH caused by a decrease in the intracellular BH4 concentration would trigger the formation of Lewy bodies. It has also been reported that BH4 availability is decreased by the treatment with MPTP (*40*). In that paper, the authors demonstrated that MPTP induces oxidative stress by inhibiting complex I of the mitochondrial respiratory chain, and causes a time-dependent depletion of BH4 (*40*). These reports support our idea of a deficiency in BH4 in the brain as a key event in the etiology of PD.

In this study, we clarified that TH, a protein specifically expressed in monoaminergic neurons, tends to form aggregates in the presence of relatively low concentrations of BH4. We suggest that TH aggregation might act as a trigger for the formation of Lewy bodies in PD. We will explore the significance of TH aggregation *in vivo* further, and clarify its association with PD, depression, and other neuropsychiatric disorders.

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