ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 315, No. 2, December, pp. 445–453, 1994

Cloning and Expression of Rabbit and Human Brain Tryptophan Hydroxylase cDNA in *Escherichia Coli*[1]

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Received June 7, 1994, and in revised form August 3, 1994

Rabbit and human brain tryptophan hydroxylase were cloned and expressed in *Escherichia coli*. Each of the respective cDNAs, including the complete coding sequence of tryptophan hydroxylase, was obtained by reverse transcription of rabbit or human brain mRNA and subcloned into the expression vector pET-3C. The expressed rabbit brain tryptophan hydroxylase activity, measured in the presence of tetrahydrobiopterin, represents approximately a 50-fold enhancement in yield (units/g tissue (wet wt) over that of a rabbit brain extract. Likewise, the level of expressed human brain tryptophan hydroxylase is approximately 57 times the average yield previously reported for a human brain homogenate and approximately 10-times the activity of homogenates of human raphe nucleus. The rabbit brain and pineal-derived tryptophan hydroxylase sequences varied by disparities in six amino acid residues (99% identity). The human carcinoid and brain peptide sequences varied by disparities in 18 amino acid residues (96% identity). Several properties of both expressed enzymes were studied and compared with those of native tryptophan hydroxylases. © 1994 Academic Press, Inc.

Key Words: **tryptophan hydroxylase; rabbit brain; human brain; molecular cloning; expression.**

Tryptophan hydroxylase (L-tryptophan, tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16) catalyzes the hydroxylation of L-tryptophan to 5-hydroxy-L-tryptophan. This reaction, which occurs in the presence of molecular oxygen and requires the natural cofactor (6R)-tetrahydrobiopterin (BH_4)[5] and iron, is generally considered to be the rate-limiting step (1, 2) in the biosynthesis of the neurotransmitter serotonin. Serotonin may affect a wide range of physiological phenomena, including sleep behavior (3) and pain perception (4). A disruption of serotonergic-mediated neural transmission has been implicated in a variety of disorders, including the associated depression and dementia in Parkinson's syndrome, the neurodegenerative symptoms of Huntington's disease, and the dementia associated with Alzheimer's disease (5).

Tryptophan hydroxylase activity is found in brain (1, 6–8) and is located peripherally in the pineal body (9, 10) and in mast cells (11) and is associated with neurons innervating the gut (12). Tryptophan hydroxylase cDNA has been cloned from a variety of sources including rabbit pineal gland (13), rat pineal gland (14) and serotonergic neurons (15), mouse mastocytoma (16), and a human carcinoid cell line (17).

Due to the low abundance of tryptophan hydroxylase in brain and peripheral sources, the enzyme has proven difficult to purify and obtain in high yield, and is, thus, the least characterized of all the aromatic amino acid hydroxylases. Previous attempts in our laboratory to screen several brain cDNA libraries did not yield tryptophan hydroxylase clones. We, therefore, employed the polymerase chain reaction (PCR, Perkin–Elmer Cetus) method to obtain amplified tryptophan hydroxylase cDNA from both rabbit and human brain. A previous report described the expression of PCR-derived rat dorsal nucleus tryptophan hydroxylase in *Escherichia coli*, but

[1] The rabbit and human brain nucleotide sequences reported herein have been submitted to the Genome Sequence DataBase and have been assigned Accession Nos. L29305 and L29306, respectively.

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[5] Abbreviations used: BH_4, tetrahydrobiopterin; 6-MPH_4, 6-methyltetrahydropterin; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosylleucylchloromethyl ketone; X-gal, 5-bromo-4-chloro-β-D-galactoside; IPTG, isopropylthio-β-D-galactoside; DTT, dithiothreitol; dNTPs, deoxynucleotide triphosphates; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; kb, kilobase; PCR, polymerase chain reaction.

0003-9861/94 $6.00
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no enzyme activity data or DNA or peptide sequence results were provided (18). Thus, we report herein the first high-level expression of catalytically active mammalian tryptophan hydroxylase in *E. coli*. In addition, some of the properties of the two expressed brain enzymes are reported and compared to those of the native rabbit and human brain tryptophan hydroxylases.

MATERIALS AND METHODS

Preparation of total RNA and production of amplified cDNAs. Total RNA was purified with the use of a kit purchased from 5 Prime-3 Prime, Inc. from rabbit midbrain and hindbrain (frozen tissue from Pelfreez) or from human midbrain, pons, and cerebellum (tissue from autopsy at 5 h, postmortem, and frozen immediately). Reverse transcription of both rabbit and human brain total RNA was performed with the use of the cDNA cycle kit (Invitrogen, see product enclosures for safe handling of mercury hydroxide) in the presence of 1–3 μg of total RNA and selected oligonucleotide primers, corresponding to regions of the published sequence for rabbit pineal (13) and human carcinoid (17) tryptophan hydroxylase cDNAs (Fig. 1, primers 2 and 6, respectively). The resulting rabbit and human brain cDNAs were amplified by two consecutive rounds of PCR reactions with the use of the Gene-Amp PCR kit (Perkin–Elmer Cetus) and an instrument manufactured by M. J. Research, Inc. The cycling protocol was as follows: Denaturation was for 1 min at 95°C, followed by re-annealing at 40°C for 2 min and extension at 72°C for 2 min. This pattern was repeated for a total of 40 cycles with a final extension time of 7 min in the last cycle.

The first round of amplifications of the rabbit brain cDNA was performed with primer pairs described in Fig. 1. The PCR reaction mixture contained 1–5 μl of cDNA, 500 μM deoxynucleotide triphosphates (dNTPs), 0.5 μM of each primer and 2.5 units of *Taq* polymerase in a total volume of 100 μl. The second round of amplification was performed using nested primer pairs (Fig. 1). The 5′-terminal primer included an *Eco*RI restriction site adjacent to an *Nde*I site at its 5′-terminus, and the 3′-terminal primer contained an *Nco*I restriction site adjacent to a *Bam*HI site at its 5′-terminus. The PCR reaction conditions were as described above except the dNTPs were present at 200 μM and the template was provided by the direct addition of 5 μl of the first-round PCR product.

The human brain tryptophan hydroxylase cDNA was also subjected to two rounds of PCR amplification. The first-round amplification was performed using primer pairs described in Fig. 1. All conditions were the same as those described for the initial amplification of rabbit brain cDNA. The second round of amplification was performed using purified cDNA template from the first set of reactions. The cDNA was purified in this instance due to the presence of small fragments (<1 kb) in the first-round PCR product. The larger fragment (1.3 kb) was isolated from a low-melting, 0.85% agarose gel (Bethesda Research Laboritories) and further purified using an Elutip column and recommended procedure (Schleicher and Schuell). The nested set of primers used for the second round of amplification are also described in Fig. 1 and, likewise, included restriction sites at their 5′-ends analogous to those of the rabbit pineal-derived second-round primers. The reamplification conditions were also as described above, except the primers were present only at 0.1 μM and the purified cDNA template was present at 0.05 ng.

Preparation of nonexpressing and expressing clones. The final PCR products derived from rabbit and human brain were sized, pooled as necessary, and treated with DNA polymerase I (New England Biolabs) in preparation for blunt-ended ligation (19, pp. 1.70–1.71) into *Sma*I-digested pGEM-7zf(−) (Promega). The resulting ligation products were used to transform the DH5α strain of *E. coli*. DNA from selected clones was purified (19, pp. 1.25–1.28), and putative tryptophan hydroxylase cDNA-containing clones were identified by restriction digestion and agarose gel analysis (19, p. 6.9). An aliquot from each of the recombinant pGEM-7zf(−) plasmids was digested with *Nde*I and *Bam*HI to generate cDNA inserts with cohesive ends for ligation (19, pp. 5.11–5.13) into similarly digested pET-3C (Novagen, Inc.). The cDNAs, obtained from these digestions, were ligated into pET-3C at 60 bases downstream from the T7 promoter site. The resulting recombinant pET-3C plasmids were used to transform the DH5 strain of *E. coli*. The stable clones established in this strain provided the recombinant plasmid cDNA (Qiagen, Inc., "midi-prep") used in the transformation of the BL21(DE3)(+plysS) strain of *E. coli* (Novagen, Inc.), which produced tryptophan hydroxylase-expressing clones.

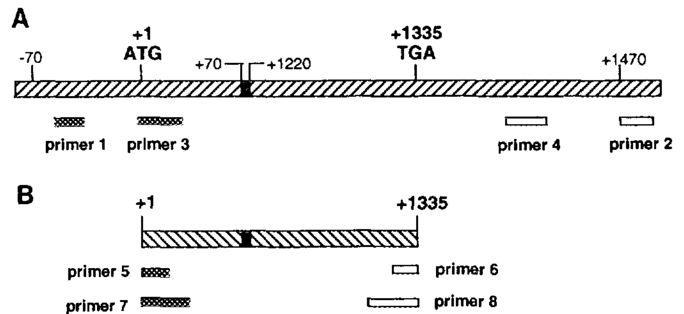

FIG. 1. Schematic representation of PCR primers used in the amplification of rabbit and human brain tryptophan hydroxylase. The position of each primer relative to the published sequence is shown. Primers 1–4 were derived from rabbit pineal (13) tryptophan hydroxylase cDNA sequence (A), and primers 5–8 were derived from the human carcinoid (17) sequence (B). Sense primers are depicted by black boxes and anti-sense primers by open boxes. Two pairs of sense and anti-sense primers, 1 + 2 and 5 + 6, were used in the first round of amplification of rabbit and human tryptophan hydroxylase cDNAs, respectively. The remaining primer pairs, 3 + 4 and 7 + 8, were used for the second set of PCR reactions. The latter two pairs of primers included restriction sites at their 5′-ends to facilitate subcloning into the expression vector (primers 3 and 7 contained an *Eco*RI site adjacent to an *Nde*I site; primers 4 and 8 contained an *Nco*I site adjacent to a *Bam*HI site). The sequences of the primers from the 5′- to 3′-end are as follows: (1) AGCCTGTCATCT-ATAGTTTC, (2) TTTAGCCTAGTGATTCATTA, (3) GAATTC-CATATGATTGAAGATAATAAA, (4) CCATGGGGATCCAAGAAA-AGCAGGGCT, (5) ATGATTGAAGACAATAA, (6) TTAGATACT-CGGCTTCC, (7)GAATTCATATGATTGAAGACAATAAGGAGAAC, (8) CCATGGATCCTTAGATACTCGGCTTCCTGCTGAC.

DNA sequence analysis of clones. Both strands of each tryptophan hydroxylase clone were sequenced with the Sequenase DNA sequencing kit and procedure (20) and with the use of selected primers based on the sequence of either the rabbit pineal (13) or the human carcinoid (17) tryptophan hydroxylase cDNA. Sequence analysis was performed on an Acugen 402 automated DNA sequencing system (EG & G Berthold, Natick, MA). The 5′- and 3′-terminal sequences of both of the hydroxylase clones in pGEM-7zf(−) were obtained with the SP6 and T7 promoter primers. The 5′-terminal sequences of both the rabbit and human brain pET-3C clones were sequenced with two 20-base primers, one recognizing the vector at 71 bp upstream from the cDNA insertion site and the other identical to the vector 35 bp upstream from the insertion site. The 3′-terminal region of the rabbit brain tryptophan hydroxylase clone in pET-3C was sequenced with two 20-base primers identical to rabbit pineal tryptophan hydroxylase cDNA at positions 1241–1260 and positions 1471–1490, respectively. The 3′-terminal portion of the human pET-3C recombinant clone was sequenced with a 20-base primer recognizing the human brain tryptophan hydroxylase cDNA at positions 1110–1129.

Expression of enzyme activity in E. coli. Recombinant clones in *E. coli* BL21(DE3),pLysS hosts were grown to mid-log phase in NZCYM (Gibco-BRL formulation of NZ Amine A, an enzymatic digest of casein, yeast extract, casamino acids, and $MgSO_4$) medium containing ampicillin

(150 μg/ml), chloramphenicol (25 μg/ml), and $FeSO_4$ (0.5 mM) and, subsequently, induced for 2 h with 0.5 mM IPTG. Frozen cell pellets were thawed in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10% (vol/vol) glycerol, 1 mM DTT, 200 μM EDTA, DNase (4 μg/ml), RNase (4 mg/ml), 0.2 mM PMSF, trypsin inhibitor (0.8 μg/ml), TLCK (4 μg/ml), pepstatin (1 μM), antipain (1 μg/ml), aprotinin (20 μg/ml), and leupeptin (0.8 μg/ml). Cells were refrozen and thawed once again to promote lysis. The resulting cell suspension was sonicated with an Ultrasonic Model W-225 sonicator, and the sonicates were centrifuged at 12,000g (4°C) for 15 min. The supernatant provided soluble enzyme, stable at −70°C for at least 2 months with little loss of activity.

Preparation and assay of expressed enzyme. For kinetic studies, the expressed tryptophan hydroxylase (from rabbit or human brain) was partially purified from *E. coli* extracts by ammonium sulfate fractionation between 29 and 42% (w/v of saturation) and subsequent gel filtration by application to a PD-10 column (Sephadex-G25, Pharmacia) in order to remove residual ammonium sulfate. These steps produced approximately a three-fold enhancement of specific activity. Tryptophan hydroxylase activity was measured by the fluorometric determination of 5-hydroxtryptophan (21) produced in the presence of 1–10 μg of protein, in a total reaction volume of 100 μl. Reactions were linear up to 12 min at 37°C. Hydroxylase activity in crude *E. coli* extracts (12,000g supernatant) was determined in the presence of 250 μM BH_4 and 100 μM tryptophan. Preliminary kinetic studies, whereby substrate and cofactor levels were systematically varied with respect to each other, resulted in the following optimized assay conditions: (i) for the determination of the apparent K_m ($S_{0.5}$) values for typtophan for the rabbit brain enzyme, tryptophan levels were varied between 10 and 1000 μM, while the BH_4 level was maintained at 250 μM and $6MPH_4$ was present at 125 μM; (ii) the apparent K_m for tryptophan for the human enzyme was determined in the presence of 10–1000 μM tryptophan and 250 μM of the respective cofactor; (iii) for determination of the K_m values for each cofactor (BH_4 or $6MPH_4$) for both the rabbit and the human enzymes, tryptophan levels were held at 100 μM while each cofactor was varied from 10 to 500 μM. Limiting levels of tryptophan (100 μM) were necessary during the K_m determinations for each cofactor due to the phenomenon of substrate inhibition of the enzyme by tryptophan (21). Since the expressed rabbit brain enzyme also demonstrated inhibition by $6MPH_4$, the level of this cofactor was maintained at 125 μM for the determination of the K_m value for tryptophan.

Other methods. The protein concentration of samples was measured by use of the Bio-Rad assay kit (22) with bovine serum albumin as a standard. Western blot analysis (23) was performed with the use of sheep anti-rat liver phenylalanine hydroxylase (24) cross-reacting antibody (1:2500 dilution) and peroxidase-conjugated (Bio-Rad) rabbit anti-sheep IgG (1:1000 dilution) as the secondary antibody. Kinetic parameters (K_m and apparent K_m ($S_{0.5}$)) were derived from two or three individual determinations (all data points in duplicate) and determined by computer-assisted, nonlinear curve fitting to the Michaelis–Menten model. DNA and peptide sequence analyses were performed with the use of the GCG Sequence Analysis Software Package (25).

RESULTS AND DISCUSSION

Cloning and expression of rabbit and human brain tryptophan hydroxylases. Due to difficulties obtaining a tryptophan hydroxylase cDNA after screening both a rabbit and human brain library, we employed a strategy using "nested" primer pairs and PCR to obtain sufficiently amplified cDNAs from rabbit and human brain mRNA. Earlier attempts to amplify the cDNA obtained from reverse transcription did not yield visible product bands. Some degree of secondary structure in the mRNA may have impeded the production of full-length cDNA. Therefore, we reverse transcribed the mRNA in the presence of a denaturant, mercury hydroxide. Since the cDNA bands obtained were visible but faint, we reamplified this material using nested primers including restriction sites at their 5′-terminii (described under Materials and Methods). The resulting cDNAs were cloned into pGEM-7zf(−) plasmid by blunt ligation. Subsequent digestion of these recombinant plasmids with *Nde*I and *Bam*HI yielded tryptophan hydroxylase cDNA fragments with cohesive ends. These fragments were ligated into similarly digested pET-3C plasmid. This strategy allowed the introduction of tryptophan hydroxylase cDNA into the pET-3C vector in the proper orientation for expression. Constructs of the rabbit and human brain tryptophan hydroxylase clones in pET-3C are shown in Fig. 2 (plasmids pjptRABB and pjptHUMB, respectively). These recombinant pET-3C plasmids were used initially to transform DH5 non-expressing hosts for establishing stable clones. In order to express tryptophan hydroxylase activity, we transformed the BL21(DE3) host strain of *E. coli,* also containing the pLysS plasmid, which confers chloramphenicol resistance and stabilization of the recombinant plasmid by inhibiting basal levels of T7 RNA polymerase production. In addition, the T7 lysozyme helps promote lysis of cells during the extraction procedure (26).

Yield of expressed enzymes. Overnight growth of transformed BL21(DE3) + pLysS cells at 30°C followed by induction and growth at 37°C produced considerable levels of tryptophan hydroxylase activity in these cells. Analysis by SDS–PAGE reveals the presence of a large amount of protein at 50 kDa in those *E. coli* lysates derived from cells transformed with recombinant plasmid (Fig. 3). Cells transformed with plasmid containing no insert are not enriched in this band. An immunoblot of the lysate from tryptophan hydroxylase expressing cells, likewise reveals a considerable amount of cross-reacting protein at 50 kDa (Fig. 3). The immunoblot of the expressed enzyme was characterized by a predominant band at 50 kDa and a smaller fragment above the 32.5-kDa marker. Since both of these bands specifically cross-reacted with antibody, it is likely that the lower band may represent some proteolytically cleaved enzyme. No cross-reactivity was observed with an *E. coli* extract from pET-3C-transformed cells lacking the tryptophan hydroxylase insert (data not shown). Although the data in Fig. 3 were derived from expressed rabbit brain enzyme, comparable results were obtained with expressed human enzyme. In both instances, a considerable amount of the antibody-cross-reacting protein in the *E. coli* lysate (50 kDa) was associated with the cell pellet (data not shown) and presumably represents inclusion body material. It was difficult to assess the amount of activity in the pelleted fraction since this material remained insoluble. However, an assay of the pellet-suspension suggests that approximately 27% of the tryptophan hydroxylase activity may be associated with the cell pellet. Various attempts to solubilize this fraction

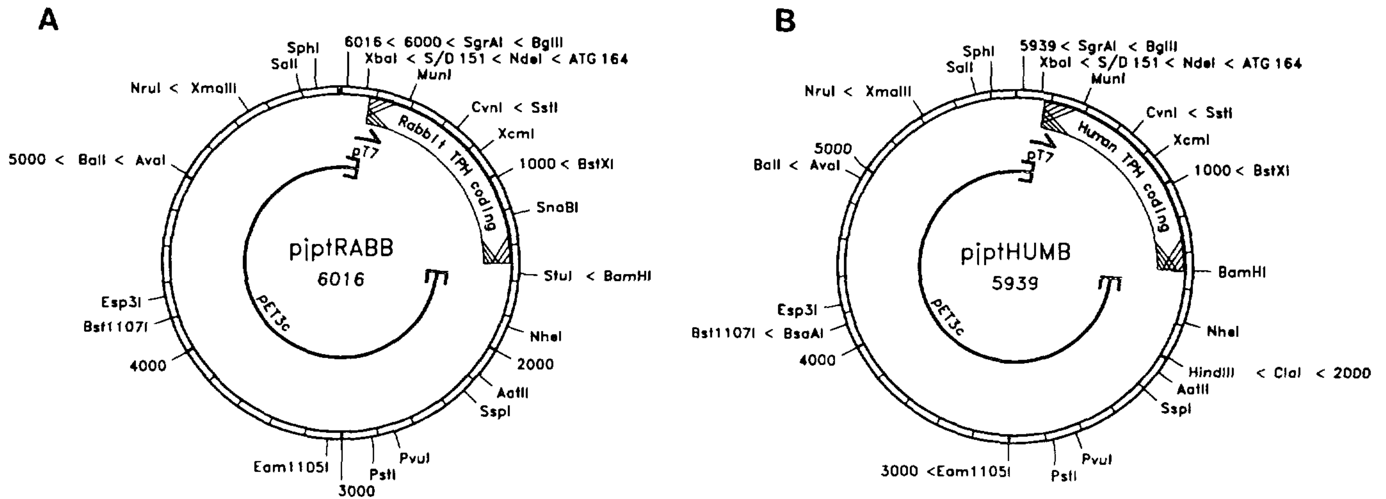

FIG. 2. Constructs of the rabbit and human brain tryptophan hydroxylase expressing clones. (A) Plasmid pjptRABB contains 1412 bp of tryptophan hydroxylase cDNA from rabbit brain. The total recombinant plasmid is 6016 bp. (B) Plasmid pjptHUMB contains 1335 bp of tryptophan hydroxylase cDNA from human brain. The total recombinant plasmid is 5939 bp. In both cases, each tryptophan hydroxylase cDNA was ligated at its 5′-terminus to the *Nde*I site of the pET-3C cloning vector and to the *Bam*HI site of the pET-3C at the 3′-terminus of the insert. The cDNA inserts are each positioned 5 bp downstream from the Shine–Delgarno region and 60 bp downstream from the T7 promoter site. Single digestion sites for specific endonucleases are indicated.

by detergent extraction or by denaturation/renaturation protocols were unsuccessful. It should be emphasized that in spite of the fact that substantial amounts of expressed enzyme are associated with the inclusion body, the amount of soluble tryptophan hydroxylase represents approximately 50-fold the amount of native enzyme found in rabbit or human brain extracts.

The levels of expressed rabbit and human brain tryptophan hydroxylase are similar. The specific activity of the expressed rabbit brain enzyme from an *E. coli* extract, measured in the presence of BH_4, was 1.51 ± 0.11 (mean ± SE) nmol min^{-1} mg^{-1}. This activity is considerably

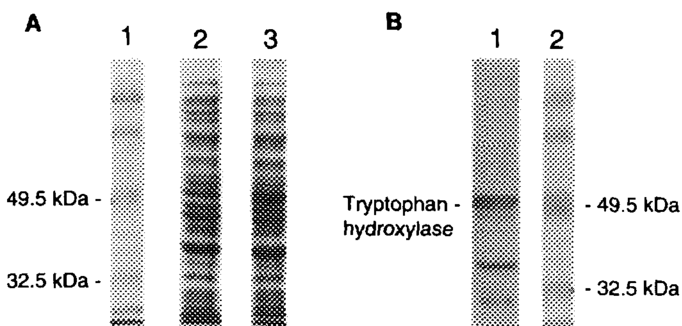

FIG. 3. SDS–PAGE and immunoblot analysis of expressed rabbit brain tryptophan hydroxylase. Lane 1 in A shows molecular weight markers. Sizes of specific markers are indicated. Lane 2 depicts a sample from a control *E. coli* lysate following gel electrophoresis. Lane 3 depicts a sample from a lysate transformed with tryptophan hydroxylase-containing plasmid. Lane 1 in B depicts an immunoblot of a similar sample. Lane 2 of B depicts transblotted molecular weight markers.

higher than that of the rabbit brain extract enzyme (0.04 ± 0.01 (mean ± SE) nmol min^{-1} mg^{-1}) assayed under similar conditions. The level of the expressed rabbit brain tryptophan hydroxylase (28.6 nmol min^{-1} g^{-1} (wet wt tissue)) compared to that of the rabbit brain extract (0.56 nmol min^{-1} g^{-1}) demonstrates that our expression system provided a 50-fold enhancement in the yield of the enzyme. The specific activity of the expressed human brain tryptophan hydroxylase, measured in the presence of BH_4, was 1.70 ± 0.02 (mean ± SE) nmol min^{-1} mg^{-1}. No comparable measurement is available for native human tryptophan hydroxylase, which has only been assayed in the presence of $6MPH_4$ (27). In order to allow comparison of our human data to those of Yamaguchi and co-workers (27), we have expressed our results in units of activity per gram of tissue (wet weight). The estimated yield of expressed human tryptophan hydroxylase activity measured in the presence of $6MPH_4$ was 15 nmol min^{-1} g^{-1}; this level represents about a 57-fold enhancement over the average activity of tryptophan hydroxylase from homogenates of several regions of human brain (0.26 nmol min^{-1} g^{-1}) and about a 10-fold enhancement over the value (1.5 nmol min^{-1} g^{-1}) reported for an isolated human raphe nucleus homogenate (27).

Properties of expressed enzymes. Ammonium sulfate fractionation of the expressed rabbit and human hydroxylases provided an approximately three-fold purification, with resulting specific activities of 4.84 ± 0.40 and 4.70 ± 0.19 nmol min^{-1} mg^{-1}, respectively. These fractions were used in kinetic analyses. Results of these studies are summarized in Table I. A comparison of the two expressed

TABLE I

A Comparison of Kinetic Parameters for the Expressed Human and Rabbit Brain Tryptophan Hydroxylases and the Native Human and Rabbit Brain Tryptophan Hydroxylases

	K_m or apparent K_m ($S_{0.5}$)* (μM)		
Enzyme source	BH_4	6-MPH_4	Tryptophan
Expressed human tryptophan hydroxylase	63	61	8*[,a] 7*[,b]
Expressed rabbit tryptophan hydroxylase	66	63*	12*[,a] 9*[,b]
Native human tryptophan hydroxylase[c]	—	216	142[b]
Native rabbit tryptophan hydroxylase[d]	31	67	50[a] 78[b]

Note. Specific assay conditions are described in the text.
[a] Assayed in the presence of BH_4.
[b] Assayed in the presence of 6-MPH_4.
[c] Values obtained from published results (27).
[d] Values obtained from published results (21).

enzymes indicates that they are almost identical with respect to kinetic parameters; both hydroxylases have similar K_m ($S_{0.5}$) values for tryptophan in the presence of either pterin cofactor and virtually identical K_m values for BH_4 or 6-MPH_4. As with the native rabbit tryptophan hydroxylase (21), both the rabbit- and human-expressed enzymes are inhibited by excess substrate in the presence of the natural cofactor, BH_4. The native human enzyme was never assayed with the natural cofactor (27). Our expressed human enzyme is slightly less responsive to increasing levels of tryptophan, in the presence of BH_4, than the expressed rabbit enzyme; at the highest level of tryptophan (1000 μM), the human hydroxylase was inhibited by approximately 35% compared to 65% inhibition of the rabbit enzyme (data not shown). Similarly, the expressed hydroxylases, like the native enzymes, are both inhibited by *p*-chlorophenylalanine (100 μM) in the presence of BH_4 (250 μM). The expressed rabbit and human enzymes were inhibited by about 57% and 53%, respectively (data not shown). These levels of inhibition are comparable to previously reported values (10).

A comparison of the expressed rabbit brain tryptophan hydroxylase to the native enzyme revealed many similarities and a few notable contrasts. The K_m value for BH_4 (66 μM) for the expressed enzyme falls within a range of observed values for the native enzyme of 31 μM (21) and 90 μM (28). Moreover, the apparent K_m for 6-MPH_4 for the expressed enzyme of 63 μM is essentially the same as the K_m for 67 μM reported for the native rabbit brain hydroxylase (21). The apparent K_m for tryptophan (12 μM) in the presence of BH_4 for the expressed rabbit brain enzyme is slightly lower than reported values of 20–50 μM for the native enzyme (21, 28, 29). In contrast, the apparent K_m for tryptophan of the expressed rabbit enzyme in the presence of 6-MPH_4 (9 μM) is only about 10% of the value reported for the native hydroxylase (78 μM). Although both the native and expressed rabbit hydroxylases have higher V_{max} values in the presence of BH_4, the V_{max} of the expressed rabbit enzyme is approximately two-fold higher than its V_{max} measured with 6-MPH_4, whereas the V_{max} of the native rabbit enzyme in the presence of BH_4 is only a 20% higher V_{max} than that obtained with 6-MPH_4 (data not shown). The expressed and native rabbit brain hydroxylases for the most part appear to share the same properties. The few exceptions noted above may reflect the relative lack of post-translational modification of the expressed enzyme obtained from *E. coli*. A more detailed examination of homogeneous preparations of both the expressed and native hydroxylases is required to assess the structural basis for these apparent differences in properties. Furthermore, since the immunoblot (Fig. 3) presents evidence of some degree of proteolysis, it is possible that the kinetic properties of the partially purified, expressed hydroxylase, as measured in this report, may reflect those of a mixture of proteolyzed and nonproteolyzed enzyme.

Kinetic comparisons between the expressed and native human brain tryptophan hydroxylases are less satisfying because data derived from assays with the natural cofactor, BH_4, are lacking for the native human hydroxylase, and because activity and kinetic data describing the native human brain enzyme are available from only a single study (27). Nonetheless, a comparison of the results obtained in the presence of 6-MPH_4 revealed some apparent differences between the native and expressed activities. The K_m values for both tryptophan and 6-MPH_4 for the expressed hydroxylase are lower than those reported for the native enzyme (27). As shown in Table I, the K_m for tryptophan for the native hydroxylase (assayed with 300 μM 6-MPH_4) is almost 20-fold greater than our apparent K_m determination for the expressed enzyme (assayed with 250 μM 6-MPH_4). Similarly, the K_m for 6-MPH_4 for the native enzyme (assayed with 400 μM tryptophan) is about 3.5-fold higher than our K_m value for the expressed human enzyme (assayed with 100 μM tryptophan). Again, it is likely that the expressed enzyme exists in a relatively unmodified state compared with the native hydroxylase. In addition, a more extensive study of both native and expressed human enzymes with the natural cofactor would shed more light on their apparent differences.

Sequence analysis of rabbit and human brain tryptophan hydroxylase clones. The nucleic acid and deduced peptide sequences of the cloned rabbit brain and human brain tryptophan hydroxylases are shown in Figs. 4 and 5, respectively. A comparison of the rabbit pineal (13) and rabbit brain cDNA sequences revealed disparities in 13 bases between the two sequences (99% identity). The deduced peptide sequences of the pineal and brain hydroxylases (99% identity) differ by disparities in six amino

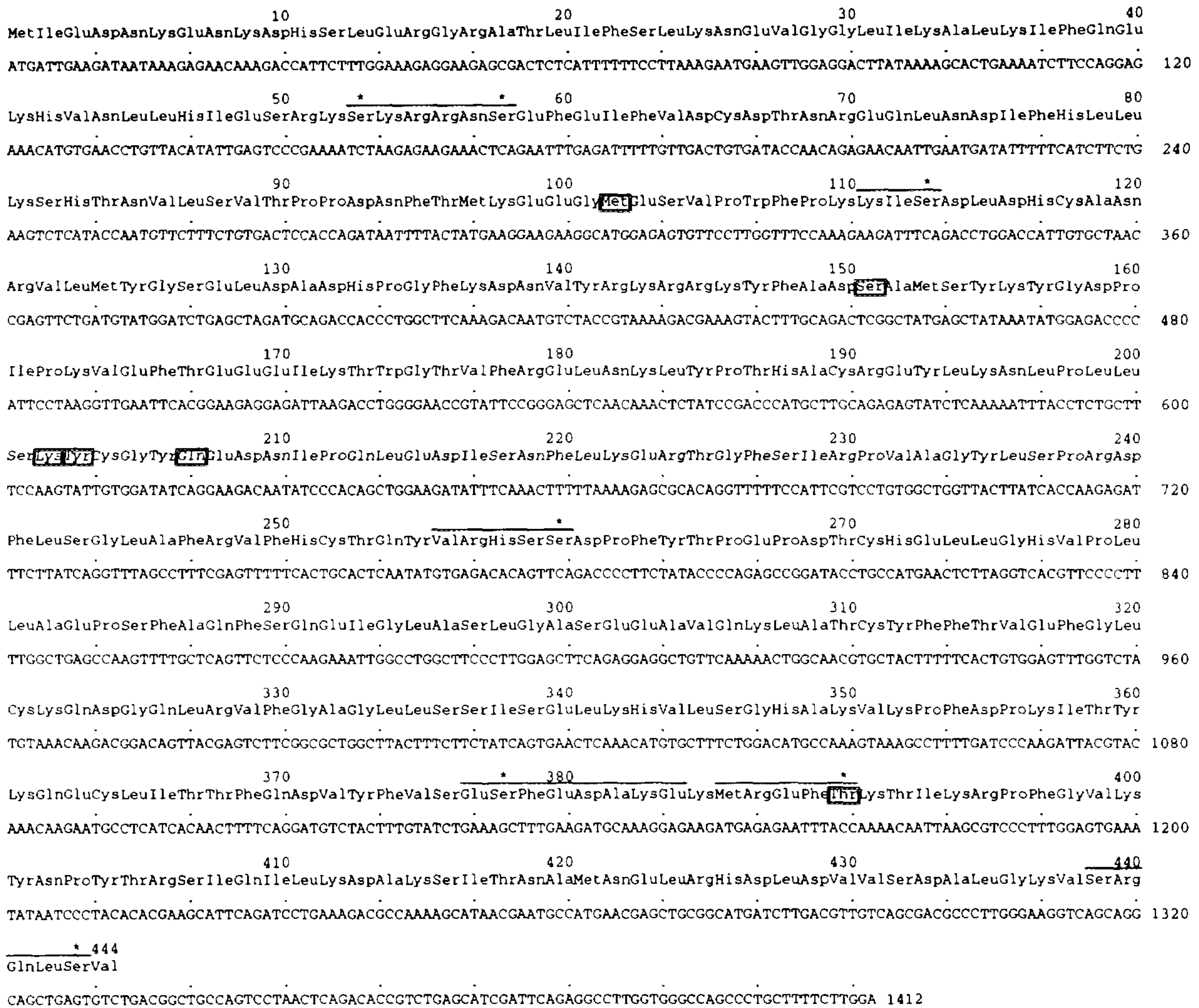

FIG. 4. Nucleic acid and peptide sequence of rabbit brain tryptophan hydroxylase. Boxed residues represent those amino acids in the rabbit brain tryptophan hydroxylase sequence which differ from the rabbit pineal sequence (13). The lines above the peptide sequence indicate residues comprising various protein kinase recognition motifs (described in text). An asterisk above specific residues indicates potentially phosphorylated amino acids.

acid residues (Fig. 4, boxed residues). The rabbit pineal and brain clones appear to be quite similar, if not identical. Presently, we have no evidence suggesting that the rabbit brain cDNA represents a unique clone. The observed differences between the rabbit pineal and brain clones could result from the occurrence of DNA polymorphisms or be due to sequencing discrepancies between laboratories. A more serious concern, however, is the possibility of base misincorporation errors (30) due to infidelity of the *Taq* polymerase during the amplification of the cDNA. An argument against this possibility is presented by the observation that all of the amino acid substitutions in the rabbit brain sequence, relative to the pineal sequence, are also found in at least one of the other clones of tryptophan hydroxylase (Fig. 6).

A comparison of the human brain and human carcinoid (17) tryptophan hydroxylase cDNA sequences revealed disparities between 99 base positions, resulting in a shared identity of 93%. The deduced peptide sequences for the two human hydroxylase clones differ by 18 amino acid residues (Fig. 5, boxed residues). Most of the amino acid disparities between the human brain and the human carcinoid tryptophan hydroxylase sequences represent conservative changes; thus, the two human peptide sequences share 96% identity. As was noted for the rabbit brain peptide sequence, the amino acid substitutions observed in the human brain tryptophan hydroxylase sequence were shared with at least one of the other tryptophan hydroxylase clones (Fig. 6), suggesting that these substitutions were not due to PCR-directed base misincorporation errors. Of interest, is the observation that the cloned rabbit brain cDNA and peptide sequences share 99%

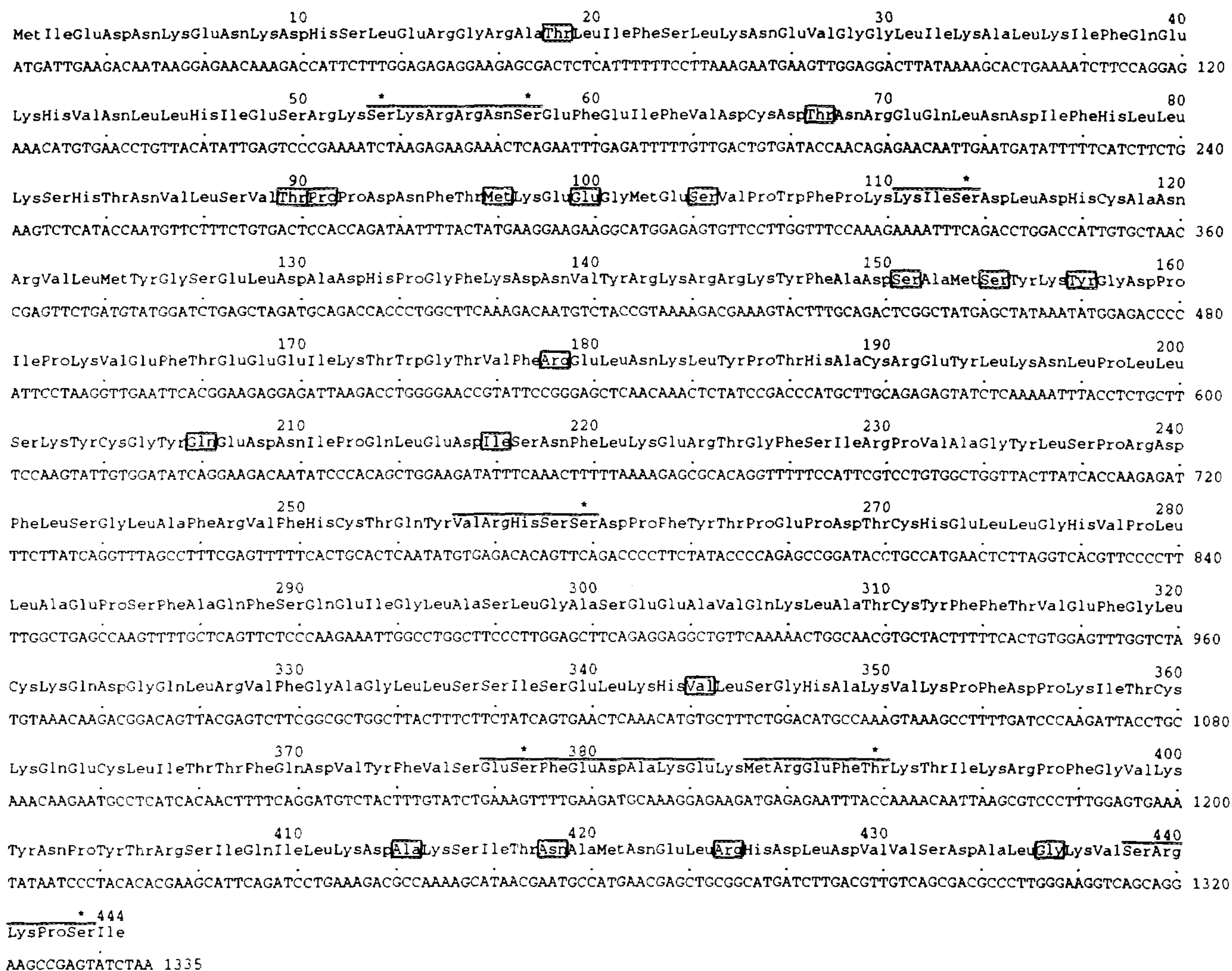

FIG. 5. Nucleic acid and peptide sequence of human brain tryptophan hydroxylase. Boxed residues represent those amino acids in the human brain tryptophan hydroxylase sequence which differ from the human carcinoid sequence (17). The lines above the peptide sequence indicate residues comprising various protein kinase recognition motifs (described in text). An asterisk above specific residues indicates potentially phosphorylated amino acids.

identity with the corresponding sequences of the human brain clone. Thus, the human brain clone appears to be more closely related to the rabbit brain and rabbit pineal clones than to the human carcinoid counterpart. Although our data do not prove that the human brain tryptophan hydroxylase cDNA is distinct from that of the human carcinoid clone, the differences presented here raise this possibility. In this regard it should be recalled that the human carcinoid clone was derived from a tumor cell line; therefore, the peptide sequence could reflect mutations of its DNA. Gross abnormalities in DNA derived from carcinoid tumors and in tumors producing biogenic amines have been observed (31).

A comparison of the known peptide sequences of mammalian clones of tryptophan hydroxylase (Fig. 6) discloses several common features. All forms of the enzyme share nine Cys residues. The mouse, rat, and two human forms of tryptophan hydroxylase share a 10th Cys residue at position 360.[6] Six of the nine shared Cys residues are also common to the other aromatic amino acid hydroxylases (not shown). Cys residues 66, 311, and 364 are unique to tryptophan hydroxylase cDNAs. Another interesting feature concerns the conservation among all six tryptophan hydroxylase clones of specific Leu residues (320, 327, 334, and 346) as candidates for involvement in "leucine-zip-

[6] The amino acid positions in Fig. 6 are offset by three residues due to the insertion sequence found in the mouse tryptophan hydroxylase sequence. Thus, for example, Cys 360, mentioned in the text, is located at position 363 in Fig. 6.

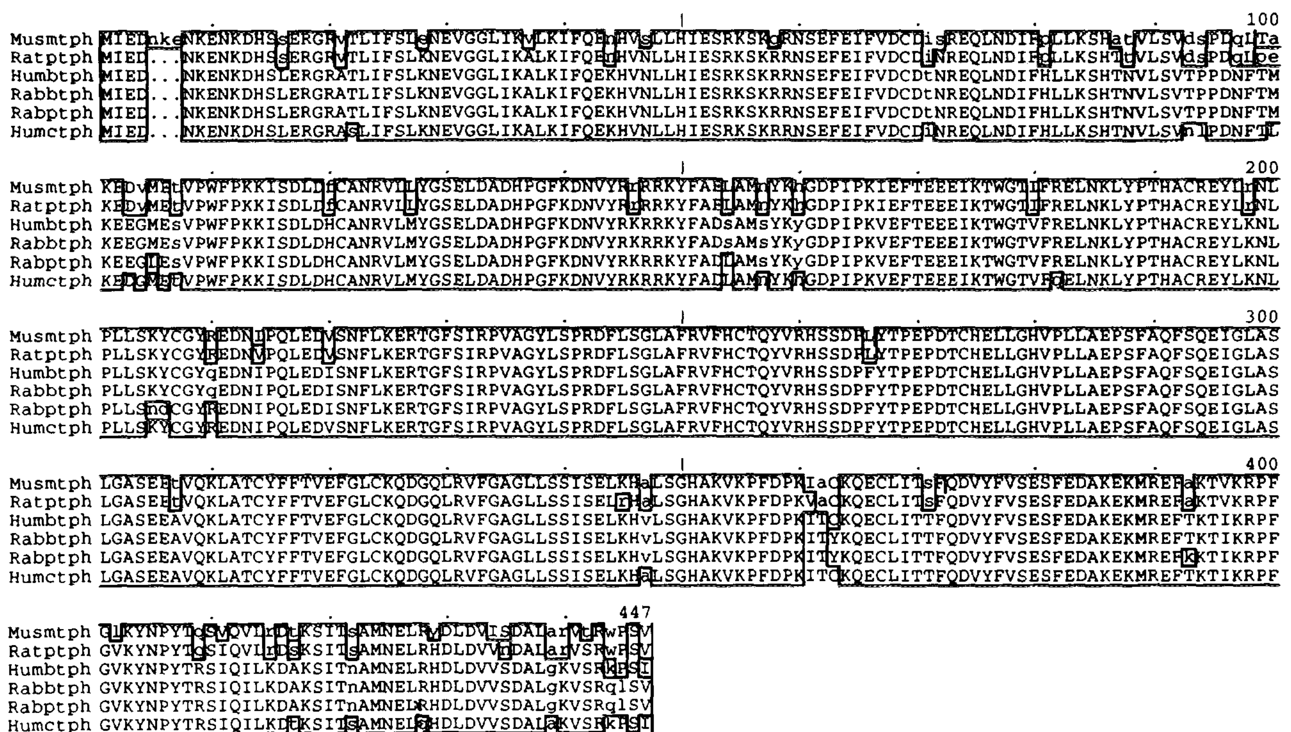

FIG. 6. Alignment of the peptide sequences of six tryptophan hydroxylase clones. Musmtph indicates murine mastocytoma tryptophan hydroxylase (16). Ratptph indicates rat pineal tryptophan hydroxylase (14), which is identical in sequence to the rat brain clone (15). Humbtph indicates human brain tryptophan hydroxylase. Rabbtph indicates rabbit brain tryptophan hydroxylase. Rabptph indicates rabbit pineal tryptophan hydroxylase (13), and Humctph indicates human carcinoid tumor tryptophan hydroxylase (17).

per" formations (32). Finally, analysis of the six tryptophan hydroxylase sequences for putative protein kinase recognition motifs (33) indicate that there are several such sites. Three Ser residues, previously identified in rat tryptophan hydroxylase (14) as consensus sites for cAMP-dependent protein kinase (Ser 58) and Ca^{2+}/Calmodulin-dependent protein kinase (Ser 260 and Ser 443), are also conserved in the rabbit and human brain clones (see highlighted regions in Figures 4 and 5). In addition, several other consensus sites are also conserved among all six clones of tryptophan hydroxylase; these include a potential protein kinase C site at Ser 53, a consensus cAMP-dependent protein kinase site at Ser 113, and a casein kinase 2 site at Ser 378 (Figs. 4 and 5). The presence of several shared, putative phosphorylation sites in all six tryptophan hydroxylase clones suggests possible regulation of the enzyme by coordinated multisite phosphorylation as has been observed for tyrosine hydroxylase (34–36).

In summary, tryptophan hydroxylase from both rabbit and human brain has been cloned in *E. coli*. These expressed enzymes exhibit many properties associated with the native enzymes, including appropriate subunit molecular weights, several shared kinetic properties, and similar responses to inhibition by substrate and the known inhibitor, *p*-chlorophenylalanine. Furthermore, these clones provide the first enriched source of brain tryptophan hydroxylase and the only enriched source of any form of the human enzyme and should facilitate the purification and further characterization of neuronal tryptophan hydroxylase.

ACKNOWLEDGMENTS

We thank Dr. Michael Brownstein for synthesis of several DNA oligomers and Dr. Nancy Rance and Dr. John Smylek for kindly providing human brain tissue. We are greatful to Ms. Cindy Falke for technical assistance and to Dr. Yuehua Wang and Ms. Margot Gibson for helpful discussions. We are also greatful to Dr. Eduardo Padlan for helpful discussions and advice.

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