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Tyrosine hydroxylase activity and extrinsic fluorescence changes produced by polyanions

Laura G. GAHN and Robert ROSKOSKI, Jr.*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA, 70119, U.S.A.

The activity of tyrosine hydroxylase *in vitro* is affected by many factors, including pH, phosphorylation by several protein kinases, and polyanions. We investigated the activation of tyrosine hydroxylase by RNA or DNA (polyanions), using purified rat PC12 cell enzyme. RNA and DNA each increased tyrosine hydroxylase activity in the presence of subsaturating (125 μM) tetrahydrobiopterin at pH 6. RNA increased enzyme activity up to 6-fold with an EC_{50} of 3 μg/ml. RNA and DNA each increased tyrosine hydroxylase activity by decreasing the K_m of the enzyme for tetrahydrobiopterin from 3 mM to 295 μM in the presence of 100 μg/ml RNA or 171 μM in the presence of 100 μg/ml DNA. We used the apolar fluorescent probe 8-anilino-1-naphthalenesulphonic acid (1,8-ANS) as a reporter group to provide the first evidence for changes in conformation related to changes in activity. At pH 6.0, 1,8-ANS bound to tyrosine hydroxylase and exhibited a characteristic fluorescence spectrum. At pH 7.2, both enzyme activity and fluorescence decreased. DNA or heparin (another polyanion) activated tyrosine hydroxylase and decreased fluorescence of the reporter group 30% at pH 6.0. This decrease suggests that these polyanions altered the conformation of tyrosine hydroxylase. The activating effects of polyanions were diminished at physiological pH (6.8–7.2) or in the presence of bivalent-cation salts (10 mM) or univalent-cation salts (100 mM). These results suggest that polyanions play a minimal role, if any, in the physiological regulation of tyrosine hydroxylase activity.

INTRODUCTION

Tyrosine hydroxylase (EC 1.14.16.2) is the rate-limiting enzyme in catecholamine biosynthesis *in vivo* (Levitt et al., 1965). It catalyses the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (dopa). Phenylalanine can also serve as a substrate (Fukami et al., 1990; Ribeiro et al., 1991). The activity of tyrosine hydroxylase *in vitro* is regulated by many factors, including polyanions such as heparin (Katz et al., 1976), salts (Kuczenski and Mandell, 1972), phospholipids (Lloyd and Kaufman, 1974), and poly(vinylsulphuric acid) (Kuczenski and Mandell, 1972). Activation by polyanions is associated with a decrease in the K_m for the tetrahydropteridine substrate (Katz et al., 1976). Nelson and Kaufman (1987) reported that RNA (also a polyanion) alters tyrosine hydroxylase activity *in vitro*. In the presence of subsaturating 6-methyltetrahydropterin (0.1 mM), they reported that a low concentration of RNA (15 μg/ml) activates the enzyme in bovine adrenal homogenates, whereas higher concentrations of RNA (50 to 1000 μg/ml) are inhibitory. Tyrosine hydroxylase exhibits a pH optimum near 6 (Nagatsu et al, 1964), and this is the pH value used for those studies noted above that were performed *in vitro*. We investigated the interaction of RNA and DNA with tyrosine hydroxylase and the effects of pH on polyanion activation.

We also investigated the interactions of the apolar fluorescent probe 8-anilino-1-naphthalenesulphonate (1,8-ANS) with tyrosine hydroxylase. Since the first use of 1,8-ANS to study the haem-binding site of myoglobin (Stryer, 1965), 1,8-ANS has been used extensively to characterize conformational changes in proteins (Saucier et al., 1985), where it serves as a hydrophobic probe (Brand and Gohlke, 1972). Improved tyrosine hydroxylase purification procedures now yield greater than 20 mg of protein (Gahn and Roskoski, 1991; Andersson et al., 1992) and made the present studies possible. These 1,8-ANS studies demonstrate, for the first time, the existence of conformational changes in tyrosine hydroxylase coinciding with changes in enzyme activity.

EXPERIMENTAL

Materials

Bovine liver RNA, salmon sperm DNA, 1,8-ANS (hemimagnesium salt) and ribonucleases were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concentrations of RNA and DNA were determined using the following relationships: $A_{260} = 1.00 \equiv 40$ μg/ml for RNA; $A_{260} = 1.00 \equiv 50$ μg/ml for DNA (Sambrook et al., 1989). Charcoal (Darco G-60) was obtained from J. T. Baker (Jackson, TN, U.S.A.). L-[3,5-^{3}H]Tyrosine was obtained from NEN Research Products (Boston, MA, U.S.A.). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin was obtained from Dr. B. Shircks Laboratories (Jona, Switzerland). Since tetrahydrobiopterin is unstable at neutral pH, 5 mM HCl was added to stock solutions, which were stored at 4 °C for no more than 1 month. Tyrosine hydroxylase was purified from PC12 cells (derived from a rat pheochromocytoma) by the method of Gahn and Roskoski (1991). The specific activity of tyrosine hydroxylase was 0.6 μmol/min per mg (at 37 °C, 100 μM tyrosine and 1 mM tetrahydrobiopterin, pH 6.0). The catalytic subunit of cyclic AMP-dependent protein kinase was purified by the method of Hartl and Roskoski (1982) to a specific activity of 10 μmol/min per mg at 30 °C with 100 μM Kemptide (LRRASLG) and 100 μM ATP, pH 7. Both enzymes were homogeneous as judged by SDS/PAGE.

Tyrosine hydroxylase activity measurements and phosphorylation

Tyrosine hydroxylase activity was determined by measuring the release of ^3H_2O during the conversion of L-[3,5-^{3}H]tyrosine into dopa by the method of Reinhard et al. (1986). Enzyme

Abbreviations used: 1,8-ANS, 8-anilino-1-naphthalenesulphonic acid; dopa, 3,4-dihydroxyphenylalanine.

* To whom correspondence should be sent.

(5–30 μg/ml) was incubated at 37 °C with 100 μM L-[3,5-^{3}H]tyrosine (0.5 μCi per assay), 125 μM (6*R*)-5,6,7,8-tetrahydro-L-biopterin, 1500 units/ml catalase, 5 mM dithiothreitol, and 50 mM Pipes buffer, pH 6.0 (unless otherwise noted) in a total volume of 30 μl. Reactions were initiated by adding tyrosine hydroxylase and terminated after 10 min by adding 300 μl of 7.5% charcoal (Darco G-60) in 1 M HCl. Charcoal with adsorbed tyrosine and catechols was sedimented by centrifugation at 13000 *g* for 2 min; aliquots (100 μl) of supernatant were removed, and ^3H_2O radioactivity was determined by liquid-scintillation spectrometry. Under the conditions employed, the reaction was linear for at least 20 min. Samples were assayed in triplicate. For the nucleic acid experiments, the RNA or DNA was added to the assay solution first, then tyrosine hydroxylase was added to start the reaction. For determination of steady-state kinetic parameters, tyrosine hydroxylase activity was determined at concentrations of 50–1200 μM tetrahydrobiopterin or 2–100 μM tyrosine. Kinetic parameters were calculated by performing a non-linear least-squares fit to [substrate] versus log velocity with the program BDATA (EMF Software, Knoxville, TN, U.S.A.). The standard errors of K_m and V_{max} were calculated from three independent determinations.

For phosphorylation of tyrosine hydroxylase, the enzyme (0.2–2 mg/ml) was incubated with 1 μM purified catalytic subunit (40 kDa) of cyclic AMP-dependent protein kinase, 100 μM ATP and 1 mM $MgCl_2$ in 50 mM Pipes buffer, pH 6.0, at 30 °C for 20 min. This procedure leads to the stoichiometric phosphorylation and activation of tyrosine hydroxylase (Roskoski et al., 1987). Control samples were incubated with $MgCl_2$ and buffer only. The samples were diluted to provide a tyrosine hydroxylase concentration of 15 μg/ml during the activity assay.

Fluorescence measurements

Fluorescence was measured using an SLM 4800D spectrofluorimeter in the ratio mode and SLM PR8002 spectrum processing software. Samples consisted of 40 μM 1,8-ANS and 100–150 μg/ml tyrosine hydroxylase in 50 mM Pipes buffer at the specified pH in a 3 mm-path-length quartz cell. For experiments in which the pH was altered, the Pipes was replaced with the buffer combination of 50 mM Mes, 50 mM acetic acid and 100 mM Tris (referred to as 0.2 M MAT buffer). The sample chamber temperature was maintained constant at 20 °C, and samples were equilibrated at this temperature for several minutes before measurements were made. Fluorescence emission was measured from 400 to 600 nm in 2 nm increments, with an excitation wavelength of 380 nm. Figure 3 shows an average of two uncorrected emission spectra. Control samples showed that Pipes, DNA and heparin had no effect on 1,8-ANS fluorescence in the absence of tyrosine hydroxylase. In addition, tyrosine hydroxylase, Pipes, DNA, and heparin had no significant intrinsic fluorescence at these wavelengths. RNA altered 1,8-ANS fluorescence; therefore RNA was not used in the fluorescence studies. Changes in the fluorescence intensity were quantified by determining the area under the emission spectrum, which is proportional to the quantum yield. Areas were calculated by integrating from 400 to 550 nm with the SLM spectrum processing software.

RESULTS

Activation of tyrosine hydroxylase by RNA and DNA

Bovine liver RNA or salmon sperm DNA produced an increase in tyrosine hydroxylase activity at pH 6.0 and subsaturating (125 μM) tetrahydrobiopterin (Figure 1). Bovine liver RNA was effective at 1 μg/ml with an EC_{50} of 3 μg/ml, and salmon sperm DNA was effective at 0.1 μg/ml with an EC_{50} of 1 μg/ml. Heparin increased tyrosine hydroxylase activity in a similar manner with an EC_{50} of 0.3 μg/ml. Heparin (1 mg/ml) produced the greatest activation of tyrosine hydroxylase (8-fold). RNA and DNA (1 mg/ml) increased activity 6-fold and 7-fold respectively. Although there was variation in the percentage stimulation from experiment to experiment, the rank order of effectiveness of the three polyanions remained constant.

We examined the efficacy of several sources of RNA in increasing tyrosine hydroxylase activity (Table 1) and found that both ribosomal RNA and transfer RNA were capable of increasing tyrosine hydroxylase activity. Since the bulk of RNA in the cell is ribosomal, we also investigated the effect of intact ribosomes on tyrosine hydroxylase activity. Ribosomes, at an RNA concentration of 20 μg/ml, increased tyrosine hydroxylase activity more than 3-fold. Although ribosomal proteins are bound to ribosomal RNA in the intact particle, much of the RNA is on the surface of the ribosome (Lake, 1985) and is still able to interact with and activate tyrosine hydroxylase.

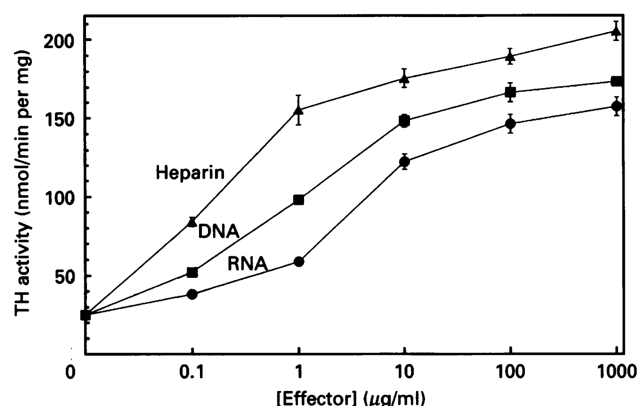

Figure 1 Concentration-dependence of nucleic acid and heparin activation of tyrosine hydroxylase

Tyrosine hydroxylase (TH) activity was assessed at pH 6.0 in the presence of various amounts of bovine liver RNA, salmon sperm DNA or heparin. Reactions were initiated by the addition of enzyme to the otherwise complete assay mixture. The absence of error bars indicates an S.E.M. smaller than the symbol size.

Table 1 Activation of tyrosine hydroxylase by various nucleic acids

Tyrosine hydroxylase activity was assessed in the presence of 20 μg/ml of the specified nucleic acid at pH 6.0 as described in the Experimental section. Ribosomes were prepared from PC12 cells by the method of Roskoski (1969), and RNA content in the ribosomes was assessed by the method of Fleck and Munro (1962).

Addition	Activity (nmol/min per mg) (mean ± S.E.M.)
None	25.0 ± 1.1
Bovine liver RNA (total)	72.8 ± 5.7
Torula yeast RNA (total)	73.6 ± 5.2
Bovine liver rRNA	111 ± 6
Bovine liver tRNA	95.7 ± 1.5
Salmon sperm DNA (total)	86.5 ± 0.7
Ribosomes	93.2 ± 2.0

Table 2 Alteration of steady-state kinetic parameters of tyrosine hydroxylase

The K_m (μM) and $V_{max.}$ (nmol/min per mg) of tyrosine hydroxylase for tyrosine and tetrahydrobiopterin (H_4 biopterin) were determined at pH 6.0 in the presence or absence of 100 μg/ml of either bovine liver RNA or salmon sperm DNA as described in the Experimental section. Tetrahydrobiopterin kinetic parameters were assessed in the presence of 100 μM tyrosine.

	Control	+RNA	+DNA
K_m (H_4 biopterin)	3130±480	295±24	171±9
$V_{max.}$ (app.) (H_4 biopterin)	857±74	411±22	332±16
K_m (Tyr)			
125 μM H_4 biopterin	17.1±2.2	20.5±2.7	19.3±1.1
1 mM H_4 biopterin	40.3±2.7	38.0±2.9	34.0±3.3
$V_{max.}$ (app.) (Tyr)			
125 μM H_4 biopterin	33.0±1.6	113±17	120±19
1 mM H_4 biopterin	174±10	183±8	147±10

Table 3 Salts attenuate RNA activation of tyrosine hydroxylase

Tyrosine hydroxylase activity was measured at pH 6.0 as described in the Experimental section.

	Activity (nmol/min per mg) (mean±S.E.M.)		
Salt (10 mM)	Control	+100 μg/ml RNA	% of control
None	23.4±0.8	104±3	442
$MgCl_2$	34.9±3.9	38.7±0.5	111
$CaCl_2$	32.2±1.0	32.7±1.0	102
$Fe(NH_4)_2(SO_4)_2$	46.0±1.5	44.5±2.7	96
KCl (10 mM)	24.5±1.3	94.4±0.8	386
KCl (100 mM)	46.4±3.7	52.1±0.6	112

Steady-state kinetic effects of nucleic acids on tyrosine hydroxylase activity

The influence of bovine liver RNA and salmon sperm DNA on the tyrosine hydroxylase K_m and $V_{max.}$ for tetrahydrobiopterin and tyrosine is summarized in Table 2. The K_m of the enzyme for tetrahydrobiopterin decreased 10-fold in the presence of RNA, and the K_m decreased 18-fold in the presence of DNA. A 50 and 60 % decrease in $V_{max.}$ was also observed in the presence of RNA and DNA respectively. Assessment of accurate values of the kinetic parameters for the untreated enzyme was limited in these experiments by the maximal concentration of tetrahydrobiopterin used (1.2 mM). Higher concentrations inhibited tyrosine hydroxylase activity and were not used. The K_m in the presence of RNA and DNA was low enough for this not to be a significant factor in the determination of these kinetic parameters. At 125 μM tetrahydrobiopterin, the K_m for tyrosine was unchanged by RNA or DNA. The polyanion-dependent increase in $V_{max.}$ with tyrosine as the varied substrate would be expected if the K_m for tetrahydrobiopterin decreased. At a higher tetrahydrobiopterin concentration (1 mM), polyanions did not significantly alter the $V_{max.}$. These results are consonant with the idea that nucleic acid activation of tyrosine hydroxylase at pH 6.0 was caused by a decrease in the K_m of the enzyme for the pterin cofactor. The K_m of the enzyme for tyrosine increased from 17 μM to 40 μM as the tetrahydrobiopterin concentration was increased from 125 μM to 1 mM (Table 2). Oka et al. (1981) also reported that the K_m for tyrosine increases at higher tetrahydrobiopterin concentrations.

Salts attenuate RNA activation of tyrosine hydroxylase

The activity of tyrosine hydroxylase is influenced by salts (Kuczenski and Mandell, 1972). This finding prompted us to explore the interrelationship of salt and RNA activation. The activation of tyrosine hydroxylase by bovine liver RNA was attenuated by 10 mM $MgCl_2$ (Table 3). Other bivalent-cation salts, such as $CaCl_2$ and $Fe(NH_4)_2(SO_4)_2$, also prevented RNA activation of the enzyme. In the presence of 10 mM $CoCl_2$, $NiCl_2$, $CuCl_2$ or $ZnCl_2$, tyrosine hydroxylase was completely inactive. Univalent-cation salts, such as KCl (10 mM), had little effect on RNA activation. At a higher concentration (100 mM), however, KCl also decreased activation. In addition to decreasing RNA activation, univalent-cation salts (100 mM) and some of the bivalent-cation salts (10 mM) significantly enhanced tyrosine hydroxylase activity. Activation of tyrosine hydroxylase and the prevention of heparin activation of the enzyme by a very high salt concentration (1 M NaCl) was previously reported (Kuczenski and Mandell, 1972). The attentuating influence of the salts may be related to inhibition of the interaction of the polyanion with tyrosine hydroxylase.

Reversal of DNA activation of tyrosine hydroxylase

Limited proteolysis activates tyrosine hydroxylase (Vigny and Henry, 1981). It was necessary to rule out the possibility that activation of tyrosine hydroxylase by RNA was due to contaminating proteinases in the RNA preparations. We therefore investigated the ability of ribonucleases to eliminate the activating effects of RNA. Bovine liver RNA (60 μg/ml) was incubated with a mixture of ribonucleases, including 30 μg/ml *Aspergillus clavatus* ribonuclease, 25 μg/ml bovine pancreatic ribonuclease A, 14 μg/ml *Aspergillus oryzae* ribonuclease T1, and 5 μg/ml mung-bean (*Phaseolus aureus*) nuclease in 5 mM Mops, pH 7.2, overnight at room temperature. Controls consisted of buffer alone, RNA in buffer, and the ribonucleases in buffer, each incubated overnight at room temperature. Tyrosine hydroxylase activity was then assessed in the presence of a 1:3 dilution of each of these solutions, with a final nominal concentration of 20 μg/ml RNA in the assay. Preincubation with ribonucleases nullified activation by RNA (Table 4). In addition, preincubation of the ribosomes with ribonucleases (with a final nominal ribosomal RNA concentration of 33 μg/ml in the assay), decreased the ability of the ribosomes to activate tyrosine hydroxylase, confirming that the RNA components, rather than the protein, of the ribosomes were responsible for the activation. By SDS/PAGE we also saw no evidence of proteolysis of the hydroxylase after incubation with 1 mg/ml bovine liver RNA for up to 1 h (results not shown), confirming that partial proteolysis was not the mechanism by which the enzyme was activated. We note furthermore that a preincubation of RNA or any other polyanion and the enzyme was not required to produce activation.

Phosphorylation of tyrosine hydroxylase by cyclic AMP-dependent protein kinase

We investigated the influence of bovine liver RNA on phosphorylated tyrosine hydroxylase. When tyrosine hydroxylase was phosphorylated by ATP and cyclic AMP-dependent protein kinase, tyrosine hydroxylase was activated (Roskoski et al., 1987), but it was not further activated by RNA (results

Table 4 Ribonucleases attenuate RNA activation of tyrosine hydroxylase

Overnight incubations of RNA and RNAases were carried out as described in the text (in duplicate), and tyrosine hydroxylase activity was determined at pH 6.0 as described in the Experimental section.

Addition	Activity (nmol/min per mg) (mean ± S.E.M.)
None	42.7 ± 2.0
+ RNAase	53.5 ± 2.5
+ 20 μg/ml bovine liver RNA	166 ± 13
+ RNA/RNAase	51.2 ± 0.2
+ 33 μg/ml RNA in ribosomes	257 ± 3
+ Ribosomes/RNAase	66.0 ± 5.1

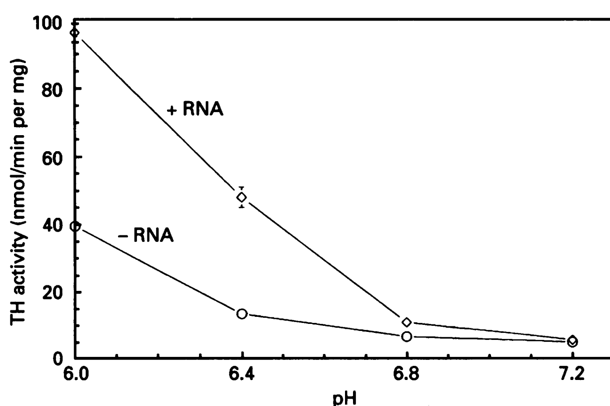

Figure 2 pH-dependence of tyrosine hydroxylase (TH) activation by nucleic acids

The activity of tyrosine hydroxylase in the presence or absence of 100 μg/ml bovine liver RNA was assessed at the specified pH. The absence of error bars indicates an S.E.M. smaller than the symbol size.

not shown). In fact, the activity of phosphorylated tyrosine hydroxylase was decreased approx. 25% by high concentrations (100–1000 μg/ml) of RNA at pH 6.0 and 125 μM tetrahydrobiopterin.

pH-dependence of polyanion activation

The degree of activation of tyrosine hydroxylase by RNA decreased markedly as the pH increased (Figure 2). In the presence of 100 μg/ml of RNA, maximal activation was observed at pH 6.0–6.4 (a 4-fold increase in activity), while less activation was observed at pH 6.8. Only a 20% increase in catalytic activity was observed at pH 7.2. Heparin activation of tyrosine hydroxylase was also more pronounced at pH 6.0 than at pH 7.2.

pH-dependence of extrinsic fluorescence properties using 1,8-ANS as a reporter group

Changes in tyrosine hydroxylase activity in response to pH and polyanions might be related to alterations in protein confor-

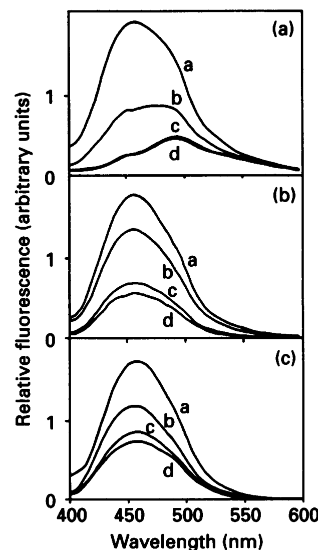

Figure 3 1,8-ANS fluorescence in the presence of tyrosine hydroxylase

(**a**) 1,8-ANS in the presence (a) or absence (c) of tyrosine hydroxylase at pH 6.0 and in the presence (b) or absence (d) of tyrosine hydroxylase at pH 7.2. (1,8-ANS control spectra have not been subtracted in this panel.) (**b**) 1,8-ANS and tyrosine hydroxylase in the absence (a) or presence (b) or 100 μg/ml DNA at pH 6.0 and in the absence (c) or presence (d) of 100 μg/ml DNA at pH 7.2. (1,8-ANS control spectra have been subtracted in this panel.) (**c**) 1,8-ANS and tyrosine hydroxylase in the absence (a) or presence (b) of 100 μg/ml heparin, at pH 6.0 and in the absence (c) or presence (d) of 100 μg/ml of heparin at pH 7.2. (1,8-ANS control spectra have been subtracted in this panel.)

mation. As a first step in examining this hypothesis, we measured the extrinsic fluorescence of the protein using 1,8-ANS as a probe. 1,8-ANS binds to the hydrophobic regions of proteins non-covalently and exhibits a characteristic fluorescence spectrum (Brand and Gohlke, 1972). If a conformational change of tyrosine hydroxylase leads to a change in the number of 1,8-ANS binding sites or an alteration of the quantum yield of the fluorescent probe, then the spectrum will reflect such conformational changes.

We examined the extrinsic fluorescence of tyrosine hydroxylase under conditions where tyrosine hydroxylase activity was altered. We first compared the spectra observed at pH 7.2 (less active) and 6.0 (more active). The fluorescence intensity was 100% greater at pH 6.0 than 7.2 (Figure 3). This fluorescence change paralleled the activity change as a function of pH (Figure 4). The emission maximum of 1,8-ANS decreased from 495 nm in buffer to 455 nm when bound to tyrosine hydroxylase at both pH values. Both the blue shift in the wavelength of the emission maximum and the fluorescence enhancement following binding to tyrosine hydroxylase represent a shift of the probe from the polar solvent to apolar regions of tyrosine hydroxylase (Brand and Gohlke, 1972).

Using the double-titration method of Horowitz and Criscimagna (1985), we determined the stoichiometry of 1,8-ANS binding. Fluorescence was measured (excitation at 380 nm, emission at 470 nm) for solutions of 30, 80 or 200 μg/ml of tyrosine hydroxylase titrated with 20–100 μM ANS, and solutions of 15, 40, 80, and 150 μM 1,8-ANS were titrated with 10–50 μg/ml tyrosine hydroxylase. We obtained maximum binding values of 212 ± 16 (pH 6.0) and 116 ± 9 (pH 7.2) molecules of 1,8-ANS ($n = 3$) per molecule of the tetrameric enzyme. The large differences in the spectra at pH 6.0 and 7.2 provide evidence for a substantial difference in conformation at the two pH

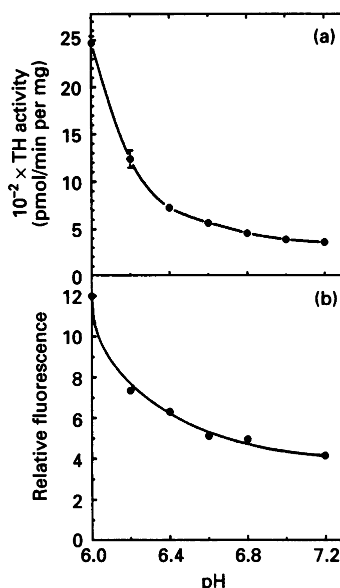

Figure 4 pH-dependence of tyrosine hydroxylase activity (a) and 1,8-ANS fluorescence in the presence of tyrosine hydroxylase (b)

Tyrosine hydroxylase activity and relative fluorescence were determined as described in the Experimental section at the designated pH.

values. There was no observable change in the K_d for 1,8-ANS (60 ± 20 μM). Since 1,8-ANS bound at many different sites on tyrosine hydroxylase, this value represents an average K_d of the binding sites. As noted in the Experimental section we used 40 μM 1,8-ANS in experiments other than these titrations. This concentration is near the K_d, so the enzyme was approximately half-saturated with 1,8-ANS.

The conformational changes produced by the shift in pH were determined to be reversible by the following experiment. To a solution of 0.25 mg/ml tyrosine hydroxylase in 0.1 M MAT buffer, 1.0 MAT buffer (pH 8.0) was added to change the pH to 7.2. Then 1.0 M MAT buffer (pH 5.0) was added to reduce the pH to 6.0. Identical samples were kept at pH 6.0 or were changed to 7.2. (All samples had a final MAT concentration of 0.38 M.) Increasing the pH to 7.2 decreased the fluorescence, and then decreasing the pH to 6.0 increased the fluorescence to the value (within 10%) of the sample kept at pH 6.0. These manipulations demonstrated the reversibility of the pH-induced conformational changes. Activity changes were also reversible. Fluorescence changes were also reversible when the pH of the solution of tyrosine hydroxylase at pH 7.2 was decreased to pH 6.0 and increased to pH 7.2.

Polyanion-induced changes in extrinsic fluorescence

DNA and heparin were more potent activators of tyrosine hydroxylase at pH 6.0 than at pH 7.2. DNA (100 μg/ml) decreased fluorescence by 30% at pH 6.0, while leaving the wavelength of maximum emission unchanged (Figure 3b). By contrast, DNA decreased fluorescence much less at pH 7.2. The greater change in extrinsic fluorescence at pH 6.0 than at 7.2 paralleled the greater degree of activation of tyrosine hydroxylase at the lower pH. The results with heparin, another polyanion, were similar. Heparin decreased the extrinsic fluorescence more at pH 6.0 than at 7.2 (Figure 3c). Note, however, that at either pH the more active enzyme was associated with decreased fluorescence. This differs from the fluorescence properties of tyrosine hydroxylase associated with pH, where more active enzyme exhibited greater extrinsic fluorescence. In control experiments without enzyme, neither DNA nor heparin altered 1,8-ANS fluorescence, so the fluorescence changes observed may be attributed to the polyanions interacting with the enzyme rather than with the 1,8-ANS directly.

DISCUSSION

Kuczenski and Mandell (1972) first demonstrated that heparin, a polyanion, increases tyrosine hydroxylase activity by decreasing the K_m of the enzyme for its pterin cofactor while not affecting the K_m of the enzyme for tyrosine. We found that the activation of tyrosine hydroxylase by nucleic acids was similar to that produced by heparin, with the nucleic acids also activating the enzyme by decreasing the K_m of the enzyme for tetrahydrobiopterin.

Our results on the effects of nucleic acids on tyrosine hydroxylase activity differed in several respects from those reported by Nelson and Kaufman (1987). They found that, at pH 6.0, purified and homogeneous rat brain and bovine adrenal-medulla tyrosine hydroxylase are inhibited by 10 μg/ml and higher concentrations of torula-yeast RNA. This is in sharp contrast with our results, where we observed activation of rat tyrosine hydroxylase purified from PC12 cells or a transplantable pheocheomocytoma. Differences in the source of enzyme, the purification procedure, or the phosphorylation state of the enzyme, may explain these discrepancies. Nelson and Kaufman (1987) reported that their purified enzyme exhibited an A_{280}/A_{260} absorbance ratio of 0.82; they suggested that their purified tyrosine hydroxylase contains about 6% (w/w) bound nucleotide. In contrast, we observed an A_{280}/A_{260} ratio of 1.63 ± 0.07 (for three preparations); our enzyme was associated with negligible amounts of bound nucleic acid. This may explain the differences in the effects of RNA on tyrosine hydroxylase activity in these two studies.

Investigators must be aware that activation by nucleic acids in vitro may produce adventitious effects in the experimental situation. After cell disruption to measure tyrosine hydroxylase activity, RNA or DNA have the potential to influence tyrosine hydroxylase, owing to cell-barrier destruction. In brain, for example, where protein represents about 8% of the total mass (McIlwain and Bachelard, 1985), the ratio of RNA to protein is 14 μg/mg of protein. Typical tyrosine hydroxylase activity determinations are performed at 1–2 mg/ml protein, and the accompanying RNA is in the range where it influences activity (the EC_{50} of RNA is 3 μg/ml at pH 6.0). On the basis of our data, RNA would activate tyrosine hydroxylase by decreasing the K_m for tetrahydrobiopterin. This is the same effect seen in the activation of tyrosine hydroxylase by cyclic AMP-dependent protein kinase phosphorylation (Vulliet et al., 1980; Roskoski et al., 1987). RNA might therefore activate basal tyrosine hydroxylase activity and mask the activating effects of protein kinases. On the basis of our data, such an effect would be maximal at pH 6.0. This is the pH of optimal enzyme activity (Nagatsu et al., 1964) and the pH at which activity studies are often performed (Kuczenski and Mandell, 1972; Nelson and Kaufman 1987). The effects of RNA were greatly decreased at pH 7.2, and activity measurements at this pH should minimize RNA effects.

The fluorescence of 1,8-ANS has enabled us for the first time to address the nature of conformational changes of the rate-limiting and chief regulatory enzyme of catecholamine biosynthesis. This fluorescent probe, which has an affinity for hydrophobic regions of proteins (Brand and Gohlke, 1972),

bound to many sites on the tetrameric tryosine hydroxylase. Decreasing the pH from 7.2 to 6.0 led to an increase in activity and an increase in fluorescence. This reversible fluorescence increase could be due to a change in the microenvironment of the reporter group or to a change in the number of bound fluorophores. Our titration studies indicated that the maximum number of 1,8-ANS molecules bound to tetrameric tyrosine hydroxylase increased from 116 (pH 7.2) to 212 (pH 6.0). Saucier et al. (1985) found that ciliary dynein binds 37 molecules of 1,8-ANS per monomer. This is similar to our result and contrasts with the binding of one molecule of 1,8-ANS to the active site of apomyoglobin (Stryer, 1965). 1,8-ANS appears to interact with tyrosine hydroxylase at many different regions of the protein. The fluorescence of 1,8-ANS increases in proportion to the hydrophobicity of its environment (Stryer, 1965). The fluorescence increase that we observed when 1,8-ANS bound to tyrosine hydroxylase is much less than the increase observed when 1,8-ANS binds to the single myoglobin site reported by Stryer (1965) (4-fold versus 200-fold). In addition, the 1,8-ANS bound with lower affinity to tyrosine hydroxylase than to myoglobin (K_d of 60 μM versus 3.4 μM). This suggests that 1,8-ANS is binding to many sites of tyrosine hydroxylase that are only weakly hydrophobic, yet more hydrophobic than the solvent.

There is evidence that changes in ionization of specific residues of tyrosine hydroxylase are necessary for substrate and inhibitor binding to tyrosine hydroxylase, thereby explaining the activity changes observed with pH changes (Fitzpatrick, 1988; Haavik et al., 1990). Our results suggest that reversible conformational changes in the protein may also contribute to the pH-dependence of tyrosine hydroxylase activity. Significant changes in tyrosine hydroxylase structure, detected by extrinsic fluorescence, occurred when pH was altered. The data suggest that, at pH 6.0, tyrosine hydroxylase displays more hydrophobic binding sites than at pH 7.2. These changes in fluorescence appear to reflect changes in the overall conformation of tyrosine hydroxylase rather than changes only at a specific site, such as a substrate or effector binding site. We previously reported that tyrosine hydroxylase, although more active at pH 6.0, is less stable at pH 6.0 than pH 7.2 (Gahn and Roskoski, 1991). Perhaps the decreased stability at pH 6.0 is related to the conformation with more exposed hydrophobic regions.

The effect of polyanions (DNA, heparin) on the fluorescence of 1,8-ANS differed from the effect of pH. When pH was altered, the more active enzyme had a greater influence, while activation of polyanions was associated with a decrease in fluorescence. Polyanions had less effect on activity at pH 7.2 than at pH 6.0. They also had less effect on the extrinsic fluorescence at pH 7.2 than at pH 6.0. Since 1,8-ANS is negatively charged at the pH values employed here, it is possible that some of the 1,8-ANS molecules are binding at the same sites to which the negatively charged DNA or heparin molecules bind. If so, then the decrease in fluorescence observed when DNA or heparin interacts with tyrosine hydroxylase may be due to displacement of some of the 1,8-ANS. It should be noted, however, that in contrast with polyanions interacting with tyrosine hydroxylase, the fluorophore has no effect of tyrosine hydroxylase activity. We would like to know the three-dimensional structure of tyrosine hydroxylase, including how it changes in response to various regulatory agents. Our initial attempts to crystallize the enzyme, however, have been unsuccessful.

In contrast with heparin, RNA occurs within neuronal and endocrine cells that contain tyrosine hydroxylase, and RNA has the potential to regulate tyrosine hydroxylase activity (Nelson and Kaufman, 1987). That the bulk of DNA and RNA is segregated from the enzyme in nerve terminals minimizes the possibility of a regulatory role for these polyanions. Moreover, nucleic acids were less effective stimulatory agents at physiological pH (7.2) than at pH 6.0. This is the reverse of the situation found for cyclic AMP-dependent phosphorylation (Markey et al., 1980; Richtand et al., 1985) and emphasizes the importance of phosphorylation in the physiological regulation of tyrosine hydroxylase. Furthermore, tyrosine hydroxylase was less sensitive to the stimulatory effects of polyanions at physiological salt concentrations. In the presence of 100 mM KCl and 50 mM Pipes, pH 7.2, tyrosine hydroxylase activity was increased only 15% by 100 μg/ml bovine liver RNA. Polyanions alter tyrosine hydroxylase activity and provide a means for studying the regulation of the enzyme *in vitro*; regulation by polyanions, however, is probably not important physiologically.

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