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ACTIVATION OF TYROSINE HYDROXYLASE BY POLYANIONS AND SALTS

AN ELECTROSTATIC EFFECT

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Summary

The activity of a partially purified preparation of tyrosine hydroxylase (EC 1.14.16.2) from the bovine caudate nucleus was increased by heparin, chondroitin sulfate, phosphatidylserine, polyacrylic acid, polyvinyl sulfuric acid, and both poly-D-, and poly-L-glutamic acids, all polyanions. A variety of salts both activated the enzyme and prevented the activation by the polyanions. The observations that activity is increased when the enzyme interacts with salts and with macromolecules of high negative charge density are used to infer a model for these interactions and for the structural change in the enzyme that accompanies activation.

Introduction

In general, the properties of enzymes that are best understood are those that can be studied with the isolated proteins in solution. Since an enzyme's activity can be modulated by its interaction with other tissue components, these properties may be of limited value in describing the behavior of an enzyme in vivo. Studies of the interaction of an enzyme with tissue components, or other macromolecules, may, therefore, give some insight into the manner in which enzyme activity may be physiologically regulated.

There is evidence that the catalytic activity of the enzyme, tyrosine hy-

* In an early paper [1], Kuczenski showed slight (25%) activation of canine hypothalamic tyrosine hydroxylase by chondroitin sulfate. In later papers [7], dealing with the rat striatal enzyme, he states that chondroitin sulfate is inactive.

Abbreviation: DOPA; L-3,4-dihydroxyphenylalanine.

droxylase (EC 1.14.16.2), can be altered by its binding to other tissue components. Kuczenski and coworkers [1—4] reported that the enzyme from the rat corpus striatum can be isolated in both soluble and particulate, presumably membrane-bound forms The distribution of the enzyme between these forms was affected by pharmacological manipulation and was, therefore, thought to be of physiological importance. The soluble enzyme, furthermore, could be activated by salts and by heparin, but not by chondroitin sulfate* or hyaluronic acid. The kinetic properties of heparin-activated tyrosine hydroxylase, at least partially, resembled those of the particulate enzyme. Therefore, interaction of the enzyme with heparin was proposed as a model for its binding to subcellular structures.

Musacchio [5], working with rat adrenal tyrosine hydroxylase, had previously shown that this enzyme, too, could be isolated in both soluble and membrane-bound forms. Here the distribution of the enzyme was reported to be sensitive to the ionic composition of the homogenizing medium. Following Kuczenski, this group found that the soluble adrenal enzyme, like that from the corpus striatum, could be activated by heparin [6].

Heparin was the only macromolecule found by Kuczenski and his coworkers to increase tyrosine hydroxylase activity significantly. Though these workers [7] have recently suggested that activation is somehow related to the polyanionic character of heparin, they did not study the structural specificity or the physical basis for enzyme activation. It was not known, for example, whether tyrosine hydroxylase recognized, or interacted with, some local stereochemical element of heparin structure, or whether interaction reflected some physical property of the activator.

Recently, Lloyd and Kaufman [8] surveyed the effects of phospholipids on the activity of a partially purified tyrosine hydroxylase from the bovine caudate nucleus and found that the enzyme could be activated by sonicated suspensions of phosphatidylserine, as well as by heparin. The effects with the bovine caudate enzyme are quantitatively smaller than those observed with the rat striatal enzyme, but are qualitatively similar.

It is evident, therefore, that tyrosine hydroxylase can be activated by interaction with heparin, phosphatidylserine, and biological membranes. These modulators differ markedly in structure and composition, but all are polyanions. If these phenomena have a common mechanistic basis, the interaction of tyrosine hydroxylase with these substances, and the resulting activation, are likely to be electrostatic effects. Following this reasoning, the present study was undertaken to explore the extent to which electrostatic forces are responsible for the interaction of tyrosine hydroxylase with other macromolecules, and for the accompanying modulation of its catalytic activity.

Materials and Methods

L-[3,5-$^3H_2$]Tyrosine, 30 Ci/mol, was obtained from the Amersham Radiochemical Center, and was purified according to the method of Ikeda et al. [9]. 3-Iodo-L-tyrosine was from the Aldrich Chemical Co.; crystalline catalase in suspension and glucose-6-phosphate dehydrogenase (Grade 1), from Boehringer

Mannheim Corp.; and 2-(*N*-morpholino)ethane sulfonic acid, NADPH (Type 1), and glucose 6-phosphate were from Sigma Chemical Co. 6-Methyltetrahydropterin was purchased from Calbiochem. CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals. Tetrahydrobiopterin was synthesized by Dr. Sheldon Milstien. Sheep liver dihydropteridine reductase was purified from sheep liver through the calcium phosphate gel step as described by Kaufman [10].

Sodium heparin (pork intestinal mucosa) was obtained from Hynson, Westcott, and Dunning, Inc. Poly-D-, and Poly-L-glutamic acids (both Type III-B, sodium salt; degree of polymerization 426 and 565, respectively) and chondroitin sulfate (Grade III, sodium salt, from whale and shark cartilage) were purchased from Sigma Chemical Co. Polyacrylic acid and polyvinyl sulfuric acid (potassium salt) were from K and K Laboratories. Phosphatidyl-L-serine (A grade, from bovine brain) was from Calbiochem or Supelco. Dipalmitoyl- and egg yolk lecithin were from Sigma and Supelco, respectively. Suspensions of lipids were prepared each day by sonication in 2-(*N*-morpholino)-ethane sulfonic acid buffer.

pH was measured with a Radiometer Titrator Model TTT 1C with a scale expander and Model GK 2302C electrode. Concentrations of tetrahydropterins were determined spectrophotometrically as discussed by Shiman et al. [11]. Protein concentrations were determined by the method of Lowry et al. [12] using bovine serum albumin as a standard.

Tyrosine hydroxylase was obtained from an aqueous extract of an acetone powder prepared from bovine caudate nucleus, and purified by chromatography on a 3-iodotyrosine-sunubstituted Sepharose column, as described by Lloyd and Kaufman [8], followed by chromatography on hydroxyapatite.

Heparin-substituted Sepharose 4B was prepared from CNBr-activated Sepharose as described by Sakamoto et al. [13]. Its effects were compared to those of activated Sepharose treated with ethanolamine.

For assay of tyrosine hydroxylase activity, incubation mixtures contained 50 mM sodium 2-(*N*-morpholino)-ethane sulfonate buffer (pH 6.2), 50 μM L-tyrosine (or tyrosine in the indicated concentration), $2 \cdot 10^5$—$3 \cdot 10^5$ cpm L-[3,5-$^3H_2$]tyrosine, 2000 units catalase, and pterin cofactor as indicated. Unless otherwise stated, the ionic strength of assay mixtures was adjusted to 0.1 M with NaCl. Except where indicated, the reaction media contained 20 mM 2-mercaptoethanol to maintain the cofactor in the reduced state. When the enzymic cofactor-regenerating system was used, the mercaptoethanol was replaced by sheep liver dihydropteridine reductase in excess, 0.01 mg/ml glucose-6-phosphate dehydrogenase, 0.17 mM NADPH, and 10 mM glucose 6-phosphate. Samples to be assayed also contained tyrosine hydroxylase in the indicated amount, and whatever materials were being examined for their effect on enzyme activity. Reaction volumes were between 0.1 and 0.5 ml. Incubation was at 30° C. Reactions were terminated by addition of trichloroacetic acid to a final concentration of 5%. The tritiated water that was formed was assayed by the method of Nagatsu et al. [14].

Both the specific activity of tyrosine hydroxylase and the extent of salt and polyanion activation observable varied somewhat from preparation to preparation. Activity, and sensitivity to activation, however, were reproducible and stable with any given preparation. Unless otherwise indicated, all experiments

were performed under conditions in which product formation increased in linear fashion with time of incubation and protein concentration.

Results

Effect of polyanions and salts on tyrosine hydroxylase activity

To test the hypothesis that activation of tyrosine hydroxylase by heparin and phosphatidylserine is an electrostatic phenomenon, we examined the effects of a number of polyanions and salts on the activity of a partially purified preparation of the bovine caudate enzyme. Under the conditions of Fig. 1, tyrosine hydroxylase activity is increased 1.8—5.1-fold by NaCl, heparin, phosphatidylserine, polyacrylic acid, polyvinyl sulfuric acid, and both poly-D-, and poly-L-glutamic acids, but not by bovine serum albumin. In other experiments, both dipalmitoyl- and egg yolk lecithin were inactive. Under the conditions of Fig. 1 (ionic strength 0.1), chondroitin sulfate was inactive at concentrations up to 1 mg/ml. In experiments performed in 0.02 M sodium 2-(*N*-morpholino)-ethane sulfonate buffer, pH 6.2, ionic strength 0.01 M, under conditions where heparin activation was greater than 10-fold (vidae infra), chondroitin sulfate did activate tyrosine hydroxylase. At ionic strength of 0.01 M, 10^{-3}, 10^{-2}, and

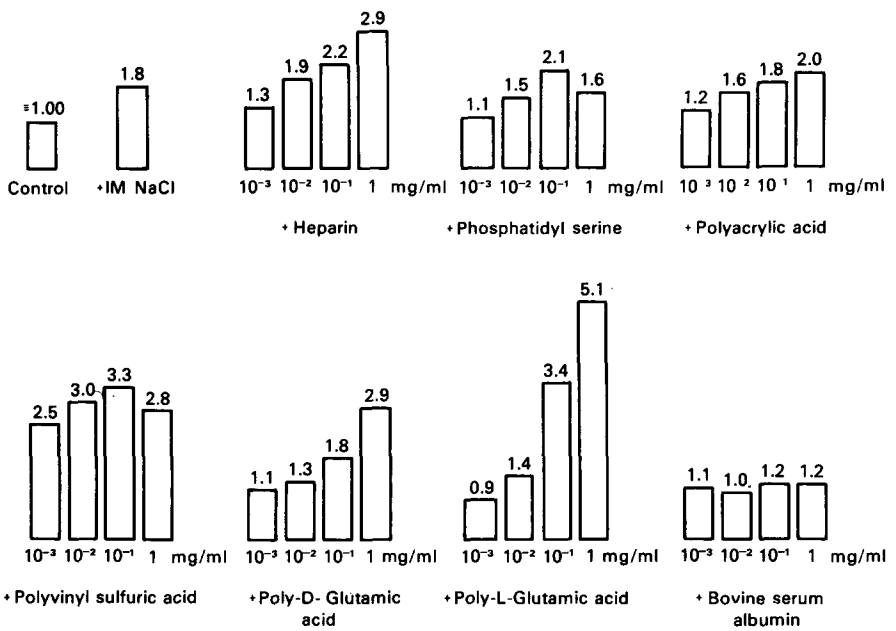

Fig. 1. Assay of tyrosine hydroxylase in the presence of several polyanions. Tyrosine hydroxylase activity was assayed as described under Materials and Methods at 200 μM 6-methyltetrahydropterin, 50 μM tyrosine. Reaction volume was 100 μl. Buffer was 0.05 M 2-(*N*-morpholino)-ethane sulfonic acid, ionic strength was adjusted to 0.1 or 1.0 M with NaCl; pH 6.2. The pH of the polyanion solutions were adjusted to maintain assay pH. Concentration of activators are given as weights of materials as commercially available; that of heparin and polyglutamic acid refer to the sodium salt; of polyvinyl sulfuric acid, to the potassium salt; and polyacrylic acid, to the acid. Incubation was for 10 min at 30°C. The control rate was 0.042 nmol tyrosine/10 min per 10 μg protein. Heights of bars are proportional to the amount of product formed in 10 min. The numbers above the bars are the ratios of the apparent rates to the control rate. Rates in the presence of each of the activators except polyvinyl sulfuric acid were linear with time.

10^{-1} mg/ml chondroitin sulfate increased enzyme activity 1.3-, 1.9-, and 2.5-fold, respectively. Though tyrosine hydroxylase can be activated both by salts and by a number of polyanions, the latter compounds are far more effective. For example, 1 mg/ml heparin corresponds to a concentration of about 10^{-4} M, and a concentration of ionized groups of 6 mM. This is clearly quite low compared to the ionic strength of the assay medium and the concentration of salt required for significant enzyme activation. Similarly, di- and trivalent anions (sulfate, phosphate, fumarate, succinate, and citrate) were inactive at equivalent concentrations well above those at which polyanion effects were maximal. When polyanions are added to the reaction medium pairwise, their effects are not additive. For example, at 1 mg/ml where heparin activates 2.9-fold, and polyacrylic acid, 2.0-fold, the two together activate only 2.2-fold. Similarly, at 0.1 mg/ml, where heparin activates 2.2-fold and phosphatidyl-serine, 2.1-fold, the two together activate 2-3-fold. These observations suggest that the polyanions share a common mechanism or site of action. Kinetic parameters for tyrosine hydroxylase in the presence of several of the above activators are given in Table I.

Salt can both activate tyrosine hydroxylase and prevent activation of the enzyme by polyanions. In the presence of each of the polyanions of Fig. 1, increasing the NaCl concentration of the medium inhibits activity. The effect of NaCl on heparin activation is shown in Fig. 2A. In the presence of 1 mg/ml heparin, enzyme activity is inhibited more than 50%, when NaCl is increased from 0.1 to 0.25 M. At 0.1 M ionic strength, heparin activates the enzyme 2.7-fold. At ionic strengths 0.25 or greater, no significant activation is seen.

TABLE I

KINETIC PARAMETERS OF TYROSINE HYDROXYLASE

Tyrosine hydroxylase activity was assayed using the chemical reducing system. Assay medium was 0.05 M sodium 2-(*N*-morpholino)-ethane sulfonate, pH 6.2. Ionic strength was adjusted to 0.1 or 1.0 M with NaCl. Incubation was for 10 min at 30°C. Kinetic parameters and standard errors were obtained using a weighted least squares program with a Wang programming calculator. Different enzyme preparations were used for determination of tyrosine and 6-methyltetrahydropterin kinetic parameters and, therefore, V values obtained by varying tyrosine and cofactor cannot be compared directly. Data for determination of the control K_m for the cofactor were restricted to cofactor concentrations above 200 μM. Below that concentration, rates were more rapid than expected for simple saturation kinetics. This probably reflects the presence of the interconvertible forms of the enzyme, as discussed below, present at equilibrium under assay conditions. We cannot, however, rigorously exclude the possibility that it is due to the presence in the enzyme preparation of some irreversibly activated enzyme. (See footnote p. 92.)

	V_{max} *	K_m (μM)
(a) 6-Methyltetrahydropterin varied; [Tyrosine] = 50 μM		
Control	0.090 ± 0.006	530 ± 70
+ 1 M NaCl	0.19 ± 0.01	340 ± 30
+ 1 mg/ml polyacrylate	0.23 ± 0.005	340 ± 20
+ 1 mg/ml heparin	0.23 ± 0.005	250 ± 10
+ 1 mg/ml phosphatidyl-serine	0.14 ± 0.005	130 ± 15
(b) Tyrosine concentration varied; [6-Methyltetrahydropterin] = 1.5 mM		
Control	0.10 ± 0.005	43 ± 5
+ 1 M NaCl	0.19 ± 0.01	37 ± 4
+ 1 mg/ml polyacrylate	0.24 ± 0.01	47 ± 5

* nmol DOPA/10 min per 10 μg protein.

The dependence of heparin activation on the ionic environment is further documented in Fig. 2B. At ionic strength of 0.025 M, 1 mg/ml heparin gives a large (greater than 8-fold), but transient, stimulation of tyrosine hydroxylation.

Table II shows the effects of several univalent electrolytes on the activity of tyrosine hydroxylase. The enzyme is activated by each of the salts. The extent of activation at 0.5 M depends upon the nature of both the cation and anion used. Activation by divalent ions can also be observed. We do not, however, find specific effects of low concentrations of calcium such as those observed by Roth et al. [15,16] with tyrosine hydroxylase activity in extracts from other brain areas and from peripheral nerve. Furthermore, neither enzyme activity nor heparin stimulation are dependent upon trace concentrations of divalent cations; both properties are unaffected by the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) to reaction mixtures.

In summary, tyrosine hydroxylase can be activated by a number of chemically dissimilar polymers which share only a high negative charge density. Salt can modulate the ability of these molecules to increase enzyme activity, presumably by a screening effect. (In high salt media, the surface electrostatic potential of the polyanions becomes less negative. The effectiveness of a polyanion as an activator apparently depends upon this potential, which is a function of both charge density and ionic environment, rather than upon charge density alone.) These results are the expected ones if activation were an electrostatic phenomenon rather than the result of specific chemical interactions. Kuczenski, as discussed above, observed activation of tyrosine hydroxylase by heparin, but not by chondroitin sulfate. He argued that this apparent specificity implied that activation could not be a pure electrostatic effect. He did not, however, consider the possibility that the relative activities of the mucopolysaccharides re-

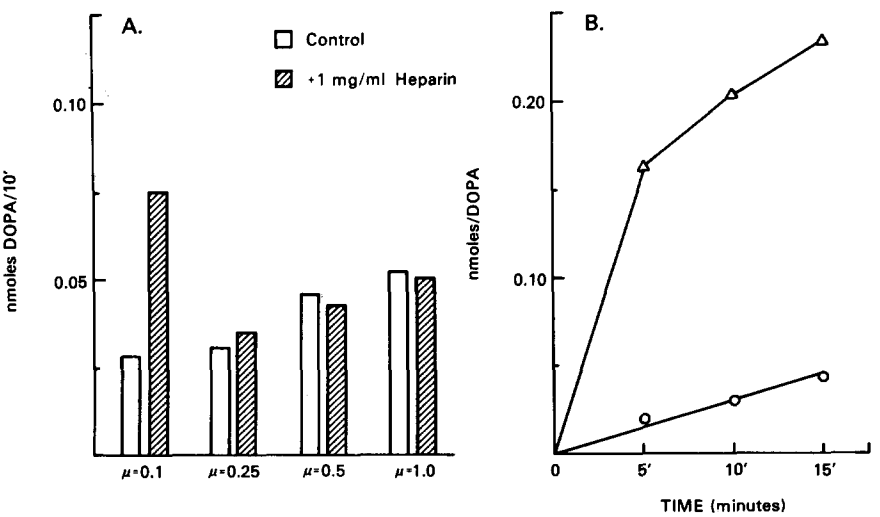

Fig. 2. Salt dependence of heparin activation. Enzyme was assayed as described under Materials and Methods at 50 μM tyrosine, 200 μM 6-methyltetrahydropterin. Protein was 10 μg/0.1 ml. Buffer was 0.05 M 2-(*N*-morpholino)-ethane sulfonic acid. In (A), ionic strength was adjusted with NaCl. In (B), no NaCl was added. The pH was 6.2. Incubation was 10 min or as indicated, at 30°C. Reactions in (A) were linear with time. ○———○, control; △———△, 1 mg/ml heparin.

TABLE II

EFFECT OF UNIVALENT IONS ON TYROSINE HYDROXYLASE ACTIVITY

Tyrosine hydroxylase activity was measured in the presence of various salts at 0.5 M concentration. Buffer was 0.05 M sodium 2-(*N*-morpholino)-ethane sulfonate; pH in reaction mixtures was 6.18 ± 0.04. Tyrosine was at 50 μM; 6-methyltetrahydropterin, at 200 μM; and 2-mercaptoethanol, at 20 mM. Reaction volume was 0.1 ml. 15 μg protein were added per tube. Incubation was at 30° C for 10 min.

	Rate (nmol/10 min per 15 μg protein)	Relative rate
Control	0.047	1.00
+ 0.5 M LiCl	0.091	1.92
+ 0.5 M KCl	0.069	1.46
+ 0.5 M NaCl	0.082	1.74
+ 0.5 M NaBr	0.116	2.46
+ 0.5 M sodium acetate	0.063	1.32

flected their charge densities. (To a first approximation, heparin has three; chondroitin sulfate, two; and hyaluronic acid, one negative charge per disaccharide unit.) Our finding that chondroitin sulfate, at low ionic strength, can activate bovine caudate tyrosine hydroxylase shows that activation by mucopolysaccharides is less specific than previously thought. In view of our other findings, the fact that chondroitin sulfate can activate is not surprising, since it, too, is a polyanion. The fact that it is less potent than heparin might have been predicted from its lower charge density.

Activation of tyrosine hydroxylase is independent of the type of tetrahydropterin-regenerating system and of the structure of the tetrahydropterin

As demonstrated by Kaufman and Fisher [17], activity of the pterin-dependent mono-oxygenases requires the presence of the cofactor in fully reduced form. Tetrahydropterins, however, are readily auto-oxidized. Under the conditions commonly employed for assay of tyrosine hydroxylase, cofactor autooxidation is rapid relative to the rate of the enzyme-catalyzed reaction. Therefore, even though the extent of enzyme-catalyzed cofactor oxidation is quite low in experiments such as these, assay of enzyme activity requires the presence of reagents capable of reducing the cofactor that has been oxidized during the course of the reaction. 2-Mercaptoethanol was used for this purpose in the above experiments. As shown in Table III, activation of tyrosine hydroxylase is observable, as well, when cofactor reduction is accomplished enzymatically with the reduced pyridine nucleotide-dependent, sheep liver dihydropteridine reductase. Although the absolute activity of the hydroxylase under these conditions is sensitive to the nature of the cofactor-regenerating system, the extent of activation is not.

All above experiments were performed with the synthetic cofactor, 6-methyltetrahydropterin. Additional experiments have examined the effects of polyacrylic acid and high concentrations of salt on tyrosine hydroxylase activity in the presence of the naturally occurring cofactor, tetrahydrobiopterin, and have demonstrated similar increases in activity. Activation of the enzyme by heparin and phosphatidylserine in the presence of tetrahydrobiopterin has previously been reported [8]. Thus, polyanion activation of tyrosine hydroxylase, in contrast to many other regulatory properties of pterin-dependent hydroxylases [17], is not critically dependent on cofactor structure.

TABLE III

COMPARISON OF CHEMICAL AND ENZYMIC TETRAHYDROPTERIN-REGENERATING SYSTEMS

Tyrosine hydroxylase was assayed both in the presence of 20 mM 2-mercaptoethanol and the enzymic reducing system as described under Materials and Methods. Tyrosine was at 50 μM, 6-methyltetrahydropterin, at 200 μM. Buffer was 0.05 M 2-(*N*-morpholino)-ethane sulfonate; ionic strength was adjusted to 0.1 or 1.0 with NaCl. pH was 6.2. Incubation was for 10 min at 30° C.

	Rate (nmol DOPA/10 min 10 μg protein)	Stimulation
Chemical reduction		
Control	0.038	—
+ 1 M NaCl	0.10	2.6
+ 1 mg/ml heparin	0.14	3.7
+ 1 mg/ml polyacrylate	0.11	2.9
Enzymic reduction		
Control	0.055	—
+ 1 M NaCl	0.16	2.9
+ 1 mg/ml heparin	0.18	3.3
+ 1 mg/ml polyacrylate	0.15	2.7

Activation of tyrosine hydroxylase by heparin-substituted Sepharose: evidence for a direct interaction of heparin and the hydroxylase

Though the enzyme preparation used in these studies was purified approx. 10-fold, it was, of course, not homogeneous. It was therefore necessary to prove that activation of tyrosine hydroxylase by polyanions was the result of a direct interaction between the two molecules, rather than an indirect effect. Table IV presents typical results of a series of experiments in which we studied the interaction of tyrosine hydroxylase with heparin immobilized on agarose beads. Tyrosine hydroxylase is both bound to and activated by heparin-substituted

TABLE IV

INTERACTION OF TYROSINE HYDROXYLASE WITH HEPARIN-SUBSTITUTED SEPHAROSE

0.1 mg tyrosine hydroxylase was preincubated for 5 min at 30° C in the presence of all components of the assay mixture except [3,5-$^3H_2$]tyrosine, in a volume of 0.5 ml. Where indicated, 25% (v/v) control, or heparin-substituted Sepharose (prepared as described under Materials and Methods, and washed extensively immediately before use), and 2 mg/ml sodium heparin were present in the preincubation mixture. 50-μl aliquots of the mixture, or 50 μl of the supernatant after centrifugation of the Sepharose were assayed for enzyme activity at 50 μM tyrosine, 1.0 mM 6-methyltetrahydropterin in a volume of 0.1 ml. Incubation was for 10 min at 30° C in 0.05 M sodium 2-(*N*-morpholino)-ethane sulfonate buffer, pH 6.2, ionic strength 0.1. The bound activity was recovered when the sedimented Sepharose wa assayed after being washed with buffer.

	Tyrosine hydroxylase activity (nmol DOPA/10 min)		Activity bound to Sepharose (%)
	Total	Supernatant	
Control	0.089		
+ control Sepharose	0.093	0.076	18
+ heparin-substituted Sepharose	0.25	0.030	88
+ heparin-substituted Sepharose + 2 mg/ml heparin	0.26	0.13	50

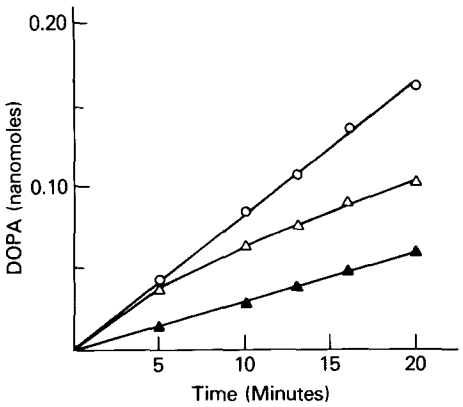

Fig. 3. Reversal of salt activation. Tyrosine hydroxylase was preincubated 5 min at 30°C at a concentration of 0.48 mg/ml in 0.05 M 2-(*N*-morpholino)-ethane sulfonic acid buffer, pH 6.2, in the presence of the enzymic cofactor-regenerating system. Ionic strength was adjusted as indicated with NaCl. The preincubation medium contained 50 μM tyrosine, 200 μM 6-methyltetrahydropterin, and all other components of the assay medium except labelled tyrosine. After preincubation, samples were diluted 10-fold and assayed in a volume of 0.1 ml at the indicated ionic strength, pH 6.2, at 50 μM tyrosine, and at 200 μM. 6-Methyltetrahydropterin also with the enzymic regenerating system. ▲———▲, preincubation at 0.1 M, assay at 0.1 M; △———△, preincubation at 1.0 M, assay at 0.1 M; ○———○, preincubation at 0.1 M, assay at 1.0 M.

Sepharose, but not by control, ethanolamine-substituted Sepharose. Activation by bound heparin is not independent of that by soluble heparin. Moreover, added heparin decreases binding to the substituted Sepharose. These results indicate that activation does result from a direct interaction of the polyanion with the enzyme.

Reversibility of the salt activation of tyrosine hydroxylase

Since tyrosine hydroxylase can be irreversibly activated by partial proteolysis [18], and under conditions that promote protein phosphorylation*, it was important to test the reversibility of salt and polyanion activation. Reversibility was demonstrated in a series of experiments in which enzyme was preincubated, in the absence of labelled tyrosine, in a reaction medium in which tyrosine hydroxylase is known to be activated, and then assayed in a control medium. Fig. 3 shows a typical experiment in which the activity of enzyme preincubated in 1 M NaCl, 0.05 M sodium 2-(*N*-morpholino)-ethane sulfonate buffer, pH 6.2, was assayed, at the same pH, at ionic strength 0.1, after a 10-fold dilution with sodium 2-(*N*-morpholino)-ethane sulfonate buffer of low ionic strength. The activity of this enzyme was compared with that of tyrosine hydroxylase preincubated and assayed after 10-fold dilution at constant ionic strengths of 0.1 and 1.0 M. At 5 min, the sample that had been preincubated at 1 M NaCl and assayed at 0.1 M NaCl (middle curve) was 2.6-fold activated relative to the low salt control (lowest curve). After 5 min, however, the rate of hydroxylation in the sample that had been preincubated at high salt and as-

* T. Lloyd and S. Kaufman (personal communication), and Morgenroth et al. [24].

sayed at low salt declined so that between 10 and 20 min, activity of the two samples was comparable. Thus, activation by high concentrations of salt is, indeed, reversible; reversal is completed only after several minutes at 30° C.

In a similar experiment, the activity of tyrosine hydroxylase in the presence of 1 mg/ml heparin was assayed at 0.25 M NaCl after a preincubation at 0.10 M salt. This material showed greater activity than the control that had been preincubated and assayed in 0.25 M NaCl only in the first 5 min of assay. Though interpretation of this experiment is more difficult than that above, heparin activation too, is apparently slowly reversible.

Discussion

The above data demonstrate activation of 10-fold purified bovine caudate tyrosine hydroxylase by a variety of macroanions. Electrostatic forces have been implicated in this interaction by two criteria. First, the ability to activate does not seem to be sensitive to the chemical nature of the activator but only to its charge density. Second, the interaction of polyanions with the enzyme can be screened by salt. Salt, furthermore, can, itself, increase enzyme activity. Each of these effects is reversible, and apparently results from a direct interaction of the enzyme with the activators.

Since protein molecules are themselves polyelectrolytes, it seems reasonable to assume that the interaction of the enzyme with polyanions and salt is, at least to a first approximation, coulombic. Based on this assumption, the above observations can be used to infer certain structural features of the enzyme: (a) Tyrosine hydroxylase must be capable of existing in two or more states, differing in catalytic activity and in either tertiary or quaternary structure, or in the distribution and extent of protonation. (b) In low salt concentrations, and in the absence of polyanions, the more catalytically active of these states must have, at least, local areas of higher positive charge density and electrostatic potential than the state of lower activity. (c) The differences in charge density between these states must be sufficiently great to give a difference in their electrostatic free energy in the absence of activators, that is significant relative to thermal energy.

It is clear from this model that polyanions would interact more strongly with the enzyme in its active state and would stabilize it. Similarly, activation by salts probably reflects their ability to decrease the electrostatic free energy of the enzyme in its active form.

As shown above, the effects of simple salts depend upon both the cation and anion added, implying that both ions interact with the enzyme. The site at which small anions interact may be the same area of high positive charge that is responsible for polyanion binding; i.e. there may be no fundamental difference between the actions of small anions and highly negatively charged polymers.

The polyanions are active at concentrations quite low compared to those of monovalent ions required for significant rate enhancements. Such apparent selectivity, in simplest terms, only implies that the charge density of the area in question on the enzyme surface be high. It is well known that multiple charged counterions are concentrated relative to univalent ions at a charged interface, and that, with high surface potentials, this charge selectivity can be enormous

[19]. The difference in apparent affinities under discussion is consistent with the simple coulombic model.

As shown in Table I, kinetic parameters for the stimulated enzyme depend upon the activator used. This dependence is not consistent with the simple two state model described above. It should be noted, however, that the microenvironments of the salt and polyanion activated enzymes must differ in a manner that reflects the properties of the bound activator. As discussed by Katchalski et al. [20], and McLaren and Packer [21], such properties as the local pH, electrostatic potential and electrostatic field felt by the functional groups of the protein are sensitive to the ionic environment and to bound macroions. These effects should, in principle, modify the observed catalytic properties of an enzyme; microenvironment effects must be superimposed on any model attempting to explain the interaction of a protein with polyelectrolytes and salts. The apparent deviations from the two state model are not surprising, and might well be due to perturbations on the simple model. In spite of these complexities, it seems reasonable to focus on the fact that the agents discussed here can all activate the enzyme. The basic mechanism operative is, most likely, similar in each case.

Charge-charge interactions, in general, may play two roles in the regulation of enzyme activity. First, if an enzyme can exist in two forms of distinct tertiary or quaternary structure that differ in electrostatic free energy, the equilibrium between them and, hence, the kinetic properties of the enzyme should be sensitive to the ionic environment. Second, since the dissociation constants of ionizable groups on an enzyme surface depend upon the potential due to nearby charges, the ion environment may influence the kinetic properties of an enzyme by changing the number or the distribution of bound protons without any other structural change.

Clearly either effect could be operative here. The observation that activation due to 1 M NaCl is only slowly reversible with dilution, suggests that a mechanism of the first type is involved in this case, since effects due to screening, or to weak ion binding, should be rapidly reversible, whereas structural changes can be relatively slow. This conclusion, however, is based upon quite indirect evidence. Confirmation of a change in tertiary or quaternary structure during activation, and experiments probing the similarity of action of simple salt and polyanions must await physical studies, requiring as yet unavailable pure enzyme.

This discussion until now has focused upon a possible mechanism for activation of tyrosine hydroxylase. The anions have been treated as mobile counterions. Kuczenski's initial observations along with the demonstration of activation by phosphatidylserine, suggests another point of view. Since many tissue components, including cell membranes are polyanionic, and since soluble tyrosine hydroxylase must be diffusable within the cytoplasm, the enzyme could act as a counterion for these other components of the cell. Certainly the interaction of the enzyme with sonicated phosphatidylserine, known to exist as single bilayer vesicles [22], is reminiscent of the Davson-Danielli model for membrane-protein binding [23]. In the experiments described here, lipid-protein interaction is electrostatic, and depends upon the negative charge of the polar head groups of the lipid. Since, according to our model, the activated

form of the enzyme has surface areas of higher charge density than the non-activated form, the activated enzyme would be a better counterion for the membrane surface, and membrane-protein interaction should lead to increased activity.

In summary, this work has confirmed and expanded our electrostatic model for the effect of polyanions on tyrosine hydroxylase activity. This is of interest both because it suggests a simple physical basis for enzyme activation, and because implicit in this model is a mechanism by which the interaction of proteins with macromolecular tissue components, including membranes, can modulate enzymic activity.

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