We have investigated the role of serine 40 (Ser-40) in tyrosine hydroxylase (TH) catalysis of basal and activated enzymes by protein kinase A (PKA)-mediated phosphorylation. Wild type and mutant TH were transiently and stably expressed in AtT-20 cells, and the enzymatic activities of the recombinant enzymes were analyzed. The specific enzymatic activity of transiently expressed TH mutants Ser-40_leucine or tyrosine (Leu-40m or Tyr-40m) was higher than that of the wild type enzyme or of other mutants in which Ser-8, -19, and -31 were replaced by leucine. The kinetic studies carried out with the stably expressed TH show that the $K_m$ for the cofactor 6-methyltetrahydropteridine is lower and the $K_i$ for dopamine is higher when the enzymatic hydroxylation is catalyzed by the Leu-40m or Tyr-40m than by the wild type enzyme. The kinetic parameters and the pH profile of the enzymatic hydroxylation catalyzed by the Leu-40m or Tyr-40m are similar to the enzyme activated by PKA-mediated phosphorylation. We suggest that Ser-40 in TH exerts an inhibitory influence on the enzymatic activity, and its replacement with another amino acid by site-directed mutagenesis or its modification by phosphorylation leads to a change in conformation with an increased enzymatic activity. The importance of Ser-40 in the activation of TH by PKA-mediated phosphorylation was investigated by comparing the activation of the wild type enzyme with that of Leu-40m or Tyr-40m. The findings that the enzymatic activity is increased by PKA-mediated phosphorylation of the wild type enzyme, but not of the Leu-40m or Tyr-40m, demonstrate that phosphorylation at Ser-40 is essential for activation of TH by PKA. The findings that addition of ATP plus cAMP to homogenates from transfected AtT-20 cells stimulates the recombinant wild type TH activity indicate that these cells contain endogenous cAMP-dependent protein kinase.

Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine which represents the first step in the biosynthesis of catecholamines. The activity of TH is subject to short and long term regulation (1, 2), and phosphorylation of the enzyme plays an important role in the activation of the enzymatic activity (3). TH is phosphorylated by different protein kinases at multiple serine-containing phosphorylation sites located at the N-terminal region of the enzyme (4, 5). At least four phosphorylation sites in TH were identified: Ser-8, -19, -31, and -40 (4, 5). TH is phosphorylated at Ser-40 by cyclic AMP-dependent protein kinase (protein kinase A (PKA)), calcium/phospholipid-dependent protein kinase (protein kinase C) (6) and calcium/calmodulin protein kinase II (7), and the phosphorylation by PKA at this site seems to play an important role in the activation of the enzymatic activity. To determine the role of Ser-40 in the regulation of basal enzymatic activity and in the activation of TH by PKA-mediated phosphorylation, we have investigated the effects of substitution of Ser-40 in TH with leucine (Leu) or tyrosine (Tyr) by site-directed mutagenesis. We have transiently and stably transfected AtT-20 cells with TH cDNA constructs and analyzed the properties of the expressed wild type and mutant enzymes. The kinetic characteristics, as well as the activation of the wild type and TH mutant enzymes by PKA-mediated phosphorylation, were studied.

**MATERIALS AND METHODS**

**Plasmid Vector Construction**—A 1.8-kb rat TH cDNA fragment, including a 1494-bp TH coding region, an 11-bp 5'-flanking end, a 284-bp 3'-flanking end, and a 12-bp linker site, was amplified by PCR using PC12 cDNA library as a template. The reaction mixture was submitted to 40 PCR cycles (Cetus-Perkin Elmer). The timing used was 1 min at 94 °C, annealing at 37 °C for 2 min, and followed by a 3-min extension at 72 °C. The construct pSP72-TH was formed by inserting a 1.8-kb Xhol fragment of rat TH cDNA into the Xhol site of pSP72 vector (Promega). The cDNA was confirmed by DNA sequencing (8). The 1.8-kb cDNA fragment obtained by Xhol digestion of pSP72-TH construct was subcloned into the Xhol site of the CDMS expression vector (9). The construct CDMS-TH is illustrated in Fig. 1.

**Site-directed Mutagenesis**—cDNA for mutants with a substitute AA for Ser at positions -8, -19, -31, and -40 was constructed by site-directed mutagenesis using the PCR overlapping extension procedure with primers containing the desired base substitutions (10). For the substitution of Ser-40 with Tyr, we synthesized the oligonucleotide 5'-CGGGCAGACTATCTCATCGAG-3' and its reverse complement. For the substitution of Ser-40 with Leu, we synthesized the oligonucleotide 5'-CGGGCAGACTATCTCATCGAG-3' and its reverse complement. Each mutation was carried out in two steps. In the first step, the fragment to be overlapped was amplified in separate reactions, together with the wild type flanking primers, was used for the PCR reaction by PKA as a substrate. One reaction 10 ng of TH cDNA served as a template. In the second step a small amount of each product from the first reactions, together with the wild type flanking primers, was used for the PCR reaction. The PCR in both steps was carried out under the.
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The enzymatic activities of wild type and Leu-40m or Tyr-40m at different pH values of the incubation medium

|        | TH activity* |
|--------|--------------|
| pH 6.0 |               |
| WT TH  | 109 ± 7      |
| Leu-40m| 133 ± 9      |
| Tyr-40m| 372 ± 18     |
| pH 7.2 |               |
| WT TH  | 14 ± 1       |
| Leu-40m| 115 ± 10     |
| Tyr-40m| 302 ± 19     |

*TH activity is expressed as nanomoles of product formed/hour/milligram of protein under the standard assay condition. The results are the means from at least three experiments ± S.E.

The specific enzymatic activities obtained from AtT-20 cells transiently transfected with TH cDNA constructs

| Wild type and TH mutants | TH activity (nmol/h/mg protein) |
|--------------------------|--------------------------------|
| WT TH                    | 1.45 ± 0.07                     |
| Leu-8m                   | 1.40 ± 0.08                     |
| Leu-19m                  | 1.47 ± 0.07                     |
| Leu-31m                  | 1.01 ± 0.06                     |
| Leu-40m                  | 7.90 ± 0.60*                    |
| Tyr-40m                  | 9.30 ± 0.75*                    |

*p < 0.001 when compared with WT TH.

Western blot analysis of wild type TH and Ser-40 mutants expressed in AtT-20 cells

The enzymatic activities of wild type and Leu-40m or Tyr-40m mutants expressed in AtT-20 cells transiently transfected with TH cDNA constructs

Fig. 1. The construct of rat TH gene in the expression vector CDMS-TH.

Fig. 2. Western blot analysis of wild type TH and Ser-40 mutants expressed in AtT-20 cells. Proteins were fractionated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. 1500 diluted rabbit anti-rat TH antibody was used in the first incubation. The nitrocellulose sheets were subsequently incubated with 1:250 goat anti-rabbit IgG conjugated with peroxidase. M, protein markers. Lane 1, wild type TH (50 μg of total protein). Lane 2, Tyr-40m (50 μg of total protein). Lane 3, Leu-40m (50 μg of total protein). Lane 4, untransfected AtT-20 cells (50 μg of total protein). Lane 5, rat striatum (50 μg of total protein). Lane 6, purified TH from PC12 cells (1 μg of total protein).

The enzyme was isolated from a medium containing 600 μg/ml of G418. Cell colonies which were G418 resistant were selected, and TH activity was determined in the homogenates.

Phosphorylation of TH—In some experiments a purified enzyme preparation was used. For purification of TH the cells were sonicated in 20 mM potassium phosphate (KP) buffer, pH 6.5, and centrifuged for 20 min at 10,000 × g. The enzyme was precipitated from the supernatant fraction by addition of (NH₄)₂SO₄ to 80% saturation. After dialysis this fraction was brought to 40% saturation with NH₄Cl, the solution centrifuged, and the supernatant discarded. The pellet was dissolved in a minimum volume KP buffer, pH 6.5, containing 10% glycerol, 0.5 mM dithiothreitol, 0.5 μM leupeptin, 0.1 μM pepstatin, 0.5 mM phenylmethylsulfonyl fluoride. The solution was filtered through a high performance liquid chromatography column (TSK 3000 SW column, 7.5 mm, inner diameter × 30 cm) which was preequilibrated with KP buffer, pH 6.5. The fractions of 0.5 ml were collected, and those with the highest specific enzymatic activity were pooled and stored at −70 °C.

The specific enzymatic activities obtained from AtT-20 cells transiently transfected with TH cDNA construct results in the expression of tumors were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 60% confluent AtT-20 cells were transfected with 20 μg of constructed plasmid DNA using the calcium phosphate precipitation method (12). 48 h after addition of plasmid DNA the cells were harvested.

To stably transfect AtT-20 cells, the following procedures were used. AtT-20 cells grown in Dulbecco’s modified Eagle’s medium containing 600 μg/ml of G418. Cell colonies which were G418 resistant were selected, and TH activity was determined in the homogenates.

Stimulation of TH Activity by Phosphorylation—In vitro phosphorylation of TH was carried out as previously described (6).

Western blot analysis—50 μg of proteins obtained from transfected cell extracts were submitted to SDS-polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose sheet. Following transfer, the sheet was preincubated at room temperature for 1 h in 5% non-fat milk. The sheet was then washed for 5 min, three times with TBS, pH 7.4, containing 0.05% Tween 20 at room temperature. After 1 h of incubation with rabbit anti-rat TH antibody (500) at room temperature, the sheet was washed as above and then incubated for 1 h with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:250). Following the third wash the proteins were stained by H₂O₂ treatment. Protein concentration was measured by Lowry’s procedure.

RESULTS

TH Activity in AtT-20 Cells Transiently Expressing Wild Type or TH Mutants—The transient transfection of AtT-20 cells with a TH cDNA construct results in the expression of TH activity (Table I), while the untransfected cells do not contain enzymatic activity. These results are in agreement with a nonmutated TH cDNA construct (wild type, WT TH) or with protein). Lane 2, Tyr-40m (50 pg of total protein). Lane 3, Leu-40m (50 pg of total protein). Lane 4, untransfected AtT-20 cells (50 pg of total protein). Lane 5, rat striatum (50 pg of total protein). Lane 6, purified TH from PC12 cells (1 μg of total protein).

following conditions: 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min for a total of 40 cycles. The PCR products were isolated on gel electrophoresis, digested with HindIII/PstI, and ligated by T4 ligase into CDMS-TH which had been deleted by its wild type HindIII/PstI fragment. All of the mutants were determined by double-stranded DNA sequencing.

DNA Sequencing—TH cDNA sequence and mutants were determined from the double-stranded template using GemSeq K/RT™ system with the chain-termination method (11).

Transfection of AtT-20 Cells—AtT-20 cells derived from pituitary tumors were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 60% confluent AtT-20 cells were transfected with 20 μg of constructed plasmid DNA using the calcium phosphate precipitation method (12). 48 h after addition of plasmid DNA the cells were harvested.

To stably transfect AtT-20 cells, the following procedures were used. AtT-20 cells grown in Dulbecco’s modified Eagle’s medium containing 600 μg/ml of G418. Cell colonies which were G418 resistant were selected, and TH activity was determined in the homogenates.

Cloning of TH—In some experiments a purified enzyme preparation was used. For purification of TH the cells were sonicated in 20 mM potassium phosphate (KP) buffer, pH 6.5, and centrifuged for 20 min at 10,000 × g. The enzyme was precipitated from the supernatant fraction by addition of (NH₄)₂SO₄ to 80% saturation. After dialysis this fraction was brought to 40% saturation with NH₄Cl, the solution centrifuged, and the supernatant discarded. The precipitate was dissolved in a minimum volume KP buffer, pH 6.5, containing 10% glycerol, 0.5 mM dithiothreitol, 0.5 μM leupeptin, 0.1 μM pepstatin, 0.5 mM phenylmethylsulfonyl fluoride. The solution was filtered through a high performance liquid chromatography column (TSK 3000 SW column, 7.5 mm, inner diameter × 30 cm) which was preequilibrated with KP buffer, pH 6.5. The fractions of 0.5 ml were collected, and those with the highest specific enzymatic activity were pooled and stored at −70 °C.

TH Activity and Kinetic Studies—TH activity was determined as previously described (13). The enzyme was incubated if not otherwise stated for 6 min at 37 °C in a mixture of 100 μl containing the following components: 200 mM Tris-acetate buffer, pH 6.0 (or KP buffer, pH 7.2), 37.5 mM 2-mercaptoethanol, 1.0 mM 6-methyltetrahydropteridine (6-MPH₂), 1.4 mM ferrous sulfate, 2000 units of catalase, and 100 μM L-tyrosine plus 0.2 μCi [3,5-3H]tyrosine (specific activity/μCi/50 nmol). The reaction was stopped by addition of 200 μl of 0.1 N NaOH, and the mixture was passed through an ion-exchange column (13). The Kₘ values were calculated from Lineweaver-Burk plots and the Kₘ values from the Dixon plots (14). Kinetic data were computed by fitting the data to linear functions by the method of least-square analysis (15).
with a previously reported study which has shown that transiently transfected AtT-20 cells with a TH cDNA construct express TH activity (16). We have also investigated the effects of transient transfection of AtT-20 cells with TH cDNA mutant constructs on the expression of the recombinant TH mutants. TH codons for Ser-8, -19, -31, and -40 were individually substituted with either the codons for Leu or Tyr. The data in Table I show that TH mutants Ser-40 -> Leu or -> Tyr (Leu-40m or Tyr-40m) have a severalfold higher specific enzymatic activity than the expressed wild type enzyme. However, the specific activities of expressed TH mutants Leu-8m, Leu-19m, or Leu-31m are approximately of the same order as the wild type enzyme (Table I).

**TH Activities in AtT-20 Cells Stably Expressing Wild Type or TH Leu-40m or Tyr-40m—**To determine the mechanisms responsible for the increased enzymatic activity of the transiently expressed Leu-40m or Tyr-40m, we have stably transfected AtT-20 cells with TH cDNA constructs in which the codon for Ser-40 was substituted with either the codon for Leu or Tyr. The stably expressed wild type TH in AtT-20 cells had approximately a 100-fold higher specific enzymatic activity than the transiently expressed enzyme (data not shown). The stably expressed TH in AtT-20 cells was utilized for immunohistochemical identification by Western blot analysis and for determination of the kinetic parameters.

**Western Blot Analysis—**The expressed TH in AtT-20 cells was identified by Western blot analysis. The AtT-20 cells stably transfected with the nonmutated TH cDNA construct (wild type) express a single protein band which is recognized by anti-TH (Fig. 2, lane 1) and has the same apparent molecular mass (58-60 kDa) as TH from rat striatum or cultured PC12 cells (lanes 5 and 6). The untransfected AtT-20 cells do not contain proteins recognized by anti-TH (Fig. 2, lane 4). The cells which were stably transfected with the Leu-40m or Tyr-40m constructs also show a single protein band which is recognized by anti-TH (Fig. 2, lanes 2 and 3) and have the same molecular mass as the wild type or TH isolated from PC12 cells or striatum.

**Effect of pH on Enzymatic Activity—**To determine whether the pH profiles of the wild type and TH Ser-40 mutants are similar to those of PC12 cells or rat striatum, we have investigated the enzymatic activities at pH 6.0 and at the physiological pH 7.2. The enzymes isolated from PC12 cells or rat striatum have a pH optimum at 6.0 (17), and the wild type enzyme expressed in AtT-20 cells also has a pH optimum at 6.0 (data not shown). The enzymes isolated from PC12 cells or striatum, when phosphorylated by PKA-mediated phosphorylation, have approximately the same activities at both pH values 6.0 and 7.2 (17), and the enzyme activities of the Leu-40m or Tyr-40m are also approximately the same at both pH values 6.0 and 7.2 (Table II).

**Table III**

| Enzyme     | pH 6.0 | pH 7.2 |
|------------|--------|--------|
|            | $K_m$  | $V_{max}$ | $K_m$  | $V_{max}$ |
| Wild type  | 0.26   | 20.4    | 0.76   | 6.06     |
| Leu-40m    | 0.09   | 71.4    | 0.11   | 66.8     |

The $K_m$ values were obtained from a single Lineweaver-Burk plot, and similar values were obtained from three separate plots.

**FIG. 3.** The Lineweaver-Burk plots of wild type and Leu-40m assayed at different concentrations of 6-MPH$_4$ at pH 6.0 and 7.2. The concentration of Tyr was 100 mM and the concentration of 6-MPH$_4$ varied from 0.06 to 1 mM. Each point represents the mean of triplicate samples from a single experiment. Similar data were obtained in three different experiments. The Lineweaver-Burk plot of Tyr-40m was almost identical to that of Leu-40m.

**FIG. 4.** Dixon plots of wild type and TH mutant activity as a function of dopamine and 6-MPH$_4$ concentration. The standard assay was carried out at the indicated concentrations of dopamine and at 0.1 and 1.0 mM of 6-MPH$_4$ for 6 min. (Dopamine was added with all other components at the beginning of the incubation and the enzymatic reaction was started immediately afterward by addition of 6-MPH$_4$.) Velocity is reported as nanomoles of product formed/hour at 37 °C. Each point represents the mean of duplicate samples.
Kinetic Studies—The kinetic parameters of tyrosine hydroxylation catalyzed by the wild type and Leu-40m were calculated from Lineweaver-Burk plots carried out at a constant concentration of the substrate Tyr and at variable concentrations of the cofactor 6-MPH, at two different pH values, 6.0 and 7.2. The $K_m$ of the wild type enzyme for 6-MPH is higher than that of the Leu-40m or Tyr-40m when the enzymatic activities were assayed either at pH 7.2 or at 6.0. The $V_{max}$ of the enzymatic reaction catalyzed by the Leu-40m or Tyr-40m is higher than that catalyzed by the wild type (Fig. 3 and Table III). The end product inhibition by dopamine of the enzymatic hydroxylation catalyzed by the wild type and by the Leu-40m was analyzed by constructing Dixon plots which were carried out at two different concentrations of 6-MPH and at variable concentrations of dopamine (Fig. 4). The $K_i$ values for dopamine calculated from two separate Dixon plots were found to be higher when the enzymatic hydroxylation was catalyzed by Leu-40m (6.0 ± 3.0 μM) as compared with the wild type enzyme (19.0 ± 4.0 μM).

Activation of TH by PKA-mediated Phosphorylation—To determine whether substitution of Ser-40 with Leu or Tyr alters the activation of TH by PKA-mediated phosphorylation, we have compared the activation by phosphorylation of the wild type TH with that of Leu-40m and Tyr-40m. The PKA-mediated phosphorylation of the wild type enzyme isolated from AtT-20 cells significantly increases the enzymatic activity. However, the activity of the enzyme obtained from Leu-40m or Tyr-40m is not increased by PKA-mediated phosphorylation (Fig. 5). These results indicate that the increase in TH activity elicited by PKA-mediated phosphorylation is due to the phosphorylation of the enzyme at Ser-40. To determine whether Ser-31 plays a role in the activation of TH by PKA-mediated phosphorylation, we have analyzed the activation of the wild type and Ser-31 -> Leu mutant (Leu-31m). The PKA-mediated phosphorylation of the Leu-31m increases the enzymatic activity by approximately the same percentage as it increases the wild type enzyme (Fig. 5).

To determine whether AtT-20 cells contain endogenous protein kinases which might activate TH, we have investigated enzyme activities in cell homogenates with and without addition of ATP plus cAMP. It is evident from Fig. 6 that TH activity in AtT-20 cell homogenates is stimulated by the addition of ATP plus cAMP, and upon addition of PKA the enzymatic activity is further stimulated. These findings indicate that homogenates from AtT-20 cells contain endogenous protein kinases which could activate TH upon addition of ATP plus cAMP. In a separate experiment we have determined whether or not TH purified from AtT-20 cells (see "Materials and Methods") still contains endogenous cAMP-dependent protein kinases. The results presented in Fig. 6 show that addition of ATP plus cAMP to purified TH does not stimulate enzymatic activity, while addition of ATP plus cAMP and PKA stimulated the enzymatic activity. Thus, the purified enzyme preparation does not contain endogenous PKA which stimulates TH.

**DISCUSSION**

The present findings that transiently expressed Leu-40m or Tyr-40m have higher specific enzymatic activities than the wild type enzyme suggest that either these mutations produce an activated form of the enzyme or that the transfection of the cDNA mutant constructs into AtT-20 cells was more efficient. To investigate whether the Leu-40m and Tyr-40m are expressed in an activated form of the enzyme, we have produced sufficient quantities of the stably expressed wild type and mutated enzyme forms for kinetic studies. Based on previously reported studies on the activation of TH by PKA or protein kinase C-mediated phosphorylation (6, 15), we assumed that if Leu-40m and Tyr-40m represent an activated form of the enzyme, then the kinetics of tyrosine hydroxylation catalyzed by these enzyme forms should differ from those catalyzed by the wild type enzyme. The present findings that the $K_m$ for the pteridine cofactor 6-MPH, is lower and the $K_i$ for dopamine is higher when the hydroxylation reaction is catalyzed by the Leu-40m and Tyr-40m, as compared with that catalyzed by the wild type, support the idea that the former enzymes are expressed in an activated form. The pH dependence of the enzymatic reactions catalyzed by the wild type also differs from that catalyzed by Leu-40m or Tyr-40m. The wild type enzyme and the nonphosphorylated enzymes derived from PC12 cells or striatum show an optimum for their activities at pH 6.0 and much lower activities at pH 7.2, while the Leu-40m and Tyr-40m, as well as the phosphorylated enzymes, have similar enzymatic activities at both pH 6.0 and 7.2.

We have compared the $K_m$ values for the pteridine cofactor of the recombinant TH with the $K_m$ values of the PC12
enzyme reported in our previous studies (15). The $K_i$ values for the pteridine cofactor of the recombinant TH (wild type) are similar to those of nonactivated PC12 TH, while those of the recombinant Leu-40m or Tyr-40m are similar to those of PC12 activated by protein kinase-mediated phosphorylation. (The $K_i$ for the pteridine cofactor of the recombinant wild type TH and PC12 TH was found to be in the range of 0.2 mM at pH 6.0 and in the range of 0.9 mM at pH 7.2, while that of the recombinant Leu-40m or Tyr-40m and of PC12 TH activated by PKA was found to be in the range of 0.09 mM at pH 6.0 and in the range of 0.1 mM at pH 7.2.) Thus, the kinetic values for the pteridine cofactor and the pH profile of the enzymatic activities of the recombinant Leu-40m or Tyr-40m suggest that these mutant enzymes are expressed in AtT-20 cells in an activated form with similar properties as the enzyme activated by PKA-mediated phosphorylation.

After this paper was submitted for publication, it was reported that recombinant wild type enzyme expressed in Escherichia coli (or in SF9 cells) is inactivated by incubation with dopamine in a time-dependent manner (18). We have therefore analyzed the effects of preincubation with dopamine on the wild type and of the Leu-40m enzyme obtained from AtT-20 cells. The addition of 10 $\mu$m of dopamine to the incubation mixture containing the wild type enzyme results in a 25% inhibition of the enzymatic activity, and the same magnitude of inhibition is observed when the enzyme is preincubated at 30 °C in KP buffer, pH 6.0, for 1–6 min. The addition of 10 $\mu$m dopamine to the incubation mixture containing the Leu-40m enzyme does not significantly alter the enzymatic activity, and no significant changes in the enzymatic activity were observed following preincubation with dopamine for 1–6 min. These results indicate that under our experimental conditions, the wild type or the Leu-40m enzymes expressed in AtT-20 cells are not inactivated by dopamine. Thus, the recombinant TH expressed in AtT-20 cells is resistant to dopamine inactivation in a similar fashion as the nonrecombinant enzyme from PC12 cells (18). The preincubation studies with dopamine are also in agreement with our kinetic results which show that dopamine less effectively inhibits the Leu-40m than the wild type enzyme.

We would like to point out that modification of Ser-40 by PKA-mediated phosphorylation, or removal of Ser-40 by mutagenesis, leads to activation of TH. One is therefore tempted to suggest that Ser-40 exerts an inhibitory effect on the catalytic activity, and modification at this site may produce a conformational change which leads to increased enzymatic activity. Since phosphorylation of TH results in a phosphate-bearing negative charge at the position of Ser-40, it is conceivable that substitution of Ser-40 with negatively charged dicarboxylic acids such as glutamic or aspartic acid will result in more pronounced activation of the enzyme.

It is noteworthy that based on several studies it was suggested that activation of TH by limited proteolysis leads to a release of an inhibitory TH peptide from the enzyme, and activation by phospholipids such as lyssolecithin leads to a reversible displacement of an inhibitory peptide (19, 20). We have shown that antibodies directed to a peptide corresponding to a TH segment containing Ser-40 (anti-TH-16) stimulate TH activity (21). The activation of TH by anti-TH-16 is also associated with a decrease in the $K_i$ for the pteridine cofactor (21). Based on experimental data we suggested that the stimulation of TH by anti-TH-16 is probably due to an interaction of anti-TH-16 with a segment of TH in the vicinity (probably upstream) of Ser-40 (21). Thus, TH may be present in the basal state in a conformational form which exhibits low enzymatic activity, and the activation of the enzyme by removal or blockade of a peptide located at the amino terminal site of the enzyme, or modification of Ser-40 either by phosphorylation or substitution with another amino acid, leads to a change in conformation to one or more forms with increased catalytic activity.

A number of studies have shown that TH is phosphorylated in vitro and in vivo at different sites by various protein kinases (4–6, 21, 22). It was reported that activation of the nigrostriatal pathway increases the phosphorylation of striatal TH at serines 19, 31, and 40 (23), and indirect evidence suggests that activation of TH by PKA involves phosphorylation at Ser-40. To further assess the role of Ser-40 in the activation of the enzyme, we have analyzed the effects of PKA-mediated phosphorylation of wild type TH and Ser-40 mutants on the activities of the respective enzymes. Our present findings that PKA-mediated phosphorylation stimulates the activity of the wild type enzyme, but not that of the Ser-40 mutant enzymes, demonstrate that activation of TH by PKA-mediated phosphorylation results from phosphorylation at Ser-40 and that phosphorylation by PKA at other Ser-containing sites does not increase enzymatic activity. Since phosphorylation at Ser-31 (22) may also contribute to the activation of TH, we have analyzed whether the activity of Ser-31 mutants is affected by PKA-mediated phosphorylation. It is evident from our studies that PKA-mediated phosphorylation increases the enzymatic activities of the wild type and that of the Ser-31 mutant to the same extent, indicating that activation of TH by PKA does not involve Ser-31. Thus, our data further establish the central role of Ser-40 in the activation of TH by PKA.

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