Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8779–8783, October 1991
Biochemistry

High-level expression of rat PC12 tyrosine hydroxylase cDNA in *Escherichia coli*: Purification and characterization of the cloned enzyme

(pheochromocytoma/molecular cloning/protein phosphorylation)

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ABSTRACT **A rat cDNA containing the complete coding sequence for rat tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2) was isolated from a rat PC12 cDNA library and subcloned in a bacterial expression plasmid, and large amounts of functional enzyme were produced in *Escherichia coli*. The recombinant enzyme was purified approximately 20-fold to a final specific activity of 1.8 μmol/min per mg of protein, with a yield of 30%. As much as 1 mg of pure protein could be obtained from 1 g of wet bacterial cells. The purified hydroxylase was shown to be homogeneous by denaturing polyacrylamide electrophoresis and isoelectric focusing. Amino acid analysis of the N terminus (25 residues) revealed 100% identity with rat PC12 tyrosine hydroxylase, as deduced from its cDNA sequence. Several of the kinetic properties of the recombinant enzyme resembled those of the native PC12 hydroxylase. However, in contrast to the native enzyme, the purified recombinant hydroxylase was shown to be in an activated form. Phosphorylation with cAMP-dependent protein kinase resulted in stoichiometric incorporation of phosphate, but the kinetic profile of the recombinant enzyme was unaffected. Several clues to these differences are considered that may provide insight into the structural features important to the regulation of tyrosine hydroxylase.**

Tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2), catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) in brain and adrenal medulla. This is the initial and rate-limiting enzyme in the biosynthesis of catecholamines, including dopamine, norepinephrine, and epinephrine, which serve important biological functions as neurotransmitters or hormones (1). Tyrosine hydroxylase belongs to a group of aromatic amino acid hydroxylases that require iron and the coenzyme (6*R*)-tetrahydrobiopterin (BH_4) for catalytic activity (2, 3). The enzyme is inhibited by its substrate, tyrosine (4–7), and by its product, L-dopa, as well as by other catechols (1, 4, 8). Phosphorylation activates tyrosine hydroxylase (3), causing a decrease in the K_m for the cofactor, a shift in the pH optimum, decreased sensitivity to product inhibition and, at neutral pH, an increase in V_{max}. Other activating factors include high salt concentrations, polyanions, and interaction with membrane phospholipids (reviewed in ref. 9), but their physiological significance is unknown.

Studies of the mechanism and properties of tyrosine hydroxylase have been hindered by the difficulty in obtaining large amounts of pure enzyme, particularly from neuronal sources. Numerous studies have reported the purification of tyrosine hydroxylase from a variety of tissues (4, 8, 10, 11), but in most cases the yield was too low, or the enzyme was insufficiently pure to allow extensive biochemical characterization. Recently, several authors have employed molecular cloning techniques to express large amounts of tyrosine hydroxylase in a variety of systems (12–16). In the present study, we describe the expression at high levels of rat PC12 tyrosine hydroxylase in *Escherichia coli*, along with the purification and characterization of the recombinant enzyme.

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MATERIALS AND METHODS

Isolation and Cloning of Rat Tyrosine Hydroxylase cDNA. A rat PC12 λgt10 cDNA library (Clontech) was grown on *E. coli* host strain C600 *hfl*, transferred onto Hybond-N nylon filters (Amersham), and hybridized (17) with γ-^{32}P-labeled oligomers to positions 12–62 (N terminus) and 1459–1508 (C terminus) of the rat tyrosine hydroxylase coding sequence (18).

Bacteriophage DNA was purified (ref. 19, pp. 2.73), and the positive cDNA inserts were excised with *Bam*HI and *Hin*dIII restriction enzymes and subsequently ligated to *Bgl* II–*Hin*dIII-digested pGEM3Z vector (Promega) to generate intermediate subclones. Fifteen cycles of PCR amplification (Perkin–Elmer) from the insert in pGEM3Z produced a 1.5-kilobase (kb) product, which was purified on an agarose gel, made blunt with the Klenow fragment of DNA polymerase, and ligated to the expression vector pET3C (20), which had been digested with *Nde* I and similarly treated and isolated and used to transform *E. coli* strain DH5. Two recombinant clones, pYHW4 and pYHW5, were obtained. The insert DNA and junctions were thoroughly confirmed by DNA sequencing (21) with T7 DNA polymerase (Pharmacia) and analyzed on an Acugen 402 automated DNA sequencing system (EG&G Berthold, Natick, MA).†

Expression of Rat Tyrosine Hydroxylase cDNA in *E. coli*. Cultures of *E. coli* host strain BL21(DE3) (20), transformed by pYHW4 and pYHW5, were grown overnight (to $OD_{600} \approx 3$) in LB–ampicillin broth (ref. 19, p. A1), diluted (1:50), grown to logarithmic phase in LB–ampicillin medium containing 0.1 mM ferrous sulfate, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and finally pelleted by centrifugation at 7500 × *g* for 10 min at 4°C. The pellets were washed with 0.01 M $MgSO_4$ for storage at −70°C.

Purification of Tyrosine Hydroxylase. *E. coli* cells (5 g) were thawed and resuspended in 40 ml of 0.05 M Tris·HCl buffer, pH 7.2, containing 0.25 M sucrose, 0.1 mM EDTA, 2.0 μM leupeptin, 0.5 μM pepstatin, and phenylmethylsulfonyl fluoride at 100 μg/ml (sonication buffer). The cells were subsequently subjected to three cycles of sonication on ice, with the use of an Ultrasonic model W-225 set at 20% of maximum

Abbreviation: BH_4, 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin [(6*R*)-tetrahydrobiopterin].
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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M73804).

output. Typically, the first cycle of sonication consisted of 4 pulses, each for 30 sec, followed by a 30-sec off period. The cell lysates were then centrifuged at 17,800 × *g* for 15 min at 4°C. The pellet was resuspended in the same volume of sonication buffer and was subjected to a second cycle of sonication, consisting of 10 pulses delivered at 30-sec intervals. This process was repeated once, and the supernatants from the second and third cycles of sonication were pooled for further purification. By excluding the first soluble fraction, generated by the shorter sonication, it was possible to remove 60% of the total protein with a 2-fold purification in tyrosine hydroxylase activity. The subsequent steps in the purification of the enzyme involved ammonium sulfate fractionation followed by heparin-Sepharose chromatography, according to the method of Fitzpatrick *et al.* (12). The final enzyme preparation was judged to be pure on the basis of SDS/polyacrylamide gel electrophoresis and densitometry.

Assay Procedures. The hydroxylation of tyrosine was measured by the tritiated H_2O release assay (22) with the modifications described by Ribeiro *et al.* (7). Standard assay conditions included catalase at 2000 units/ml, dihydropteridine reductase (Sigma) at 50 milliunits/ml, 0.1 mM NADH, 200,000 cpm of [^{3}H]tyrosine, pure tyrosine hydroxylase (0.2 μg), and 200 mM sodium acetate buffer, pH 6.0. When the pH was varied, the buffer was changed either to 50 mM 2-[*N*-morpholino]ethanesulfonic acid (Mes) (pH 5–7.2) or 50 mM Hepes (pH 7–8.5). The reaction mixture was incubated for 5 min at 37°C with 100 μM 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin (BH_4).

The phosphorylation of tyrosine hydroxylase was carried out in a mixture containing 40 mM potassium phosphate buffer, pH 6.6, 10 mM $MgCl_2$, 0.3 mM ATP, 1.5 mM dithiothreitol, 10% (vol/vol) glycerol, 10 μg of tyrosine hydroxylase, and 35 picomolar units (0.6 μg) of cAMP-dependent protein kinase catalytic subunit (Sigma), in a total volume of 250 μl. After a 10-min reaction at 30°C, the samples were placed in ice and aliquots containing approximately 0.2 μg of phosphorylated tyrosine hydroxylase were immediately assayed for enzyme activity. In experiments measuring the incorporation of [^{32}P]phosphate into tyrosine hydroxylase, 0.3 mM [γ-^{32}P]ATP replaced unlabeled ATP, and the amount of labeled enzyme was measured as described previously (23).

Electrophoresis and Western Blot Immunoanalysis. Standard SDS/polyacrylamide electrophoresis and isoelectric focusing were performed according to the specifications of NOVEX, the manufacturer of the equipment, and stained with Coomassie blue or silver reagent (Bio-Rad). Densitometry of gels was performed with the IMAGE software package (24) and a peripheral COHU model 4815 solid-state video camera. Western blot immunoanalysis was carried out as described (25), with mouse anti-tyrosine hydroxylase monoclonal antibody (Boehringer Mannheim) at 20 μg/100 ml and peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim) at 25 μg/100 ml.

Other Methods. The hydroxylation of phenylalanine was assayed by the method of Katz *et al.* (5), as described by Ribeiro *et al.* (7). N-terminal amino acid sequence analysis was performed on an Applied Biosystems 470 A gas-phase sequencer equipped with an on-line 120A phenylthiohydantoin amino acid analyzer and a 900A controller and data analysis system. Pure tyrosine hydroxylase from cultured PC12 cells was obtained by the procedure of Kuhn and Billingsley (26), as described by Ribeiro *et al.* (7). Protein was measured by the method of Bradford (27) with the Bio-Rad assay kit and bovine serum albumin as a standard. All kinetic parameters were determined by direct fit to the Michaelis–Menten equation with the EZ-FIT curve-fitting program (F. W. Perrella, DuPont).

RESULTS

Isolation and Cloning of Rat PC12 Tyrosine Hydroxylase cDNA. Approximately 1 × 10^6 phages from the rat PC12 cDNA library were screened with two probes corresponding to both extreme 5′ and 3′ ends of the tyrosine hydroxylase coding sequence. Twelve clones retained positive signals to both probes after three rounds of screening and isolation.

For directly cloning rat tyrosine hydroxylase cDNA in expression vector pET3C, PCR was utilized to facilitate amplification and purification of the desired gene. A tyrosine hydroxylase gene fragment was amplified, with intermediate subclones as templates, and directly inserted into the *Nde* I site of the pET3C vector such that the spacing between the Shine–Dalgarno ribosome-binding site and the translation initiation codon would be in the optimal range for maximum protein production (28). Two plasmids, pYHW4 and pYHW5 (Fig. 1), containing the cDNA for the complete tyrosine hydroxylase in the appropriate orientation, were carried forward and the promoter region is shown in Fig. 2.

Expression of Tyrosine Hydroxylase in *E. coli*. The plasmids pYHW4 and pYHW5 were used to transform *E. coli* strain BL21(DE3) to test for expression. Bacteria containing recombinant tyrosine hydroxylase cDNA or a negative control plasmid were grown under inducing conditions and in the presence of 0.1 mM ferrous sulfate. Addition of low concentrations of ferrous sulfate to the growing cultures was necessary to ensure maximal enzyme activity (data not shown). Different pre- and postinduction growth periods were tested to optimize the yield and specific activity of tyrosine hydroxylase. A representative sample of the experiments is shown in Fig. 3. The results indicate that 2-hr pre- and postinduction incubations produced the highest yield, whereas long-term growth (4 hr) lowered the relative recovery of tyrosine hydroxylase activity.

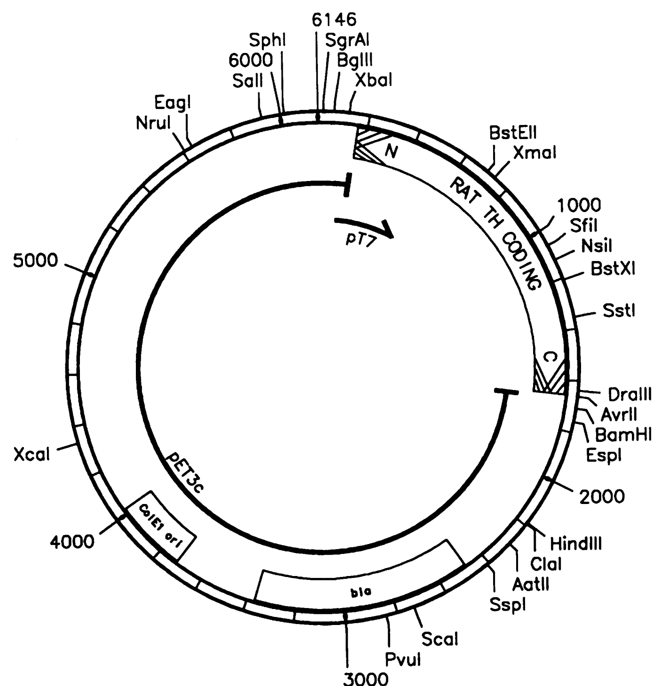

FIG. 1. The rat PC12 tyrosine hydroxylase (TH) clone. Plasmid pYHW5 contains 1497 base pairs (bp) of rat PC12 cDNA and a total of 6146 bp. Immediately upstream of this coding sequence is the promoter pT7, which is identified specifically by T7 RNA polymerase. This plasmid contains a β-lactamase gene (*bla*), which confers resistance to ampicillin. The cleavage sites of all commercially available restriction endonucleases that cleave the pYHW5 clone only once are shown.

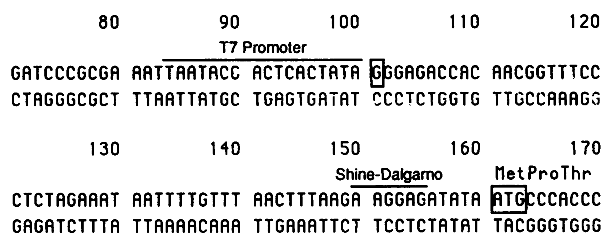

FIG. 2. Transcription and translation initiation region of pYHW5. The T7 RNA polymerase and *E. coli* ribosome recognition (Shine–Dalgarno) sites are indicated. The coordinates shown correspond to the plasmid map in Fig. 1. The G residue at position 101 represents the start site of transcription, and the ATG at position 161 defines the N terminus of the rat tyrosine hydroxylase. The junction between the vector, pET3c, and the tyrosine hydroxylase cDNA insert is located at residues 160/161.

Crude extracts from induced recombinant *E. coli* were assayed for tyrosine hydroxylase activity and analyzed by both SDS/polyacrylamide gel electrophoresis and Western blotting (Fig. 4 *A* and *B*). All of the strains that carried the recombinant cDNA contained high levels of tyrosine hydroxylase activity, and all displayed an intense protein band with an apparent molecular weight of 60,000. This same band reacted positively and specifically with a monoclonal antibody raised against pheochromocytoma tyrosine hydroxylase.

Extraction and Purification of Tyrosine Hydroxylase. The recombinant tyrosine hydroxylase in *E. coli* was expressed in a highly insoluble form, which could not be readily recovered by simple sonication. One cycle of low-intensity sonication, consisting of 4 pulses at 30-sec intervals, followed by centrifugation of cell lysates, yielded less than 35% of total enzyme activity. The remaining activity was associated with the insoluble protein pellet. Various methods of chemical and mechanical lysis were used in attempts to solubilize the tyrosine hydroxylase. The addition of low levels of Triton X-100 (0.2%) to the sonication buffer significantly improved the recovery of soluble tyrosine hydroxylase. In contrast, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) had no apparent effect, and lysozyme caused a severe loss in enzyme activity. The most effective extraction method consisted of repeated sonication of the pellets through consecutive cycles of centrifugation and resuspension in buffer. Two such cycles of sonication were generally sufficient to solubilize 95% of all measurable tyrosine hydroxylase activity.

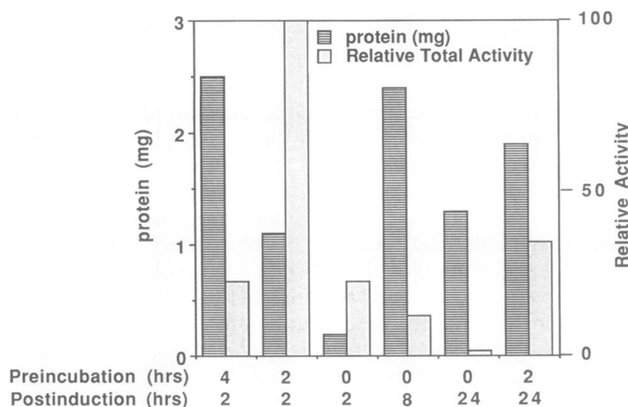

FIG. 3. Induction conditions. *E. coli* cultures in early logarithmic phase were preincubated for the times indicated before isopropyl thiogalactoside was added to induce expression for the postinduction period prior to harvesting. Crude extracts were assayed for tyrosine hydroxylase activity and protein. The activity data are expressed as percentages of maximum recovery.

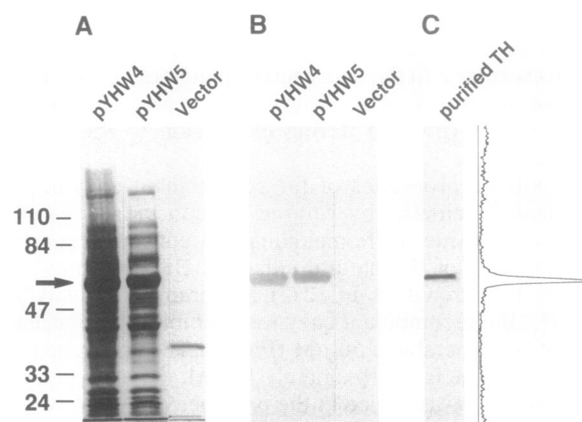

FIG. 4. Rat PC12 recombinant tyrosine hydroxylase analyzed by SDS/polyacrylamide gel electrophoresis and Western transfer. Crude extracts from bacteria transformed with pYHW4, pYHW5, or pET3c vector without insert were electrophoresed on an SDS/10% polyacrylamide gel. (*A*) Coomassie blue-stained protein. (*B*) Western blot developed with anti-tyrosine hydroxylase. (*C*) Pure recombinant tyrosine hydroxylase analyzed by SDS/polyacrylamide gel electrophoresis and densitometry. The hydroxylase subunit appears as the only prominent band, with an estimated molecular weight of 60,000, as determined by the relative motility of suitable protein standards ($M_r \times 10^{-3}$ is indicated on the left).

Following extraction from the recombinant *E. coli*, tyrosine hydroxylase was purified to apparent homogeneity by the procedure of Fitzpatrick *et al.* (12). On the basis of activity measurements, the enzyme was purified nearly 20-fold to a final specific activity of 1.8 μmol/min per mg, when assayed at pH 6.0 and at 37°C for 2 min, with 1 mM 6-methyltetrahydropterin and 0.1 mM L-tyrosine. The estimated recovery was 30%. One purification procedure yielded approximately 5 mg of pure tyrosine hydroxylase from 5 g of *E. coli* cells. Fig. 4*C* shows a typical SDS/polyacrylamide gel of the purified enzyme and establishes the purity of the preparation. Tyrosine hydroxylase appears as the only prominent band on the gel, with a corresponding single peak on the densitometric profile. The identity of the band was established by Western analysis, with the use of a monoclonal antibody raised against pheochromocytoma tyrosine hydroxylase (data not shown).

Characterization of the Pure Recombinant Tyrosine Hydroxylase. Several of the chemical and physical properties of pure recombinant tyrosine hydroxylase were determined. The molecular weight (M_r) of the enzyme subunit was estimated as 60,000, based on the relative mobilities of protein standards on SDS/polyacrylamide gels. The N-terminal amino acid sequence (25 residues) of the recombinant enzyme was 100% identical with that of rat PC12 tyrosine hydroxylase, as deduced from its cDNA sequence. When analyzed by isoelectric focusing, the pure recombinant enzyme produced a single band in the pH range of 5.8–5.9, under conditions where the isoelectric point for pure PC12 tyrosine hydroxylase was 5.3 (results not shown). In addition, the pure recombinant hydroxylase incorporated 1 mol of $^{32}P_i$ per mol of subunit after a 10-min incubation with cAMP-dependent protein kinase under standard phosphorylating conditions.

Tyrosine hydroxylase is known to contain between 0.5 and 1 mol of iron per mol of subunit, and the iron is required for catalytic activity (11, 29, 30). During expression in *E. coli*, the high demand for iron may be sufficient to deplete endogenous stores, resulting in the production of an iron-deficient enzyme. In the present study, this was easily corrected by the addition of low concentrations (0.1 mM) of ferrous sulfate to growing cultures of recombinant *E. coli*. The exogenously

supplied iron was taken up rapidly, nearly tripling the specific activity of the crude enzyme. After purification, the addition of ferrous sulfate to the assay mixture did not cause a further increase in activity, suggesting that enough exogenous iron had been incorporated during expression to yield a fully active enzyme.

The kinetic properties of the recombinant enzyme were investigated, initially, by varying the concentrations of the substrate tyrosine while maintaining a constant concentration (100 μM) of the natural cofactor, BH_4. As previously reported for adrenal (4), PC12 (7), and brain tyrosine hydroxylase (5), the recombinant enzyme is inhibited by concentrations of tyrosine above 50 μM (Fig. 5). The apparent K_m or $S_{0.5}$ for tyrosine is approximately 11 μM. When the concentration of BH_4 was varied in the presence of 40 μM tyrosine the K_m for BH_4 was found to be 45 μM. Phenylalanine is also a substrate for the recombinant enzyme. In the presence of 100 μM BH_4, the K_m for the hydroxylation of phenylalanine to tyrosine and dopa is 55 μM, and there is no apparent substrate inhibition up to 300 μM phenylalanine (Fig. 5). Finally, varying the pH at 40 μM tyrosine and 100 μM BH_4 indicated that the enzyme is most active within a broad range of pH values (pH 6.3–7.0); the pH optimum is approximately 6.5.

Activation of tyrosine hydroxylase by phosphorylation, polyanions, or salts or by interaction with membranes is known to alter several kinetic properties of the enzyme (3, 31, 32). Some of these well-established kinetic changes were investigated with pure recombinant enzyme, and the results are summarized in Table 1. Phosphorylation of tyrosine hydroxylase catalyzed by cAMP-dependent protein kinase caused a slight decrease in the K_m for BH_4 at pH 7.2, but not at pH 6.0. Furthermore, the V_{max} was unaffected by phosphorylation, remaining at approximately 1 μmol/min per mg of protein under phosphorylating and nonphosphorylating conditions at both pH values. The pH optimum of the cloned hydroxylase shifted slightly with phosphorylation, from a broad optimal pH range of 6.3–7.0 to a sharper pH optimum of 7.5. In contrast, the inhibition of tyrosine hydroxylase by its product L-dopa was unchanged after phosphorylation, as evidenced by a constant IC_{50} value of 50 μM. Finally, whereas the native enzyme can be activated by phosphatidyl-L-serine, which lowers the K_m for BH_4 severalfold (32), the recombinant hydroxylase is essentially unaffected by this phospholipid. The K_m for BH_4 at pH 6.3 is approximately 60 μM, both in the presence and in the absence of 0.5 mM phosphatidyl-L-serine (data not shown).

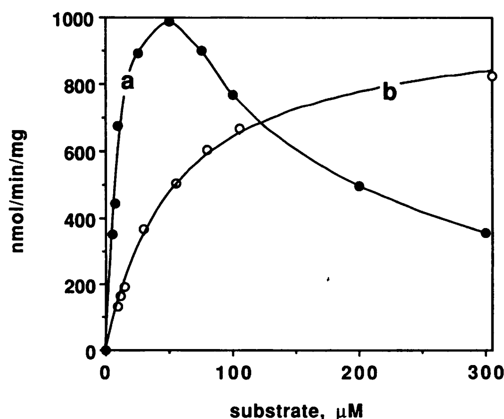

FIG. 5. Activity of pure recombinant tyrosine hydroxylase as a function of tyrosine (curve a) or phenylalanine (curve b) concentration. Substrate inhibition by excess tyrosine occurs at concentration greater than 50 μM. No inhibition was observed with phenylalanine in this concentration range. The hydroxylation of tyrosine to L-dopa was followed with the tritium release assay, whereas the hydroxylation of phenylalanine to tyrosine and L-dopa was assayed by thin-layer chromatography. Each reaction was carried out for 5 min at 37°C and in the presence of 100 μM BH_4.

Table 1. Effect of phosphorylation with cAMP-dependent protein kinase on the kinetic profile of recombinant tyrosine hydroxylase

	K_m BH_4, μM		V_{max}, μmol/min per mg		IC_{50} L-dopa,	pH
Hydroxylase	pH 6.0	pH 7.2	pH 6.0	pH 7.2	μM	optimum
Phosphorylated	40	20	0.9	1.0	50	7.5
Nonphosphorylated	60	50	1.0	0.9	50	6.3–7.0

DISCUSSION

Rat PC12 tyrosine hydroxylase was cloned in the pET3C vector and expressed in *E. coli*. These recombinant cells could synthesize tyrosine hydroxylase protein actively and selectively at levels of 5% of total *E. coli* protein. With such high-level expression, it is not uncommon that normally soluble eukaryotic proteins appear as partially insoluble products (33). This was the case in the present study, where sonication yielded less than 35% of total tyrosine hydroxylase activity, with the remaining enzyme being associated with a low-speed insoluble pellet. The reasons for this insolubility may involve self-aggregation of excess foreign protein, or perhaps co-aggregation of the protein with bacterial membrane components (13).

The recovery of insoluble tyrosine hydroxylase was a major step in the purification of this enzyme. Procedures for the solubilization of cloned proteins typically rely on harsh methods of denaturation and renaturation or enzymic lysis (34), which often lead to irreversible modification of protein structure (35). In the present study, we tested a variety of disruption procedures and found that repeated cycles of short-pulse, low-intensity sonication were sufficient to yield over 95% of all tyrosine hydroxylase in the soluble fraction with minimal loss of activity. The enzyme could then be purified to apparent homogeneity, with an overall purification of approximately 20-fold, which is consistent with tyrosine hydroxylase representing at least 5% of the total *E. coli* protein.

The physical and kinetic properties of pure recombinant tyrosine hydroxylase were determined in this study. Several physical properties are analogous to those of native tyrosine hydroxylase. The recombinant enzyme has the same subunit molecular weight and the same N-terminal amino acid sequence as the PC12 hydroxylase. Similarly, the requirement for iron was characteristic of native tyrosine hydroxylase (9, 11, 29, 36), as was the rapid incorporation of phosphate with cAMP-dependent protein kinase (6, 8, 10, 11). Other properties, however, suggest that subtle structural differences exist between the recombinant and native enzymes. The isoelectric point for the recombinant enzyme (5.8–5.9) exceeds that of the native PC12 hydroxylase (5.3) (data not shown), suggesting that the ratio of negatively charged residues to positively charged residues in the PC12 enzyme may be greater than that for the recombinant enzyme. The structural basis for these differences is not known, nor is it known if the change in negative charge reflects post-translational modifications in the bacterium or in the PC12 cell. There have been multiple forms of tyrosine hydroxylase reported in the adrenal (pI = 5.5–6.0) (11) and in PC12 cells (pI = 5.4, 5.6, and 5.9) (8, 26). Covalent modifications such as phosphorylation or deamidation, possibly combined with altered electrostatic interactions, may be responsible for the differences in both isoelectric points and regulatory properties.

The elucidation of these differences awaits further investigation.

The recombinant hydroxylase exhibits biphasic kinetics with tyrosine and typical substrate inhibition at concentrations higher than 50 μM (4–7). In addition, this enzyme has the same ability to hydroxylate phenylalanine to tyrosine and dopa as the native hydroxylase from PC12 cells, adrenal medulla, or brain (4, 5, 7). Other kinetic properties, however, resembled those of activated tyrosine hydroxylase (3). Typically, activation is accompanied by a number of kinetic changes that include a substantial decrease in the K_m for the cofactor BH_4, an increase in V_{max} at neutral pH, a shift in the pH optimum from approximately 6.0 to a physiological range of 7.0–7.5, and an increase in the K_i for dopa and dopamine, indicating a diminished sensitivity to product inhibition (3).

In the present study, however, these kinetic parameters were generally in the same range as those for the activated hydroxylase, and further attempts to activate the recombinant enzyme by either phosphorylation with cAMP-dependent protein kinase (3) or interaction with phosphatidylserine (31) had virtually no effect. At pH 6.0, the K_m for BH_4 is in the same range as that of activated PC12 or adrenal tyrosine hydroxylase (8, 11, 37, 38), and neither phosphorylation nor exposure to phosphatidylserine led to the expected 2- to 4-fold decrease in K_m value (8). Similarly, at pH 7.2, the K_m for BH_4 is comparable to that of the activated hydroxylase, and although phosphorylation lowered the K_m even further, from 50 μM to 20 μM, this decrease is considerably less than that of nearly 7-fold described for the PC12 enzyme (8). Furthermore, phosphorylation did not affect the V_{max} for BH_4 at pH 7.2, although a stimulation of up to 10-fold might have been expected (3, 8, 39), and phosphorylation caused but a slight shift in the pH activity profile, from a broad optimal range of pH 6.3–7.0 to approximately 7.5. The IC_{50} for L-dopa inhibition was also unchanged by phosphorylation, in contrast to the expected increase of up to 7-fold in the K_i for product inhibition (8). Collectively, the results suggest that the purified cloned enzyme is at least partially activated.

The mechanism(s) underlying this apparent activation of the purified recombinant hydroxylase are currently unknown. The possibility exists that the cloned enzyme was activated during purification by any one of a number of factors that are known to activate tyrosine hydroxylase *in vitro*, including limited proteolysis (40) and interaction with polyanions or salts (31). Alternatively, the activation could be a direct consequence of expression in *E. coli* and could reflect the occurrence or absence of yet-undetermined post-translational modifications.

In summary, we have shown the expression at high levels of rat PC12 tyrosine hydroxylase and have described several properties of the cloned enzyme. The kinetic behavior of this hydroxylase is similar to that of the native PC12 enzyme, except that the purified, cloned tyrosine hydroxylase appears to be at least partially activated. Since phosphorylation of the enzyme at the cAMP-dependent kinase site leads to only modest changes in its catalytic properties, our results also suggest that other mechanisms are responsible for the observed activation of the enzyme. Further investigation will be necessary to elucidate the structural and functional changes that mediate the activation of tyrosine hydroxylase.

We thank Michael Brownstein for synthesizing DNA oligomers, Joanne Gutierrez for amino acid sequencing, Jennifer Tipper and Robert Pohlman for helpful discussions, Emma Suggs for technical assistance, and Marge Schnackenberg for secretarial support.

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