Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6659–6663, July 1994
Biochemistry

High-level expression and deletion mutagenesis of human tryptophan hydroxylase

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Contributed by Seymour Kaufman, April 12, 1994

ABSTRACT **Human tryptophan hydroxylase has been expressed as a soluble and active form in *Escherichia coli* by fusion with an affinity tag, maltose-binding protein. The fusion protein has been purified to near homogeneity by affinity chromatography on crosslinked amylose resin. The purified fusion protein has a specific activity of 86 nmol of 5-hydroxytryptophan per min per mg of fusion protein. A series of truncation mutants have also been made to explore the domain organization of tryptophan hydroxylase. All deletion mutants were subject to affinity purification and kinetic characterization. While removal of the N-terminal 164 amino acids completely inactivates the enzyme, deletion of the first 91 residues results in a 7-fold reduction in specific activity. From the C terminus, deletion of 36, 55, or 112 amino acids abolishes the activity, whereas deletion of 19 residues decreases the specific activity by ≈11-fold. These results are consistent with a model for tryptophan hydroxylase in which the enzyme consists of an N-terminal regulatory domain, a catalytic core, and a small C-terminal region of uncertain but important function.**

Tryptophan hydroxylase [TPH; tryptophan 5-monooxygenase; L-tryptophan, tetrahydrobiopterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], containing nonheme iron, catalyzes the pterin-dependent hydroxylation of L-tryptophan to form 5-hydroxytryptophan (5-HTP) (1). Within the central nervous system, this reaction is the first and rate-limiting step in biosynthesis of the neurotransmitter 5-hydroxytryptamine (serotonin) (2). Serotonin in the central nervous system has possible roles in regulation of body temperature and control of different behaviors as well as in the etiology of affective disorders (3). In the endocrine pineal gland, the hydroxylation of tryptophan by TPH is the first but not rate-limiting step in the biosynthesis of melatonin (4), which is a physiologically important bioregulatory molecule (5).

TPH is one member of a family of pterin-dependent aromatic amino acid hydroxylases, which also includes phenylalanine hydroxylase [PAH; phenylalanine 4-monooxygenase; L-phenylalanine, tetrahydrobiopterin:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] and tyrosine hydroxylase [TH; tyrosine 3-monooxygenase; L-tyrosine, tetrahydrobiopterin:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] (1, 6–8). Due to its limited availability and extreme instability, biochemical characterization of TPH has been hampered (9, 10). In contrast, the other two enzymes, PAH and TH, have been relatively well studied (11, 12). Successful expression of PAH and TH in heterologous systems, such as *Escherichia coli* and insect cells, has been helpful in biochemical studies of both enzymes (13–16).

TPHs from brain, pineal gland, and mast cells exhibit distinct biochemical characteristics (17, 18). Biochemical differences between TPHs from rat brain and pineal gland are due to posttranslational modification (17). cDNA clones encoding TPH have been isolated from rabbit and rat pineal gland (19, 20), rat brain (17), and mouse mastocytoma and human carcinoid cell lines (21, 22). TPH cDNA, recently isolated from human brain in this laboratory, has been expressed in *E. coli*, but the expressed protein is largely insoluble (J. Tipper, B. A. Citron, P. Ribeiro, and S.K., unpublished data). Efforts attempting to denature and renature insoluble TPH under different conditions were unsuccessful (unpublished results). The present report describes the high-level expression of human TPH by fusion with an affinity tag, maltose-binding protein (MBP), and the affinity purification of fused human TPH, which is soluble and active. We also describe the construction and characterization of a series of deletion mutants to assess the structural basis of the functional domain organization of human TPH.†

MATERIALS AND METHODS

Materials. Plasmid H1A, containing TPH coding sequence isolated from human brain, and goat polyclonal antibodies to rat PAH were generously provided by J. Tipper (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). Horseradish peroxidase-labeled rabbit anti-goat IgG was purchased from Pierce. D-Glucose 6-phosphate, D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteriodes*, 5-HTP, and dihydropteridine reductase were obtained from Sigma. Catalase was from Boehringer Mannheim. Tween 20 and L-tryptophan were purchased from Calbiochem. (6*R*)-5,6,7,8-Tetrahydrobiopterin (BH_4) and 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$) were from B. Schirks Laboratories (Jona, Switzerland). Factor Xa protease and crosslinked amylose resin were bought from New England Biolabs. All the other materials were obtained as indicated below.

Oligonucleotide-Directed Mutagenesis. A phagemid *in vitro* mutagenesis kit (Bio-Rad) was used. Single-stranded uracil-rich DNA templates of phTPH1 or phTPH2 were prepared as described (23). Mutagenic primers used were as follows: o227, 5′-GAG GGA AGG ΔATG ATT GAA G-3′; o302, 5′-GGC GCT GGC TAA CTT TCT TCT ATC-3′; o304, 5′-GTT CTT TCT GTG GAA TTC CCA GAT AAT TTT-3′; o306, 5′-ATG AGA GAA TTC TAA AAA ACA ATT AAG C-3′; o307, 5′-ACA CGA AGC ATT TAA GGA TCC TGA AAG-3′; o338, 5′-TG AAC GAG CTG CAG TAA TGA TCT TGA CGT-3′. The deleted nucleotide is indicated by Δ and the substituted or inserted one is underlined. All mutants were selected by restriction digestion or by DNA sequence analysis of double-stranded plasmids on an Acugen model

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Abbreviations: TPH, tryptophan hydroxylase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; MBP, maltose-binding protein; BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin; $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin; 5-HTP, 5-hydroxytryptophan.

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†A preliminary report of this work was presented at the 25th Annual Meeting of the American Society for Neurochemistry, March 5–9, 1994, Albuquerque, NM.

402 automated DNA sequencing system (EG & G Bethod, Natick, MA).

Construction of TPH Expression Plasmids. phTPH1 was constructed by insertion of the 1.4-kb *Nde* I/*Bam*HI fragment of H1A between the *Xmn* I and *Bam*HI sites of pMAL-c2 (New England Biolabs). phTPH2 was derived from phTPH1 by deletion of an extra nucleotide with mutagenic primer o227. A 0.85-kb fragment of phTPH2 was cloned into pMAL-c2 to yield phTPH7. Site-directed mutagenesis with o304, o304/o307, and o304/o338 was used to convert phTPH2 to plasmids phTPH4, phTPH9, and phTPH16, respectively; 1.0-kb *Eco*RI/*Bam*HI fragments from phTPH4, phTPH9, and phTPH16 were then ligated to *Eco*RI/*Bam*HI-digested pMAL-c2 to obtain phTPH10, phTPH11, and phTPH17, respectively. Primers o302, o306, o307, and o338 were used to introduce termination codons into the TPH coding sequence in phTPH2, which yielded phTPH3, phTPH5, phTPH6, and phTPH15, respectively.

Expression and Purification of Human TPH. Cultures of *E. coli* strain DH5 (GIBCO/BRL), transformed by phTPH2 or its derivatives, were grown and induced for expression as described (13, 24). The cell pellet was washed once with 0.85% NaCl and stored at −20°C until use.

The purification procedure was carried out at 4°C. The pellet (≈4 g) was resuspended in 100 ml of buffer A [50 mM NaHepes, pH 6.8/0.2 M NaCl/0.4 mM $FeSO_4$/1 mM EDTA/0.5 mM L-tryptophan/0.1% Tween 20/10% (vol/vol) glycerol] supplemented with 1 mM phenylmethylsulfonyl fluoride and immediately sonicated for 15 pulses, each for 30 sec, followed by a 30-sec off period. The sonicated homogenate was centrifuged at 27,000 × *g* for 10 min and the supernatant was diluted to 150 ml with buffer A. The diluted crude extract was loaded onto a column (3 × 12 cm) packed with 20 ml of crosslinked amylose resin at 0.5–1 ml/min. The column was washed extensively with buffer A. The enzyme was eluted with buffer A containing 5–10 mM maltose at 0.5 ml/min. The enzyme came off immediately after the void volume. About 10 mg of TPH fusion protein was obtained from 4 g of cells. The eluted enzyme fractions were either used directly or dialyzed against buffer A without tryptophan. The dialyzed enzyme was used within 1 week when stored at −80°C. Cleavage of fused TPH was carried out at 4°C or room temperature by addition of factor Xa protease.

TPH Enzymatic Assay. TPH activity was measured by the fluorometric determination of the product 5-HTP as described (25) with a few modifications. A regular assay mixture contained 50 mM Tris·HCl (pH 7.5), 125 μM L-tryptophan, 1 mM dithiothreitol, 1 mM NADH, 0.39 unit of dihydropteridine reductase per ml, 10 mM D-glucose 6-phosphate, 5 milliunits of D-glucose-6-phosphate dehydrogenase per ml, and 0.2 mg of catalase per ml, in a final vol of 0.1 ml. The reaction was initiated by addition of TPH or cofactor (BH_4 or $6MPH_4$), incubated at 37°C for 10–15 min, terminated with 0.1 ml of 10.5 M HCl/1.3 M $HClO_4$, chilled on ice for 5 min, and finally centrifuged at room temperature for 5 min. Upon excitation at 295 nm, the fluorescence of the supernatant at 538 nm was measured on an LS-3B fluorescence spectrometer (Perkin–Elmer) with 5-HTP as the standard. The 5-HTP standard solutions were prepared in the same manner as assay mixtures except that the incubation step at 37°C was omitted. Under these conditions, the rate of 5-HTP formation was proportional to the added amount of purified TPH (up to 60 milliunits). One unit of TPH activity is defined as the amount of TPH that catalyzes formation of 1 nmol of 5-HTP per min under the conditions specified. Probably because of the presence of inhibitory materials, it was difficult to accurately determine TPH activity in crude extracts.

Solutions of BH_4 and $6MPH_4$ were prepared in 5 mM HCl as described (26). Protein concentrations were determined with the BCA reagent (Pierce) using bovine serum albumin as the standard. To minimize the interference by tryptophan in the BCA method, protein samples containing tryptophan were pretreated by acetone precipitation. The precipitates were washed with 75% ethanol and redissolved in 0.9% SDS/36 mM NaOH.

Other Methods. Western immunoanalyses were performed as described (27). To obtain kinetic parameters, experimental data were fitted to the Michaelis–Menten equation with a Macintosh program, Enzyme Kinetics 1.0c, developed by D. G. Gilbert (Indiana University). In the case of inhibition by high concentrations of substrate or cofactor, data obtained at low concentrations were used to calculate apparent K_m values.

RESULTS

Expression and Purification of Human TPH. To achieve high-level expression and permit affinity purification of active TPH, a fusion protein expression and purification system was used (28). Plasmid phTPH2 was constructed to express a fusion protein consisting of MBP, affinity tag at the N terminus, followed by a linker peptide, and human TPH at the C terminus (Fig. 1). The single-stranded form of phTPH2 could be rescued for site-directed mutagenesis. Plasmid phTPH2, therefore, provided a convenient vector for expression, mutagenesis, and purification of human TPH.

Because of the well-documented instability of TPH (9, 10, 29), buffer A was introduced to stabilize the enzyme. Iron(II) was included in buffer A to prevent iron loss from TPH. Due to the presence of Fe^{2+}, fresh buffer A had a greenish color. Total and soluble bacterial protein extracts from phTPH2-containing DH5 were analyzed by SDS/PAGE and revealed an induced band of 91 kDa, which remained in the soluble protein extract (Fig. 2*A*). The molecular mass of this band was close to the expected value of TPH fusion protein. This band was estimated to represent 3–5% of soluble bacterial proteins. To ensure the identity of this induced band, Western immunoanalysis of the bacterial proteins was performed. Since TPH was reported to crossreact with antibodies to

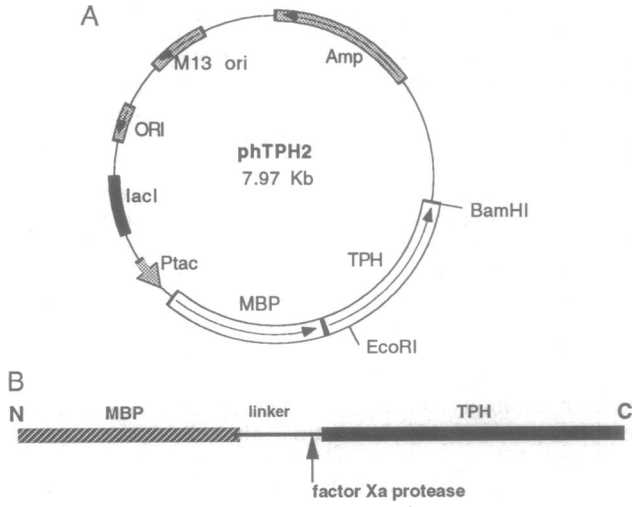

FIG. 1. Schematic illustration of expression vector phTPH2 (*A*) and the fusion protein (*B*). (*A*) Coding sequences for MBP and TPH are fused and placed downstream from the tac promoter (Ptac). phTPH2 can be induced to express the fusion protein. (*B*) The recognition sequence (Ile-Glu-Gly-Arg) for factor Xa protease is at the C terminus of the linker. The protease is expected to cleave the bond between the arginine residue in the tetrapeptide and the first residue of TPH to release intact authentic TPH. Sizes calculated from amino acid sequences are as follows: fusion protein, 91 kDa; MBP, 40 kDa; linker, 2 kDa; TPH, 49 kDa.

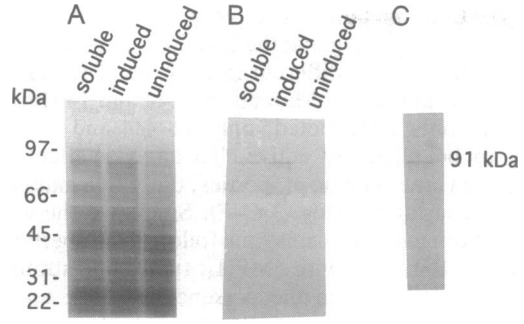

FIG. 2. TPH fusion protein analyzed by SDS/PAGE and Western blotting. (*A*) Bacterial protein extracts separated by SDS/PAGE followed by Coomassie blue staining. (*B*) Bacterial protein extracts analyzed by Western immunoanalysis with polyclonal antibodies to rat PAH. (*C*) Purified TPH fusion protein analyzed by SDS/PAGE.

PAH (30), the available anti-rat PAH antibodies were used. The antibodies reacted with the induced 91-kDa band as well as with several smaller bands (Fig. 2*B*).

Soluble protein extracts from *E. coli* cells, grown under different conditions, were assayed for TPH activity. The specific activity of the soluble extract from DH5 harboring phTPH2 was estimated to be >1.7 units per mg, whereas no activity was detected from protein extracts made from the same host transformed with vector pMAL-c2. Human TPH, therefore, was expressed from phTPH2 as a soluble and active fusion protein.

TPH fusion protein was expressed in 2- to 4-liter cultures of *E. coli* DH5, transformed with plasmid phTPH2, and purified by affinity chromatography on amylose resin. TPH precipitated when the enzyme purified in buffer A was dialyzed against the same buffer with a lower concentration of Tween 20 (0.01%), indicating that Tween 20 is important for TPH solubility. When buffer A was used, the purified enzyme retained ≈60% activity after 3 months at −80°C. As judged by SDS/PAGE (Fig. 2*C*), the fusion protein was close to homogeneity, with a major band of 91 kDa (≈80%) and some minor smaller bands. The 91-kDa band as well as some minor bands crossreacted with anti-rat PAH polyclonal antibodies.

Attempts were made to cleave intact TPH from the purified fusion protein by digestion with factor Xa protease (Fig. 1*B*). Probably because of nonspecific digestion by the protease at sites partially homologous to its recognition sequence, only partial digestion of the fusion protein gave intact TPH. Cleaved MBP was removed by ammonium sulfate fractionation, which yielded a mixture of cleaved and fused forms of TPH. Efforts aimed at separating these two forms were unsuccessful (data not shown). Since TPH was reported to be a tetramer (10), the cleaved and fused forms of TPH might exist as tight heterocomplexes, which made it very difficult to separate the two forms. We were also unsuccessful in attempting to cleave the fusion protein while bound to amylose resin. Furthermore, digestion of fused TPH by factor Xa protease promoted formation of precipitates consisting of the cleaved and fused forms of TPH. Because of the difficulties we experienced in obtaining cleaved TPH, the fused form was used for the experiments described below.

Kinetic Properties of Fused TPH. Different from the results for rabbit and mouse brain TPHs (18, 25), human TPH is more active with BH_4 than with $6MPH_4$. In the presence of the natural cofactor, BH_4, the purified fusion protein has a specific activity of 86 units per mg of fusion protein, which is equal to a calculated value of 160 units per mg of TPH. When the synthetic analog, $6MPH_4$, was used as the cofactor, the specific activity was found to be 62 units per mg of TPH, which is close to the reported value of 82 units/mg for the less active form of the rat brain enzyme but lower than the value of 374 units/mg for the more active one (10).

As reported for the rabbit brain enzyme (25, 31), fused TPH exhibits typical substrate inhibition by L-tryptophan (>0.13 mM) in the presence of BH_4 (Fig. 3*A*). Inhibition by excess L-tryptophan is much less evident when $6MPH_4$ is substituted for BH_4 (Fig. 3*A*). Slight inhibition is evident at high concentrations of BH_4 or $6MPH_4$ (Fig. 3*B*). The derived K_m or apparent K_m values are summarized in Table 1. Different K_m values have been reported for TPH from different sources: 14–50 μM for L-tryptophan (BH_4); 27–87 μM for L-tryptophan ($6MPH_4$); 22–294 μM for BH_4; 50–119 μM for $6MPH_4$ (10, 18, 29, 31, 32). This wide range of values may be partially explained by the fact that different buffer systems were used for the determinations. To test this possibility, the K_m for L-tryptophan was determined in different buffers. In the presence of BH_4, the K_m of fused TPH for L-tryptophan was found to be 26 μM in 50 mM Tris·HCl (pH 7.5) and 80 μM in 50 mM NaHepes (pH 6.8). This is also consistent with the report that the K_m value of TH for $6MPH_4$ is 24 μM in Tris·HCl and 86 μM in another buffer (33).

Effects of Truncations on TPH Activity. Proteolysis and deletion mutagenesis have well documented the conclusion that only the C-terminal two-thirds of PAH or TH is essential for catalytic activity, with the N-terminal one-third being the regulatory domain (34–37). TPH shares highly homologous regions with PAH and TH in the C-terminal two-thirds (19, 20) (Fig. 4). It has been demonstrated that the C-terminal 380 residues of mouse TPH are sufficient for catalytic activity (21). To further analyze the domain organization of TPH, a series of plasmids (Fig. 4) were constructed from phTPH2 to express mutants lacking peptides from one or both termini of human TPH.

Like full-length TPH, deletion mutants were expressed as MBP fusion proteins and purified by affinity chromatography

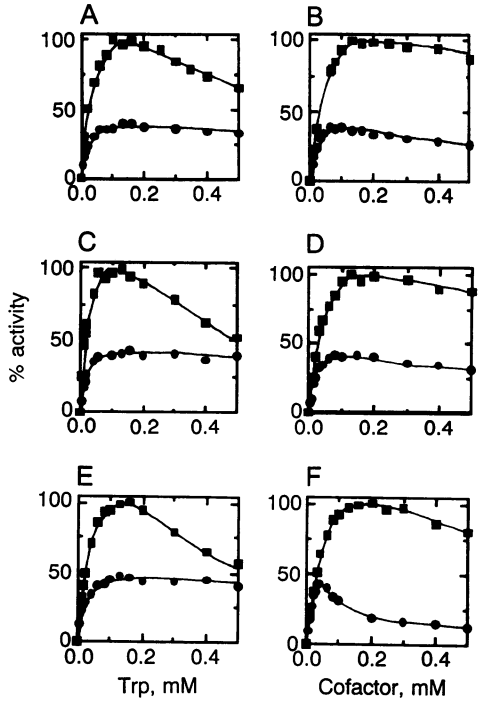

FIG. 3. Effects of substrate (*A*, *C*, and *E*) and cofactor (*B*, *D*, and *F*) concentrations on TPH activity of the wild-type (1–444, *A* and *B*) and mutant (92–444, *C* and *D*; 1–425, *E* and *F*) enzymes. TPH activity, assayed in the presence of BH_4 (■) or $6MPH_4$ (●), is expressed as percentage of maximal activity of each enzyme with BH_4. The following cofactor concentrations were used: BH_4, 0.25 mM (*A*, *C*, and *E*); $6MPH_4$, 0.125 mM (*A* and *C*) and 0.04 mM (*E*). In *B*, *D*, and *F*, L-tryptophan concentration was fixed at 0.125 mM.

Table 1. Steady-state kinetic parameters of wild-type (WT) and mutant forms of human TPH

Parameter	WT 1–444	92–444	1–425
$K_{app,Trp}$ (BH_4), μM	26	28	16
$K_{m,Trp}$ ($6MPH_4$),* μM	23	13	18
V_{max} ($6MPH_4$), min^{-1}	3.2	0.36	0.32
K_{app,BH_4},† μM	48	39	33
$K_{app,6MPH_4}$,‡ μM	26	25	30

K_{app}, apparent K_m; a proper value could not be obtained because of strong substrate inhibition. $K_{app,Trp}$ was obtained by varying L-tryptophan concentration from 10 to 150 μM with a constant BH_4 concentration of 250 μM. V_{max} values are expressed as mol of 5-HTP formed per min per mol of subunit. For 1–444, the value of 3.2 min^{-1} is equivalent to 62 units per mg of TPH.

*Concentration of $6MPH_4$ was 125 μM for 1–444 and 92–444 and 40 μM for 1–425.

†L-Tryptophan concentration was constant at 125 μM and the concentration of BH_4 was varied between 10 and 150 μM.

‡While L-tryptophan concentration was fixed at 125 μM, the concentration of $6MPH_4$ was increased from 5 to 90 μM for 1–444 and 92–444 and from 5 to 40 μM for 1–425.

on amylose resin. As judged by SDS/PAGE (data not shown), the mutants were almost as pure as full-length TPH, with major bands of the expected sizes. The purified mutants crossreacted with polyclonal antibodies to rat PAH (data not shown). The relative activities of the mutants are included in Fig. 4.

To analyze the regulatory function of the N-terminal region of human TPH, deletion mutants lacking the first 91 and 164 residues were constructed. While deletion of the N-terminal 91 residues (mutant 92–444) retains ≈14% of the specific activity of full-length TPH (1–444), further deletion of 73 residues (mutant 165–444) abolishes the activity, which indicates that residues between 92 and 164 are essential for catalysis. Similarly, it was shown for mouse TPH that the C-terminal 380 but not 279 amino acids are sufficient for catalytic activity (20).

To study a possible regulatory role of the TPH C terminus, truncation mutants 1–425, 1–408, 1–389, and 1–333 were made (Fig. 4). Whereas mutants 1–389 and 1–333 display no detectable activity, 1–425 and 1–408 have 9% and 1.3% of the specific activity of 1–444, respectively. Therefore, the C-terminal region of TPH is important for maximal activity.

It was shown that removal of the C-terminal 43 amino acids inactivated rat TH and that further deletion of 157 residues from the N terminus restored about one-half of the activity (37). To extend this study to TPH, deletions were made from both termini of TPH to create mutants 92–408 and 92–425 (Fig. 4). Both mutants have very low or no detectable activity, demonstrating that the N- and C-terminal regions of TPH are functionally different from those of rat TH.

Kinetic Parameters of Mutants 90–444 and 1–426. Among all the mutants constructed, only 92–444 and 1–425 were shown to be relatively active. To analyze effects of the deletions on other kinetic properties, these two mutants were characterized further (Fig. 3 *C–F*). Similar to the wild-type enzyme, both mutants display the following properties: more active with BH_4 than with $6MPH_4$; typical substrate inhibition by L-tryptophan in the presence of BH_4; no evident substrate inhibition by L-tryptophan in the presence of $6MPH_4$; and slight cofactor inhibition by high concentrations of BH_4. Unlike 1–444 and 92–444, however, the C-terminal deletion mutant 1–425 shows a very strong inhibition by $6MPH_4$ (>40 μM) at a constant concentration of L-tryptophan.

Kinetic data similar to those shown in Fig. 3 were collected to derive K_m or apparent K_m values for the substrate and cofactors. As shown in Table 1, all the K_m values of the two deletion mutants are very close to those of the wild-type enzyme. The two deletions, therefore, appear to have minimal effects on binding of substrate L-tryptophan and cofactor BH_4 or $6MPH_4$.

DISCUSSION

The results presented in this paper demonstrate that soluble active TPH has been expressed and purified by fusion with MBP. Without MBP affinity tag, the expressed polypeptide of authentic human TPH is mainly insoluble in *E. coli* (J. Tipper, B. A. Citron, P. Ribeiro, and S.K., unpublished data). It is unclear why fusion with MBP helps expression of the active and soluble TPH. One possible explanation is that MBP interacts with chaperonin SecB, which in turn helps to prevent aggregation of the fusion protein. It has been reported that SecB binds to the MBP precursor (38).

Just like the native rabbit brain enzyme (31), fused TPH, in the presence of BH_4, exhibits biphasic kinetics with strong inhibition by L-tryptophan at concentrations >130 μM. By contrast, with $6MPH_4$ as the cofactor, there is only slight substrate inhibition even at L-tryptophan concentrations as high as 0.5 mM. It has been observed that, in the presence of BH_4, PAH (39) and TH (13, 40) also show inhibition by high concentrations of the substrates phenylalanine and tyrosine, respectively. Unlike PAH (11) and TH (37), however, human TPH may have a higher rate of hydroxylation when BH_4 rather than $6MPH_4$ is used as the cofactor.

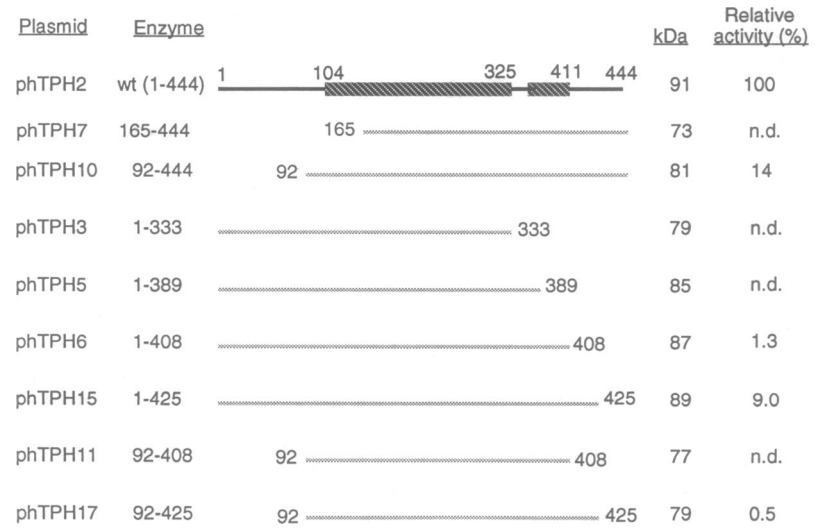

FIG. 4. Schematic illustration of wild-type and mutant forms of TPH. For simplicity, only TPH regions of fusion proteins are shown. For wild-type enzyme, variable and conserved regions among aromatic amino acid hydroxylases are indicated by heavy lines and hatched boxes, respectively. For mutants, dotted lines are used to depict TPH regions, whose relative N and C termini are denoted by adjacent numbers. Molecular activities, assayed in the presence of BH_4, are expressed as percentage of that of 1–444. n.d., Not detectable.

Among the mutants constructed to define the TPH domain organization, only the N-terminal deletion mutant 92–444 and the C-terminal deletion mutant 1–425 are relatively active, which suggests that the catalytic domain of TPH may be located between residues 92 and 425. In this respect, human TPH is analogous to PAH (34) and TH (37). However, since 92–444 is less active than 1–444, the N-terminal region, residues 1–91, may be necessary to keep the catalytic core (residues 92–425) in the most active conformation. This is different from regulation mechanisms of PAH and TH, where the N-terminal peptides are believed to serve as inhibitory domains (34–37, 41).

Unlike 1–444 and 92–444, 1–425 shows strong inhibition by $6MPH_4$ (>40 μM; see Fig. 4). It is unclear why deletion of the C-terminal 19 amino acids makes TPH behave so differently, but this result may suggest that the C-terminal region is involved in functional regulation of the central catalytic domain.

Since 1–425 is less active than 1–444, the N-terminal 2-kDa region may also help the catalytic core, residues 92–425, maintain the most active conformation. This is consistent with the reports that deletions of the C-terminal 43 and 20 amino acids of rat TH decrease V_{max} by 3- and 4-fold, respectively (37, 42). Based on these results and amino acid sequence homologies among the three hydroxylases (19, 20), we propose that these enzymes are constituted with three functional domains: an N-terminal regulatory domain, a catalytic core, and a small C-terminal region of uncertain but important function. An "inhibitor peptide" model was previously proposed to explain the inhibitory function of the N-terminal peptide of rat liver PAH (34, 43). It was also suggested that rat TH consists of two functional domains: the N-terminal regulatory region and the catalytic center (36). Partial proteolysis indicated that a 5-kDa C-terminal fragment of rat liver PAH is important for the tetramerization (34). It was also reported that the C terminus of rat TH is responsible for tetramer formation (42). Studies on the multimerization of human TPH remain to be done.

Further investigation is needed to understand how the N- and C-terminal regions of human TPH functionally affect the central catalytic domain. It is possible that the three domains may interact with each other, which is consistent with the deletion analysis of rat TH (37). Based on amino acid sequences of mammalian aromatic amino acid hydroxylases, Liu and Vrana (44) have predicted that these enzymes have potential leucine zippers in the catalytic domains and coiled-coil motifs in the C-terminal regions (45). In addition, we believe that there are putative coiled-coil motifs in the N-terminal regulatory domain of human TPH. It is not yet known whether these putative motifs in different domains mediate the domain interaction.

It is obvious that conclusions drawn from fused TPH may not reflect the real nature of the native enzyme, but this system should be useful for certain purposes before the native hydroxylase becomes easily accessible.

In summary, human TPH has been expressed and purified as an active fusion protein. It has also been demonstrated that the structural determinants of catalytic activity of TPH are somewhat different from those of PAH and TH. In particular, there is no indication that the N-terminal region of TPH exerts negative control over its catalytic activity in the same way that the corresponding regions in the other two hydroxylases do over their catalytic activities.

We wish to thank J. Tipper for providing plasmid H1A; M. Brownstein for oligonucleotide synthesis; P. Ribeiro for help with the TPH assay; and C. Falke, E. Suggs, and J. Powell for technical support.

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