THE BIOSYNTHESIS OF PHOSPHORYLATED TYROSINE HYDROXYLASE BY ORGAN CULTURES OF RAT ADRENAL MEDULLA AND SUPERIOR CERVICAL GANGLIA

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Summary. Tyrosine hydroxylase has been synthesized in organ cultures of rat adrenal medulla and superior cervical ganglia and isolated by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis. When the cultures were grown in radioactive phosphate the tyrosine hydroxylase contained radioactivity. Superior cervical ganglia from animals injected with nerve growth factor made more tyrosine hydroxylase and proportionately more phosphate was incorporated into the enzyme than in ganglia from control animals.

In recent years there have been a number of studies which showed that phosphorylating conditions alter the kinetic properties of the enzyme tyrosine hydroxylase (1-3). These alterations include a decrease in the K_M for its pteridine cofactor, an increase in the V_{max}, and a shift in the pH optimum of the activity. It was also noted that cAMP, ATP, and Mg^{++} were all required and that cAMP or ATP alone were ineffective in producing these alterations in kinetics of tyrosine hydroxylase action. These experiments strongly suggest that tyrosine hydroxylase can be phosphorylated by a cAMP-dependent protein kinase and that such phosphorylation can regulate the action of the enzyme.

On the other hand, direct attempts to demonstrate phosphorylation of tyrosine hydroxylase have failed (1,4). It has been suggested that the enzyme itself is not phosphorylated, but that some protein activator

Abbreviations used are: SDS, sodium dodecylsulfate; SCG, superior cervical ganglion.

is the site of addition of phosphate (4). However, no direct evidence for such an activator has appeared. Recent experiments have demonstrated that liver phenylalanine hydroxylase, an enzyme with a cofactor requirement and a mechanism of action similar to that of tyrosine hydroxylase, is phosphorylated, and the phosphorylated form of the enzyme has been isolated (5,6).

It seems possible, and even likely, that both enzymes exist in a phosphorylated form, and that the failure to observe phosphorylated tyrosine hydroxylase after exposure to phosphorylating conditions is due to some experimental failure or to an unusual lability of the phosphate group. In order to investigate this problem we have measured the incorporation of radioactive phosphate into the enzyme during its biosynthesis in organ culture. The biosynthesis of tyrosine hydroxylase by adrenal medulla has previously been studied by Chuang et al. in vivo (7), and more recently in this laboratory in organ cultures of adrenal medulla and superior cervical ganglia from young rats (8). We now report that the tyrosine hydroxylase isolated from these organs cultured in the presence of radioactive phosphate contains phosphate groups.

MATERIALS AND METHODS

Adrenal medullae were removed from 10-day old Sprague-Dawley rats and placed in organ culture. Four medullae were used per dish with 0.30 ml of BGJ_b medium, Fitton-Jackson modification, without phenol red (GIBCO), and supplemented with 0.1% bovine serum albumin and an antibotic-antimycotic mixture which included penicillin, streptomycin, and fungizone at 100 units, 100 µg, and 0.25 µg per ml, respectively. The cultures were maintained at 37° for 16-20 hrs in a humidified atmosphere of 95% O_2 and 5% CO_2 with either 50 µCi of ^{3}H-L-leucine (New England Nuclear, 57.4 Ci/mmole) or 0.5 mCi of ^{32}P-potassium monophosphate (New England Nuclear, 500 mCi/mmole). At appropriate times the medullae were removed from culture, rinsed with 0.25 M sucrose, and homogenized in 0.7 ml of 5 mM Tris, pH 7.4 containing 0.1% Triton X-100. The homogenates were centrifuged at 20,000 x g for 20 min.

Five-day old Sprague-Dawley rats were injected subcutaneously with 2.5 S nerve growth factor (10 µg/g body weight) prepared by the method of Bocchini and Angeletti (9). The animals were sacrificed 24 hrs later and the superior cervical ganglia were removed. The ganglia were cleaned, decapsulated, and placed in culture as described above for adrenal medulla except that 250 µCi of ^{3}H-leucine and 2.1 mCi of ^{32}P-potassium monophosphate were used in the cultures.

Portions of the supernatant fractions, usually equivalent to one medulla or one ganglion were brought to a volume of 1.0 ml and a concentration of 1.0% Triton X-100 and 1.0% deoxycholate. Sufficient carrier tyrosine hydroxylase from the 150,000 x g supernatant of adult rat adrenal was added to adjust the final tyrosine hydroxylase concentration to 2.5 units (1 unit = 1 nmole of tyrosine hydroxylated per hour). Monospecific sheep antiserum against chymotrypsin-treated tyrosine hydroxylase (10) was added. The samples were incubated at 30° for 1 hr and then overnight at 4°.

The antigen-antibody mixtures were layered onto 0.5 ml cushions of 1.0 M sucrose, 0.15 M NaCl, 1% Triton X-100, 10 mM and sodium phosphate, pH 7.0, and centrifuged at 16,000 x g for 15 min. The top of the cushion was washed twice gently with phosphate buffered saline, and the pellet itself was then washed 2 or 3 times, each time with vortex mixing followed by centrifuging. The pellet was then solubilized by boiling for 15 min in a volume of 60 µl containing sodium phosphate, pH 7.0, 28 mM, SDS, 2.8%, and 2-mercaptoethanol, 0.4%. Electrophoresis was carried out on 7.5% SDS polyacrylamide gels for 4 hrs at 8 ma per gel (11). The gels were sliced into 1.2 mm sections; the sections were solubilized in 1 ml portions of hydrogen peroxide containing 1% ammonium hydroxide at 55° overnight. The samples were then counted in 15 ml of Aquasol. The procedures for the biosynthesis and quantitation of tyrosine hydroxylase in similar organ cultures of sympathetic ganglia are discussed in more detail in a recent publication (8).

Tyrosine hydroxylase was assayed by the procedure of Nagatsu et al. (12). The incorporation of ^{3}H-leucine or ^{32}P-potassium monophosphate into acid-insoluble material was carried out as previously described (8) by precipitation with 10% TCA after the addition of 250 µg of bovine serum albumin.

RESULTS

As shown in Fig. 1A, tyrosine hydroxylase synthesized in adrenal medulla cultured in the presence of ^{3}H-leucine incorporated radioactivity. Fig. 1B shows that tyrosine hydroxylase isolated from medullae cultured in the presence of $^{32}P_i$ was also labeled. A calculation based on the amount of radioactive leucine incorporated into the enzyme molecule and its specific activity in the medium indicates that, in a typical experiment, 0.4 pmol of tyrosine hydroxylase was synthesized per medulla. This is on the order of 1% of the soluble protein synthesized in the culture (8). Comparable calculations based on the phosphate incorporation indicated that about 11% of the molecules contained $^{32}P_i$. This estimate is based on the assumption that leucine comprises about 10% of the tyrosine hydroxylase protein and that one mole of phosphate is incorporated per mole of enzyme.

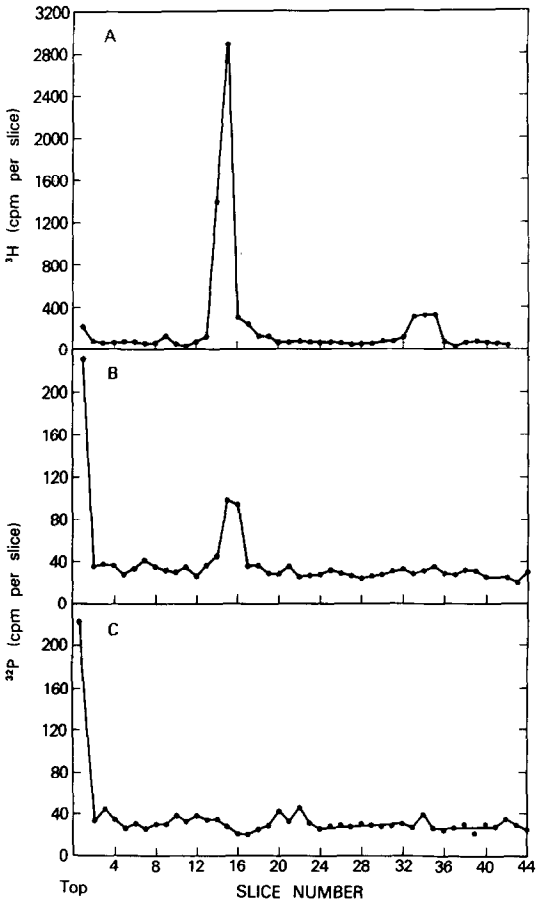

Figure 1. SDS-Acrylamide gels of antibody-antigen precipitates from rat adrenal medullae labeled in culture with (^{3}H)-leucine (A), with (^{32}P)-phosphate (B) or with (^{32}P)-phosphate added following homogenization of the tissue (C).

The possibility that the appearance of radioactivity in the tyrosine hydroxylase peak was due to non-specific coprecipitation was ruled out by several control experiments. First, as shown in Figure 1C, the addition of ^{32}P-potassium monophosphate to the homogenate following culture did not yield radioactivity in the tyrosine hydroxylase peak. Second, SDS- polyacrylamide gel electrophoresis of the ^{32}P-labeled medulla supernatants before immunoprecipitation showed that the major peak of radioactivity was in slices 19 and 20. Since this peak was not

TABLE I

Incorporation of ^{3}H-L-leucine and ^{32}P-potassium monophosphate into tyrosine hydroxylase of superior cervical ganglia of 5-day old rats with and without pretreatment of the animals with nerve growth factor.

Treatment	^{3}H-Leucine incorporation into tyrosine hydroxylase	^{32}P-Phosphate incorporation into tyrosine hydroxylase	Phosphorylation*	^{32}P-Phosphate incorporation into acid-insoluble material
	pmol/SCG	pmol/SCG	%	pmol/SCG
Control	3.05	0.034	11.1	649
Nerve growth factor-treated	6.15	0.065	10.6	687

*based on the assumption that leucine comprises 10% of the tyrosine hydroxylase molecule

present in the tyrosine hydroxylase samples, it seems reasonable to conclude that the ^{32}P in the tyrosine hydroxylase peak was not due to non-specific coprecipitation. Finally, the presence of cycloheximide in the culture decreased ^{3}H-leucine incorporation by more than 95% and ^{32}P incorporation by 90%.

Experiments were designed to investigate whether phosphate incorporation into newly synthesized tyrosine hydroxylase of sympathetic ganglia could also be demonstrated. Treatment with nerve growth factor, which has been shown (13) to stimulate the specific synthesis of tyrosine hydroxylase in this organ, was used to induce the enzyme. The results are shown in Table I. Pretreatment of the animal with nerve growth factor stimulated tyrosine hydroxylase synthesis *in vitro* about 2-fold as judged by ^{3}H-leucine incorporation. Incorporation of ^{32}P into tyrosine hydroxylase is stimulated to the same extent. No significant increase in ^{32}P incorporation into the total acid-precipitable material was observed. The percent of the tyrosine hydroxylase molecules phosphorylated, based on the calculations described above, was about 11% in each case, control or nerve growth factor-treated.

DISCUSSION

These results show that tyrosine hydroxylase is a phosphoprotein, as is phenylalanine hydroxylase, and also offer an explanation for the many observations that phosphorylating conditions alter the characteristics of the enzymatic action of purified tyrosine hydroxylase. Further, these experiments provide a methodology for the further study of the effects of physiological stimuli on the level of phosphorylation of this key enzyme in norepinephrine biosynthesis.

Less than stoichiometric amounts of phosphate were found in the tyrosine hydroxylase. This may be due to the presence of phosphate cleavage or transfer enzymes in the tissue. Another explanation may be that the phosphate group found in tyrosine hydroxylase is labile under the conditions employed. Alternatively, this result may be a consequence of the slow equilibration of the $^{32}P_i$ with the intracellular phosphate(s). On the other hand, it is difficult to know what the actual extent of tyrosine hydroxylase phosphorylation in the cell should be since the physiological meaning of the phosphorylation remains unknown.

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