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MECHANISM OF TYROSINE HYDROXYLASE ACTIVATION BY PHOSPHORYLATION

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SUMMARY : cAMP-dependent phosphorylation of native tyrosine hydroxylase from bovine adrenal medulla increased the enzyme activity and induced a shift of its pH optimum from 5.8 to 6.4. These effects are similar to those observed on adding anions such as heparin, dextran sulfate or morpholino ethane sulfonic acid. These effects of phosphorylation have been interpreted in terms of the polyelectrolyte theory, as proposed previously for activation by anions (Vigny, A., and Henry, J. P. (1981) J. Neurochem. 36, 483-489). We postulated the existence on the enzyme of a cationic regulatory site exerting a negative control on the active site Phosphorylation would modify covalently the charge of this regulatory site. This interpretation is further supported by the observation that the active fragment obtained by limited proteolysis of the enzyme has a pH optimum at 6.4 and cannot be activated by anions or phosphorylation.

Tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of catecholamines in central and peripheral nervous systems and in chromaffin cells of the adrenal medulla. Many observations have shown that anions are involved in a short term regulation of catecholamines synthesis (1, 2, 3). Anions such as heparin, dextran sulfate and anionic MES buffer induce a shift in the pH optimum and a decrease of the K_M of native tyrosine hydroxylase for the cofactor pterin (4). These results have been previously interpreted in term of polyelectrolyte theory (4). It was suggested that the active site of tyrosine hydroxylase was under the negative control of a cationic regulatory site. The cationic site generated a local positive electrostatic potential ψ which affected the microenvironment of the active site. The local proton concentration around the active site $(H^+)_{in}$ was lower than that of the bulk of the solution $(H^+)_{out}$ and pH_{in} was given by the Debye-Hückel theory (5), $pH_{in} = pH_{out} + 0.43\ (\frac{\varepsilon \psi}{kT})$, were ε is the charge of the proton. In absence of

ABBREVIATIONS : MES, 2-(N-morpholino) ethane sulfonic acid ; $DMPH_4$, 6,7-dimethyltetrahydropterin.

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anions, maximal activity was observed at an external pH (pH_{out}) of 5.8, corresponding to a local pH (pH_{in}) of 6.4. Anions interacting with the cationic site (4) cancelled the potential ψ and under these conditions pH_{in} became equal to pH_{out}, accounting for the anion induced pH optimum change. This interpretation also accounted for the K_M change observed in presence of anions, since the cationic site affected the local concentration not only of protons but also of a positively charged substrate such as the pterin $DMPH_4$. The apparent K_M for $DMPH_4$ was expressed as $K_M = K_M^\circ \exp(+\frac{z\varepsilon\psi}{kT})$, where $Z\varepsilon$ was the charge of the substrate. The positive electrostatic potential near the active site increased the K_M for $DMPH_4$. Recently, it has been shown that phosphorylation of tyrosine hydroxylase also decreases the K_M of the enzyme for the pterin (6-11). To investigate the possibility of similar mechanisms of regulation by activatory anions and by phosphorylation, the effects of phosphorylation on the activity and the pH-dependency of bovine adrenal medulla tyrosine hydroxylase were studied. The proposed interpretation was further studied by proteolysis experiments.

MATERIALS AND METHODS : L-(3,5 - ^3H) tyrosine, 40 mCi/mmole, was obtained from Amersham and further purified on a Dowex 50 W-X4 (H^+) column. Protein kinase (from beef heart), cAMP, theophylline and trypsin inhibitor from pancreas were purchased from Sigma. ATP was from Calbochiem and $DMPH_4$ from Aldrich. Creatine phosphate, creatine kinase and trypsin were obtained from Boehringer.

Native tyrosine hydroxylase from bovine adrenal medulla was prepared by gel filtration on Sepharose 6 B (11), and stored at -80° C until used. The enzyme was assayed as described previously (4), usually in 100 mM MES buffer pH 6.4. One unit of tyrosine hydroxylase activity corresponds to one nmole of dopa formed per minute.

Tyrosine hydroxylase was phosphorylated by incubation for 20 min at 30° C in the following medium : phosphate buffer (pH 7.0) 50 mM, NaF 16 mM, theophylline 5 mM, creatine phosphate 2 mM, creatine kinase 1 mg/ml, cAMP 10 µM, $MgCl_2$ 5 mM, protein kinase (from beef heart), 30 µg/ml, ATP 400 µM ; it was diluted 10 times in the assay mixture.

Limited proteolysis of native tyrosine hydroxylase was obtained by incubation for 10 min at 37° C in Tris-HCl buffer pH 7.4 in the presence of various amounts of trypsin The proteolysis was stopped by addition of sufficient amounts of trypsin inhibitor from pancreas.

Linear 5 - 20 % sucrose gradients were centrifuged at 4° C for 15 hours at 40 000 rpm in an SW 41 rotor.

RESULTS AND DISCUSSION : Phosphorylation shifted the pH optimum of the hydroxylation reaction from 5.8 to 6.4 (Fig. 1a) and the change of the pH profile was similar to that induced by anions (4). Phosphorylation also increased

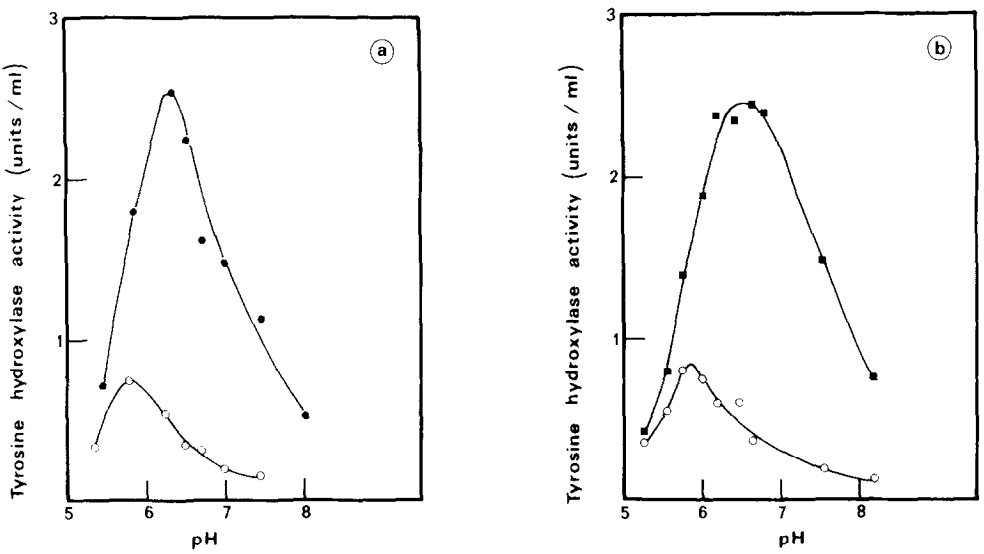

Figure 1 a - Effect of phosphorylation on the pH-profile of tyrosine hydroxylase. Native (o) or phosphorylated (●) tyrosine hydroxylase was assayed in 100 mM Tris-maleate buffer with 60 µM $DMPH_4$ as the co-factor (subsaturing conditions).

b - pH-profile of native (o) and partially proteolyzed (■) enzyme. Tyrosine hydroxylase was proteolyzed with 6 µg/ml of trypsin and was assayed as described in Figure 1 a.

the enzyme activity measured at the pH optimum (Fig. 1a). This activation was observed at both saturating and subsaturating concentrations of the co-factor $DMPH_4$. As in the case of anions, two effects were involved, an increase of the Vmax and a decrease of the K_M for the cofactor, since the activation was more pronounced at low $DMPH_4$ concentration. The similarity of the effects of phosphorylation and specific anions such as heparin, dextran sulfate or MES suggests a common mechanism underlying both types of activation This mechanism might also explain the shift of pH optimum and the activation observed after proteolysis of tyrosine hydroxylase (Fig. 1 b).

When the native enzyme was incubated for various lengths of time in the phosphorylation medium, tyrosine hydroxylase activity measured in Tris-maleate buffer increased with time and reached a plateau value approximately equal to the activity of the non-incubated enzyme assayed in MES buffer (Fig. 2). Tyrosine hydroxylase activity measured in MES buffer was the same for the incubated and the non-incubated enzyme. Analysis in the two buffers of the time course of dephosphorylation gave the same results (data not shown). These results supported the possibility that the effects of phosphorylation and of the interaction with anions, which were not additive,

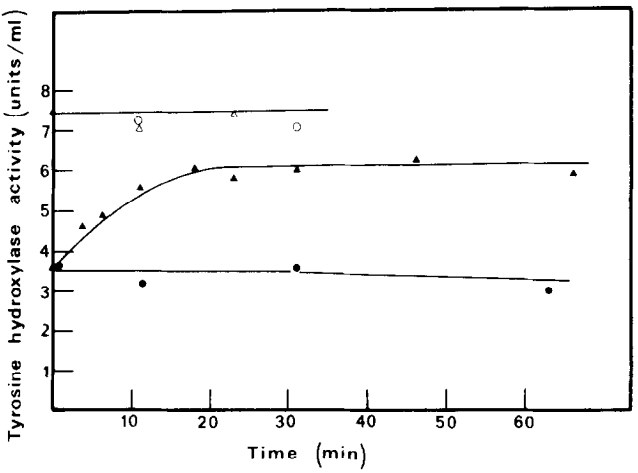

Figure 2 - Kinetics of activation of tyrosine hydroxylase by phosphorylation. Native tyrosine hydroxylase was phosphorylated as described in methods (▲ , △). Controls (●, o) were incubated in 50 mM Tris-HCl buffer (pH 7.4) at the same temperature. Aliquots were withdrawn at intervals and immediately assayed for tyrosine hydroxylase activity either in Tris-maleate (▲ ,●) or in MES (△, o) 100 mM buffer at pH 6.4.

occured at a common site. It should be noted that the activation by proteolysis observed in Tris-maleate buffer was not seen either in the presence of anions such as MES, or after incubation under phosphorylating conditions (Table 1), thus indicating that proteolysis was not additive with the other processes of activation.

The activation observed at pH 6.4 after incubation of the enzyme under phosphorylating conditions was not merely mediated by the anions present

Table 1. Percentage of tyrosine hydroxylase activity after proteolysis

		Trypsin (mg)						
Phosphorylation	Assay buffer	0	$4\ 10^{-4}$	$8\ 10^{-4}$	$2\ 10^{-3}$	$4\ 10^{-3}$	$4\ 10^{-2}$	$2\ 10^{-1}$
–	Tris-maleate	100	130	180	205	190	150	11
–	MES	200	210	200	195	185	160	4
+	Tris-maleate	200	210	225	–	200	130	
+	MES	200	220	220	–	200	145	

Various amounts of trypsin were added to tyrosine hydroxylase (3 mg of protein, 1.6 units) in 200 µl final volume. After a 10 min incubation, trypsin inhibitor (1 mg/1.5 mg trypsin) was added and an aliquot was assayed for tyrosine hydroxylase activity while another was incubated under phosphorylating conditions before being assayed. Assays were performed as described in methods, either in MES or in Tris-maleate buffer at pH 6.4.

Table 2. Percentage of enzymatic activity after incubation under phosphorylating conditions.

Preincubation medium	Assay medium: Tris - maleate	Assay medium: MES
1. Control	100	200
+ cAMP	108	200
+ ATP-Mg^{2+}	124	180
+ ATP-Mg^{2+} + cAMP	130	200
+ protein kinase	104	208
2. Complete	190	204
- ATP-Mg^{2+}	90	180
- creatine phosphate, - creatine kinase	190	220
- creatine phosphate, - creatine kinase, - ATP-Mg^{2+}	90	186

Tyrosine hydroxylase (7.5 mg/ml of protein, 10 units/ml) was preincubated in buffer (control), complete or incomplete phosphorylation medium and assayed for activity at pH 6.4 either in Tris-maleate or in MES buffer.

in the medium, as shown by control experiments (Table 2). It did not originate either in proteolysis during incubation under phosphorylating conditions since after phosphorylation the sedimentation coefficient of tyrosine hydroxylase was unchanged (data not shown) ; limited proteolysis should have induced a variation of the sedimentation coefficient from 9.6 to 3.3 S. In addition, the peak at 9.6 S was 1.7 times more active when assayed in MES buffer than in Tris-maleate, indicating partial dephosphorylation during centrifugation and excluding the possibility of proteolysis (see Table 1).

The activation by incubation in the phosphorylating medium, which is strictly ATP-dependent, is thus likely to result from phosphorylation of the protein ; indeed, others (9, 10, 12) have demonstrated ^{32}P incorporation into purified tyrosine hydroxylase subunits. We propose that phosphorylation by a covalent modification of the protein affects the charge of the cationic regulatory site and suppresses its electrostatic effect on the active site as do anions. Phosphorylation thus has the same effect as proteolysis which separates the regulatory and the active sites (4). The same regulatory effects could

thus be obtained by three different processes : interaction with anions, partial proteolysis and phosphorylation. In each case, we propose a similar mechanism involving a change in the local charges near the active site.

The pH shift induced by phosphorylation, anions or proteolysis of tyrosine hydroxylase is about 0.6 pH units, corresponding to an electrostatic potential ψ at the active site of 37 mV. In aqueous solutions characterized by a dielectric constant of 80, such a potential might be generated for example, by two positive charges 10 Å away from the active site. This calculation indicates that the number of unit charges carried by the positive regulatory site might not be high, thus allowing its neutralization by phosphorylation.

We consider phosphorylation of tyrosine hydroxylase as covalently modulating the electrostatic effects of a regulatory cationic site. It is tempting to generalize this molecular mechanism to some other enzymes regulated by phosphorylation. Basic aminoacids have been repeatedly identified in the vicinity of the phosphoryl acceptor residue (13), which might represent the postulated cationic regulatory site. This characteristic has also been observed in the case of another hydroxylase using the pterin cofactor : the phenylalanine hydroxylase (14). This cationic site could have various types of effects on the active center microenvironment depending on the nature of the charged species involved (H^+, positively or negatively charged substrate . . .). Phosphorylation might either activate enzymes reacting with positively charged substrates (15, 16) or inhibit those reacting with negatively charged ones (17, 18). In all cases, the regulatory effects would involve electrostatic forces and consequently interaction of anions with the cationic site or proteolysis of the fragment bearing this site should mimic the transition induced by phosphorylation.

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