Tyrosine Hydroxylase Inactivation Following cAMP-Dependent Phosphorylation Activation

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Abstract: Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is activated following phosphorylation by the cAMP-dependent protein kinase (largely by decreasing the $K_m$ of the enzyme for its pterin co-substrate). Following its phosphorylation activation in rat striatal homogenates, we find that tyrosine hydroxylase is inactivated by two distinct processes. Because cAMP is hydrolyzed in crude extracts by a phosphodiesterase, cAMP-dependent protein kinase activity declines following a single addition of cAMP. When tyrosine hydroxylase is activated under these transient phosphorylation conditions, inactivation is accompanied by a reversion of the activated kinetic form (low apparent $K_m$, 50.2 mM) to the kinetic form characteristic of the untreated enzyme (high apparent $K_m$, 31.0 mM). This inactivation is readily reversed by the subsequent addition of cAMP. When striatal tyrosine hydroxylase is activated under constant phosphorylation conditions (incubated with purified cAMP-dependent protein kinase catalytic subunit), however, it is also inactivated. This second inactivation process is irreversible and is characterized kinetically by a decreasing apparent $V_{max}$ with no change in the low apparent $K_m$ for pterin co-substrate (0.2 mM). The latter inactivation process is greatly attenuated by gel filtration which resolves a low-molecular-weight inactivating factor(s) from the tyrosine hydroxylase. These results are consistent with a regulatory mechanism for tyrosine hydroxylase involving two processes: in the first case, reversible phosphorylation and dephosphorylation and, in the second case, an irreversible loss of activity of the phosphorylated form of tyrosine hydroxylase. Key Words: Tyrosine hydroxylase—Phosphorylation activation—cAMP-dependent—Protein kinase—Dephosphorylation. Vrana K. E. and Roskoski R. Jr. Tyrosine hydroxylase inactivation following cAMP-dependent phosphorylation activation. J. Neurochem. 40, 1692–1700 (1983).

Tyrosine hydroxylase (EC 1.14.16.2) is generally regarded as the enzyme catalyzing the rate-limiting reaction in catecholamine biosynthesis (Levitt et al., 1965). Given the rate-limiting nature of this enzyme, it is natural that tyrosine hydroxylase is subject to apparent regulation by a wide variety of processes. In reference to long-term regulation, for example, biosynthesis of tyrosine hydroxylase is induced in vivo by cold stress (Gordon et al., 1966b; Chuang and Costa, 1974), nerve stimulation (Thoenen et al., 1969), and treatment with reserpine (Zigmond et al., 1974). In reference to short-term regulation, experiments have demonstrated the prompt enhancement of tyrosine hydroxylase activity and concomitant norepinephrine synthesis following increased nerve activity in vivo (Gordon et al., 1966a) and in vitro (Alousi and Weiner, 1966).

Furthermore, acute administration of neuroleptic drugs produces an increase in tyrosine hydroxylase activity (Zivkovic and Guidotti, 1974; Zivkovic et al., 1974), while chronic treatment with neuroleptics produces a decrease in enzyme activity (Lerner et al., 1977b). Tyrosine hydroxylase is directly controlled by end-product feedback inhibition (Ikeda et al., 1966), and both phospholipids (Lloyd and Kaufman, 1974; Lloyd, 1979) and anions (Kuczenski and Mandell, 1972) activate the enzyme. Both ionic strength and ionic composition, moreover, may also be important factors in determining tyrosine hydroxylase activity (Kuczenski, 1981).

Reports from several laboratories have shown that tyrosine hydroxylase is activated by protein phosphorylation conditions (Lovenberg et al., 1975; Morgenroth et al., 1975; Goldstein et al., 1976;
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Ames et al., 1978; Vrana et al., 1981). This activation has been correlated with the direct phosphorylation of tyrosine hydroxylase by the cyclic AMP-dependent protein kinase (Joh et al., 1978; Yamauchi and Fujisawa, 1979; Vulliet et al., 1980). Phosphorylation activates tyrosine hydroxylase, in part, by decreasing the apparent $K_m$ of the enzyme for its pterin co-substrate (Lovenberg et al., 1975; Lloyd and Kaufman, 1975; Morita and Oka, 1977; Ames et al., 1978; Vrana et al., 1981). This is physiologically important in light of the existence of low co-substrate concentrations in tissues (Guroff et al., 1967; Lloyd and Weiner, 1971). Phosphorylation of tyrosine hydroxylase also results in an increase in the $K_i$ of the enzyme for its inhibitor, dopamine (Lerner et al., 1977a; Ames et al., 1978; Vrana et al., 1981), thus providing a mechanism for releasing the enzyme from feedback inhibition. Furthermore, increases in tyrosine hydroxylase activity following treatment with neuroleptic drugs in vivo are also associated with a decreased $K_m$ for pterin co-substrate and a decreased affinity (increased $K_i$) for feedback inhibitors (Zivkovic et al., 1974).

We have reported that tyrosine hydroxylase is activated and subsequently inactivated in the supernatant of a rat corpus striatal homogenate in response to cyclic AMP-dependent protein phosphorylation conditions (Vrana et al., 1981). The nature of this inactivation phenomenon is the subject of the present paper. Our findings suggest that inactivation involves two independent mechanisms.

Our experiments were conducted with the 40,000 x g supernatant of a homogenate of rat corpus striatum. In this manner, we hoped to preserve as many regulatory factors as possible. As will be shown, gel filtration has allowed us to resolve and identify two mechanisms for the inactivation of tyrosine hydroxylase, as well as factors critical to the modulation of tyrosine hydroxylase activity. A preliminary account of this work has previously been presented (Vrana and Roskoski, 1982).

MATERIALS AND METHODS

Homogenate preparation and column chromatography

Crude homogenates of rat corpus striatum were prepared as follows. The tissues were used immediately following dissection or following storage in liquid nitrogen. The tissue was homogenized with a glass mortar and Teflon pestle in 3 volumes of 50 mM PIPES (pH 6.0) containing 0.2% Triton X-100. Following homogenization and centrifugation (40,000 x g), the pellet was rehomogenized in one additional volume of buffer. Following centrifugation, the supernatants were combined, vortexed, divided into aliquots, and stored in liquid nitrogen. Enzyme prepared and stored in this manner showed no detectable loss of activity over a 3-month period. For the gel filtration studies, 200 µl (approximately 2.5 mg of protein) of the striatal supernatant was applied to a Sephadex G-15 (medium) or G-100 (medium) column (1 x 24 cm) equilibrated with 25 mMPIPES (pH 6.0). Fractions (0.5 ml) were collected and the two fractions containing maximal tyrosine hydroxylase activity were pooled and used in subsequent experiments.

Tyrosine hydroxylase enzymatic assay

tyrosine hydroxylase activity was determined radio metrically following an enzymatically coupled DOPA decarboxylase reaction and low-voltage paper electrophoresis as previously described (Vrana et al., 1981). Briefly, the tyrosine hydroxylase activity was measured in the presence of 125 µM 6-methyltetrahydropterin (6-MPH$_2$) and 100 µM [14C]tyrosine in the presence of partially purified hog kidney aromatic amino acid decarboxylase and sheep liver dihydropteridine reductase along with necessary cofactors. The reaction mixture was incubated at 37°C for 15 min, after which the reaction was terminated. Then the dopamine product was resolved from precursor tyrosine by paper electrophoresis, and the radioactive product was quantitated by liquid scintillation spectrometry.

Protein phosphorylation studies

tyrosine hydroxylase preparations were preincubated under phosphorylation conditions prior to measurement of tyrosine hydroxylase activity. These conditions included 13 mM magnesium acetate, 0.67 mM ATP, 0.5 mM theophylline, 0.08 mM EGTA, and 10 mM PIPES, pH 6.0 (in a final volume of 200–600 µl). Cyclic AMP-dependent protein kinase activity was introduced by either of two means. The endogenous striatal protein kinase was activated by including 0.13 mM cAMP in the phosphorylation preincubation. In some instances, exogenous purified bovine brain cAMP-dependent protein kinase catalytic subunit was added (as noted) to the preincubation. At the specified times following the initiation of the preincubation reaction (37°C), 10-µl samples were removed from the preincubation mixture, placed on ice, and subsequently assayed for tyrosine hydroxylase activity as described above. It was expected that all processes involved in the preincubation (phosphorylation, dephosphorylation, phosphodiesterase activity) would continue to act during the assay procedure. No attempts were made, therefore, to inhibit the protein kinase activity prior to assaying for TH activity, for example, so as not to produce unopposed phospha tease activity.

Protein kinase activity was determined by the method of Witt and Roskoski (1975), with the use of the synthetic serine peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly as the phosphate-accepting substrate and phosphoric acid elution (Hartl and Roskoski, 1982). Cyclic AMP was added only during the phosphorylation preincubation, as noted in the figure legends. Under experimental conditions in which exogenous, purified protein kinase catalytic subunit was added, there was no need for cAMP, since the catalytic subunit is cAMP-independent and constitutively active (having been resolved, during purification, from the regulatory subunit).

To measure phosphodiesterase activity, striatal extracts were incubated under phosphorylating conditions as described above, with the addition of [3H]cAMP (0.13 mM; 770 Ci/mmol). At selected times following addition of the striatal extract, 10 µl of the mixture was added to 10 µl of 0.5 M formic acid containing 10 mM piperidine. The mixture was then applied to a Sephadex G-15 column (1 x 24 cm) equilibrated with 25 mM PIPES (pH 6.0). Fractions (0.5 ml) were collected and the two fractions containing maximal tyrosine hydroxylase activity were pooled and used in subsequent experiments.
5'-AMP and 10 mM cAMP. The nucleotides were then resolved by ascending chromatography on polyethyleneimine (PEI) thin-layer plates, with 25 mM potassium phosphate—100 mM NaCl (pH 3.5) as solvent. The cAMP and 5'-AMP were visualized with ultraviolet light, and their respective radioactivities were quantitated by liquid scintillation spectrometry.

The results presented here have been replicated (unless otherwise noted) in at least three different striatal preparations and are representative of a minimum of three separate experiments. The kinetic experiments have been replicated at least three times. All data points are the mean of duplicate determinations.

Materials

Enzymes were prepared and materials obtained as previously described (Vrana et al., 1981). [3H]cAMP (37 Ci/mmol) was obtained from Amersham (Amersham, England). PEI-impregnated cellulose thin-layer plates (PEI-cellulose) were obtained from Brinkmann Instruments, Inc. (Westbury, NY). Sephadex G-15 and G-100 resins were obtained from Sigma Chemical Co. (St. Louis). The serine peptide phospho-accepting substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was obtained from Sigma Chemical Co. and Boehringer Mannheim Biochemicals (Indianapolis). Cyclic AMP-dependent protein kinase catalytic and regulatory subunits were purified to electrophoretic homogeneity from bovine brain by the method of Beavo et al. (1974) as modified by Hartl and Roskoski (1982).

RESULTS

Cyclic AMP dependence of the phosphorylation activation and subsequent inactivation of tyrosine hydroxylase

As previously reported (Vrana et al., 1981), exposure of striatal supernatants to protein phosphorylation conditions (MgATP, cAMP) leads to an increase in tyrosine hydroxylase activity as determined in a subsequent 15-min assay at subsaturating pterin co-substrate concentrations. This activation is cAMP-dependent and is mediated, in part, by a decrease in the apparent Michaelis constant (K_m) of tyrosine hydroxylase for its co-substrate. Following extended preincubation under these conditions, this initial activation is followed by a decrease in tyrosine hydroxylase activity to levels approximating those seen in the absence of MgATP and cAMP.

The inactivation of tyrosine hydroxylase following phosphorylation activation can be explained by the loss of cAMP-dependent protein kinase activity and the subsequent dephosphorylation of the tyrosine hydroxylase. As shown in Fig. 1, the initial loss of tyrosine hydroxylase activity is accompanied by a parallel loss of protein kinase activity. This decline in protein kinase activity is caused by conversion of cAMP to 5'-AMP, which is mediated by endogenous phosphodiesterase activity. All of the preincubation mixtures contained 500 µM theophylline as a phosphodiesterase inhibitor. As Fig. 1 demonstrates, this concentration is insufficient to prevent the hydrolysis of the cAMP. It does, however, greatly slow the loss of cAMP as compared with the same conditions in the absence of theophylline (not shown). The addition of cAMP following tyrosine hydroxylase inactivation results in the transient reactivation of protein kinase and tyrosine hydroxylase activities. Reactivation could also be accomplished using purified cAMP-dependent protein kinase catalytic subunit (not shown). It is unclear why the tyrosine hydroxylase activity following reactivation approaches only 50% of the initial level of activation following the addition of the same concentration of cAMP. As shown in Fig. 1, protein kinase activity is fully recovered with the second addition of cAMP. Reactivation comparable to the initial activation can be achieved, however, by using greater amounts of cAMP or an excess of the purified protein kinase catalytic subunit to effect the reactivation (Vrana et al., 1981).

We expected that treatment of tyrosine hydroxylase under conditions of continuous protein kinase activity would maintain the tyrosine hydroxylase in the active state. To test this, tyrosine hydroxylase was activated using the purified...
Tyrosine hydroxylase inactivation under conditions of constant protein kinase activity. Striatal tyrosine hydroxylase was activated using MgATP (0.67 mM) and purified bovine brain protein kinase catalytic subunit (12.5 μg/ml). At 30 min, C-subunit was added to double the protein kinase activity. Tyrosine hydroxylase activity (C—M, + MgATP, + C-subunit; O—O, -CAMP, -MgATP) and protein kinase activity (■—■, + MgATP, + C-subunit) were determined.

The ability to reactivate tyrosine hydroxylase is a function of the phosphorylation state of the system. (A) Tyrosine hydroxylase (■—■) was activated with MgATP (0.67 mM) and purified protein kinase catalytic subunit (10 μg/ml) for 3 min, at which time an excess of purified protein kinase regulatory subunit (25 μg/ml) was added to terminate the protein kinase activity (■—■). Following further incubation (35 min), cAMP (0.13 mM) was added to reactivate the protein kinase. (B) Tyrosine hydroxylase (■—■) was activated as in A, except that the protein kinase activity (■—■) was not terminated until 44 min. Following incubation for an additional 25 min, cAMP (0.13 mM) was added.

Tyrosine hydroxylase activity is inactivated under conditions of phosphorylation. The catalytic subunit of the cAMP-dependent protein kinase (Fig. 2). The catalytic subunit, during purification, has been resolved from its regulatory subunit and therefore exists in an active state which is independent of cAMP. Although protein kinase activity remains active and stable throughout the 30 min of preincubation, tyrosine hydroxylase is nevertheless inactivated following activation. This inactivation is not reversed by the further addition of protein kinase catalytic subunit. Similar results were obtained using the endogenous protein kinase maintained in the active state, either by the repeated addition of cAMP or by the addition of cAMP in the presence of the additional phosphodiesterase inhibitors papaverine and isobutylmethylxanthine (300 μM). In both cases, protein kinase activity remained elevated and tyrosine hydroxylase was transiently activated but subsequently inactivated (not shown). This inactivation was also not reversed by the addition of purified catalytic subunit.

The ability to reactivate the tyrosine hydroxylase appears, therefore, to depend on the phosphorylation state of the extract and the length of time that the tyrosine hydroxylase is exposed to phosphorylation conditions. This relationship was examined through the use of purified kinase regulatory subunit (R subunit). Tyrosine hydroxylase was activated by using purified protein kinase catalytic subunit and MgATP. The protein kinase activity was then terminated by the addition of purified protein kinase regulatory subunit, which results in the formation of the inactive protein kinase holoenzyme. As indicated in Fig. 3, the addition of the regulatory subunit immediately inhibits protein kinase activity in the absence of cAMP. The addition of the regulatory subunit at the point of maximal tyrosine hydroxylase activation (3 min) is associated with a rapid inactivation of tyrosine hydroxylase (Fig. 3A). This inactivation levels off at a basal activity approximating that seen in the absence of phosphorylating conditions (not shown).

The rapid loss of tyrosine hydroxylase activity suggests that there is a high level of phosphatase activity present in the striatal homogenate. This activity must be high enough to dephosphorylate and inactivate the tyrosine hydroxylase during the initial minutes of the activity assay (15 min), thus producing the apparent rapid inactivation. The subsequent addition of cAMP to activate the protein kinase produces renewed activation-inactivation of tyrosine hydroxylase. As demonstrated in Fig. 3B, however, several differences are seen when tyrosine hydroxylase is inactivated under constant phosphorylating conditions. First, the inactivation is much slower than that observed when the protein kinase activity is abruptly terminated following activation. Second, tyrosine hydroxylase activity declines to undetectable levels, in contrast to the basal level obtained when protein kinase is inhibited prior to inactivation. Finally, when protein kinase is inhibited with regulatory subunit following the inactivation of tyrosine hydroxylase, tyrosine hydroxylase activity is not restored by the subsequent addition of cAMP. Reactivation of tyrosine hydroxylase fails to occur even though the addition of cAMP reactivates protein kinase activity, as in Fig. 3A. Compared with the inactivation illustrated in Fig. 2, the inactivation in Fig. 3B occurs more slowly (under very similar conditions). This variation represents differences in striatal preparations and emphasizes the need to compare qualitative rather than quantitative changes in these experiments. It appears,
therefore, that tyrosine hydroxylase activated by phosphorylation can be inactivated by two, distinct processes.

**Enzyme kinetic analysis of the two mechanisms of tyrosine hydroxylase inactivation**

Studies were undertaken to characterize the steady state kinetic nature of the inactivation of tyrosine hydroxylase following its phosphorylation activation. As shown in Fig. 4, the activation of striatal tyrosine hydroxylase following exposure to cAMP and ATP produces a biphasic curve for data expressed in double-reciprocal form. At low concentrations of 6-MPH₄, the activated tyrosine hydroxylase exhibits a reduced $K_m$ for pterin as compared with the untreated enzyme. At high concentrations of 6-MPH₄, however, a second kinetic component is manifested. This observation of biphasic double-reciprocal plots has been extensively characterized for adrenal tyrosine hydroxylase (Masserano and Weiner, 1979; 1981; Masserano et al., 1981) and recently described for rat striatal tyrosine hydroxylase (Lazar et al., 1982). The biphasic phenomenon can be explained by postulating a mixed population of enzyme molecules consisting of low-$K_m$ and high-$K_m$ forms (Masserano and Weiner, 1979). Two conclusions can therefore be drawn from these data. First, as previously described, activation of tyrosine hydroxylase is mediated, in part, by a reduced $K_m$ of the enzyme for its pterin co-substrate (Lovenberg et al., 1975; Lloyd and Kaufman, 1975; Morita and Oka, 1977; Ames et al., 1978; Vrana et al., 1981). Second, the levels of cAMP-dependent protein kinase in the striatal homogenate are not sufficient to effect total activation of the tyrosine hydroxylase present in the same preparation. This latter point is indicated by the nonlinear reciprocal plots observed following treatment with cAMP and MgATP.

We next investigated the nature of the time-dependent inactivation of tyrosine hydroxylase following activation with cAMP and MgATP. At several time points following the addition of cAMP and MgATP to the striatal preparation, the enzyme was analyzed to determine its kinetic state (Fig. 5). The loss of enzyme activity is associated with the conversion of the enzyme from a predominantly low-$K_m$ form to a high-$K_m$ form. At 15 min following initial activation, moreover, the tyrosine hydroxylase exhibits a monophasic double-reciprocal plot, with a $K_m$ approximating that of the untreated enzyme. The inactivation of tyrosine hydroxylase following activation with cAMP and MgATP is accomplished, therefore, by the reversion of the low-$K_m$ (activated) form of the enzyme to its high-$K_m$ (less active) form.

The kinetic mechanism for inactivation of tyrosine hydroxylase under constant phosphorylating conditions is different from that just described (Fig. 6). First, addition of excess, exogenous cAMP-dependent protein kinase catalytic subunit is sufficient to totally activate the tyrosine hydroxylase present in the striatal extract. This is demonstrated by the linear double-reciprocal plot representing a single low-$K_m$ form of the enzyme. Second, tyrosine hydroxylase inactivation is not mediated by reversion

![FIG. 4. Treatment of striatal extracts with cAMP and MgATP results in the partial activation of tyrosine hydroxylase. Striatal extracts were prepared in the presence (●) of cAMP, + MgATP or absence (○) of cAMP, + MgATP) or absence (●) of cAMP, - MgATP) of phosphorylation conditions, as described in Materials and Methods. These preparations were then assayed, without preincubation, for tyrosine hydroxylase activity in the presence of varying concentrations of 6-MPH₄ (67 μM to 2 mM) plus 100 μM tyrosine.](image)

![FIG. 5. Inactivation of tyrosine hydroxylase following activation with cAMP and MgATP is mediated by reversion of the enzyme from the low-$K_m$ to high-$K_m$ form. Striatal extracts were prepared under phosphorylation conditions in the presence of cAMP and MgATP as described in Materials and Methods. Following selected periods of preincubation at 37°C, the preparations were assayed as in Fig. 4: (○) 6 min; (●) 7 min; (□) 15 min.](image)
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FIG. 6. Inactivation of tyrosine hydroxylase under constant phosphorylation conditions is mediated by a decreasing $V_{\text{max}}$ with no change in the apparent $K_m$. Striatal extracts were phosphorylated in the presence of C-subunit and MgATP as described in Materials and Methods. Following preincubation at 37°C (0, 0 min; ●, 6 min), the preparations were assayed as in Fig. 4.

FIG. 7. Gel filtration to remove low-molecular-weight component(s) eliminates the inactivation process observed under conditions of constant protein kinase activity. (A) Striatal supernatant was filtered on a Sephadex G-15 column as described in Materials and Methods. Tyrosine hydroxylase (●—●) was then activated as described in Fig. 1, with cAMP and MgATP. Protein kinase activity (■—■) was determined simultaneously. (B) The same filtered extract was activated, using MgATP (0.67 mM) and purified protein kinase catalytic subunit (12.5 μg/ml). Tyrosine hydroxylase activity (●—●) and protein kinase activity (■—■) were then determined.

to the less active form, but by a decreasing $V_{\text{max}}$. This decrease in $V_{\text{max}}$ occurs in spite of the fact that the $K_m$ remains in its high-affinity state. Following incubation of the striatal extract under these constant phosphorylating conditions for 12 min, there is no detectable tyrosine hydroxylase activity, even at the highest co-substrate concentrations (not shown). These results are explained by a mechanism in which the phosphorylated tyrosine hydroxylase is susceptible to terminal loss of enzyme activity. Under constant phosphorylation conditions, therefore, the enzyme exhibits a low $K_m$ consistent with activation; but, with time, the $V_{\text{max}}$ decreases as the number of active enzyme molecules is reduced. Ultimately all of the enzyme activity is lost, even though activation conditions have been maintained.

Effect of gel filtration of tyrosine hydroxylase inactivation

As reported previously (Vrana et al., 1981), gel filtration of a striatal extract abolishes the inactivation process. When striatal supernatants are passed over a Sephadex G-100 column and are then exposed to protein phosphorylation conditions, they lose the ability to inactivate tyrosine hydroxylase as compared with an unfiltered preparation. Gel filtration, therefore, fails to alter the first mode of tyrosine hydroxylase inactivation. Gel filtration, then, resolves a factor from tyrosine hydroxylase which is involved in the inactivation process following phosphorylation activation. Subsequent experiments have determined that this inactivating factor is a low-molecular-weight substance that comigrates with glucose on a Sephadex G-15 column (not shown).

We next investigated which of the two inactivation processes (or both) was abolished by the removal of this low-molecular-weight factor. This line of investigation was aided by the fact that we could resolve the inactivation factor from the higher-molecular-weight enzymes by means of a Sephadex G-15 column. As depicted in Fig. 7, striatal extracts were filtered on a Sephadex G-15 column and exposed to phosphorylation conditions designed to distinguish between the two previously described inactivation processes. When the tyrosine hydroxylase was activated using endogenous protein kinase activity (addition of cAMP and MgATP, Fig. 7A), it was subsequently inactivated as described earlier. As before, this inactivation of tyrosine hydroxylase is associated with a loss of protein kinase activity mediated by the conversion of cAMP to 5'-AMP. Additionally, this inactivated tyrosine hydroxylase can be reactivated with either cAMP or the purified protein kinase C-subunit. Gel filtration, therefore, fails to alter the first mode of tyrosine hydroxylase inactivation. If the filtered extract is exposed to purified protein kinase catalytic subunit (Fig. 7B), the inactivation is greatly attenuated or even eliminated. The low-molecular-weight factor resolved by gel filtration is, therefore, involved in the inactivation process observed under continuous phosphorylation conditions. The attenuation of this inactivation of tyrosine hydroxylase following gel filtration is dependent on the amount of added protein kinase C-subunit (not shown). Addition of larger amounts of C-subunit yields higher levels of tyrosine hydroxylase activity, but some loss of this activity becomes evident. It should be emphasized, though, that this loss of activity is much slower than that seen in the absence of gel filtration.
DISCUSSION

We have investigated the regulation of tyrosine hydroxylase activity by protein phosphorylation in rat striatal preparations. We have previously reported (Vrana et al., 1981) that striatal tyrosine hydroxylase is activated following exposure to protein phosphorylation conditions and that it is subsequently inactivated during continued phosphorylation treatment. In the studies presented here, we investigated the mechanism underlying this inactivation process. Based on these studies, we propose a regulatory scheme for tyrosine hydroxylase in striatal homogenates as illustrated in Fig. 8. Initial experiments showed that the inactivation of tyrosine hydroxylase following activation with cAMP and ATP was associated with the loss of protein kinase activity mediated by the conversion of cAMP to 5'AMP. The loss of cAMP results in the reassociation of the inactive, tetrameric holoenzyme of the cAMP-dependent protein kinase (R_2C_2). In the absence of protein kinase activity, a phosphatase could then dephosphorylate tyrosine hydroxylase and convert it to its less active form. This inactivation process should be readily reversed by the addition of cAMP or purified protein kinase catalytic subunit, as we have observed experimentally. Preliminary efforts in our laboratory to inhibit the phosphatase, however, have been unsuccessful, but Lazar et al. (1978) demonstrated the enzymatic dephosphorylation of purified, phosphorylated adrenal tyrosine hydroxylase by striatal extracts. Similar findings were reported by Yamauchi and Fujisawa (1979). These results support the hypothesis that tyrosine hydroxylase may be actively regulated by a phosphodiesterase/phosphatase system.

Additionally, we find that treatment of striatal tyrosine hydroxylase under constant phosphorylating conditions results in the inactivation of the enzyme, following an initial activation. This inactivation occurs in spite of the fact that protein kinase activity does not decline. Furthermore, tyrosine hydroxylase activity cannot be salvaged by additional protein kinase activity. It appears, then, that this second form of inactivation is a property of the phosphorylated form of tyrosine hydroxylase. Vitto and Mandell (1981) have reported that the phosphorylated tryptophan hydroxylase is rendered more susceptible to proteolysis by phosphorylation. Lazar et al. (1981) have reported that the purified tyrosine hydroxylase, once phosphorylated, becomes more thermally labile than the native enzyme. Our experiments suggest that striatal tyrosine hydroxylase, when treated under conditions designed to maintain the enzyme in the phosphorylated state, is irreversibly inactivated. This process corresponds to the enzyme form TH-X in Fig. 8. This inactivation may be due, inter alia, to decreased thermal stability or increased susceptibility to proteolysis.

Enzyme kinetic evidence supports the existence of two mechanisms for the inactivation of tyrosine hydroxylase following its activation by protein phosphorylation. The first process, associated with the reversible inactivation or deactivation of striatal tyrosine hydroxylase following activation with cAMP and MgATP, involves the reversion of the activated, low-K_m enzyme form to its less active, high-K_m counterpart. Following this inactivation process, the enzyme kinetically resembles the untreated, control enzyme. This return of tyrosine hydroxylase to a form similar to the untreated enzyme accounts for the possibility of reactivating the enzyme with renewed phosphorylation conditions. In the second instance, inactivation under constant phosphorylation conditions is associated with a decline in V_max during which tyrosine hydroxylase is maintained in the low-K_m form. These results are consistent with an irreversible loss of enzyme activity, which occurs after tyrosine hydroxylase has been phosphorylated.

Utilizing gel filtration, we have resolved a low-molecular-weight factor involved in the inactivation of tyrosine hydroxylase that is observed under constant phosphorylating conditions. Filtration to remove this factor, followed by phosphorylation with exogenous protein kinase catalytic subunit, results in a form of tyrosine hydroxylase that is activated but not subsequently inactivated. Therefore, the transition to the inactive enzyme form (TH-X) is somehow blocked by filtration.

The first, phosphodiesterase-associated inactivation process is consistent with the role of cAMP as a short-lived second messenger. Following a stimulus-induced increase in cAMP levels, an increase in protein kinase activity and tyrosine hydroxylase activity would occur. After the stimulus is withdrawn and cAMP synthesis decreases, cAMP phosphodiesterase can reverse the process. This is a common, reversible regulatory scheme which occurs throughout intermediary metabolism.
and Weiner (1981) report that following a single electroconvulsive shock in rats, adrenal cAMP levels, protein kinase activity, and tyrosine hydroxylase activity rise and fall in a manner similar to that observed in our invitro system. Furthermore, if the initial shock is followed some time later by an additional shock, the process of activation-inactivation is repeated. Although the data do not show a causal relationship between cAMP levels and tyrosine hydroxylase activity, the evidence is at least consistent with the model we propose.

At the present time, it is unclear what role the second, apparently irreversible, inactivation process serves in vivo. The work reported by Lazar et al. (1981) on the reduced thermal stability of the phosphorylated tyrosine hydroxylase, however, is consistent with our findings.

Acknowledgments: The authors acknowledge the technical assistance of David J. Tate and the excellent secretarial assistance of Karen L. Vrana. This work was supported by USPHS Grant NS-15994 (R.R.) and NIH Training Grant T32HL07098 (K.V.).

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