Effects of Phosphorylation on Binding of Catecholamines to Tyrosine Hydroxylase: Specificity and Thermodynamics†

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ABSTRACT: As the catalyst for the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters, the activity of tyrosine hydroxylase is tightly regulated. A principle means of posttranslational regulation is reversible phosphorylation of serine residues in an N-terminal regulatory domain. Phosphorylation of serine 40 has been shown to have a large effect on the rate constant for dissociation of dopamine and a much smaller effect on that for DOPA [Ramsey, A. J., and Fitzpatrick, P. F. (1998) *Biochemistry 37*, 8980−8986]. To determine the structural basis for the differences in affinity and to further test the validity of the previously proposed model for regulation, the effects of phosphorylation of serine 40 on the affinities for a series of catechols have been determined. The affinities of the unphosphorylated enzyme vary by 3 orders of magnitude due to differences in the rates of dissociation. The highest affinities are found with catecholamines which lack a carboxylate. The affinities of the phosphorylated enzyme show a much smaller range. In the case of binding of dihydroxyphenylalanine, the decrease in affinity upon phosphorylation is due primarily to a decrease in the enthalpy of the interaction. Based upon these results, a structural model for the effect of phosphorylation is proposed.

The biosynthesis of the catecholamine neurotransmitters begins with the hydroxylation of tyrosine by tyrosine hydroxylase to form dihydroxyphenylalanine (DOPA).[1] This is generally thought to be the rate-limiting step, with the subsequent formation of dopamine, norepinephrine, and epinephrine depending upon the presence of the required enzymes within a specific cell. Tyrosine hydroxylase is a mononuclear non-heme iron protein, as are the related enzymes phenylalanine and tryptophan hydroxylase. When tyrosine hydroxylase is purified, the iron is ferric, even though activity requires ferrous iron, due to the facile oxidation of the iron by oxygen (*1*−*5*). Tetrahydropterins will reduce the iron, suggesting that the oxidation state within the cell is a mixture of ferric and ferrous forms (*5*).

As the enzyme catalyzing the initial step in a vital biosynthetic pathway, the activity of tyrosine hydroxylase is heavily regulated (*6*). At the posttranslational level, the primary mechanism of regulation is reversible phosphorylation of serine residues in an N-terminal regulatory domain (*1*, *7*). Phosphorylation occurs on residues which correspond to serines 19, 31, and 40 in the rat enzyme (*8*, *9*). In cells, increased calcium levels result in increased phosphorylation of Ser19, increased activity of the phosphoinositol/protein kinase C pathway results in increased phosphorylation of Ser31, and increased cAMP levels result in increased phosphorylation of Ser40 (*10*−*13*). Similar results have been obtained with pure proteins, in that calmodulin-dependent protein kinase phosphorylates Ser19, the kinases ERK1 and ERK2 phosphorylate Ser31, and cAMP-dependent protein kinase A phosphorylates Ser40 (*8*, *9*). There is substantial evidence that phosphorylation of Ser40 increases the activity of tyrosine hydroxylase (*14*−*20*); the evidence regarding the effects of phosphorylation of the other two sites is more equivocal, at least in part due to the lack of absolute specificity of the relevant kinases (*16*, *19*−*24*).

Interpretation of the effects of phosphorylation on tyrosine hydroxylase has been complicated by the presence of substoichiometric amounts of catecholamines in enzyme purified from nonrecombinant sources (*25*, *26*). A substantial effect on enzyme activity upon phosphorylation of Ser40 is only seen for enzyme with a bound catecholamine (*17*). We have recently shown that phosphorylation has no effect on inhibition of the ferrous enzyme by dopamine, but decreases the affinity of the ferric form by about 300-fold (*27*). Based upon these results, the regulatory mechanism in Scheme 1 has been proposed. In this model, the active ferrous form can be oxidized to the inactive ferric form by molecular oxygen. Activity is restored upon reduction of the iron by tetrahydrobiopterin. If a catecholamine binds to the ferric form, the enzyme is trapped in an inactive species which cannot be rereduced. Phosphorylation activates the enzyme by increasing the rate of dissociation of bound catecholamines, allowing reduction to the ferrous enzyme to proceed. In the previous study, only two catecholamines were characterized, dopamine and DOPA. The binding of dop-

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[1] Abbreviations: DOPA, dihydroxyphenylalanine.

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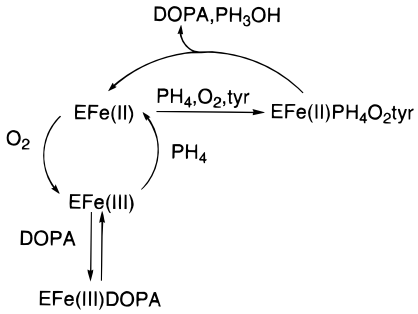
Scheme 1



amine to the ferric enzyme is about 3 orders of magnitude tighter than the binding of DOPA, while the binding affinities of the ferrous enzyme for the two compounds differ by less than 20-fold. The present study extends these analyses to a series of catecholamines and related compounds to gain insight into the structural bases for the differences in binding affinity. As a further probe of the structural differences resulting from phosphorylation of Ser40, the effects of phosphorylation on thermodynamic parameters for binding of DOPA have been determined.

EXPERIMENTAL PROCEDURES

[3,5-^{3}H]Tyrosine was purchased from Amersham Corp. and purified before use by the method of Ikeda et al. (*28*). 6-Methyltetrahydropterin was from B. Schircks Laboratories; stock solutions in 5 mM HCl were prepared on the day of use. 2,3-Dihydroxynaphthalene, dihydroxyphenylalanine, and dopamine were purchased from Sigma Chemical Co. Dihydroxyphenylserine, norepinephrine, epinephrine, 3-(3,4-dihydroxyphenyl)-2-methyl-2-hydrazinopropionate (carbidopa), and dihydroxyphenylacetic acid were purchased from Research Biochemicals International. 4-Methylcatechol was from Aldrich Chemical Co.

Rat tyrosine hydroxylase was expressed in *E. coli* and purified as described previously (*5*). The purified enzyme was concentrated by precipitation with 50% ammonium sulfate; the pellet was dissolved in 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1, and dialyzed against a 300-fold excess of the same buffer for 9 h. To obtain enzyme containing a stoichiometric amount of iron, the purified enzyme was similarly precipitated with 50% ammonium sulfate, and the pellet was dissolved in 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1, at a final concentration of about 9 mg/mL. After dialyzing the sample for 1 h against 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1, a stoichiometric excess of ferric sulfate was added while stirring the sample rapidly. Excess iron was then removed by extensive dialysis against 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1. Enzyme stoichiometrically phosphorylated at Ser40 was prepared by phosphorylation with cAMP-dependent protein kinase followed by purification using a Q-Sepharose column, as previously described (*27*). The activity of tyrosine hydroxylase was determined using an assay based on the release of tritium from [3,5-^{3}H]tyrosine as previously described (*4*). The protein content of enzyme samples was determined using an $A^{1\%}_{280\ nm}$ value of 10.4 (*2*). The iron content of the purified enzyme was determined using a Perkin-Elmer model 2380 atomic absorption spectrophotometer equipped with a graphite furnace as described previously (*5*). All enzyme concentrations are given in terms of enzyme-bound iron. UV−Visible spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer.

The rates of association of individual catechols with tyrosine hydroxylase were determined by mixing 10−20 μM (final concentration) tyrosine hydroxylase with varied amounts of the catechol 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 diethylenetriaminepentaacetate, 10% glycerol, pH 7.1. The absorbance changes over time were fit to eq 1, where ΔA is the absorbance change and A_f is the final absorbance after complete reaction. While the rate constant determined in this fashion is rigorously equal to the sum of the rate constants for the forward and reverse reactions, the contribution of the reverse reaction was sufficiently small that it could be ignored. To determine the rate constants for dissociation of catechols, 20−25 μM enzyme was mixed with an excess of the catechol of interest in 50 mM Hepes, 100 mM KCl, 0.2 mM diethylenetriaminepentaacetate, 10% glycerol, pH 7.1. After incubation of the enzyme and catechol for 15 min at 4.5 °C, 2,3-dihydroxynaphthalene was added to give a final concentration of 1 mM. The formation of the enzyme−dihydroxynaphthalene complex was then monitored at 550 nm. The absorbance changes were fit to eq 1 to determine the dissociation rate constant. When the rate constant for dissociation was less than 10^{-5} s^{-1}, it was not possible to follow the dissociation to completion due to competition from protein denaturation. Consequently, the absorbance of the enzyme−dihydroxynaphthalene complex determined in a separate experiment was used as an end point in fitting the time course data to eq 1.

$$k_{obs} = A_f + \Delta A e^{-kt} \quad (1)$$

RESULTS

Effect of Catecholamine Structure on Kinetics of Binding. The initial characterization of the effects of phosphorylation on catecholamine binding to tyrosine hydroxylase considered only two catecholamines, DOPA and dopamine (*27*). Since these two catecholamines showed very different rates of binding and effects of phosphorylation, the binding of a series of different catecholamines was analyzed to probe the structural features responsible. The compounds chosen for analyses included the remaining physiological catecholamines epinephrine and norepinephrine as well as a series of related catechols (Figure 1). Binding of a catecholamine to the resting ferric form of tyrosine hydroxylase results in a binary complex characterized by a broad absorbance band around 700 nm with an extinction coefficient of about 2 mM^{-1} cm^{-1} (*29*). The rate of the change in absorbance at 690 nm when the enzyme is mixed with different concentrations of catecholamines was consequently used to determine the rates of formation of the binary complex. In all cases, the absorbance changes exhibited first-order kinetics. A representative plot of the observed rates of complex formation as a function of the concentration of dihydroxyphen-

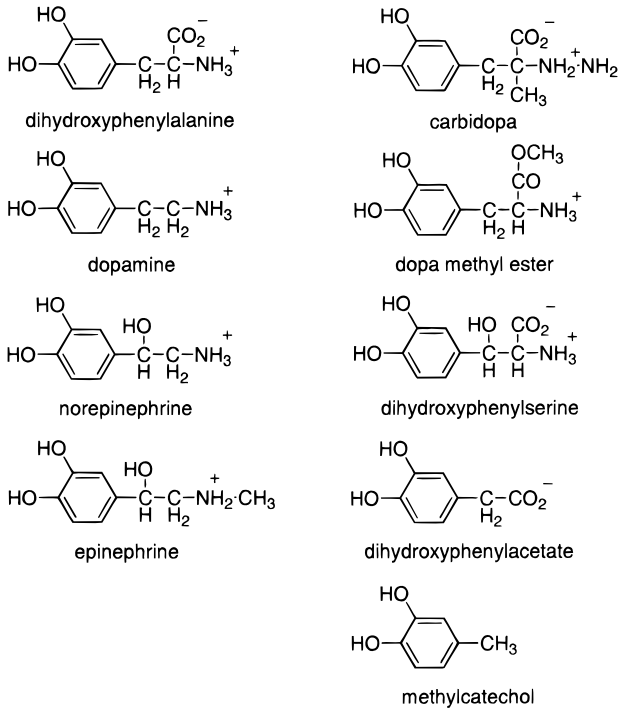


FIGURE 1: Structures of catechols in Table 1.

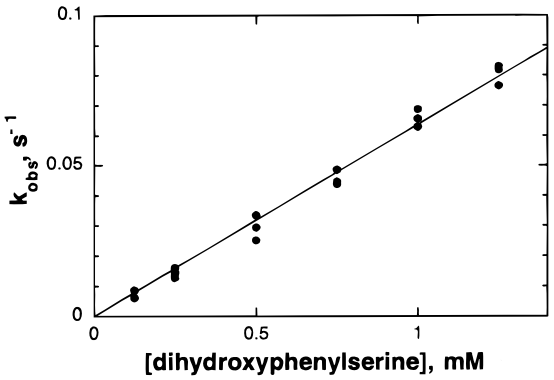


FIGURE 2: Concentration dependence for dihydroxyphenylserine binding to unphosphorylated tyrosine hydroxylase. The rate of change in absorbance at 690 nm due to the iron−catechol charge-transfer interaction was determined at the indicated concentrations of dihydroxyphenylserine at 4.5 °C in 50 mM Hepes, 100 mM KCl, 0.2 mM diethylenetriaminepentaacetate, 10% glycerol, pH 7.1.

ylserine is shown in Figure 2. The second-order rate constants for binding of catecholamines to tyrosine hydroxylase were determined from the slopes of the lines in this and similar plots. These values are given in Table 1 both for unphosphorylated enzyme and for enzyme which had been phosphorylated on Ser40 by cAMP-dependent protein kinase. No complex formation could be detected with tyrosine, 3-aminotyrosine, or 3-chlorotyrosine (results not shown).

The rate constant for dissociation of each catecholamine from the complex can in theory be determined from plots such as shown in Figure 2. However, the precision with which the *y*-intercept can by determined from Figure 2 is clearly too low to allow such an approach to be used. Instead, the dissociation was monitored directly. 2,3-Dihydroxynaphthalene binds to tyrosine hydroxylase with a K_d value of about 15 nM regardless of whether the enzyme is phosphorylated on Ser40 (*27*). The dihydroxynaphthalene−enzyme complex absorbs maximally at 550 nm (*27*), in contrast to the maximal absorbance around 690 nm seen with the compounds analyzed in these studies (results not shown). This difference in absorbance maxima was utilized to monitor dissociation of the latter compounds. The binary complex was formed by incubating the enzyme with a concentration of the ligand of interest which was at least 100 times its K_d value. Dihydroxynaphthalene was then added to a final concentration of 1 mM. Under these conditions, any free enzyme formed when the catechol dissociates is rapidly trapped by the dihydroxynaphthalene in an effectively irreversible complex. Consequently, the rate of the increase in absorbance at 550 nm equals the rate of catechol dissociation. A representative plot of the data obtained from such an analysis with dihydroxyphenylserine is shown in Figure 3. The dissociation rate constants determined in this fashion using both unphosphorylated and phosphorylated enzyme are summarized in Table 1. The independently determined association and dissociation rate constants were used to calculate the equilibrium constants for binding of the compounds in Figure 1 to both forms of tyrosine hydroxylase.

Thermodynamics of Binding of DOPA to Tyrosine Hydroxylase. The effects of temperature on the association and dissociation rate constants for binding of DOPA were determined using both unphosphorylated and phosphorylated tyrosine hydroxylase. Arrhenius plots of the data are shown in Figure 4. The independently determined rate constants could be used to determine the equilibrium constant for binding at each temperature. A van't Hoff plot of these K_a values is shown in Figure 5. The ΔH and ΔS values determined from these data are given in Table 2.[2]

DISCUSSION

The mechanism in Scheme 1 was initially proposed to describe the different redox states of tyrosine hydroxylase (*5*). The active ferrous form of the enzyme is readily oxidized to the inactive ferric form, which can be trapped by a catecholamine. A major effect of phosphorylation of Ser40 is a decrease in the affinity of the ferric form for catecholamines; this decrease in affinity is due almost exclusively to an increase in the rate constant for dissociation (*27*). With both DOPA and dopamine, the magnitude of the increase in the K_d value is significantly greater than the relatively small changes in steady-state kinetic parameters previously described upon phosphorylation of this enzyme (*14*−*20*), suggesting that modulation by phosphorylation of catecholamine binding to ferric tyrosine hydroxylase is a critical regulatory mechanism regulating catecholamine biosynthesis.

[2] To avoid complications due to denaturation of the enzyme, measurement of the binding kinetics was carried out at 4.5 °C, where some of the rates are quite slow. In the case of DOPA, measurements were also made at temperatures up to 25 °C. At the latter temperature, phosphorylation results in a decrease in the half-life of the enzyme−DOPA complex from 160 s to only 8 s. It can be calculated from the data in Figure 4B that these values would decrease a further 3-fold at 37 °C. If the temperature dependence of the binding of dopamine is similar to that of DOPA, a reasonable assumption, phosphorylation would result in a decrease in the half-life of the enzyme dopamine−complex at 25 °C from almost 8 days to only 4 min, with a further decrease to about 90 s at 37 °C. Studies of the effects of activation of tyrosine hydroxylase in intact cells show that the maximum increase in activity is achieved after about 2−3 min (*30*, *31*). Thus, the values in Table 1 are consistent with modulation of the dissociation rates by phosphorylation being physiologically relevant.

Table 1: Effect of Phosphorylation on the Affinity of Tyrosine Hydroxylase for Catechols

	unphosphorylated enzyme			phosphorylated enzyme			
catechol	k_{on}[a] (mM^{-1} s^{-1})	$10^4 \times k_{off}$[b] (s^{-1})	K_d (μM)	k_{on} (mM^{-1} s^{-1})	$10^4 \times k_{off}$ (s^{-1})	K_d (μM)	effect on K_d
DOPA	0.39 ± 0.068	4.6 ± 1.02	1.2 ± 0.33	0.64 ± 0.059	107 ± 8	16.6 ± 2.0	14 ± 4.2
D-DOPA	0.116 ± 0.001	0.96 ± 0.28	0.83 ± 0.24	0.25 ± 0.003	15 ± 0.21	6.0 ± 0.16	7.2 ± 2.1
dopamine	0.82 ± 0.026	0.0011 ± 0.0005	0.0013 ± 0.00059	1.4 ± 0.028	3.6 ± 0.08	0.26 ± 0.008	200 ± 91
norepinephrine	0.40 ± 0.009	0.013 ± 0.0009	0.0032 ± 0.00023	0.83 ± 0.014	1.4 ± 0.026	0.17 ± 0.004	53 ± 4.0
epinephrine	0.28 ± 0.0073	0.013 ± 0.0012	0.0046 ± 0.00021	0.49 ± 0.011	1.54 ± 0.023	0.31 ± 0.008	67 ± 3.5
carbidopa	0.12 ± 0.0065	3.1 ± 0.025	2.6 ± 0.14	0.23 ± 0.007	48 ± 0.83	21 ± 0.73	8.1 ± 0.52
DOPA methyl ester	0.39 ± 0.014	0.041 ± 0.0071	0.0105 ± 0.0019	0.72 ± 0.041	15 ± 0.06	2.1 ± 0.12	200 ± 38
dihydroxyphenylserine	0.067 ± 0.0032	1.6 ± 0.06	2.4 ± 0.15	0.12 ± 0.004	10.3 ± 0.14	8.5 ± 0.27	3.5 ± 0.24
dihydroxyphenylacetate	0.0040 ± 0.00048	4.1 ± 0.25	104 ± 14	0.0028 ± 0.00078	6.5 ± 0.14	230 ± 63	2.2 ± 0.67
4-methylcatechol	0.23 ± 0.0046	1.4 ± 0.08	0.63 ± 0.037	0.36 ± 0.002	24 ± 1.3	6.7 ± 0.37	10.6 ± 0.86

[a] 10−20 μM tyrosine hydroxylase, 33 μM catechol, 0.2 mM diethylenetriaminepentaacetate, 100 mM KCl, 10% glycerol, 50 mM Hepes, pH 7.1, 4.5 °C. [b] 20−25 μM tyrosine hydroxylase, 33 μM catechol, 1 mM 2,3-dihydroxynaphthalene, 100 mM KCl, 10% glycerol, 50 mM Hepes, pH 7.1, 4.5 °C.

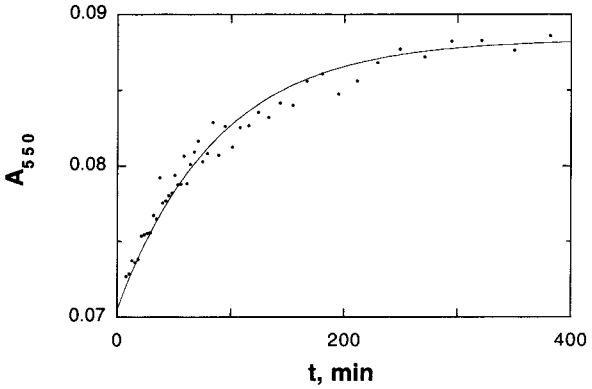


FIGURE 3: Dissociation of dihydroxyphenylserine from unphosphorylated tyrosine hydroxylase. The enzyme−catechol complex was formed by adding dihydroxyphenylserine (final concentration 33 μM) to 20 μM tyrosine hydroxylase in 50 mM Hepes, 100 mM KCl, 0.2 mM diethylenetriaminepentaacetate, 10% glycerol, pH 7.1, at 4.5 °C. After 15 min, 2,3-dihydroxynaphthalene was added to a final concentration of 1 mM, and the binding of dihydroxynaphthalene to the free enzyme was followed at 550 nm.

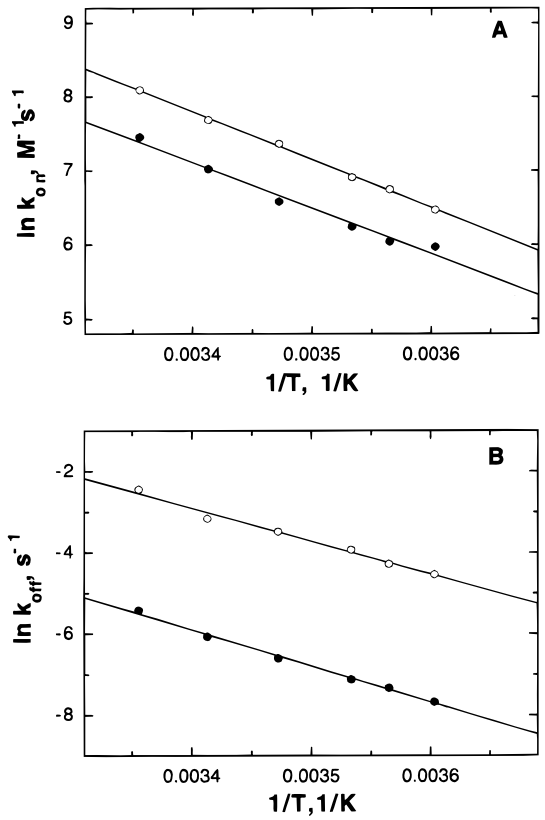


FIGURE 4: Effects of phosphorylation on the temperature dependences of the association (A) and dissociation (B) rate constants for binding of DOPA to tyrosine hydroxylase: filled symbols, unphosphorylated enzyme; open symbols, enzyme phosphorylated on Ser40. The conditions were as described for Figures 2 and 3.

The previous study (*27*) determined the effects of phosphorylation on binding of only two catecholamines, DOPA and dopamine. These two compounds show very different affinities for tyrosine hydroxylase and very different effects of phosphorylation. DOPA binds to ferric tyrosine hydroxylase with a K_d value of about 1 μM; this increases 15−20-fold upon phosphorylation. In contrast, dopamine binds with a K_d value of about 1 nM; this increases several hundredfold upon phosphorylation. The data in Table 1 extend these previous observations significantly. As was the case with the more abbreviated analysis, phosphorylation of Ser40 has only a moderate effect on the rate constants for association, with an increase of about 2-fold in almost all cases. A much more impressive effect is seen on the rate constants for dissociation, so that the changes in the K_d values mirror the increases in off rates. The magnitudes of the resulting decreases in affinity are correlated with the initial affinities, in that those compounds which bind most tightly to the unphosphorylated enzyme show the largest decreases in affinity upon phosphorylation. As a consequence, a much smaller range of affinities is seen with the phosphorylated enzyme.

One goal of the studies described here was to elucidate the critical structural features of catecholamines responsible for these differences in affinity. The values do indeed provide significant insight into the effects of catecholamine structure on affinity. The values in Table 1 show that the side chain has a dominant effect on the specificity and affinity of catechol binding to tyrosine hydroxylase and allow evaluation of the relative contributions of the amino and carboxyl residues. The presence of an amino group is required for tight binding, since all of the compounds with high affinity are amines. Methylation of the amino group does not alter the affinity, since epinephrine and norepinephrine have the same affinities. The presence of a carboxylate decreases the affinity of unphosphorylated tyrosine hydroxylase for a

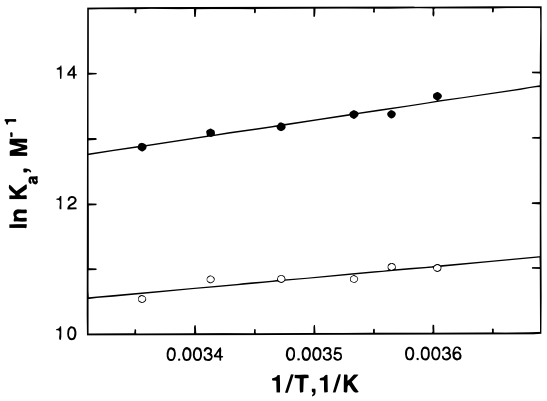


FIGURE 5: Effects of phosphorylation on the temperature dependence of the association constants for binding of DOPA to tyrosine hydroxylase: filled symbols, unphosphorylated enzyme; open symbols, enzyme phosphorylated on Ser40. The conditions were as described for Figures 2 and 3.

Table 2: Effect of Phosphorylation on Thermodynamic Parameters for Binding of DOPA to Tyrosine Hydroxylase[a]

kinetic parameter	theromdynamic parameter	unphosphorylated	phosphorylated
k_{on}	k_{act} (kcal/mol)	12.3 ± 0.8	12.9 ± 0.2
k_{off}	k_{act} (kcal/mol)	17.8 ± 0.6	16.2 ± 0.9
K_a	ΔH (kcal/mol)	−5.4 ± 0.6	−3.2 ± 0.9
K_a	ΔS [cal/(mol·deg)]	7.5 ± 2.2	10.2 ± 3.0

[a] The conditions were as described for Figures 2 and 3.

catechol by about 2 orders of magnitude. This conclusion is based upon comparison of the affinities of DOPA and DOPA methyl ester, of dihydroxyphenylserine and norepinephrine, and of methylcatechol and dihydroxyphenylacetate. Comparison of the affinities of dopamine to norepinephrine or of DOPA to dihydroxyphenylserine shows that the presence of a benzyl hydroxyl has little effect on the affinity of the unphosphorylated enzyme, but attenuates the decrease in affinity upon phosphorylation.

No structure is yet available for catecholamines bound to any form of tyrosine hydroxylase. However, a structure is available for catecholamines bound to the catalytic domain of human phenylalanine hydroxylase (*32*); the catalytic domain of tyrosine hydroxylase is homologous to that of phenylalanine hydroxylase, and the active sites are superimposeable (*33*, *34*). The phenylalanine hydroxylase−catechol structures show both catechol oxygens to be bound to the active site iron. Such an interaction was previously suggested from spectroscopic studies of catechol complexes of both tyrosine and phenylalanine hydroxylase (*25*, *29*, *35*). While there are several conserved amino acid residues in the active site which would have the potential for interacting with a positively charged amine, no interactions can be seen between the protein and the side chains of the catechols DOPA, dopamine, epinephrine, and norepinephrine. There is no evidence that binding of catechols to phenylalanine hydroxylase is physiologically relevant, so that the only interactions are those expected between a ferric atom and a catechol. In contrast, binding of catecholamines to tyrosine hydroxylase appears to be a critical regulatory mechanism. Given the lack of interactions between residues in the active site of phenylalanine hydroxylase and the side chains of catechols, the difference in regulatory properties must be ascribed to the distinct regulatory domains of the two enzymes. This conclusion is supported by the results of limited proteolysis as a probe of the structural changes which occur upon binding of dopamine or phosphorylation of Ser40 in tyrosine hydroxylase (*36*). The region of the regulatory domain of tyrosine hydroxylase between residues 33 and 50 is extremely sensitive to proteolysis. Binding of dopamine greatly decreases the sensitivity, while phosphorylation of Ser40 increases the sensitivity by a comparable amount. These results have been interpreted as evidence for a direct interaction of this portion of the regulatory domain with the amino group of a catecholamine bound in the active site and for a conformational change of this portion of the protein upon phosphorylation. Further evidence for such a model comes from the results of Abate and Joh (*37*), who have shown that deletion of the first 39 residues of tyrosine hydroxylase resulted in enzyme which was not inhibited by preincubation with dopamine. The specificity studies described here suggest that the critical interaction determining the affinity for a catecholamine is between the regulatory domain and the amino moiety of the catecholamine. Catecholamines which lack a carboxylate interact most strongly with the regulatory domain, so that the catecholamine neurotransmitters have the highest affinity. The conformational changed caused by phosphorylation would weaken this interaction, leaving the interaction of the catechol oxygens with the metal as the dominant interactions, resulting in the much smaller differences in the affinities of the phosphorylated enzyme for different catechols.

The effects of phosphorylation on the thermodynamics of DOPA binding are consistent with such a model. The most significant change is a decrease in the enthalpy of the interactions, consistent with the loss of an electrostatic interaction between the protein and DOPA. There may also be a larger increase in entropy when DOPA binds to the phosphorylated enzyme. A dominant factor in determining the entropic change when ligands bind to proteins is often the release of water molecules to bulk solvent. Binding of DOPA to the active site cleft of tyrosine hydroxylase would be expected to displace water molecules from the hydrophobic cleft, with an attendant increase in the overall entropy. In contrast, interaction of the regulatory domain with a bound catecholamine would be expected to decrease the conformational entropy of the protein, compensating for the increase due to water release. The loss of such an interaction could result in a greater increase in the total entropy change for catecholamine binding to the phosphorylated enzyme, as is seen.

The results described here provide significant new details regarding the effects of phosphorylation of Ser40 on the activity of tyrosine hydroxylase. They establish the critical importance of the amino moiety of catecholamines in the magnitude of the effect of phosphorylation. They also establish that the magnitude of the effect of phosphorylation is due to differences in the rates of dissociation of catechols from the unphosphorylated enzyme. They will provide a firm quantitative basis both for analyzing the effects of phosphorylation on the remaining sites, Ser19 and Ser30, and for evaluating the effects of site-directed mutagenesis of residues which may be critical for these regulatory properties.

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