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Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: Mechanism of enzyme activation

(purification/kinetics/tyrosine monooxygenase)

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ABSTRACT **Tyrosine hydroxylase [tyrosine monooxygenase, L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] was highly purified from rat caudate nuclei. When the pure hydroxylase was phosphorylated by incubation with cyclic AMP-dependent protein kinase and [^{32}P]ATP, ^{32}P and tyrosine hydroxylase activity were detected after polyacrylamide gel electrophoresis in a single protein band. After sodium dodecyl sulfate gel electrophoresis, ^{32}P was detected only in a probable active subunit of tyrosine hydroxylase of molecular weight 62,000. Phosphorylation of the hydroxylase increased its activity by 2-fold, and was associated with an increase in V_m without any change in K_m for either substrate or cofactor. We propose that the pool of native tyrosine hydroxylase is composed of a mixture of enzyme molecules in both active and probably inactive forms, that the active form is phosphorylated, and that phosphorylation produces an active form of the enzyme at the expense of an inactive one.**

An important, if not major, action of the cyclic nucleotides is to initiate the phosphorylation of intracellular proteins via the activity of protein kinases (1–3). This phosphorylation, which in many instances converts a biologically inert molecule into an active one, has been proposed to serve as a final common pathway for a variety of agents regulating cellular function (1–4). It remains to be established if phosphorylation is a mode of regulation of the activity of a class of proteins essential for neuronal function; namely, the enzymes that catalyze the biosynthesis of neurotransmitters.

In brain, the enzyme tyrosine hydroxylase [tyrosine monooxygenase, L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating, EC 1.14.16.2] catalyzes the first and presumed rate-limiting step in the biosynthesis of the catecholamine neurotransmitters, dopamine and norepinephrine (5). Over the past several years the demonstration that the activity of the enzyme is altered by cyclic nucleotides (6–11) has raised the question as to whether Tyr hydroxylase may be regulated by phosphorylation, and if so, whether it is the enzyme itself which is phosphorylated. Subjection of impure enzyme, either partially purified or in tissue extracts, to conditions of phosphorylation has resulted in activation of the enzyme, attributed variously to an increased affinity of enzyme to cofactor (6, 9, 10) or reduction of inhibition by a natural inhibitor(s) (6, 12). However, since pure Tyr hydroxylase was not available, it was not possible to determine whether the molecule itself was directly phosphorylated (13) or whether the effect was due to phosphorylation of some other regulatory mechanism.

In the present study, using highly purified Tyr hydroxylase, we have sought to determine if the enzyme can be directly phosphorylated. We shall demonstrate that the enzyme can be phosphorylated by a cyclic AMP (cAMP)-dependent protein kinase, that such phosphorylation increases the catalytic activity of the enzyme, and that the kinetics of activation suggest that the effect is due to the conversion of inactive to active enzyme.

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MATERIALS AND METHODS

Materials. L-Tyrosine, Trizma base, ATP, cAMP, and sodium dodecyl sulfate ($NaDodSO_4$) were purchased from Sigma Chemical Co.; 6-methyltetrahydropteridine ($MePH_4$) from Calbiochem; NCS Tissue Solubilizer from Amersham/Searle Corp.; [γ-^{32}P]ATP tetra(triethylammonium) salt (2–10 Ci/mmol), L-[U-^{14}C]tyrosine (380 Ci/mol), and Bray's solution from New England Nuclear Corp.; phenyl-Sepharose and DEAE-Sephadex from Pharmacia Fine Chemicals, Inc.; DEAE-cellulose (DE-52) from Whatman Ltd.; Bio-Gel P-300 from Bio-Rad Laboratories; and acrylamide and *N,N'*-methylenebisacrylamide from Eastman Kodak Co.

Enzyme Assay. Tyr hydroxylase activity was assayed by modification (14) of the method of Coyle (15), with 1 mM $MePH_4$. For studies of the kinetics of the enzyme, the initial velocity was determined by assaying for 5 min. Protein kinase activity was assayed by the method of Rubin *et al.* (16).

Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the methods of Davis (17) and Ornstein (18), and $NaDodSO_4$/polyacrylamide gel ($NaDodSO_4$ gel) electrophoresis by the method of Weber and Osborn (19).

Determination of Molecular Weights by $NaDodSO_4$ Gel Electrophoresis. The molecular weights of protein bands of purified Tyr hydroxylase on $NaDodSO_4$ gels were determined by comparison of their electrophoretic mobility with those of three standard proteins: bovine serum albumin (67,000), egg albumin (45,000), and chymotrypsinogen A (27,000).

Purification of Tyr Hydroxylase from Rat Caudate Nucleus. Caudate nuclei (33.9 g) were removed from the brains of 530 Sprague–Dawley rats killed by cervical dislocation and decapitation, pooled, and homogenized in 20 mM potassium phosphate buffer (pH 7.0) with a Polytron tissue homogenizer. The buffer used throughout the purification was potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 39,000 × g for 30 min. Powdered ammonium sulfate was added to the supernatant to 80% saturation, and the precipitate was collected by centrifugation at 15,000 × g for 15 min. The precipitate was dialyzed against 20 mM buffer and the protein was further fractionated by ammonium sulfate from 25 to 50% saturation. The fraction was then dialyzed against 20 mM buffer and equilibrated with the same buffer containing 20% ammonium sulfate.

A phenyl-Sepharose column (1.5 × 12 cm) was equilibrated with 20 mM buffer containing 20% ammonium sulfate, and the

Abbreviations: Tyr hydroxylase, tyrosine hydroxylase; cAMP, cyclic AMP; $NaDodSO_4$, sodium dodecyl sulfate; $MePH_4$, 6-methyltetrahydropteridine.

dialyzed protein was applied to the column. The column was washed with 20 mM buffer containing 20% ammonium sulfate, and the protein was then eluted sequentially, first by buffer containing 5% ammonium sulfate, next by 5 mM buffer, and, finally, by water. More than 90% of the total Tyr hydroxylase activity was eluted with 5 mM buffer representing approximately a 6-fold purification of the enzyme.

The eluted enzyme was concentrated and put on a DEAE-cellulose column (0.9 × 6 cm), which was equilibrated with 20 mM buffer. The protein was again eluted by sequential application of buffer containing either 70, 120, or 200 mM sodium chloride. Fifty percent of the total Tyr hydroxylase activity was recovered by elution with the buffer containing 120 mM sodium chloride, representing a further 3.3-fold purification. The active enzyme was then put on a second DEAE-cellulose column (0.9 × 4.5 cm) and eluted with a linear concentration gradient of sodium chloride between 50 and 200 mM. In this step, approximately 70% of the enzyme activity was recovered, producing greater than a 2-fold purification. A summary of the purification steps is given in Table 1.

Purification of cAMP-Dependent Protein Kinase from Rat Heart. Protein kinase was purified from rat heart by the method of Rubin *et al.* (16). In summary, 150 rat hearts were homogenized in 40 mM potassium phosphate buffer, pH 6.1/2 mM EDTA/4 mM mercaptoethanol, and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was brought to 55% saturation with ammonium sulfate and dialyzed against 50 mM Tris buffer, pH 7.6/10 mM sodium chloride/4 mM mercaptoethanol. Protein kinase was further purified by DEAE-Sephadex (batch type), DEAE-cellulose column chromatography, and finally Bio-Gel P-300 column chromatography. The specific activity of purified protein kinase was 295 nmol/mg per min.

Phosphorylation of Tyr Hydroxylase and Separation of Phosphorylated Hydroxylase from Protein Kinase. Tyr hydroxylase recovered from the second DEAE-cellulose column was used for the enzyme phosphorylation. The phosphorylation conditions were as follows. To 400 μl containing 300 μg of the hydroxylase, the following incubation mixture was added to make a final volume of 1.0 ml: 15 μg of protein kinase purified from rat heart, 50 mM potassium phosphate buffer (pH 7.0), 50 μM ATP containing 1.5 nmol of [^{32}P]ATP, 10 μM cAMP, and 10 mM $MgSO_4$. The mixture was incubated for 5 min at 30°.

The phosphorylated Tyr hydroxylase was then separated from protein kinase by polyacrylamide gel electrophoresis for 4 hr at 3 mA per tube, with 7.5% acrylamide gel and 0.4 M glycine/Tris buffer, pH 8.2. In order to identify the location of Tyr hydroxylase on the gels, we incubated the same reaction mixture with unlabeled ATP and placed it on another gel. After staining, the single protein band containing hydroxylase activity was identified. The region of radiolabeled ^{32}P corresponding to authentic enzyme was isolated and pooled, and enzyme protein was eluted overnight in a cold room (4°C) with 0.1 M sodium acetate buffer (pH 5.6). Electrophoretic separation was essential because protein kinase was completely removed from the system so that pure phosphorylated Tyr hydroxylase could be identified.

In order to demonstrate that electrophoretic separation of Tyr hydroxylase from other contaminant protein yields completely pure and homogeneous hydroxylase, we subjected the eluate of the second DEAE-cellulose column to the same electrophoresis. The hydroxylase band was isolated and pooled, and enzyme protein was eluted in a manner similar to that described above. This step recovered approximately 20% of Tyr hydroxylase activity, with a 1.6-fold further purification.

In the kinetic studies of enzyme activation, Tyr hydroxylase eluted from gels was used to ascertain the purity of the enzyme used for the activity assay. The phosphorylation was carried out by incubation of 500 μl (15 μg) of the hydroxylase with the same incubation mixture without [^{32}P]ATP as described above.

RESULTS

Homogeneity and Protein Subunits of Tyr Hydroxylase. Electrophoresis of the eluate of the second DEAE-cellulose column, which was used for the enzyme phosphorylation, yielded one major and one faint minor band (Fig. 1, gel a). Since electrophoretic separation of Tyr hydroxylase from protein kinase after enzyme phosphorylation was an essential step in identifying the purity and homogeneity of phosphorylated hydroxylase, it was necessary to demonstrate the purity of the hydroxylase eluted from the gels after electrophoresis. Electrophoresis of Tyr hydroxylase eluted from these gels showed only a single protein band, which contained hydroxylase activity (Fig. 1, gel b). $NaDodSO_4$ gel electrophoresis demonstrated that this pure Tyr hydroxylase consists of three protein subunits with molecular weights equivalent to 52,000, 62,000, and 68,000 (Fig. 1, gel c). In order to demonstrate that all protein subunits of Tyr hydroxylase are common to all forms of the hydroxylase from different tissues, the enzyme was purified from human neuroblastoma cell lines (SK-N-BE-2) by similar purification procedures. This, however, yielded only a single protein band of molecular weight 62,000 (Fig. 1, gel d), suggesting that a protein unit of molecular weight 62,000 is a universal and active unit of Tyr hydroxylase.

Phosphorylation of Tyr Hydroxylase. In our initial study we utilized a commonly used, commercially available protein kinase prepared from bovine heart (Sigma Chemical Co.). However, we discovered that if this protein kinase (probably protein kinase type II) were incubated with [^{32}P]ATP and the

Table 1. Purification of Tyr hydroxylase from rat caudate nuclei*

Purification step	Total protein, mg	Total activity,† units	Specific activity,‡ units/mg protein	Yield, %
80% ammonium sulfate precipitate	889.6	23,191	26.1	100
25–50% ammonium sulfate fraction	194.1	15,196	78.35	65.5
Phenyl-Sepharose column (hydrophobic)	30.5	13,966	457.9	60.2
DEAE-cellulose column (stepwise)	5.08	7,676	1511.1	33.1
DEAE-cellulose column (gradient)	1.71	5,426	3173.3	23.4
Polyacrylamide gel electrophoresis	0.2§	1,010	5045	4.4

* Starting material: 530 rat caudate nuclei, 33.9 g.

† Units are expressed in nmol of dopa formed for a 20-min assay. Tyr = 0.2 mM; $MePH_4$ = 1 mM, 30°, pH 5.9.

‡ Protein concentration was determined by the method of Lowry *et al.* (20).

§ Since protein concentration was highly diluted in the elution buffer, this value is approximate. Therefore, specific activity is also approximately estimated. Activity was determined by use of 50 μM tyrosine. In this stage the enzyme was unstable, and when the protein was concentrated, the specific activity drastically decreased.

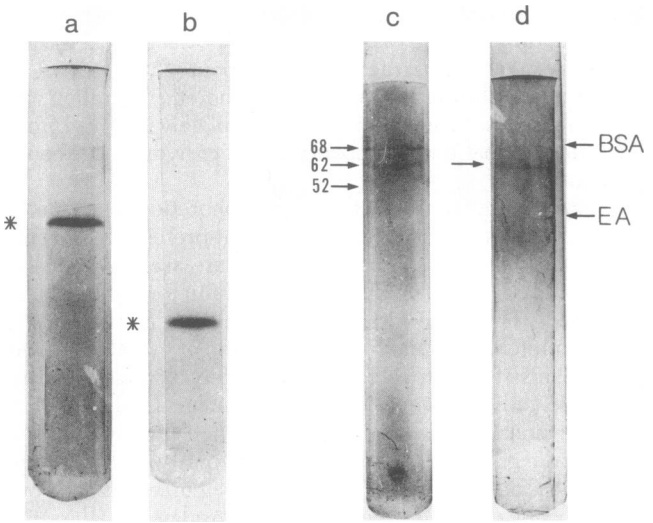

FIG. 1. Regular polyacrylamide gel electrophoretic patterns of Tyr hydroxylase: (Gel a) Eluate of second DEAE-cellulose column chromatography. To show entire protein profile, we carried out electrophoresis for 2 hr and stained the protein by 0.1% amido black in 7% acetic acid. Hydroxylase activity was detected only in a major protein band (asterisk). One minor protein band was also detected. (Gel b) Pure enzyme eluted from gels after electrophoresis as described above. Electrophoresis was for 4 hr to examine further separation of the protein band. However, only a single protein band was detected. $NaDodSO_4$ gel electrophoretic patterns of pure Tyr hydroxylase: (Gel c) Pure enzyme as in gel b. Three protein subunits were detected with molecular weights equivalent to 52,000, 62,000, and 68,000, as indicated. (Gel d) Pure enzyme from cultured cells of human neuroblastoma (SK-N-BE-2). Only one protein unit of molecular weight 62,000 was detected (arrow). Mobility of two standard proteins, bovine serum albumin (BSA) and egg albumin (EA), is indicated.

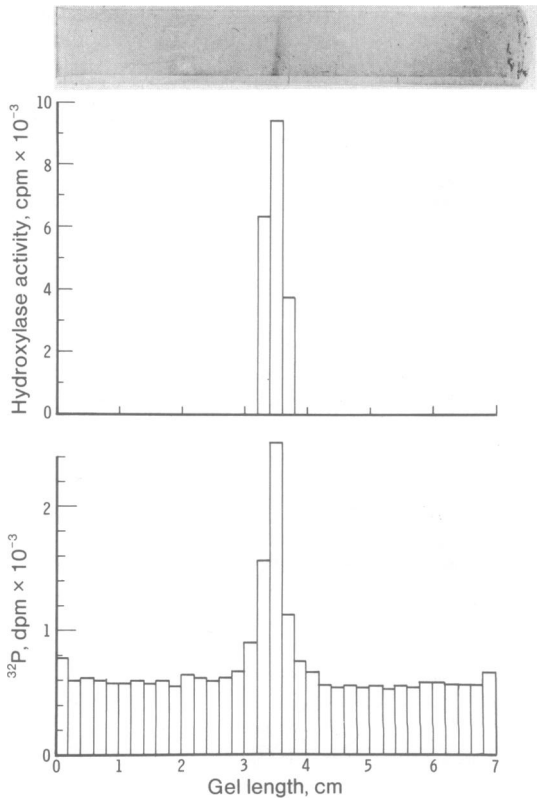

FIG. 2. Distribution of Tyr hydroxylase activity and ^{32}P in a polyacrylamide gel after electrophoresis of phosphorylated and purified hydroxylase. Note that both the enzymatic activity and ^{32}P were detected only in the hydroxylase band. Conditions for the electrophoresis were similar to those described for Fig. 1; 2-mm gel slices were used for determination of hydroxylase activity and ^{32}P.

incubation mixture subjected to polyacrylamide gel electrophoresis, ^{32}P was detected in several protein bands on the gel. One of these radiolabeled bands migrated a distance identical to that which authentic Tyr hydroxylase migrates under comparable conditions, therefore making it impossible to isolate phosphorylated Tyr hydroxylase from this other phosphorylated protein. On the other hand, when protein kinase purified from rat heart (probably type I) was comparably treated, no phosphorylated bands could be detected on the gel. Hence, for all studies of phosphorylation of Tyr hydroxylase, it was essential to use rat heart protein kinase.

After phosphorylation, the hydroxylase was subjected to polyacrylamide gel electrophoresis to separate phosphorylated hydroxylase from protein kinase. The region of radiolabeled ^{32}P corresponding to authentic enzyme was isolated and pooled, and the enzyme protein was eluted and concentrated. Aliquots of the eluted ^{32}P-labeled hydroxylase were then subjected to polyacrylamide or $NaDodSO_4$ gel electrophoresis.

Polyacrylamide gel electrophoresis yielded only one protein band, which was the only source of both Tyr hydroxylase activity and ^{32}P in the gel (Fig. 2). On $NaDodSO_4$ gel electrophoresis, ^{32}P was detected only in the protein band corresponding to the subunit of molecular weight 62,000 (Fig. 3). Addition of cAMP to the reaction mixture was necessary for phosphorylation of the hydroxylase; without cAMP, ^{32}P did not appear in the hydroxylase band on regular polyacrylamide or $NaDodSO_4$-containing gels. These findings demonstrate that Tyr hydroxylase, and especially the protein unit of molecular weight 62,000, can be phosphorylated by a cAMP-dependent protein kinase.

Activation of Tyr Hydroxylase by Phosphorylation and Kinetics of Activated Enzyme. When Tyr hydroxylase, highly purified by gel electrophoresis, was incubated with purified protein kinase, cAMP, ATP, and Mg^{2+} for 5 min (see *Materials and Methods*), hydroxylase activity increased more than 2-fold

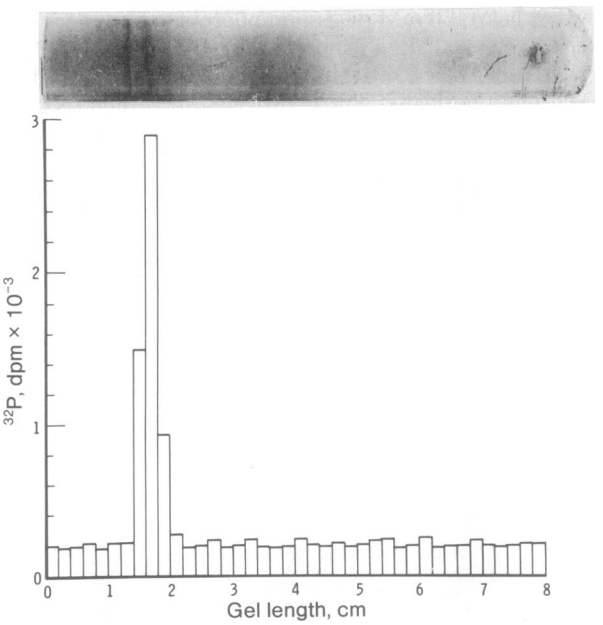

FIG. 3. Distribution of ^{32}P-labeled Tyr hydroxylase in $NaDodSO_4$ gel after electrophoresis of phosphorylated hydroxylase. ^{32}P was detected only in a subunit of molecular weight 62,000. Conditions for $NaDodSO_4$ electrophoresis were similar to those described for Fig. 1; 2-mm gel slices were used for determination of ^{32}P.

(Fig. 4). No change in enzyme activity occurred when cAMP was deleted from the reaction mixture.

Kinetic analysis of activated enzyme demonstrated that the increase in Tyr hydroxylase activity produced by phosphorylation was associated with an increase in V_m without change in K_m for either substrate or cofactor (Fig. 4). The Lineweaver–Burke plot of Tyr hydroxylase activity of both untreated and phosphorylated enzyme was linear, indicating thereby an absence of allosteric changes or substrate inhibition of the enzyme.

DISCUSSION

The present study, as its first objective, sought to determine if Tyr hydroxylase could be phosphorylated by a cAMP-dependent protein kinase. The hydroxylase was highly purified from caudate nucleus of rat brain, and the enzyme was phosphorylated by cAMP-dependent protein kinase purified from rat heart (probably type I). The homogeneity of both pure Tyr hydroxylase and pure phosphorylated enzyme was established by the demonstration that, when subjected to polyacrylamide gel electrophoresis, only one protein band was detected and that this solitary band had Tyr hydroxylase activity. However, $NaDodSO_4$ gel electrophoresis of pure enzyme yielded three protein bands whose molecular weights corresponded to 52,000, 62,000, and 68,000, suggesting that the enzyme consists of three dissimilar protein subunits. The fact that three protein bands of identical molecular weights can be isolated by $NaDodSO_4$ gel electrophoresis of Tyr hydroxylase purified from other brain regions and the adrenal gland of rats (21), and that $NaDodSO_4$ gel electrophoresis of Tyr hydroxylase purified from a human neuroblastoma cell line (Fig. 1, gel d) and rat pheochromocytoma by others (22) yields only a single protein band of molecular weight of 62,000, implies that the protein unit of molecular weight 62,000 is catalytically active. The two other units may represent structural or regulatory subunits.

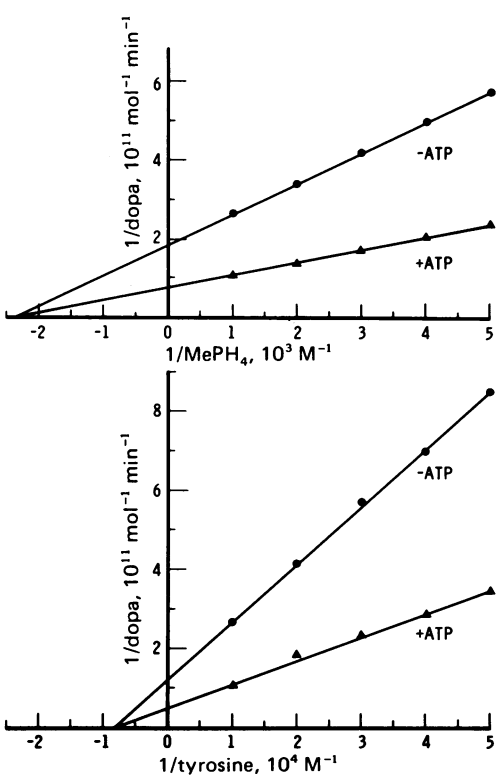

FIG. 4. Kinetic comparison between pure Tyr hydroxylase and pure phosphorylated hydroxylase. Note K_m for either substrate or cofactor does not change by enzyme phosphorylation. K_m values for $MePH_4$ and tyrosine were 4.4×10^{-4} and 1.25×10^{-4} M, respectively. Initial velocity was measured for 5 min at 30°. Tyrosine (1×10^{-4} M) and $MePH_4$ (1×10^{-3} M) were used for determination of K_m for $MePH_4$ and tyrosine, respectively. Tyr hydroxylase activity used for this experiment was as follows: without ATP, with cAMP: 78.6 pmol/20 min; without cAMP, with ATP: 79.3 pmol/20 min; with both cAMP and ATP: 171.4 pmol/20 min. Concentrations of tyrosine and $MePH_4$ were 2×10^{-4} and 1×10^{-3} M, respectively.

By incubating Tyr hydroxylase with [^{32}P]ATP, cAMP, and protein kinase and isolating pure ^{32}P-labeled Tyr hydroxylase, we were able to demonstrate that the enzyme could be phosphorylated and that phosphorylation depended on the presence of cAMP and increased enzyme activity by 2-fold. The finding that the distribution of ^{32}P, after separation of the phosphorylated enzyme by $NaDodSO_4$ gel electrophoresis, was restricted to a band corresponding to the subunit of Tyr hydroxylase of molecular weight 62,000 suggests that activation of the enzyme was the result of phosphorylation of this catalytically active subunit.

An important finding in the present study was that, with pure Tyr hydroxylase, enzyme phosphorylation was associated with an increase in V_m without any change in the apparent K_m for either substrate or cofactor. An increase in V_m without any change in K_m probably reflects an increase in the amount of enzyme, the additional molecules having kinetic characteristics similar to those of the original ones. Since, in our system, the total amount of hydroxylase was fixed, an increase in the amount of enzyme must therefore represent an increase in the amount of the catalytically active form of the enzyme from inactive enzyme molecules. This concept therefore implies that in tissue the pool of native Tyr hydroxylase is composed of a mixture of enzyme molecules in both active and probably inactive forms, that the active form is phosphorylated, and that phosphorylation produced an active form of the enzyme at the expense of the inactive one. On this basis it may be possible to calculate the ratio of the amount of active to inactive form in pure Tyr hydroxylase from the difference of V_m between the native and phosphorylated forms by the Lineweaver–Burke plots (Fig. 4).

The kinetics of pure Tyr hydroxylase produced by phosphorylation differ from those which follow exposure of crude tissue preparation or partially purified enzyme to comparable phosphorylating conditions (6–10). As reported by others (6–10), phosphorylation of impure enzymes results in a decrease in the K_m for the pteridine cofactor with or without a change in V_m. Such kinetics have been interpreted as representing either an increase in the affinity of the cofactor to enzyme (6, 9, 10) or a reduction in inhibition of the hydroxylase by natural inhibitor(s) in tissue (6, 12). The difference between the kinetics produced by using pure or impure preparations of the hydroxylase most probably demonstrates the fact that the apparent K_m in crude enzyme preparation represents a mixed kinetic parameter of K_m and K_i, since such preparations contain natural inhibitors (12). Therefore, the change in the apparent K_m by enzyme phosphorylation is due to a change in the mixed kinetic parameter rather than any change in the real K_m of Tyr hydroxylase. The recent finding by Ames *et al.* (12), that when the inhibitor was removed from the system K_m change was minimal, supports this contention.

This study, therefore, clearly establishes the fact that phosphorylation of Tyr hydroxylase by cAMP-dependent protein kinase can regulate the activity of the enzyme *in vitro*. This evidence adds support to the hypothesis that one mode of regulation of the hydroxylase could be via a sequence from a membrane receptor-mediated increase in intracellular cAMP levels, to activation of a cAMP-dependent protein kinase, to an increase in the amounts of the active form of Tyr hydroxylase

by phosphorylation of enzyme in an inactive pool, ultimately to an increase in the biosynthesis and availability of the catecholamine neurotransmitter. Whether such regulation in fact occurs *in vivo* remains to be proved. However, our recent discovery (23) that cholinergic stimulation of central noradrenergic neurons can produce a prolonged increase in the activity of Tyr hydroxylase as a consequence of a change in the catalytic activity of the enzyme rather than the number of enzyme molecules, and that the activation of enzyme is due to changes of V_m without changes of K_m, suggests that such a mechanism may be operative in brain.

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