Phosphorylation of Human Recombinant Tyrosine Hydroxylase Isoforms 1 and 2: An Additional Phosphorylated Residue in Isoform 2, Generated Through Alternative Splicing*

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The single human tyrosine hydroxylase (TH) gene generates four different mRNA species through alternative splicing events. TH-1 and TH-2 mRNAs are expressed mostly in the brain. We have produced large amounts of the corresponding proteins in Escherichia coli to analyze their respective molecular characteristics. The polypeptides have molecular weights similar to those of TH expressed in Xenopus oocytes and react with antibodies to TH. The two isoforms were purified with a purity of 90% using a three-step procedure. The phosphorylation sites have been determined in the two isoforms after labelling with [γ-32P]ATP in the presence of cAMP-dependent protein kinase (PKA) or calmodulin-dependent protein kinase II (CaM-PK II). In both isoforms, Ser-40 was found to be phosphorylated by PKA, and Ser-19 and Ser-40 were found to be phosphorylated by CaM-PK II. The putative phosphorylation site generated by alternative splicing (Ser-31) was phosphorylated specifically by CaM-PK II in TH-2 only. The kinetic properties of the two isoforms in the presence of various concentrations of the substrate (tyrosine) and of the natural cofactor ((6R)-tetrahydrobiopterin) were also analyzed. TH produced in E. coli is unphosphorylated but nevertheless active. At 50 μM tyrosine and 300 μM (6R)-tetrahydrobiopterin, the specific activities of TH-1 and TH-2 are 1300 and 620 nmol of dihydroxyphenylalanine/min/mg, respectively. Phosphorylation of TH-1 and TH-2 by PKA activates both isoenzymes as shown by the increase in the affinity for the cofactor. No changes in kinetic parameters of the isoenzymes were observed after phosphorylation by CaM-PK II. Phosphorylation of specific residues in the regulatory domain of TH activity and the adjacent serine (Ser-31) represents a regulatory domain. Phosphorylation of specific residues in this domain has been shown to modulate TH activity and form the basis for the short term regulation of the enzyme. These phosphorylations involve multiple kinases, such as the cyclic AMP-dependent protein kinase (PKA) and the calmodulin-dependent protein kinase II (CaM-PK II), and several phosphorylated serine residues have been identified in vitro and in vivo (5, 7–9). In addition to this posttranslational activation, an induction mechanism that involves the synthesis of new TH molecules has also been shown to modulate TH activity in vivo (10–12).

In humans, additional regulation may also occur through alternative splicing events. Indeed four different TH-mRNAs (TH-1 to TH-4) have been characterized in human pheochromocytoma tumors (13, 14). These forms differ by the combinational insertion in the amino-terminal regulatory domain of two exons coding for 4 and 27 amino acid residues, respectively. S1 mapping analysis has revealed that the distribution of these four mRNAs is tissue-specific (13, 14). TH-1 and TH-2 being the predominant forms in the locus coeruleus and the substantia nigra of the human brain. It also should be noted that the insertion of the four additional amino acids in TH-2 generates a consensus sequence for CaM-PK II (-R-X-X-S-) (15), and the adjacent serine (Ser-31) represents a putative site of phosphorylation by CaM-PK II (Fig. 4). Such

Tyrosine hydroxylase (TH, tyrosine 3-monooxygenase, EC 1.14.16.2), the key enzyme in the biosynthesis of catecholamine, is a tetramer composed of four subunits with similar sequences (1, 2). Each subunit consists of at least two structural domains. Biochemical data from the bovine enzyme and analysis of the primary structure deduced from the rat TH-cDNA nucleic sequence have revealed that the active site is located in the carboxyl-terminal region of the protein (3, 4). The amino-terminal region, which has been demonstrated to contain several sites of phosphorylation (5, 6), represents a regulatory domain. Phosphorylation of specific residues in this domain has been shown to modulate TH activity and form the basis for the short term regulation of the enzyme. These phosphorylations involve multiple kinases, such as the cyclic AMP-dependent protein kinase (PKA) and the calmodulin-dependent protein kinase II (CaM-PK II), and several phosphorylated serine residues have been identified in vitro and in vivo (5, 7–9). In addition to this posttranslational activation, an induction mechanism that involves the synthesis of new TH molecules has also been shown to modulate TH activity in vivo (10–12).

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a differential splicing of the TH-pre-mRNA has been excluded in rat or bovine tissues (16, 17).

The kinetic properties of the TH isoforms require individual purification. This analysis is not possible using the isoenzymes from tissues, because the different TH isoforms have similar molecular weights and more than one form is expressed in all catecholaminergic tissues analyzed to date. In this paper, we have circumvented this difficulty by producing the TH-1 and the TH-2 isoforms in Escherichia coli using a bacterial expression system developed by Studier and Moffatt (18) in which the desired recombinant cDNA is transcribed with the T7 RNA polymerase. An additional advantage of this system is that eukaryotic proteins are not subjected to phosphorylation because bacteria lack most of the known mammalian protein kinases. Milligram amounts of both isoenzymes were purified and were found to display high TH specific activity when reconstituted with iron. To better elucidate the role of phosphorylation events in modulating the TH activity in humans, we have determined the kinetic parameters of TH-1 and TH-2 unphosphorylated and following phosphorylation by PKA or CaM-PK II. Thymidine acid sequence data for the phosphorylation site generated by alternative splicing in TH-2 is phosphorylated in vitro by the CaM-PK II.

**EXPERIMENTAL PROCEDURES**

**Construction of Tyrosine Hydroxylase Expression Vectors**—To express human TH in E. coli, CDNAS encoding TH-1 and TH-2 were inserted downstream from a T7 promoter between the NdeI and BamHI restriction sites of pET-3a (19), a bacterial expression vector (Fig. 1). To obtain the appropriate distance between the Shine-Dalgarno sequence that controls the initiation of translation in prokaryotes and the first TH-AUG codon, an NdeI restriction site was created 5’ adjacent to this codon. This modification was performed by site-directed mutagenesis as described previously (20) using a 25-mer mutagenic oligonucleotide (5’-GAATTCACCTGAGATGATGACCACCC-3’), the modified nucleotides are underlined. The effectivness of the actual mutation was verified by DNA sequence analysis after the termination method (21). The pET-TH constructions were introduced in E. coli BL21(DE3) (18, 19). In this system, the pl35S plasmid encoding lazyme also was present to control the basal activity of the T7 RNA polymerase (22).

**Expression and Purification of Recombinant Human Tyrosine Hydroxylase Isoforms**—Expression of TH, BET121 (20) containing the TH-expression vectors as well as the T7 RNA polymerase genes fuses to a lac operator and promoter were grown in LB broth with ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) at 37°C. At 0.6 A600, induction of expression was initiated by adding isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. Cells were harvested 2 h later and resuspended in 1 mM dithiothreitol, 1 mM MgSO4, 0.2 mM phenylmethylsulfonyl fluoride, 100 μg/ml of DNase, and 20 mM Tris-HCl, pH 7.5. The cells were lysed by sonication at 4°C and centrifuged for 40 min at 20,000 x g. Supernatants were loaded onto a DEAE-Sepharose column (15 x 2.6 cm, Pharmacia) equilibrated with 1 mM dithiothreitol, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, column buffer. The columns were washed with 3 bed volumes of the same buffer. TH isoforms were eluted with a linear gradient from 0 to 0.3 M NaCl in the column buffer. A major peak of TH activity was eluted at about 0.2 M NaCl. The corresponding fractions were applied on a heparin-Sepharose column (1 X 10 cm, Pharmacia) equilibrated with the same column buffer. TH isoforms were eluted with a linear gradient from 0.1 to 0.4 M KCl in the column buffer. Fractions containing TH activity were pooled (30 ml) and concentrated to 3 ml by ultrafiltration (CX 30, Millipore). The concentrated solution was applied on a ACA-34 Ultracel column (1.6 x 60 cm, IBF, Villelave-la-Garenne, France) equilibrated with 150 mM NaCl, 20 mM Tris-HCl, pH 7.5. About 10 and 7 mg of TH-1 and TH-2 were obtained, respectively, and kept at ~80°C until use. Protein concentrations were determined according to the method of Bradford (23) using bovine serum albumin as the standard.

**Tyrosine Hydroxylase Enzymatic Activity**—TH activity was assayed by measuring the amount of [3H]H2O formed from [3H]tyrosine. 1-35-[3H]Tyrosine (56 Ci/mmol; Commissariat à L’Energie Atomique, Saclay, France) was purified using HPLC (24). The enzymatic assay was performed as described (25) with some modifications. Standard assay conditions were 0.1 μg of purified TH in the presence of 50–200 μM L-tyrosine (Sigma) and 500,000 cpm of [3H]tyrosine, 500 μM (6R)-tetrahydrobiopterin (BH4, Schircks Laboratory, Jona, Switzerland), 100 μM ammonium sulfate, 100 μM MES, pH 7.0, in a volume of 0.1 ml. The reaction was started by adding BH4 and was stopped after 15 min at 37°C by adding 1 ml of 1 M HCl containing 7.5% of activated charcoal (Prolabo, Paris, France), according to the method of Reinhard et al. (26). After centrifugation, an aliquot (220 μl) was mixed with 5 ml of a scintillation mixture (aqueous counting scintillant, Amerham Corp.) to measure the amount of [3H]H2O formed by [3H]tyrosine hydroxylation. TH activity is expressed in nanomoles of [3H]H2O formed per min per mg of protein.

**Phosphorylation of Human Tyrosine Hydroxylase Isoforms**—Phosphorylated isoforms were phosphorylated with either PKA or CaM-PK II. Conditions used in the phosphorylation reaction (total volume 30 μl) were 0.5 mM EDTA, 50 mM NaF, 10 mM MgCl2, 25 mM Hepes, pH 7.0, 2 μg of purified TH isoforms, 2 mM ATP (magnesium salt, Sigma) and γ-[32P]ATP (1500 cpm/pmole, Amersham), appropriate amounts of the purified kinase preparation and incubation for 30 min at 30°C. In addition, 1 mM CaCl2 and 50 μg/ml calmodulin were included in the CaM-PK II assay, and 0.5 mM EGTA was included in the PKA assay. Purified protein kinases were used in the following amounts: 10 μg of the catalytic subunit of PKA (from bovine heart, Sigma) and 10 μg of CaM-PK II (from bovine heart, provided by Dr. S. El Mestikawy, C. N. R. S.). Using 10 μl of CaM-PK II, 0.25 mol of phosphate could be incorporated per mol of histone. In both cases, the reactions were initiated by the addition of ATP and stopped by adding 0.8 ml of 25% (w/v) trichloroacetic acid containing 50 μg/ml bovine serum albumin. The tubes were centrifuged and pellets were washed twice with 25% trichloroacetic acid. Total phosphate incorporation into TH isoforms were determined by Cerenkov counting.

**Isolation of Peptides and Amino Acid Sequencing**—About 100 μg of phosphorylated TH were submitted to exhaustive trypsic digestion for 18 h at 37°C (1% of porcine pancreas trypsin w/w, Sigma). Anion exchange chromatography was used for the first screening of radiolabeled acidic peptides (27). Radiolabeled peptides were purified first by an aquapore anion exchange column (2.1 mm inside diameter, 10 cm length, Brownlee) equilibrated with 20 mM Tris-HCl, pH 7.5. Peptides were eluted with a linear gradient from 0 to 500 mM NaCl. The peptides were analyzed further with a Brownlee microgradient HPLC equipped with a C8 reverse phase column (2.1 mm inside diameter, 22 cm length) equilibrated in 0.1% (v/v) trifluoroacetic acid and developed with a linear gradient from 0 to 80% water/acetonitrile in the presence of 0.1% trifluoroacetic acid. An automatic gas-liquid liquid chromatometer (Applied Biosystem, CA) was used for Edman degradation. Amino acid sequences were analyzed with an on-line phenylthiohydantoin analyzer (120 A, Applied Biosystem).

**RESULTS**

**Expression and Purification of TH**

A bacterial expression system was used to purify large amounts of TH-1 and TH-2. Each cDNA coding sequence was placed under the control of the bacteriophage T7 late promoter (Fig. 1) at the appropriate distance from a Shine-Dalgarno signal to optimize the efficiency of translation in the pET-3a expression vector (18, 19). In this system, the T7 RNA polymerase expression is induced with isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. Cells were harvested 2 h later and resuspended in 1 mM dithiothreitol, 1 mM MgSO4, 0.2 mM phenylmethylsulfonyl fluoride, 100 μg/ml of DNase, and 20 mM Tris-HCl, pH 7.5. The cells were lysed by sonication at 4°C and centrifuged for 40 min at 20,000 x g. Supernatants were loaded onto a DEAE-Sepacel column (15 x 2.6 cm, Pharmacia) equilibrated with 1 mM dithiothreitol, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, column buffer. The columns were washed with 3 bed volumes of the same buffer. TH isoforms were eluted with a linear gradient from 0 to 0.3 M NaCl in the column buffer. A major peak of TH activity was eluted at about 0.2 mM NaCl. The corresponding fractions were applied on a heparin-Sepharose column (1 X 10 cm, Pharmacia) equilibrated with the same column buffer. TH isoforms were eluted with a linear gradient from 0.1 to 0.4 M KCl in the column buffer. Fractions containing TH activity were pooled (30 ml) and concentrated to 3 ml by ultrafiltration (CX 30, Millipore). The concentrated solution was applied on a ACA-34 Ultracel column (1.6 x 60 cm, IBF, Villelave-la-Garenne, France) equilibrated with 150 mM NaCl, 20 mM Tris-HCl, pH 7.5. About 10 and 7 mg of TH-1 and TH-2 were obtained, respectively, and kept at ~80°C until use. Protein concentrations were determined according to the method of Bradford (23) using bovine serum albumin as the standard.
Promoter and T7 transcription terminator is inserted in the BamHI site.

A NdeI-EcoRI fragment including the modified first ATG of the coding sequence (NdeI restriction site) and the 3' untranslated region of the full-length human TH-1 and TH-2 cDNAs were cloned into the NdeI-BamHI sites of the pET-3a plasmid. The ase expression vector: PET-TH. A NdeI-EcoRI fragment including the modified first ATG of the coding sequence (NdeI restriction site) and the 3' untranslated region of the full-length human TH-1 and TH-2 cDNAs were cloned into the NdeI-BamHI sites of the pET-3a plasmid. The pET-3a plasmid. The ase expression vector: PET-TH.

To determine optimal phosphorylation conditions, the TH isoforms were incubated for 30 min at 30 °C with PKA and CaM-PK I1 followed by trypsic digestion and reverse phase HPLC analysis, and the amino acid sequences of phosphorylated peptides were performed as described under Experimental Procedures. After phosphorylation of TH-1 and TH-2 in the presence of PKA, radioactivity incorporated in each of the TH isoforms was recovered as a single peak for both TH isoforms corresponding to a single peptide (peptide A: RGSESSLIEDAR). After phosphorylation of TH-1 and TH-2 in the presence of CaM-PK II, incorporated radioactivity was found in two and three peptides derived from TH-1 and TH-2, respectively. Two of these peptides are present in both TH isoforms and correspond to peptide A and peptide B (AVSELDA...). The third peptide, present only in TH-2, corresponds to the phosphorylation site generated by alternative splicing (peptide C: CQSPR). Peptides A, B, and C correspond to the phosphorylation of the three serine residues, 40, 19, and 31, respectively (numbers corresponding to TH-1 amino acids, represented in Fig. 4).

Kinetic Parameters of Human Tyrosine Hydroxylase Isoforms

To be as close as possible to physiological conditions, the kinetic properties of TH isoforms were analyzed with the natural cofactor BH, as substrate preferentially to a biopterin analog as used previously (20). As shown in Fig. 5, the activities of both isoforms were maximal at pH 7.0, in agreement with other studies (28, 29). All following experiments have been performed at this pH. In these conditions, both isoforms were activated by incubation with 0.1 mM ferrous ammonium sulfate (for more detail see Ref. 30).

Determination of the Km and Vmax Values for Tyrosine and BH4 of TH-1 and TH-2—The effect of increasing tyrosine concentrations (from 6 to 100 μM) was tested at 0.2 mM BH4. Substrate inhibition is seen above 60 μM tyrosine with both TH-1 and TH-2 (data not shown). This substrate inhibition...
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FIG. 4. Position of the residues phosphorylated by PKA (closed triangle) and CaM-PK II (asterisk) in TH-1 and TH-2 proteins. The numbering of the phosphorylated serines is related to the amino acids sequence of TH-1. The position of the active site is indicated (AS).

FIG. 5. pH dependence of TH-1 and TH-2 activities. TH-1 (filled circle) and TH-2 (open circle) activities were measured in 0.1 mM MES buffer at varying pH values. Saturating concentration of BH4 (200 µM) and tyrosine (200 µM) were used in these experiments.

has been reported in the presence of the natural cofactor with the rat TH activity (29, 31, 32). At tyrosine concentrations of less than 60 µM, TH-1 and TH-2 exhibited typical Michaelis-Menten kinetics, as shown in Fig. 6A by a Lineweaver-Burk analysis performed with a nonlinear regression data analysis program (Enzfit, Elsevier Biosoft, Amsterdam, The Netherlands). The $K_m$ values for tyrosine of TH-1 and TH-2 were 37 and 17 µM, respectively, and represent significantly different affinities (Table I).

Then TH activity was measured at 50 µM tyrosine with increasing concentrations of BH4 ranging from 10 to 300 µM. A typical Michaelis-Menten kinetics was observed, as shown by the Lineweaver-Burk analysis (Fig. 6B). TH-1 and TH-2 exhibited almost similar $K_m$ values for BH4 (73-95 µM) but displayed significantly different $V_{max}$ values (1300 and 620 nmol of DOPA/min/mg; Table I).

Effect of Phosphorylation of TH-1 and TH-2 on Their Affinity for BH4—Using the same conditions of assay, the effect on enzyme kinetics of the phosphorylation by PKA and CaM-PK II was determined. The $K_m$ and $V_{max}$ values were measured for each TH with different concentrations of BH4, from 10 to 300 µM at 50 µM tyrosine. The kinetic parameters for TH-1 and TH-2 (results from three independent experiments) are compared in Table II. Following phosphorylation with PKA, both enzymes showed a decrease in their $K_m$ for the bioperin cofactor (from 95 to 51 µM for TH-1 and from 73 to 48 µM for TH-2), but their $V_{max}$ values were unchanged. No change in the affinity for BH4 or in the $V_{max}$ values was observed for either enzyme after Ca2+/calmodulin-dependent phosphorylation.

Effect of Phosphorylation on TH Inhibition by Dopamine

Previous studies on the rat TH (31, 33, 34) have shown that the feedback inhibition of TH activity by catecholamines is decreased after phosphorylation by PKA, suggesting that this phosphorylation modifies the TH-catecholamines binding (35). This effect is reflected by a 3- to 7-fold increase in the $K_i$ or dopamine of the rat TH.

The $K_i$ values for dopamine were determined for each of the two isofoms by Dixon plots (36) at 50 µM tyrosine. In these conditions, the effects of increasing concentrations of dopamine (from 0 to 10 µM) were analyzed on both the unphosphorylated and phosphorylated enzymes at different fixed concentrations of BH4 (0, 10, 25, 75 µM). The results of

![FIG. 6. Lineweaver-Burk plots of TH-1 (filled circle) and TH-2 (open circle) activity as a function of BH4 concentration in the presence of 50 µM tyrosine (A) and tyrosine concentration in the presence of 200 µM BH4 (B). Each point represents the mean of duplicate samples.](image-url)

**TABLE I**

|       | $K_m$ (µM) | $V_{max}$ (nmol DOPA/min/mg) |
|-------|------------|------------------------------|
| TH-1  | 37 ± 2     | 1300 ± 43                    |
| TH-2  | 17 ± 2     | 620 ± 2                      |

* Determined at 50 µM tyrosine and 300 µM BH4.

**TABLE II**

|       | $K_m$ (µM) | $V_{max}$ (nmol DOPA/min/mg) |
|-------|------------|------------------------------|
| TH-1  | 73 ± 5     | 1300 ± 43                    |
| TH-2  | 95 ± 5     | 620 ± 2                      |

* Determined at 50 µM tyrosine at pH 7.0.

* Determined at 200 µM BH4 at pH 7.0.
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Table II

Summary of the kinetic properties and the dopamine inhibition of TH-1 and TH-2 after phosphorylation by PKA and CaM-PK II

Results represent the mean ± SE from three experiments, except in the case of K, values for dopamine which were determined at 50 μM tyrosine and at 10, 25, and 75 μM BH, using seven different concentrations of dopamine.

| Isoform          | BH, K, μM | Vmax, nmol DOPA/min/mg | Dopamine, K, μM |
|------------------|-----------|------------------------|-----------------|
| TH-1 nonphosphorylated | 94.8 ± 2.7 | 1300 ± 43 | 0.5 ± 0.2 |
| TH-1 phosphorylated by PKA | 51.5 ± 2.4 | 1235 ± 43 | 1.5 ± 0.2 |
| TH-1 phosphorylated by CaM-PK II | 98.1 ± 2.0 | 1226 ± 10 | 0.5 ± 0.2 |
| TH-2 phosphorylated | 72.9 ± 2.9 | 620 ± 2 | 0.3 ± 0.1 |
| TH-2 phosphorylated by PKA | 48.4 ± 0.9 | 624 ± 35 | 1.2 ± 0.2 |
| TH-2 phosphorylated by CaM-PK II | 74.4 ± 14.6 | 644 ± 44 | 0.7 ± 0.1 |

the experiments analyzed by Dixon plots are compared in Table II. The phosphorylation by PKA increased the K, from 0.5 to 1.5 μM and from 0.3 to 1.2 μM for TH-1 and TH-2, respectively. The phosphorylation by CaM-PK II did not modify the K, value for TH-1, but the K, value of TH-2 was increased from 0.3 to 0.7 μM.

Discussion

Purification of the TH-1 and TH-2 Isoenzymes—In this paper we have described the purification of the four isoenzymes of human tyrosine hydroxylase, TH-1 and TH-2, which are encoded by the same gene and whose diversity originates through alternative splicing. TH-1 and TH-2 are present in the highest concentrations in human catecholaminergic tissues of the brain such as locus ceruleus and substantia nigra, as well as in the adrenal glands. The use of a bacterial expression system allowed the production of milligram amounts of the recombinant TH-1 and TH-2 proteins. A polyclonal rabbit antibody prepared against a rat TH recognized both the recombinant TH-1 and TH-2 isoenzymes and the native human enzyme (from pheochromocytoma) (Fig. 2). The purified recombinant proteins corresponded to tetramers as indicated by gel filtration analyses (data not shown). Each TH-1 and TH-2 subunit has an apparent molecular mass of 62 and 64 kDa, respectively, in agreement with previous observations in Xenopus oocytes (1, 20). Identical molecular weight protein species are detected in Western blot analysis of human pheochromocytoma proteins (Fig. 2), illustrating the diversity of TH isoforms in the tumor, as suggested previously (13, 14). The kinetic parameters of both isoenzymes are consistent with the K, values described previously for human native TH (37, 38). It is interesting to note that the K, values of the purified human TH isoforms produced in E. coli are significantly higher than the K, of purified rat TH produced using the baculovirus system (29). Although the isolated TH enzymes correspond to two different species, the amino acid sequences of the human TH-1 and the single rat enzyme are strongly homologous, suggesting that this difference might result from the different systems used to express the enzymes. It is noteworthy that in the baculovirus system, the rat enzyme is phosphorylated completely (29), whereas in E. coli the enzymes are not phosphorylated. This hypothesis is confirmed by the decrease in the K, values following phosphorylation of the TH isoforms by one protein kinase, PKA. Taken together, these results indicate that the recombinant TH isoforms produced in bacteria display biological characteristics of the native enzymes. Similar findings have been reported by Ledley et al. (39) when expressing human phenylalanine hydroxylase in bacteria.

Analysis of the Kinetic Parameters of Human TH Isoforms—The enzymes produced in bacteria are not phosphorylated (data not shown) but have high specific activities. Therefore phosphorylation events are not required to initiate TH activity, clarifying thereby an unresolved issue. Indeed, in some instances, purification of rat TH has revealed the existence of an inactive pool of TH which was attributed to unphosphorylated forms of the enzyme (40, 41). The molecular basis of this phenomenon therefore might rely on other associated mechanisms.

Previous data have shown that the specific activities, measured in the presence of a bipterin analog, of the different isoforms expressed in oocytes (20) as well as in COS cells (42) differed and that the activity of TH-1 was higher than that of TH-2. We have chosen to study the kinetic parameters of the isoenzymes in the presence of the natural cofactor, BH, to mimic physiological conditions. In this situation, substrate inhibition was observed above 60 μM tyrosine with both TH isoforms, as found previously with the rat enzyme (29, 32). Our determinations of kinetic parameters confirm and extend the initial observations in the oocyte and COS systems (20, 42) and show that, in the presence of BH, the Vmax value of purified TH-1 is higher than that of purified TH-2 (Table I). Interestingly, TH-2, which exhibits the lowest activity (Fig. 2), is translated from the most abundant TH-mRNA species in the catecholaminergic tissues, as determined by S1 mapping experiments (13, 14). However, in the presence of BH, the affinity of TH-2 for tyrosine is higher than that of TH-1. The physiological implication of this observation is unclear because tyrosine concentration does not seem to be a limiting factor in biosynthesis of catecholamines (32).

The K, values for dopamine of the nonphosphorylated TH-1 and TH-2 (0.5 and 0.3 μM) were lower than those reported for the native enzymes (600-fold lower than that of human TH isolated from pheochromocytoma (37) and 12- to 20-fold lower than that of rat striatal TH (31, 33)). This difference may be attributed to the inhibition by endogenous catecholamines in physiological conditions. This inhibition probably does not occur in our study, because E. coli contain no catecholamines. In support of this observation, solutions of purified recombinant TH-1 and TH-2 remain unconcoled (30), in contrast to the typical blue-green color observed in purification of native TH, which is attributed to a charge-transfer interaction between catecholamines and the Fe(III)-TH complex (35).

Effect of Phosphorylation on Kinetic Properties of TH Isoforms—Phosphorylation of rat and bovine TH have been observed in various conditions including electrical stimulation, neuroleptic treatment and unilateral electrolytical de-structure (10, 40, 43). These phosphorylations have been shown to be mediated by second messengers such as Ca2+ and cAMP, and at least six various protein kinases, including PKA and CaM-PK II, have been reported to phosphorylate rat TH in vitro (7, 8). In the present study we have focused on our analyses on TH phosphorylation by PKA and CaM-PK II, two kinases which have been shown to be involved in the activation of the rat TH enzyme in vivo, their effects being additive (44). This activation of the enzyme results from an increase in its affinity for the cofactor (31, 33, 45, 46), as well as from a decrease in the feedback inhibition by catechols (31, 33, 34). The availability of recombinant TH protein in a nonphosphorylated state and without the presence of cate-
cholamine provided us with a convenient system to analyze the effect of kinases as well as the feedback inhibition.

In our in vitro study of the human enzymes, we have determined that, in both TH-1 and TH-2, Ser-19 is phosphorylated only by CaM-PK II, whereas both PKA and CaM-PK II phosphorylate Ser-40. These residues have also been reported to be phosphorylated in vitro and in vivo in rat TH (5, 8, 9), reflecting the conservation of the consensus amino acid sequences of both phosphorylation sites in rat and human.

However, most importantly, in addition to these two sites, in humans, Ser-31 is phosphorylated by CaM-PK II in TH-2 but not in TH-1. This additional site results from the presence of an arginine residue within the insertion of four amino acids adjacent to the Ser-31 of TH-2. Thus, alternative splicing may affect the susceptibility to post-translational modifications of the enzyme by CaM-PK II. A similar situation has been observed, for example, in the mouse major histocompatibility antigen H-2Dd (47).

The CaM-PK-dependent phosphorylation caused an increase in the affinity of both TH-1 and TH-2 for the cofactor, as revealed by a 2-fold decrease in the $K_\text{M}$ values, whereas no change in the $V_{\text{max}}$ values was observed. We observed a 3- to 4-fold increase in the $K_\text{M}$ for dopamine in the inhibition of TH-1 and TH-2, an inhibition known to be competitive with BH$_2$ in humans (35). Similar results have been observed with the rat enzyme (29, 46), suggesting that the physiological mechanisms described in the rat to modulate the CaM-PK-dependent phosphorylation of TH also operate in humans.

The phosphorylation of the two human TH isoforms by CaM-PK II does not modify their $K_\text{M}$ values for the cofactor, but in vivo in the rat striatum this kinase has been shown to mediate the effect of depolarization by potassium (44). This discrepancy may be explained in the context of the findings of Atkinson et al. (48) and of Yamauchi et al. (49) which showed that the effect of the kinase requires in vitro the presence of an activator protein recently characterized (50). Further experiments using such an activator factor will be required to establish whether the additional phosphorylation in TH-2 does activate this form.

Studies of the affinity for dopamine have revealed further differences between TH-1 and TH-2 following phosphorylation by CaM-PK II. The phosphorylation of TH-1 by CaM-PK II does not modify the $K_\text{M}$ value for dopamine as described for the rat enzyme, whereas a 2-fold increase in the $K_\text{M}$ for dopamine was observed for TH-2. Therefore, the introduction of four amino acids in TH-2 modified not only the $V_{\text{max}}$ of the reaction to produce catecholamines but also its short term reaction to potassium (44). This study of the human enzymes, we have revealed that the phosphorylation of the reactions which confer susceptibility to the disease is located close to or within the TH gene. Genetic defects may alter the posttranscriptional control of the TH gene, its splicing pattern, as well as the sequences which play a role in the phosphorylation of the protein.

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