Proc. Nat. Acad. Sci. USA
Vol. 72, No. 8, pp. 2955–2958, August 1975
Biochemistry

ATP, cyclic AMP, and magnesium increase the affinity of rat striatal tyrosine hydroxylase for its cofactor*

(protein phosphorylation/catecholamines/dopamine/enzyme regulation)

WALTER LOVENBERG, ELEANOR A. BRUCKWICK, AND INGEBORG HANBAUER

Section on Biochemical Pharmacology, Hypertension-Endocrine Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Sidney Udenfriend, May 14, 1975

ABSTRACT **Treatment of rat striatal tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] with conditions optimal for protein phosphorylation results in the reduction of the tyrosine hydroxylase K_m for the cofactor 6-methyltetrahydropterin from 0.50 mM to 0.16 mM. This reaction is dependent upon ATP, 3′:5′-cAMP, and Mg^{++} and causes a marked decrease in the sensitivity to end-product inhibition. Other brain regions and the adrenal gland show a similar response.**

The rate of synthesis of catecholamines *in vivo* is controlled by the initial enzyme in the pathway, tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2]. As part of the first characterization of this enzyme, Udenfriend *et al.* (1) found that the end-products of the pathway, dopamine and norepinephrine, are powerful competitive inhibitors of the reaction with respect to the reduced pterin cofactor. Although it has long been accepted that end-product inhibition represents perhaps the most important regulatory mechanism for tyrosine hydroxylase, several recent studies have revealed experimental situations where both the content and rate of synthesis of catecholamines increase simultaneously. Nigro-striatal lesions cause a rapid increase in both content (2) and synthetic rate (3) of dopamine in the rat striatum. This phenomenon is inhibited by apomorphine, a dopamine receptor agonist, and stimulated by haloperidol, a dopamine receptor blocker (3). Regulation of tyrosine hydroxylase through changes of its affinity constants for the cofactor has been demonstrated recently (4). Furthermore, Zivkovic *et al.* (5) observed that tyrosine hydroxylase isolated from rats treated with haloperidol had a markedly lowered K_m for the reduced cofactor. We recently reported (6) that this phenomenon results from alteration of a macromolecule, since the effect is retained after chromatography on Sephadex G-25.

The recent discovery of a dopamine-sensitive adenylate cyclase (7) and its inhibition by dopamine receptor blockers (8–10) suggest that this enzyme may be the "dopamine receptor" and that cAMP may participate in the interconversion of the two forms of the tyrosine hydroxylase system having high and low Michaelis constants for the reduced pterin cofactor.

Abbreviation: $6MPH_4$, 6-methyltetrahydropterin.

* A preliminary account of some of this work was presented at the annual meeting of the American College of Neuropsychopharmacology, San Juan, December 1974, and at the Federation of American Societies of Experimental Biology, Atlantic City, N.J., April 1975.

The current experiments show that addition of components required for optimal protein phosphorylation substantially reduces the apparent K_m for 6-methyltetrahydropterin in solubilized tyrosine hydroxylase from rat striata. However, no direct incorporation of phosphate from ATP into the enzyme has been demonstrated.

MATERIALS

Male Sprague–Dawley rats were obtained from Zivic Miller Inc. and maintained on standard laboratory chow with a 12-hr light-dark cycle. The animals used in the following experiments weighed between 150 and 200 g.

6-Methyltetrahydropterin ($6MPH_4$) and biopterin were obtained from the Regis Chemical Co. These compounds were dissolved in 0.01 N HCl in a Thunberg tube to make a solution approximately 0.01 M. After the addition of a few mg of platinum oxide to 3 ml of solution the tube was alternately evacuated and flushed with hydrogen. After each experiment the tube was reequilibrated with hydrogen and frozen. Such a cofactor solution could be used repeatedly for several weeks with no loss of activity. Each cofactor solution was standardized spectrophotometrically, by means of the molar extinction coefficient at 264 nm of 1.6×10^4. The substrate L-[3,5-^{3}H]tyrosine was purchased from the New England Nuclear Corp. and was further purified by adsorption and elution from a 0.7 × 6.5 cm Dowex-50-X4 (200–400 mesh) column prior to use. The tritiated L-tyrosine was stored at 4° in H_2O containing 30% ethanol, and an appropriate aliquot was lyophilized immediately before each experiment. Adenosine [γ-^{32}P]triphosphate, 610 Ci/mol, was purchased from the New England Nuclear Corp. Rabbit antiserum directed against bovine adrenal tyrosine hydroxylase was kindly provided by Dr. D. M. Chuang and Dr. Erminio Costa of the National Institute of Mental Health. All other reagents were purchased from the usual commercial sources and used without further purification.

METHODS

The enzyme used in most of the studies was prepared from rat striata just prior to each experiment. The rats were decapitated, the brains were removed, and the striatal regions were quickly dissected from each hemisphere of several rats and homogenized in a conical glass homogenizer in 4 volumes of 0.05 M sodium acetate, pH 6.2, containing 0.2% Triton X-100. The homogenate was centrifuged at 40,000 × *g* for 10–15 min and the supernatant fraction was used as the enzyme source. All steps in preparing the enzyme were done at 0–5°. Tyrosine hydroxylase activity was measured

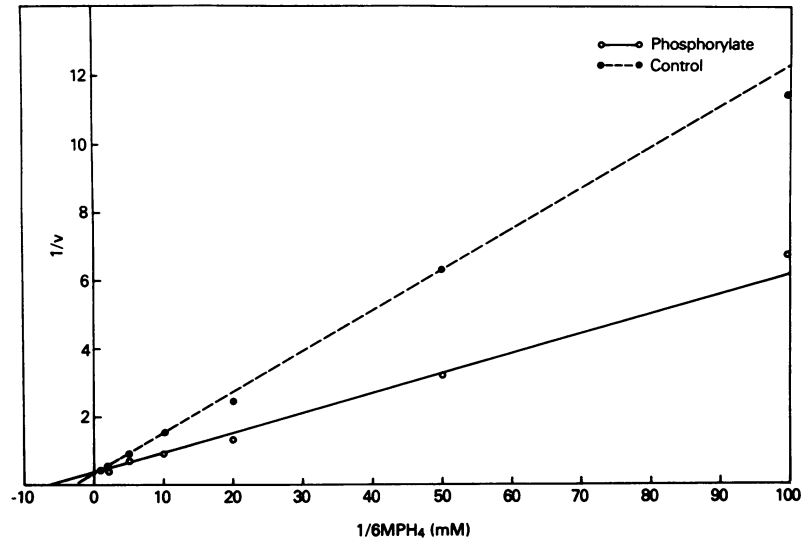

FIG. 1. The effect of phosphorylating conditions on the kinetic properties of tyrosine hydroxylase with respect to $6MPH_4$. Each assay contained 0.24 mg of protein. Experimental details are described in *text*. v = velocity.

by a modification of the method of Cicero *et al.* (11). Total volume of the reaction mixture was usually 160 μl, of which 30 μl was striatal supernatant fraction. In addition, the control reaction contained 10 nmol L-[3,5-^{3}H]tyrosine containing about 450,000 dpm, 12 μmol of 2-mercaptoethanol, 10 nmol of ferrous ammonium sulfate, 1.6 μmol of $6MPH_4$, and 20 μmol of sodium acetate, pH 6.0. The components other than the enzymes were usually combined into a cocktail and adjusted to pH 6.0 before the start of the reaction. The hydroxylation reaction was terminated by the addition of 400 μl of 5% trichloroacetic acid. The tritiated water released following the reaction is collected in the effluent of a 0.6 × 3.0 cm Dowex-50-X4 column (200–400 mesh) by the usual technique (11) and counted by liquid scintillation spectrometry. Reaction rates are calculated on the assumption that only one of the two tritium atoms on the tyrosine is released, and rates are expressed as nmol of tyrosine hydroxylated per mg of protein per hour.

In experiments where phosphorylating conditions were used, the following components were added to the reaction mixture: magnesium acetate, 2 μmol; NaF, 2 μmol; theophylline, 80 nmol; ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 12 nmol; ATP, 50 nmol; 3':5'-cAMP, 10 nmol; phosphocreatine, 0.4 mg; and creatine kinase, 1 mg. The latter two components were only needed when concentrations of ATP were less than 0.1 mM.

The incorporation of ^{32}P-labeled phosphate ([γ-^{32}P]ATP, 100 Ci/mol) into striatal tyrosine hydroxylase was measured by specific immunoprecipitation. The homogenization and centrifugation of the striata was carried out as described above. The supernatant fraction (200 μl) was incubated for 5 min at 30° after addition of phosphorylating agents described above. The reaction mixture was transferred to an ice bath and subsequently chromatographed on a Sephadex G-25 column (0.5 × 10 cm). The fraction containing tyrosine hydroxylase was then incubated with rabbit antiserum specific for tyrosine hydroxylase for one hour at 37° and, thereafter, for 12 hr at 4° (12). The reaction mixture contained 140 mM KCl, 3 mM MgCl, and 0.2% Triton X-100 (medium A) and the amount of antibody required to precipitate quantitatively the tyrosine hydroxylase. This amount has been determined by immunochemical titration. Rabbit serum to make a 2.5% solution and sheep anti-rabbit serum (GIBCO, Grand Island, N.Y. 14072) were next added and the solution was incubated for 2 hr at 4° to complete the immunoprecipitation. After centrifugation at 10,000 × *g* at 4° for 15 min the precipitates were washed four times with medium A containing 0.2% bovine serum albumin. The washed precipitates were dissolved in 1.0 ml of NCS (Amersham Searle) and the radioactivity was determined in Aquasol by liquid scintillation spectrometry. Two types of blanks were used in this determination. In the first type normal rabbit serum instead of rabbit antiserum for tyrosine hydroxylase was added to the mixture. In the second type tyrosine hydroxylase was removed by immunoprecipitation prior to the incubation with phosphorylating agents.

RESULTS

The addition of phosphorylating components had little effect on the rate of tyrosine hydroxylation under optimal reaction conditions. However, in the presence of limiting concentrations of $6MPH_4$ (0.1 mM) stimulation of as much as 3-fold control values was observed. The data shown in Fig. 1 indicate that ATP and phosphorylating components reduce the K_m of $6MPH_4$ to 1/3 the control value (0.50 mM reduced to 0.16 mM) while having no effect on V_{max}. Comparable experiments done with tyrosine concentrations ranging from 5×10^{-6} M to 5×10^{-4} M at a constant cofactor concentration (10^{-3} M) indicate that phosphorylation does not change the K_m for tyrosine (control = 44 ± 1 μM and phosphorylated 46 ± 3 μM).

The dependency of the alteration in the kinetic properties of tyrosine hydroxylase on the individual components of the phosphorylating mixture is shown in Table 1. It is apparent that the stimulation is totally dependent upon ATP and partially dependent upon cAMP and Mg^{++}. Cyclic GMP cannot replace cAMP. Since in several experiments the addition of purified protein kinase was without additional effect and since the brain is a good source of protein kinase, it was assumed that sufficient activity was present in the enzyme preparation and purified protein kinase was not added in the standard experiments. In the presence of phosphorylating conditions other nucleotides such as GTP and ADP had slight stimulating effects using 5×10^{-4} M. However, if the concentration was reduced to 0.1 mM they had no effect

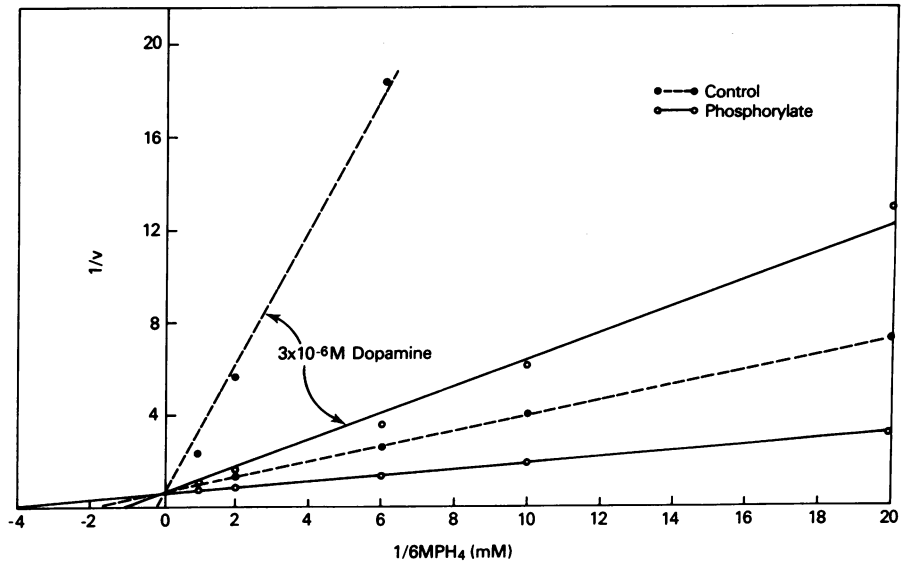

FIG. 2. Inhibition of tyrosine hydroxylase by dopamine under standard and phosphorylating conditions. Experiments were done with 0.03 ml of enzyme solution containing 0.24 mg of protein for each point as described in the *text*.

while ATP or ADP in the presence of the regenerating system were fully active at this concentration. The effect of the standard phosphorylating conditions was just as pronounced when tetrahydrobiopterin replaced $6MPH_4$ as the cofactor. The addition of 0.5 mM ATP, 0.1 mM cAMP, or 20 mM magnesium acetate alone had no effect on either the rate of reaction or the K_m for either cofactor or substrate.

The effect of phosphorylating agents on end-product inhibition is shown in Fig. 2. Since dopamine is a competitive inhibitor with respect to the cofactor, a reduction in the cofactor K_m dramatically alters the degree of inhibition by any one concentration of end-product.

In order to examine whether these changes in enzyme kinetics were due to direct phosphorylation of the enzyme molecule we determined immunochemically whether ^{32}P from [^{32}P]ATP was incorporated into tyrosine hydroxylase. No specific incorporation of phosphate into tyrosine hydroxylase was detected under the experimental condition which reduced the K_m for the cofactor. Both tyrosine-hydroxylase-containing samples and samples from which tyrosine hydroxylase had been precipitated with its antibody prior to incubation with phosphorylating agents incorporated 0.6 pmol ^{32}P per mg of protein.

In the experiments reported by Zivkovic *et al.* (5, 13) neuroleptics caused a shift in the affinity constant of the enzyme for the cofactor only in the striatum. It was therefore of interest to examine the effect of phosphorylating agents on the enzyme obtained from other brain regions and the adrenal glands. The enzyme was assayed in the presence of limiting amounts of $6MPH_4$ (0.1 mM), and the results of such an experiment are shown in Table 2. While the degree of activation varies, it is clear that phosphorylating agents activate the enzyme from any brain region or the adrenal gland.

Table 1. Requirements for the stimulation of tyrosine hydroxylase activity*

Condition	Tyrosine hydroxylase activity, nmol dopa/mg per hr
Control	2.74 ± 0.08
Phosphorylating conditions	6.27 ± 0.15
— ATP	2.71 ± 0.12
— cAMP	4.25 ± 0.21
— cAMP + cGMP	4.24 ± 0.19
— Mg^{++}	3.95 ± 0.08

* The activity was measured under standard assay conditions using 0.1 mM $6MPH_4$ as cofactor. The results are expressed as the mean ± SEM from three separate enzyme preparations assayed in duplicate. No creatine phosphate or creatine kinase were used in this experiment.

Table 2. Phosphorylating conditions activate tyrosine hydroxylase activity from various brain regions and the adrenal gland*

Tissue	Tyrosine hydroxylase activity, nmol dopa/mg protein per hr	
	Control	Experimental
Striatum	2.16 ± 0.28	4.56 ± 0.21
Septum	0.36 ± 0.04	0.50 ± 0.05
Hypothalamus	0.37 ± 0.02	0.61 ± 0.06
Forebrain	0.13 ± 0.02	0.23 ± 0.02
Adrenal	3.20 ± 0.29	5.61 ± 0.62

* Each tissue was homogenized in 4 volumes of the homogenizing buffer and centrifuged as described in *Methods*. The values are the mean of four separate experiments assayed in duplicate in the presence of 0.1 mM $6MPH_4$.

DISCUSSION

Several experimental conditions have been reported that will alter the kinetic properties of tyrosine hydroxylase *in vitro* and *in vivo*. Kuczenski and Mandell (14, 15) have shown that either heparin sulfate or other anions can cause an apparent reduction in the K_m of the cofactor. Likewise, Lloyd and Kaufman (16) have described a stimulation of partially purified tyrosine hydroxylase by phosphatidyl serine. These *in vitro* changes show a striking resemblance to the changes observed in rat striatal tyrosine hydroxylase following treatment with neuroleptic drugs (4, 13). Both effects obtained *in vitro* and *in vivo* are similar to that caused by phosphorylating agents. It appears, therefore, that an appropriately placed anionic group is necessary for high-affinity binding of the cofactor. This may be created by phosphorylation of the enzyme or of a specific activator molecule, and can per-

haps be mimicked by a variety of anions with the appropriate steric configuration.

Harris *et al.* (17) reported that cAMP activates tyrosine hydroxylase by lowering the K_m of both the cofactor and the substrate. In our current study we find that cAMP alone has no effect on the kinetic properties of tyrosine hydroxylase with regard to either the substrate or the cofactor. The reason for this difference may be due to differences in the concentrations of substrate and cofactor used and the fact that these workers used a pteridine reductase regenerating system. Furthermore, the stimulation by cAMP alone observed by these workers may have been due to the presence of unmetabolized high-energy phosphates in their extracts. Roth *et al.* (18) have recently suggested that the cAMP effect is mediated by a phosphorylation reaction.

The experiments with immunoprecipitation of tyrosine hydroxylase after reaction with labeled ATP did not show direct incorporation of phosphate into the enzyme. A potential explanation of this negative result is that the antibody may not form a precipitating complex with putative phosphorylated enzyme because of changes in the charge and conformation of the protein. It is also possible that phosphatases may quantitatively remove the phosphate during the long incubations required. It would appear that active phosphatases and ATPases are present in brain extracts, since relatively high concentrations of ATP or an ATP-regenerating system is required to observe the effect of phosphorylation. Therefore, rapid loss of phosphate from the protein during the immunoprecipitation step may occur and mask the potential phosphorylation of tyrosine hydroxylase. Further experiments are necessary to obtain evidence for or against direct phosphorylation of tyrosine hydroxylase.

Dopamine receptor blockers are inhibitors of the dopamine-sensitive adenylate cyclase (8–10). Moreover, it is known that these compounds convert tyrosine hydroxylase to the more active form *in vivo* (4, 5, 13); it is expected that the high K_m form would be found under phosphorylating conditions and the low K_m form would be present under dephosphorylating conditions. The opposite was observed. This suggests that in the striatum the role of cAMP is not that of simply activating a protein kinase but may also be involved in activating phosphatases.

The importance of the above findings from a physiological point of view is that, since the reduced cofactor is in suboptimal concentration, a rapid and substantial change in the catecholamine synthetic rate can occur even when concentrations of catecholamines remain unchanged. If it is assumed that the tyrosine hydroxylating system is in the dephosphorylated configuration in the resting control animal and that the levels of tetrahydrobiopterin and dopamine are approximately 0.1 mM (6), the system would be operating at less than 1% of its capacity. The enzyme activity measured in the above experiments is over 700 ng/g of tissue per hr, whereas the values observed for *in vivo* synthesis are about 1–2% of this rate (3, 19). A decrease in the K_m of the cofactor would result in a proportional increase in *in vivo* synthesis. Activation of a protein kinase or inactivation of a protein phosphatase would result in a rapid decrease in the K_m for tetrahydrobiopterin. This may be the phenomenon which occurs when a lesion is placed in the nigro-striatal pathway (2) or when a neuroleptic drug is given (4, 5, 13). Based on such a molecular mechanism, receptor-mediated feedback inhibition could easily be explained.

1. Udenfriend, S., Zaltzman-Nirenberg, P. & Nagatsu, T. (1965) *Biochem. Pharmacol.* **14**, 837–845.
2. Anden, N. E., Bedard, P., Fuxe, K. & Ungersted, V. (1972) *Experientia* **28**, 300–301.
3. Carlsson, A., Kehr, W. & Lindqvist, M. (1974) *Adv. Biochem. Psychopharmacol.* **12**, 135–142.
4. Costa, E., Guidotti, A. & Zivkovic, B. (1974) *Adv. Biochem. Psychopharmacol.* **12**, 161–175.
5. Zivkovic, B., Guidotti, A. & Costa, E. (1974) *Mol. Pharmacol.* **10**, 727–735.
6. Lovenberg, W. & Bruckwick, E. A. (1975) in *Pre- and Post-Synaptic Receptors*, eds. Usdin, E. & Bunney, W. E. (Marcel Dekker, New York), pp. 149–169.
7. Kebabian, J. W., Petzold, G. L. & Greengard, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2145–2149.
8. Clement-Cormier, Y. C., Kebabian, J. W., Petzold, G. L. & Greengard, P. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1113–1117.
9. Miller, R. J., Horn, A. S. & Iversen, L. L. (1974) *Mol. Pharmacol.* **10**, 759–766.
10. Karobath, M. & Leitich, H. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2915–2918.
11. Cicero, T. J., Sharpe, L. G., Robins, E. & Grote, S. S. (1972) *J. Neurochem.* **19**, 2241–2243.
12. Chuang, D. M. & Costa, E. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4570–4574.
13. Zivkovic, B. & Guidotti, A. (1974) *Brain Res.* **79**, 505–509.
14. Kuczenski, R. T. & Mandell, A. J. (1972) *J. Biol. Chem.* **247**, 3114–3122.
15. Kuczenski, R. T. & Mandell, A. J. (1972) *J. Neurochem.* **19**, 131–137.
16. Lloyd, T. & Kaufman, S. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1262–1269.
17. Harris, J. E., Morgenroth, V. H., Roth, R. H. & Baldessarini, R. J. (1974) *Nature* **252**, 156–158.
18. Roth, R. H., Walters, S. R., Murrin, L. & Morgenroth, V. H., III (1975) in *Pre- and Post-Synaptic Receptors*, eds. Usdin, E. & Bunney, W. E. (Marcel Dekker, New York), pp. 5–48.
19. Yamabe, H., de Jong, W. & Lovenberg, W. (1973) *Eur. J. Pharmacol.* **22**, 91–98.