Effects of Phosphorylation of Serine 40 of Tyrosine Hydroxylase on Binding of Catecholamines: Evidence for a Novel Regulatory Mechanism†

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ABSTRACT: The effects of phosphorylation at Ser40 of rat tyrosine hydroxylase on the affinities of catechols have been determined with both the ferric and ferrous forms of the enzyme. Phosphorylation had no effect on the K_i value for the inhibition of the ferrous enzyme by either dopamine or DOPA when the initial rate of turnover was measured in assays. However, phosphorylation of the ferric enzyme resulted in a 17-fold decrease in affinity for DOPA and a 300-fold decrease in the affinity for dopamine, while the affinity for dihydroxynaphthalene was unchanged. The changes in binding affinity for the two catecholamines were almost exclusively due to large increases in the dissociation rate constants upon phosphorylation. These results support a novel mechanism for regulation in which phosphorylation affects binding of catecholamines to the catalytically inactive ferric form of the tyrosine hydroxylase.

Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters, the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA).[1] Because of its central role in a critical physiological pathway, the enzyme is tightly regulated. The primary means of short-term regulation at the posttranslational level appears to be reversible phosphorylation of serine residues (*1*). Four phosphorylated serine residues have been identified in neuronal cells grown in the presence of [^{32}P]-phosphate, at positions 8, 19, 31, and 40 (*2*). Of these, all but Ser8 are increasingly phosphorylated under conditions that result in increased formation of catecholamines. Only in the case of Ser40 is there clear evidence for significant (greater than 2-fold) activation of tyrosine hydroxylase by phosphorylation. cAMP-Dependent protein kinase appears to be responsible for phosphorylation of Ser40 (*3*). The amount of activation reported has varied greatly over the years, depending upon the purity of the enzyme preparation among other factors (*4*). By using recombinant enzyme, Daubner et al. (*5*) were able to show that a large effect is only seen if the enzyme is in the form of a complex with a catecholamine such as dopamine. In that case, phosphorylation resulted in an increase of 10−20-fold in activity, identical to that seen with nonrecombinant enzyme purified from rat pheochromocytoma cells. Such an observation was consistent with the fact that the enzyme is isolated from nonrecombinant sources as a catecholamine complex (*6*, *7*). These results suggested that the primary effect of phosphorylation at Ser40 was to decrease the enzyme's affinity for catecholamines. However, there have been several reports of the effects of phosphorylation by protein kinase A on the inhibition of recombinant rat and human tyrosine hydroxylase by catecholamines. In general, these have shown very small changes in affinity, typically increases of only 2−3-fold in K_i values (*8*). Changes of this magnitude are not consistent with the much larger increases seen with nonrecombinant enzyme and the large increase in activity of the recombinant enzyme−catecholamine complex.

Tyrosine hydroxylase contains a single iron atom per subunit, which must be in the ferrous form for catalytic activity (*9*). We have recently shown that the iron is readily oxidized to the ferric form during catalytic turnover; the ferric iron can be rereduced by a tetrahydropterin (*10*). Alternatively, the oxidized enzyme can be trapped by the product dihydroxyphenylalanine. Scheme 1 shows the model that was proposed as a result of these studies. This model explicitly proposes that dihydroxyphenylalanine, and by implication other catecholamines, acts by binding to the oxidized form of tyrosine hydroxylase. Thus, this model predicts that phosphorylation would affect the binding of catecholamines to the ferric rather than the ferrous enzyme. In this report we describe experiments to test this hypothesis by measuring the effects of phosphorylation on the affinities of the ferric and ferrous forms of tyrosine hydroxylase. The results support the model of Scheme 1 and resolve the quandary posed by reports of small effects of phosphorylation on the affinity of the enzyme for catecholamines.

EXPERIMENTAL PROCEDURES

[3,5-$^3H_2$]Tyrosine was purchased from Amersham Corp. and purified before use by the method of Ikeda et al. (*11*). 6-Methyltetrahydropterin was synthesized as previously described (*12*, *13*); stock solutions in 5 mM HCl were prepared on the day of use. 2,3-Dihydroxynaphthalene,

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[1] Abbreviations: DOPA, dihydroxyphenylalanine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); DHN, 2,3-dihydroxynaphthalene; DTPA, diethylenetriaminepentaacetic acid; ERK, extracellular signal-regulated protein kinase.

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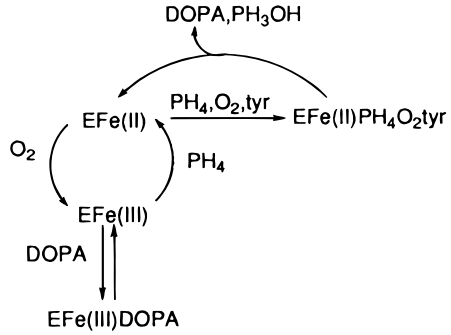
Scheme 1



dihydroxyphenylalanine, and dopamine were purchased from Sigma Chemical Co. Ultrafree-MC filter units were obtained from Millipore Corp. [ring-2,5,6-$^3H_3$]DOPA (20−40 Ci/mmol) was from Andotek Line Sciences Co, and [*ring*-5,6-$^3H_2$]dopamine (30−60 Ci/mmole) was from American Radiolabeled Chemicals, Inc. cAMP-Dependent protein kinase was purified from bovine heart by the method of Flockhart and Corbin (*14*). Rat tyrosine hydroxylase was expressed in *Escherichia coli* and purified as described previously (*5*, *10*). All enzyme concentrations are given in terms of enzyme-bound iron. The iron content of the purified enzyme was determined by using a Perkin-Elmer Model 2380 atomic absorption spectrophotometer equipped with a graphite furnace as described previously (*10*). UV−Visible spectra were obtained by using a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence experiments were performed on a SLM Instruments SLM 8000 fluorometer at 10 °C with 1 cm path length cuvettes.

To phosphorylate tyrosine hydroxylase, approximately 200 mL of 5 μM enzyme in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, was incubated at 4 °C with 43 μM ATP, 6 mM $MgCl_2$, and the catalytic subunit of cAMP protein kinase at a final concentration of about 1 μg/mL. After 2 $^1/_2$ h, additional ATP, $MgCl_2$, and protein kinase were added to give final concentrations of 60 μM, 8.5 mM, and 1.4 μg/mL, respectively. The degree of phosphorylation was monitored by loading an aliquot of the reaction mixture onto a MonoQ column eluted with a gradient from 180 to 500 KCl in 50 mM Hepes and 5% glycerol, pH 7.1. The unphosphorylated enzyme eluted at 11.7 mL and the phosphorylated enzyme at 14.5 mL. When the reaction was complete, the reaction mixture was loaded onto a Q Sepharose column (2.5 × 16 cm) equilibrated with 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1. The column was washed with 100 mL of this buffer, and the phosphorylated tyrosine hydroxylase was eluted with a gradient of 180−500 mM KCl in the same buffer. The peak fractions were concentrated by the addition of ammonium sulfate to 50% saturation, and the pellet was dissolved in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1. Ferric sulfate was added to give an Fe/subunit ratio of 1.2:1. The enzyme was then dialyzed against 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, overnight with several changes.

Initial rates of 6-methyltetrahydropterin oxidation were determined by a coupled assay with dihydropterin reductase as previously described (*15*). The conditions were 50−600 μM 6-methyltetrahydropterin, 200 μM phenylalanine, 90 μg/mL catalase, 200 μM NADH, ∼0.5 unit/mL dihydropteridine reductase, 50 mM Hepes, 80 mM KCl, and 8% glycerol pH 7.1, at 25 °C. Inhibition data were fit to eq 1 for competitive inhibition using the KinetAsyst software (IntelliKinetics, State College, PA).

$$v = \frac{V_{max}S}{K_m\left(1 + \frac{I}{K_{is}}\right) + S} \quad (1)$$

The kinetics of association of dihydroxynaphthalene with tyrosine hydroxylase were determined by adding excess enzyme (0.2−1.6 μM in iron) to a sample of 50 nM dihydroxynaphthalene in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, at 10 °C. The quenching of the fluorescence of DHN upon binding was monitored with an excitation wavelength of 324 nm and an emission wavelength of 342 nm. The rate of the change in fluorescence was determined by fitting the fluorescence as a function of time to eq 2. Here, S_0 is the fluorescence emission intensity or absorbance at zero time, S_t is the observed intensity or absorbance at the indicated time, and S_∞ is the fluorescence or absorbance when the reaction is complete. The data were corrected for any photobleaching of the fluorophore using the rate of bleaching determined in the absence of enzyme.

$$S_t = S_\infty + (S_0 - S_\infty)e^{-kt} \quad (2)$$

To determine the rate of dissociation of DHN from the DHN/enzyme complex, 50−100 nM dihydroxynaphthalene was incubated with a 2-fold excess of enzyme in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, at 10 °C, for 30 min in the dark to enable the complex to form. Dopamine was then added to a final concentration of 2.5−5 μM for the unphosphorylated enzyme and 30−100 μM for the phosphorylated enzyme, and the increase in fluorescence with time due to free DHN was monitored. The fluorometer shutters were closed between readings to prevent photobleaching of the DHN. The rate constants were derived by fitting the data to eq 2.

The rate constants for binding of DOPA or dopamine to tyrosine hydroxylase were determined by mixing approximately 15 μM (final concentration) tyrosine hydroxylase with 125−2000 μM catecholamine in an Applied Photophysics SX-18MV stopped-flow spectrophotometer. The formation of the enzyme−catecholamine complex was monitored by following the increase in absorbance at 690 nm with time. The experiments were performed at 4.5 °C in 50 mM Hepes, 100 mM KCl, 0.2 mM DTPA, and 10% glycerol, pH 7.1. The absorbance data were fit to eq 2.

To determine the rate of dissociation of DOPA or dopamine from the enzyme−catecholamine complex, approximately 20 μM tyrosine hydroxylase was incubated with a 50% stoichiometric excess of catecholamine for 15 min to form the complex. An exception was made in the study of the phosphorylated enzyme−DOPA complex, where 110 μM DOPA was added to 25 μM enzyme. DHN was then added to give a final concentration of 1 mM, and the increase in absorbance at 550 nm was monitored. The rate constants were obtained by fitting the experimental data to eq 2. The experiments were performed at 4.5 °C in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1.

Alternatively, dissociation rate constants were measured by a radiochemical technique. Tritium-labeled DOPA or dopamine (0.2−1.1 μM) was incubated with a 10% stoichiometric excess of enzyme in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, at 4.5 °C for 2.5 h. Next, 50−100 μM of unlabeled dopamine was added to the reaction mixture. Aliquots (0.1 mL) of the reaction mixture were drawn from the sample at intervals and placed in a 30 000 Da cutoff Ultrafree-MC centrifuge filter unit. The filter unit was then centrifuged at 4.5 °C for 3 min. The unit was washed twice with 0.1 mL of 50 mM Hepes and 100 mM KCl, pH 7.1, and the filter was centrifuged for 3 min after each addition of buffer. The enzyme was then removed from the filter unit with two 0.1 mL washes of 1% SDS and the radioactivity of the enzyme−SDS sample measured. The dissociation rate constants were determined by fitting the data to eq 2.

RESULTS

Inhibition of Reduced Tyrosine Hydroxylase by Catecholamines. Catecholamines are usually reported to be competitive inhibitors versus the tetrahydropterin, the first substrate to bind in the tyrosine hydroxylase kinetic mechanism (*16*, *17*). Since the active form of tyrosine hydroxylase has the iron in the ferrous state (*9*), measuring the K_i value of a catecholamine as a competitive inhibitor versus a tetrahydropterin as the variable substrate would be expected to give the K_d value for binding of the catecholamine to the ferrous form of the enzyme. However, measurements of the affinity of tyrosine hydroxylase for catecholamines have typically been done by determining the K_i values by using end-point assays. We have recently shown that the ferrous iron in tyrosine hydroxylase is readily oxidized by atmospheric concentrations of oxygen to the inactive ferric form, as shown in Scheme 1. The iron can be rereduced by a tetrahydropterin, so that it is maintained in an active form during turnover. However, in the presence of a catechol, the ferric enzyme can be trapped in an inactive catechol complex (*10*). Consequently, the enzyme rapidly loses activity in the presence of catechols; this has the potential to result in artifactually low rates when end-point methods are used to determine activity.

To circumvent the problems of the rapid loss of activity in the presence of added catecholamines, the initial rate of 6-methyltetrahydropterin consumption was measured by using a coupled assay with dihydropterin reductase. Phenylalanine was used as the amino acid substrate to minimize production of DOPA. In addition, assays were allowed to reach a steady state in the absence of inhibitor, to ensure that all of the iron was reduced, before the inhibitor was added. Figure 1 illustrates the effect of dopamine on the rate of catalysis of tyrosine hydroxylase when the activity is measured by such an assay. The rate in the absence of catecholamines is linear for several minutes. In contrast, in the presence of dopamine the initial rate is constant for only 10−20 s before it decreases significantly. Clearly, end-point methods that measure the total amount of product formed after several minutes will significantly underestimate the initial rate. By use of this continuous assay, the K_i values for dopamine and DOPA versus 6-methyltetrahydropterin were determined both with unphosphorylated enzyme and with enzyme which had been phosphorylated at Ser40 by

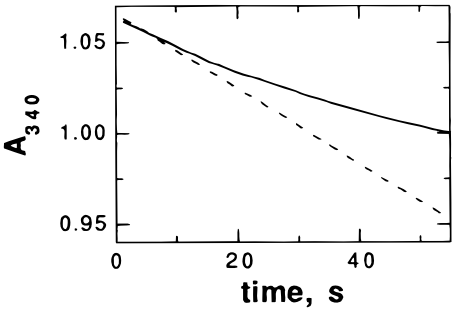


FIGURE 1: Effect of 4 μM dopamine on catalytic turnover by tyrosine hydroxylase. Unphosphorylated enzyme (final concentration 144 nM) was incubated for 30 s with 133 μM 6-methyltetrahydropterin, 200 μM phenylalanine, 90 μg/mL catalase, 190 μM NADH, 0.5 unit/mL dihydropteridine reductase, 40 mM Hepes, 80 mM KCl, and 8% glycerol, pH 7.1, at 25 °C. At time zero, water (- - -) or dopamine (—) was added.

Table 1: Effect of Phosphorylation on Catecholamine Inhibition of Tyrosine Hydroxylase Activity[a]

	K_i (μM)	
catecholamine	unphosphorylated enzyme	phosphorylated enzyme
dihydroxyphenylalanine	60 ± 18	37 ± 7.5
dopamine	33 ± 10	44 ± 20

[a] K_i values were determined in initial rate assays with 50−600 μM 6-methyltetrahydropterin, 200 μM phenylalanine, 90 μg/mL catalase, 50 mM HEPES, 80 mM KCl, and 8% glycerol, pH 7.1, 25 °C.

cAMP-dependent protein kinase. The K_i values obtained by such an approach are significantly higher than those previously reported from end-point assays over several minutes (Table 1). More importantly, there are no significant changes in the K_i values upon phosphorylation, although decreases in affinity of less than 2-fold cannot be ruled out due to the low precision resulting from the very rapid loss of activity during these assays.[2] These results strongly suggest that phosphorylation of tyrosine hydroxylase has little effect on the affinity of the active ferrous enzyme for catecholamines.

Binding of Dihydroxynaphthalene to Oxidized Tyrosine Hydroxylase. Tyrosine hydroxylase when purified contains all of its iron in the ferric form (*10*, *18*). The catechol 2,3-dihydroxynaphthalene binds to this form of the enzyme with almost complete quenching of the fluorescence of the ligand (*10*). The affinity of the ferric form of the enzyme for DHN was determined by taking advantage of this quenching. The pseudo-first-order rate constant for quenching of the DHN fluorescence by an excess of ferric enzyme was determined over a range of concentrations of tyrosine hydroxylase. Results obtained with the unphosphorylated enzyme are shown in Figure 2A. The changes in fluorescence fit well to a single exponential, consistent with a pseudo-first-order reaction. A replot of the rate constants obtained at different concentrations of enzyme gave a straight line, consistent with a one-step binding reaction (Figure 2B). An association rate

[2] While the data were analyzed assuming competitive inhibition, the rapid inactivation in the presence of catecholamines restricted the concentrations of the inhibitors that could be used. Consequently, it was not possible to use inhibitor concentrations significantly above the K_i values in Table 1, a requirement for conclusively determining the type of inhibition. Still, the observation that there was no large effect of phosphorylation on the inhibition of the reduced enzyme by dopamine or DOPA was not affected by the type of inhibition pattern assumed.

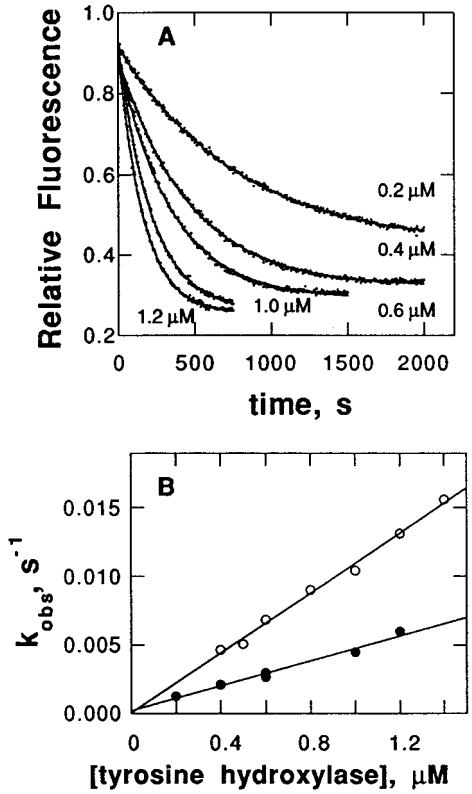


FIGURE 2: Fluorescence changes upon binding of DHN to unphosphorylated tyrosine hydroxylase. (A) Fluorescence of DHN was monitored at 10 °C after the addition of 0.2−1.2 μM tyrosine hydroxylase (based on the concentration of enzyme-bound iron) to 50 nM DHN in 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1. The lines are fits of the experimental data to eq 2. (B) Dependence of the rate of fluorescence quenching on the concentration of unphosphorylated (●) or phosphorylated (○) tyrosine hydroxylase.

constant of 4.6 (± 0.3) mM^{-1} s^{-1} could be calculated from the slope of the line.

The rate of dissociation of DHN was too slow to be measured accurately by such a method, since it is obtained from the *y*-intercept of plots such as that in Figure 2B (Table 2). Consequently, the dissociation rate constant was determined directly. The DHN−enzyme complex was formed by mixing enzyme with a substoichiometric amount of DHN to ensure that all of the catechol was bound to the enzyme. A large excess of dopamine was then added, so that any free enzyme that formed would be trapped as the nonfluorescent enzyme−dopamine complex. As a result, the release of DHN from the enzyme could be followed by monitoring the increase of fluorescence due to free DHN (Figure 3). The increase in fluorescence showed simple first-order kinetics, with a rate of 6.3 (± 0.6) × 10^{-5} s^{-1}; this rate was unaffected by doubling the dopamine concentration. This value could be used with the association rate constant determined previously to calculate the value of the equilibrium dissociation constant for DHN binding to unphosphorylated tyrosine hydroxylase as 13.5 ± 1.3 nM.

This analysis was repeated with phosphorylated enzyme; addition of an excess of the phosphorylated enzyme to a solution of DHN again resulted in a monophasic quenching of the fluorescence. The effect of the enzyme concentration on the rate of quenching is shown in Figure 2B. From the data an association rate constant of 10.9 (± 0.3) × mM^{-1} s^{-1} was determined. The increase in fluorescence as DHN dissociated from the phosphorylated enzyme in the presence of a large excess of dopamine gave a value for the dissociation rate constant of 1.92 (± 0.01) × 10^{-4} s^{-1}; this value was unaffected by a 3-fold increase in the dopamine concentration. A value of 17.5 ± 0.5 nM for the K_d value of the phosphorylated enzyme for DHN could be calculated from these rate constants.

Table 2: Effects of Phosphorylation on Kinetics of Binding of Catechols to Ferric Tyrosine Hydroxylase

	k_{on} (mM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
		Unphosphorylated Enzyme	
DHN[a]	4.6 ± 0.3	6.3 (± 0.6) × 10^{-5} [b] 2.8 (± 2.3) × 10^{-4} [d]	0.0135 ± 0.0013 [c]
dopamine[e]	0.803 ± 0.011	5.7 (± 1.1) × 10^{-7} [b] 6.9 (± 2.1) × 10^{-7} [f]	7.1 (±1.3) × 10^{-4}
DOPA[e]	0.336 ± 0.006	3.63 (± 0.09) × 10^{-4} [b] 2.06 (± 0.21) × 10^{-4} [f] 6.5 (± 6.3) × 10^{-3} [d]	1.08 ± 0.03
		Phosphorylated Enzyme	
DHN	10.9 (± 0.3)	1.92 (± 0.01) × 10^{-4} [b] 0.27 (± 3.1) × 10^{-4} [d]	0.0175 ± 0.0005
dopamine	1.36 ± 0.03	3.0 (± 0.11) × 10^{-4} [b] 1.76 (± 0.11) × 10^{-4} [f]	0.22 ± 0.01
DOPA	0.594 ± 0.020	1.1 (± 0.003) × 10^{-2} [b] 5.0 (± 2.3) × 10^{-2} [d]	18.4 ± 0.8

[a] Conditions: 50 mM Hepes, 100 mM KCl, 10% glycerol, and 0.2 mM DTPA, pH 7.1, 10 °C. [b] Determined from the fluorescence (DHN) or absorbance (dopamine and DOPA) changes upon displacement by excess dopamine (DHN) or DHN (dopamine or DOPA). [c] Calculated from the k_{on} and k_{off} rates. [d] Determined from kinetics of formation of the catechol−enzyme complex. [e] Conditions: 50 mM Hepes, 100 mM KCl, 10% glycerol, and 0.2 mM DTPA, pH 7.1, 4.5 °C. [f] Determined from the rate of dissociation of the radiolabeled inhibitor.

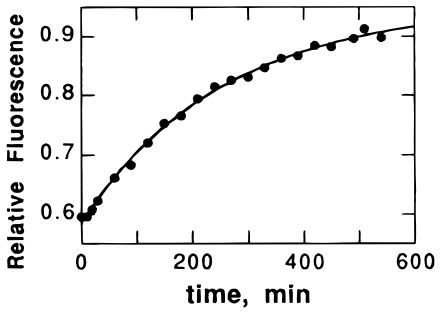


FIGURE 3: Dissociation of DHN from tyrosine hydroxylase. Tyrosine hydroxylase (100 nM enzyme bound iron) was mixed with 50 nM DHN and left in the dark for 30 min. Dopamine (final concentration 2.5 μM) was then added, and the fluorescence of the sample was determined at the indicated times. Conditions: 10 °C, 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, using an excitation wavelength of 324 nm and an emission wavelength of 342 nm.

Phosphorylation of the enzyme resulted in a bathochromic shift in the absorbance spectrum of the enzyme−DHN complex. The absorbance maximum of the enzyme−DHN complex increased from 550 to 580 nm upon enzyme phosphorylation (results not shown). This spectral change is probably due to an alteration in the environment of the DHN binding site upon phosphorylation.

Binding of DOPA and Dopamine to Reduced Tyrosine Hydroxylase. Binding of catechols to tyrosine hydroxylase is characterized by a broad absorbance band around 700 nm due to a charge transfer interaction between the catechol and the ferric iron in the enzyme active site (*6*). Representative

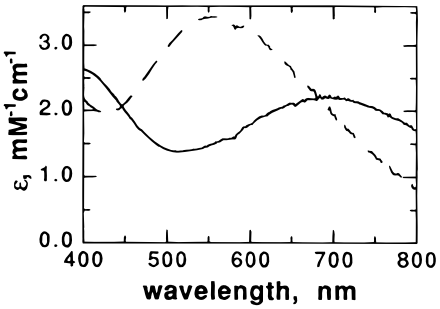


FIGURE 4: Visible absorbance spectra of enzyme−dopamine and enzyme−DHN complexes. A 3-fold excess of dopamine (——) or DHN (– – –) was added to 38 μM unphosphorylated tyrosine hydroxylase. The samples were incubated for 5 min before the absorbance spectra were determined. The conditions were 50 mM Hepes, 100 mM KCl, and 10% glycerol, pH 7.1, at 25 °C.

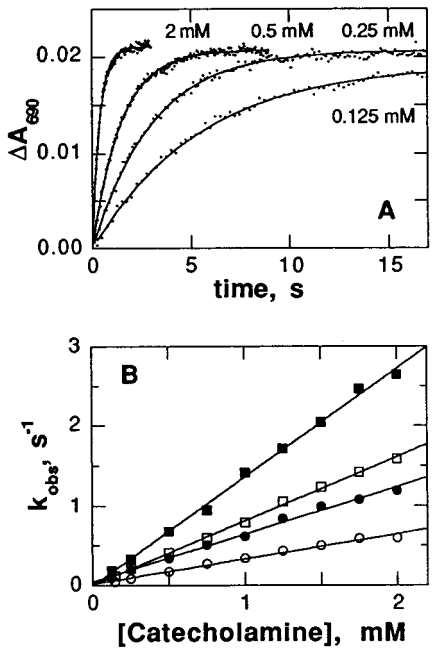


FIGURE 5: Concentration dependence for the reaction of tyrosine hydroxylase with catecholamines. (A) Phosphorylated tyrosine hydroxylase (17 μM final concentration) was mixed with the indicated final concentrations of dopamine. The formation of the tyrosine hydroxylase−dopamine complex was monitored at 690 nm in an Applied Photophysics SX-18 MV stopped-flow spectrophotometer. The lines are from fits of the data to eq 2. (B) Replot of the observed rates as a function of the concentration of dopamine (squares) or DOPA (circles) for unphosphorylated (open symbols) or phosphorylated (filled symbols) tyrosine hydroxylase. Conditions: 4.5 °C in 50 mM Hepes, 100 mM KCl, 0.2 mM DTPA, and 10% glycerol, pH 7.1.

visible absorbance spectra of enzyme with DHN or dopamine bound are shown in Figure 4. (The DOPA complex has a very similar spectrum to that of the dopamine complex.) The rate of appearance of this long-wavelength absorbance was used to monitor the binding of dopamine and DOPA to the ferric form of tyrosine hydroxylase. When the enzyme was mixed with either catecholamine, there was a first-order increase in absorbance at 690 nm, as is shown for dopamine in Figure 5A. The observed rate constant for formation of the enzyme−catechol complex varied directly with the concentration of the catecholamine (Figure 5B). The values for the second-order rate constants for the association of the enzyme with each catecholamine could be determined from the effect of catecholamine concentration on the observed

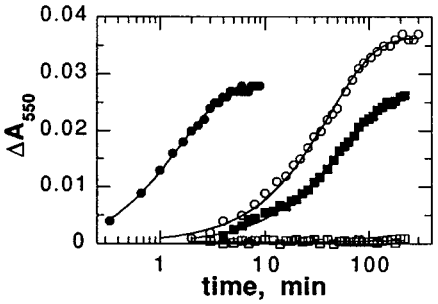


FIGURE 6: Effects of phosphorylation on rates of dissociation of catecholamines from tyrosine hydroxylase. Unphosphorylated (open symbols) or phosphorylated (filled symbols) tyrosine hydroxylase was incubated with excess dopamine (squares) or DOPA (circles) for 15 min. DHN was then added to give a final concentration of 1 mM, and the increase in absorbance at 550 nm was monitored. The lines are from fits of the data to eq 2. Conditions: 4.5 °C in 50 mM Hepes, 100 mM KCl, 0.2 mM DTPA, and 10% glycerol, pH 7.1.

rate constants. The association rate constants for the two catecholamines differed by less than 3-fold (Table 2). The analyses were repeated with enzyme that had been phosphorylated at Ser40 by cAMP-dependent protein kinase. Phosphorylation of the enzyme increased the association rate constants for both catecholamines by approximately two-thirds (Figure 5B and Table 2).

The dissociation rate constants for dopamine determined from these analyses were indistinguishable from zero (results not shown), while the precision was low for the values with DOPA (Table 2). Consequently, these values were measured independently. The absorbance spectrum of the DHN−tyrosine hydroxylase complex differs from those of the enzyme with DOPA or dopamine in that it has an absorbance maximum at 550 nm instead of 690 nm (Figure 4). Moreover, DHN binds tightly to both the phosphorylated and unphosphorylated forms of enzyme with dissociation constants of approximately 15 nM. This allowed DHN to be used as a displacing ligand in the direct determination of dissociation rate constants. The enzyme−DOPA or enzyme−dopamine complex was first allowed to form by incubating 20−25 μM enzyme with a slight excess of catecholamine. A large excess of DHN (1 mM) was then added. This resulted in a monophasic decrease in the absorbance beyond 690 nm and a simultaneous increase at 550−580 nm. Because of the large excess of DHN present, the rates of this spectral change should be limited by the rate of dissociation of the catecholamine. The data obtained for the phosphorylated and unphosphorylated enzyme with both catecholamines are shown in Figure 6 using a logarithmic time scale to accommodate the wide range in the dissociation rates. The respective rate constants determined from these date are summarized in Table 2.

The unphosphorylated enzyme−dopamine complex was so stable that an accurate value of its rate constant could not be determined by this method. A second technique was used to obtain this rate and to confirm the values obtained using the DHN displacement method. The enzyme−catecholamine complexes were formed with 1 μM enzyme and a substoichiometric amount of [^{3}H]DOPA or [^{3}H]dopamine. Once the complex had formed, 100 μM unlabeled dopamine was added. Aliquots were removed over time and the unbound catecholamine removed by filtration. The amount

of label still bound to the enzyme was then determined by scintillation counting. In the case of the unphosphorylated enzyme, only 20% of the labeled dopamine had dissociated after 80 h (results not shown). The dissociation of the phosphorylated enzyme−DOPA species proved to be too rapid to be measured accurately by the radiochemical technique. Still, the values for the dissociation rate constants determined by this approach agreed well with those determined spectrophotometrically (Table 2).

The values of the association and dissociation rate constants were used to calculate the effects of phosphorylation on the K_d values for binding of DOPA and dopamine to tyrosine hydroxylase. The large changes in the dissociation rate constants seen upon phosphorylation resulted in comparable increases in the K_d values (Table 2).

DISCUSSION

As the rate-controlling enzyme in the biosynthesis of the catecholamine neurotransmitters, the activity of tyrosine hydroxylase is heavily regulated. The principal means of posttranslational regulation appears to be reversible phosphorylation of serine residues (*1*). Depending upon the conditions, the enzyme can be phosphorylated in vivo at serines 8, 19, 31, and 40 (*2*, *19*). At least in the case of the last three sites, this results in increased biosynthesis of catecholamines (*20*−*23*). Different protein kinases appear to be responsible for phosphorylating each site. Serine 19 is phosphorylated by calmodulin-dependent protein kinase II, serine 31 by ERK, and serine 40 by cAMP-dependent protein kinase (*24*). Increases in the activity of tyrosine hydroxylase upon phosphorylation at serines 19, 31, and 40 have been demonstrated directly (*25*−*28*), although activation by calmodulin-dependent proton kinase II is reported to also require a separate protein (*26*). Only in the case of phosphorylation of serine 40 has greater than 2-fold activation been demonstrated with purified protein in the absence of other proteins.

There have been a number of studies of the effects on the kinetic parameters of tyrosine hydroxylase upon phosphorylation of serine 40, with conflicting results. Early efforts utilizing crude or partially purified enzyme were complicated by changes in the reported effects of phosphorylation as the enzyme was purified (*29*). Still, even with pure enzyme from rat or bovine sources, phosphorylation has been reported to result in decreases up to 10-fold or no effect at all on the K_M value for the tetrahydropterin (*30*−*33*), increases up to 3-fold or no effect on the V_{max} value (*27*, *32*), and/or increases in the K_i values for catecholamines (*30*, *32*). Some of the variations in the effects on individual parameters were undoubtedly due to variable phosphorylation of the enzyme when isolated (*19*, *34*); however, that would not seem to explain the variation in the kinds of effects seen. An additional complicating factor became apparent when Andersson and co-workers (*6*, *7*, *35*) showed that the enzyme purified from adrenal gland or pheochromocytoma cells contains significant but substoichiometric amounts of catecholamines bound to the active-site iron. The possible presence of bound inhibitors had not been considered in previous studies. More recent studies have utilized cloned rat or human enzyme, making it possible to study the properties of enzyme that does not contain endogenous phosphate or catecholamine. The reported effects of phosphorylation on the cloned enzymes are generally quite modest. The K_M value for tetrahydropterin decreases 2−3-fold, with little effect on the K_M value for tyrosine or on the V_{max} value (*5*, *8*, *36*). The affinity for dopamine has been reported to decrease 3-fold or about 10-fold (*8*, *17*), while the K_i value for DOPA is reported to be unaffected (*36*). None of these effects would seem to be of the magnitude expected for a significant regulatory mechanism.

The presence of bound catecholamine at the active site of tyrosine hydroxylase would be expected to have significant effects on the enzyme's activity. This was demonstrated directly by Daubner et al. (*5*), who showed that dopamine will form a tight complex with the recombinant rat enzyme, significantly inhibiting the activity. Critically, the kinetic parameters of this inhibited enzyme were identical to those of the nonrecombinant rat enzyme, including the effects of phosphorylation. This suggested that the primary effect of phosphorylation at serine 40 was to relieve the enzyme from inhibition by tightly bound catecholamines.

Recent studies of the redox state of the iron in tyrosine hydroxylase during catalytic turnover have led to the model shown in Scheme 1 (*10*). For catalysis, the enzyme-bound iron must be ferrous (*9*, *37*), but it can be readily oxidized to the ferric form by molecular oxygen. Tetrahydropterins can reduce this inactive ferric enzyme back to the ferrous form, allowing catalysis to continue (*10*). Alternatively, catechols such as DOPA can trap the ferric enzyme as an inactive complex. Thus, the activity that is measured in the presence of catechols reflects a competition between the trapping of the oxidized enzyme by the catechol and the reduction of the iron by the tetrahydropterin. If catalysis is initiated with the ferrous enzyme, as is typically done, the enzyme rapidly loses activity in the presence of catechols, with the final activity depending upon the concentrations of the catechol and the tetrahydropterin. Previous studies of the inhibition of tyrosine hydroxylase by catecholamines have invariably used end-point assays over times of 5−15 min (*8*, *38*). As is clear in Figure 1, while the rate of product formation in the absence of inhibitors is linear for several minutes, the activity rapidly decreases in the presence of a catecholamine. Clearly, K_i values obtained under such conditions would not accurately reflect the affinity of active form of the enzyme for the inhibitor. Indeed, the results presented here show that K_i values determined from initial rates are unaffected by phosphorylation of serine 40, in contrast to previous reports of the effect of phosphorylation on catecholamine inhibition. Thus, phosphorylation does not significantly affect binding of dopamine or DOPA to the active ferrous form of tyrosine hydroxylase.

Instead, phosphorylation dramatically affects the binding of DOPA and dopamine to the ferric form of the enzyme. While the association rate constants only increase about 2-fold upon phosphorylation, there are large increases in the dissociation rate constants. These result in comparable changes in the overall dissociation constants for binding to the ferric enzyme. The dissociation constant for DOPA increases 17-fold and the K_d value for dopamine increases approximately 300-fold upon phosphorylation. Indeed, because of the high affinity of the unphosphorylated enzyme for dopamine, the K_d value for this interaction must be considered an upper limit. The magnitude of these effects

means that the combined effects of catecholamine inhibition and phosphorylation can potentially modulate the activity of the enzyme over a very wide range.

There is clearly specificity in the effects of phosphorylation on binding of catechols, in that the effect on dopamine binding is the greatest, while binding of DHN is unaffected. While more extensive analyses with a series of catechols are required to accurately define the specificity, a clear difference among the three catechols is their charges. Haavik et al. (*39*) have previously reported that catecholamines will dissociate from tyrosine hydroxylase isolated from bovine adrenal gland if the pH is decreased, with an apparent pK_a value of 5.3. This observation is consistent with the presence of an amino acid residue that could interact with the positively charged amino group of catecholamines. Phosphorylation would thus mimic the effect of protonation of this residue, decreasing the affinity for catecholamines. DOPA has a much lower affinity for tyrosine hydroxylase compared to dopamine; this may be due to the presence of a carboxylate residue in the former. The active-site iron in tyrosine hydroxylase is located at the bottom of a hydrophobic pocket (*40*); the charge on such a carboxylate would be expected to decrease its affinity for a hydrophobic binding site. Finally, the binding of the neutral DHN is unaffected by phosphorylation. Since DHN lacks an amino group, such a model predicts that its affinity would be unchanged.

The results presented here are fully consistent with the model in Scheme 1. The large effects on the affinities for catecholamines of the ferric enzyme in the absence of effects on the affinities of the ferrous enzyme provides evidence that the redox cycle between the ferric and ferrous forms is physiologically relevant. This regulatory mechanism, in which phosphorylation relieves the enzyme by decreasing end-product binding to an inactive form, appears to be a novel regulatory mechanism. It provides a mechanism by which the rate of tyrosine hydroxylation can be regulated both by end-product inhibition and by the external demands on the cell.

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