Effects of Substitution at Serine 40 of Tyrosine Hydroxylase on Catecholamine Binding†

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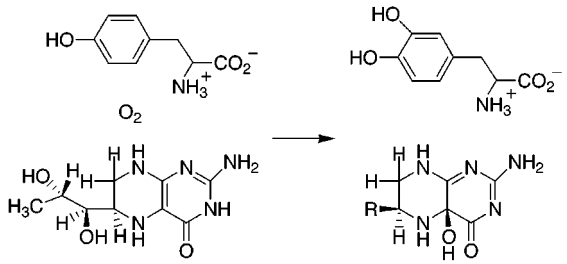
ABSTRACT: Phosphorylation of Ser40 in the regulatory domain of tyrosine hydroxylase activates the enzyme by increasing the rate of dissociation of inhibitory catecholamines [Ramsey, A. J., and Fitzpatrick, P. F. (1998) *Biochemistry 37*, 8980−8986]. To probe the structural basis for this effect and to ascertain the ability of other amino acids to functionally replace serine and serine phosphate, the effects of replacement of Ser40 with other amino acids were determined. Only minor changes in the V_{max} value and the K_m values for tyrosine and tetrahydropterin were seen upon replacement of Ser40 with alanine, valine, threonine, aspartate, or glutamate, in line with the minor effects of phosphorylation on steady-state kinetic parameters. More significant effects were seen on the binding of dopamine and dihydroxyphenylalanine. The affinity of the S40T enzyme for either catecholamine was very similar to that of the wild-type enzyme, while the S40E enzyme was similar to the phosphorylated enzyme. The S40D enzyme had an affinity for DOPA comparable to the phosphorylated enzyme but a higher affinity for dopamine than the latter. With both catecholamines, the S40V and S40A enzymes showed intermediate levels of activation. The results suggest that the serine hydroxyl contributes to the stabilization of the catecholamine-inhibited enzyme. In addition, the S40E enzyme will be useful in further studies of the effects of multiple phosphorylation on tyrosine hydroxylase, while the alanine enzyme does not provide an accurate mimic of the unphosphorylated enzyme.

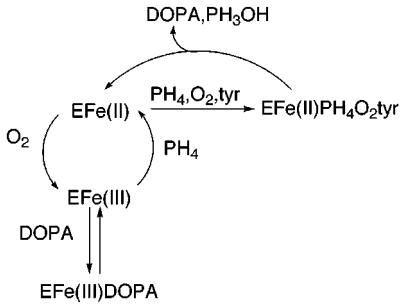
The enzyme tyrosine hydroxylase (TyrH)[1] catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA); the other two substrates in the reaction are molecular oxygen and tetrahydrobiopterin (Scheme 1) (*1*). This is the rate-limiting step in the production of the catecholamine neurotransmitters dopamine, epinephrine, and norepinephrine. The enzyme is a homotetramer containing one non-heme iron atom per subunit, bound to a 2-histidine-1-carboxylate facial triad (*2*, *3*). TyrH belongs to a family of aromatic amino acid hydroxylases which also includes phenylalanine hydroxylase and tryptophan hydroxylase. These three enzymes contain homologous C-terminal catalytic cores and distinctive N-terminal regulatory domains (*4*, *5*). To date, the three-dimensional structures of the catalytic domain of tyrosine hydroxylase and of the combined catalytic and regulatory domains of phenylalanine hydroxylase have been described (*2*, *6*).

As the catalyst for a rate-limiting step in neurotransmitter production, tyrosine hydroxylase is highly regulated. The posttranslational regulatory mechanism involves reversible phosphorylation of serine residues in the regulatory domain. These are located at positions 19, 31, and 40 in the rat enzyme and in the human isoform hTH1 (*7*−*9*). Only in the case of Ser40 has a large change in activity upon phosphorylation been demonstrated. Our present understanding of the effects of Ser40 phosphorylation on the activity of TyrH is summarized in Scheme 2. For catalytic activity, the active site iron must be in the ferrous state, but the ferrous enzyme is readily oxidized to the ferric state (*10*). This process can be reversed by rereduction of the iron; tetrahydrobiopterin is probably the physiological reductant. Catecholamines bind tightly to the iron of the ferric enzyme; this traps the enzyme

Scheme 1



Scheme 2



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[1] Abbreviations: TyrH, tyrosine hydroxylase; DOPA, 3,4-dihydroxyphenylalanine; DHN, 2,3-dihydroxynaphthalene.

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Table 1: Oligonucleotides Used in Mutagenesis of Serine 40[a]

mutation	oligonucleotide	restriction enzyme screen
S40A	5′-CGG CGA CAG GCT CTC ATC GAG G-3′	loss of *Hin*fI site
S40D	5′-GG CGA CAG GAC CTC ATC GA-3′	loss of *Hin*fI site
S40E	5′-CGG CGA CAG GAG CTC ATC GAG G-3′	loss of *Hin*fI site
S40T	5′-GG CGA CAG ACT CTC ATC GA-3′	loss of *Bsm*AI site
S40V	5′-CGG CGA CAG GTA CTC ATC GAG G-3′	loss of *Hin*fI site

[a] The altered codon for Ser40 is underlined.

in an inactive form. Phosphorylation of Ser40 results in a large increase in the rate of dissociation of catecholamines from the ferric enzyme, allowing reduction to the active enzyme (*11*). The quantitative effect of phosphorylation on the rate of dissociation depends on the catecholamine; the affinity for DOPA is decreased 15−20-fold upon phosphorylation and that for dopamine by 3 orders of magnitude. Phosphorylation has no effect on the affinities of DOPA and dopamine for the ferrous enzyme (*11*).

A number of studies to date suggest that phosphorylation of Ser40 disrupts an interaction with the regulatory domain which is necessary for tight binding of catecholamines. Limited proteolysis has been used as a probe of the structural changes in the regulatory domain which occur upon phosphorylation and dopamine binding; the results are consistent with increased mobility of residues 33−50 upon phosphorylation and decreased mobility when dopamine is bound (*12*). The amino group of a catecholamine is required for tight binding of a catechol to TyrH; this suggests that the regulatory domain interacts directly with the amino group (*13*). Finally, the structure of the catalytic domain of phenylalanine hydroxylase with a catecholamine bound shows no interactions between the amine group of the ligand and the protein, suggesting that any such interactions involve the regulatory domain (*14*). As a further probe of the structural basis for the effects of phosphorylation of Ser40, we describe here the properties of several site-directed mutants of Ser40.

EXPERIMENTAL PROCEDURES

Plasmids coding for the Ser40-mutated forms of rat tyrosine hydroxylase were constructed using the Kunkel method (*15*) of site-directed mutagenesis. A plasmid directing the expression of S40ATyrH has already been described (*16*). The remaining mutants in this study were constructed in the same manner, using the synthetic oligonucleotides in Table 1 to direct mutagenesis. Changes in the digestion products of restriction enzymes were used to detect successful mutagenesis prior to subcloning into the expression vector pET3b. DNA sequencing of the entire coding regions was performed to ensure that no unintentional mutations were included. Plasmids containing the wild-type (pETYH8) and mutated TyrH genes (pETOH1S40E, pETOH1S40D, pETOH1S40A, pETOH1S40V, and pETOH1S40T) were transformed into C41DE3 *Escherichia coli*. Cell growth and enzyme purification were carried out as previously described for the wild-type enzyme (*16*). For enzyme that would be used in catecholamine binding studies, 5 mM ferrous ammonium sulfate was added to the purified enzyme to give a 1.2:1 molar ratio of Fe(II) to enzyme (*13*). This mixture was incubated on ice for 30 min and then dialyzed against 50 mM HEPES, 10% glycerol, and 100 mM KCl, pH 7, to remove any excess iron. The amount of iron present in the enzyme was determined by atomic absorption spectroscopy as previously described (*10*).

cAMP-dependent protein kinase was purified by the method of Flockhart and Corbin (*17*). Phosphorylation of tyrosine hydroxylase was performed by the method of Ramsey and Fitzpatrick (*11*). Steady-state kinetic measurements used a colorimetric assay that measures the amount of DOPA produced (*18*). Conditions were 0.1−0.2 μM enzyme, 50 mM HEPES, 100 μg/mL catalase, 10 μM ferrous ammonium sulfate, and 1 mM dithiothreitol, pH 6.7, 32 °C, for all activity assays. For determining K_{MPH_4} values, the concentration of tyrosine was 100−125 μM, and the concentration of 6-methyltetrahydropterin (MPH_4) was varied from 0 to 300 μM. When determining K_{Tyr} values, the tyrosine concentration was varied from 0 to 200 μM, and an MPH_4 concentration of 340 μM was used. Steady-state data were fit directly to the Michaelis−Menten equation. Catecholamine binding studies were conducted using the procedures of Ramsey and Fitzpatrick (*11*). The concentrations of enzymes used for these studies are given in terms of enzyme-bound iron. To measure the rate constants for association of DOPA and dopamine to TyrH, enzyme (∼15 μM after mixing) was mixed with various concentrations of catecholamine in an Applied Photophysics SX-18MV stopped-flow spectrophotometer. Experiments were performed in 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. The increase in absorbance at 690 nm was monitored over time at each catecholamine concentration, and the data were fit to eq 1 using the program Kaleidagraph (Adelbeck Software), where A_t is the observed absorbance at 550 nm at time t, A_∞ is the absorbance after the reaction is complete, and A_0 is the absorbance at time zero. To measure dissociation rate constants, enzyme (20−40 μM) was incubated with 100−150 μM DOPA or 45 μM dopamine for approximately 15 min in 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. Formation of the enzyme−catecholamine complex was monitored at 690 nm to ensure that the reaction was complete. 2,3-Dihydroxynaphthalene (DHN) was then added to give a final concentration of 1 mM, and the A_{550} was monitored over time. The data were fit to the equation:

$$A_t = A_\infty + (A_0 - A_\infty)e^{-kt} \quad (1)$$

RESULTS

Kinetic Characterization of Mutant Enzymes. Site-directed mutagenesis was used to construct TyrH mutants enzymes in which Ser40 was replaced with alanine, threonine, valine, glutamate, or aspartate. In all cases, cell growth and enzyme purification could be successfully carried out using protocols

Table 2: Kinetic Parameters of Wild-Type and Mutant Tyrosine Hydroxylases[a]

enzyme	K_{Tyr} (μM)[b]	K_{MPH_4} (μM)[c]	V_{max} (min^{-1})[c]
wild type	48 ± 6	47 ± 16	139 ± 4
S40T	60 ± 9	57 ± 14	157 ± 6
S40A	66 ± 9	36 ± 12	183 ± 4
S40V	50 ± 7	38 ± 7	182 ± 4
S40D	49 ± 7	38 ± 11	141 ± 3
S40E	47 ± 6	37 ± 12	165 ± 17
phosphorylated	59 ± 8	29 ± 0.1	108 ± 1

[a] Conditions: 0.1−0.2 μM enzyme, 50 mM HEPES, 100 μg/mL catalase, 1 mM dithiothreitol, and 10 μM ferrous ammonium sulfate, pH 6.7, at 32 °C. [b] 340 μM MPH_4 with [tyrosine] varied from 0 to 200 μM. [c] 100 or 125 μM tyrosine with [MPH_4] varied from 0 to 300 μM.

developed for the wild-type enzyme. The yields of pure enzymes were from 70 to 120 mg from 6 L of culture. All of the mutant enzymes were readily reconstituted with iron to yield stoichiometries of 0.9−1 iron atom per subunit.

Steady-state kinetic parameters were determined with tyrosine and 6-methyltetrahydropterin as substrates for each of the mutant proteins and for unphosphorylated and phosphorylated wild-type TyrH. The results are summarized in Table 2. Previous studies have shown that phosphorylation results in no change in the K_m value for tyrosine, a slight increase in the V_{max} value, and a decrease in the K_{PH_4} value of about 2-fold (*16*). The kinetic properties of the mutant enzymes are consistent with the previous results. The V_{max} and K_{tyr} values are essentially unaffected by mutation of Ser40 to any of the amino acid residues selected. With the exception of the S40T enzyme, mutagenesis of Ser40 appears to result in a slight but insignificant decrease in the K_m value for tetrahydropterin. Thus, just as phosphorylation of Ser40 does not have dramatic effects on the steady-state kinetic parameters of the wild-type enzyme, mutagenesis of Ser40 has only slight effects. This result is consistent with the mutations having no significant effect on the overall structural integrity of the enzyme.

Catecholamine Binding. In contrast to the small effects of Ser40 phosphorylation on the steady-state kinetic parameters of TyrH, there is a dramatic change in the affinity of the ferric form of the enzyme for catecholamines upon phosphorylation (*11*). The magnitude of the change varies with the specific catecholamine used (*13*). There is a decrease in affinity of 3 orders of magnitude with dopamine, epinephrine, and norepinephrine, the downstream products of the pathway initiated with TyrH. In contrast, the affinity for the immediate product of the TyrH reaction, DOPA, decreases only about 20-fold. The effects of mutagenesis of Ser40 on catecholamine binding were analyzed with both DOPA and dopamine. While the large change in affinity seen with dopamine provides a larger dynamic range with which to evaluate the effects of individual mutants, the dissociation from the wild-type unphosphorylated enzyme is difficult to measure accurately because it is so slow. In contrast, the rates of dissociation of DOPA from the unphosphorylated and phosphorylated wild-type enzyme are readily measured, but the range of effects is much smaller, potentially obscuring subtle differences between different mutations. Because of the slow dissociation of catecholamines from the unphosphorylated enzyme and because of the high affinity of catecholamines for the enzyme, binding affinities were

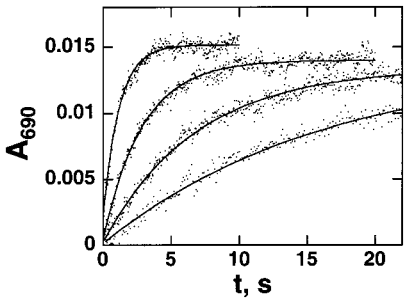

FIGURE 1: Binding of DOPA to S40T tyrosine hydroxylase. S40T TyrH (15 μM) was combined with various concentrations of DOPA, and the absorbance at 690 nm was monitored. Starting with the uppermost curve, the final concentrations of DOPA used were 985, 320, 160, and 65 μM. The curves are fits of the data to $A_t = A_\infty + (A_0 - A_\infty)e^{-kt}$. Conditions: 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C.

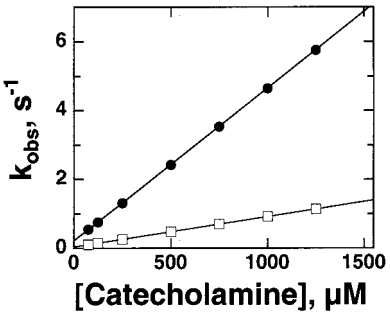

FIGURE 2: Concentration dependence for the reaction of S40T tyrosine hydroxylase with DOPA (□) or dopamine (●). The conditions were as described for Figure 1.

determined by measuring rate constants for association and dissociation separately.

When ferric TyrH is mixed with DOPA or dopamine, there is formation of a complex exhibiting a broad absorbance band centered at 690 nm. With dopamine, the ϵ_{690} value is 2000 M^{-1} cm^{-1} (*19*). Representative traces of the absorbance changes upon binding are given in Figure 1 for the reaction of S40T TyrH and DOPA. These and all of the data for binding of both DOPA and dopamine to the enzyme were fit well with a simple exponential, consistent with a straightforward one-step binding reaction. To determine the second-order rate constant for association of DOPA and dopamine with each mutant protein, the pseudo-first-order rate constants determined in experiments such as that illustrated in Figure 1 were replotted as a function of catecholamine concentration. Figure 2 shows such replots of the data for binding of both catecholamines to S40T TyrH. The second-order rate constants for association of DOPA and dopamine to the individual mutant proteins determined from these analyses are summarized in Tables 3 and 4. Consistent with the small effect that phosphorylation has on the association rate constants (*11*), mutagenesis of Ser40 to any of the amino acids used here resulted in increases in the values of less than 2-fold.

In theory, both the association rate constant and the dissociation rate constant for a single-step binding reaction can be determined from analysis of data such as that in Figures 1 and 2. The dissociation rate constant is given by the *y*-intercept in a replot of the pseudo-first-order rate constants as shown in Figure 2. However, as Figure 2

Table 3: Effect of Mutagenesis of Serine 40 on Binding of DOPA to Tyrosine Hydroxylase

enzyme	k_{on}[a] ($mM^{-1}\,s^{-1}$)	$10^3 k_{off}$[b] (s^{-1})	K_a[c] (mM^{-1})
wild type	0.65 ± 0.06	1.7 ± 0.1	370 ± 40
S40T	0.89 ± 0.01	1.80 ± 0.05	490 ± 10
S40A	1.03 ± 0.08	5.6 ± 0.3	180 ± 20
S40V	1.09 ± 0.06	11.9 ± 0.3	92 ± 6
S40D	1.20 ± 0.05	27 ± 1	45 ± 2
S40E	1.2 ± 0.2	17.8 ± 0.4	70 ± 10
phosphorylated	1.1 ± 0.3	46 ± 9	24 ± 8

[a] Conditions: 15 μM enzyme, 75−1250 μM DOPA, 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. [b] Conditions: 30 μM enzyme, 1 mM DHN, 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C; 150 μM DOPA for S40E, S40D, and phosphorylated enzymes, but 100 μM for all other enzymes. [c] Calculated from the corresponding k_{on} and k_{off} values.

Table 4: Effect of Mutagenesis of Serine 40 on Binding of Dopamine to Tyrosine Hydroxylase

enzyme	k_{on}[a] ($mM^{-1}\,s^{-1}$)	$10^5 k_{off}$[b] (s^{-1})[c]	K_a[c] (μM^{-1})
wild type	5.2 ± 0.3	0.069 ± 0.007	7500 ± 900
S40T	5.0 ± 1	0.19 ± 0.01	2600 ± 500
S40A	5.8 ± 0.8	4.4 ± 0.1	130 ± 20
S40V	5.4 ± 0.6	4.4 ± 0.01	120 ± 10
S40D	6.9 ± 0.8	12 ± 1	58 ± 8
S40E	6.5 ± 1	97 ± 9	7 ± 1
phosphorylated	7.9 ± 0.8	166 ± 4	4.8 ± 0.5

[a] Conditions: 15 μM enzyme, 75−1250 μM DOPA, 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. [b] Conditions: 30 μM enzyme, 1 mM DHN, 45 μM dopamine, 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. [c] Calculated from the corresponding k_{on} and k_{off} values.

illustrates, the dissociation rate constants for catecholamines are too slow to be determined from such an analysis, since the *y*-intercepts are not significantly different from zero in many cases. Consequently, the dissociation rate constants were measured directly, taking advantage of the fact that the complex formed by TyrH and DHN has a maximum absorbance at 550 nm (*10*). Enzyme (20−40 μM) was incubated with 100−150 μM DOPA or 45 μM dopamine until binding equilibrium was attained, as evidenced by formation of an absorbance peak at 690 nm. DHN was then added to give a final concentration of 1 mM. The K_d value for DHN is approximately 15 nM for both the phosphorylated and unphosphorylated enzymes (*11*), so that any free enzyme formed upon dissociation of DOPA or dopamine will be trapped as the enzyme−DHN complex. The pseudo-first-order rate constant for binding of 1 mM DHN to free TyrH is 5 s^{-1}, increasing to 11 s^{-1} in the phosphorylated enzyme. This is sufficiently rapid that the rate of formation of the DHN complex will be determined solely by the rate of dissociation of the bound DOPA or dopamine. Since the enzyme−DHN complex absorbs at 550 nm compared to the maximum of 690 nm seen with dopamine or DOPA, the conversion to the enzyme−DHN complex can be followed by monitoring the first-order increase in absorbance at 550 nm. Figure 3 shows representative data from such analyses for dissociation of dopamine from each of the mutant enzymes, as well as the unphosphorylated and phosphorylated wild-type enzymes. The first-order rate constants for dissociation of both DOPA and dopamine from each of the

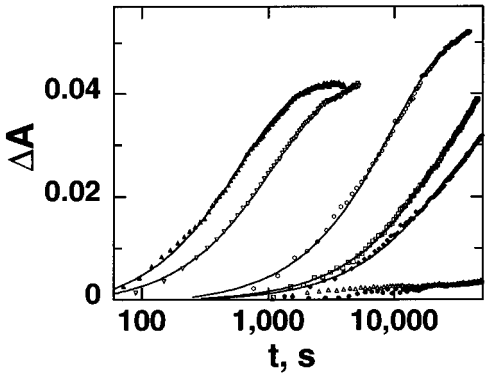

FIGURE 3: Dissociation of dopamine from phosphorylated and mutant tyrosine hydroxylases. Enzyme (20−40 μM) was incubated with 45 μM dopamine for approximately 15 min in 50 mM HEPES, 10% glycerol, 100 mM KCl, and 0.2 mM diethylenetriaminepentaacetic acid, pH 7, at 15 °C. DHN was then added to give a final concentration of 1 mM, and the increase in absorbance at 550 nm was monitored. Curves are fits of the data to $A_t = A_\infty + (A_0 - A_\infty)e^{-kt}$. The curves are for the wild-type enzyme (△), S40T TyrH (◇), S40A TyrH (◆), S40V TyrH (□), S40D TyrH (○), S40E TyrH (▽), and TyrH phosphorylated at Ser40 (▲). For clarity of presentation, most (75−80%) of the data points have been omitted.

enzymes are summarized in Tables 3 and 4. Qualitatively, the effects of the individual mutations were the same with DOPA and dopamine. S40T was indistinguishable from the wild-type enzyme, the largest dissociation rate constants were seen with the S40D and S40E enzymes, and the S40A and S40V enzymes exhibited intermediate values. The individual association and dissociation rate constants were used to calculate the equilibrium constants for DOPA and dopamine binding to each mutant enzyme. These are summarized in Tables 3 and 4.

DISCUSSION

The activation of TyrH upon phosphorylation by cAMP-dependent protein kinase was first described over 2 decades ago (*20*−*22*). Subsequent studies showed a direct relationship between increased phosphorylation of TyrH and increased catecholamine biosynthesis in adrenal cells following increases in cAMP levels (*23*), establishing phosphorylation as an important mechanism for posttranslational regulation of this enzyme. These observations were extended with the demonstration that TyrH is phosphorylated on multiple residues in cells (*24*) and that the extent of phosphorylation at individual sites varies with the different conditions used to increase catecholamine biosynthesis (*24*, *25*). A great deal of effort since then with purified enzymes and with intact cells has resulted in a relatively complete understanding of the relationship between regulatory pathways and phosphorylation of specific amino acid residues in TyrH (*1*, *8*, *26*, *27*). Increased intracellular calcium levels result in phosphorylation of Ser19. Agents which act through the phosphoinositol/protein kinase C pathway increase the level of phosphorylation of Ser31. Agents which increase cAMP levels result in increases in the level of phosphorylation of Ser40. Still, correlation of enzyme activity with phosphorylation at specific residues has proved difficult. The kinases specific for each site have been tentatively identified by studies with the pure enzymes. Calmodulin-dependent protein kinase II phosphorylates Ser19 (*28*), the MAP kinases ERK1 and ERK2 phosphorylate Ser40 (*29*), and cAMP-dependent

protein kinase phosphorylates Ser40 (*7*). However, while cAMP-dependent kinase is specific for Ser40, stoichiometric phosphorylation of Ser19 or Ser31 with other kinases also results in some phosphorylation of Ser40 (*7*, *9*, *28*, *30*).

A systematic examination of the effects of phosphorylation on the properties of TyrH requires enzyme specifically and completely phosphorylated at each site. This is most easily done in the case of Ser40, and we have been able to prepare and characterize enzyme which is phosphorylated at this site (*11*). A major effect of phosphorylation of this residue is a large increase in the rate of dissociation of catecholamines from the ferric enzyme rather than any large change in the kinetic parameters of the ferrous enzyme (*11*, *13*). Extending these analyses to enzymes specifically phosphorylated at serines 19 and 31 is problematic because of the lesser specificities of kinases for these sites. One approach to this problem is to use site-directed mutagenesis to replace all but one phosphorylation site with alanine, thereby preventing unwanted phosphorylation. Alternatively, a commonly used approach has been to replace the serine of interest with either aspartate or glutamate in order to mimic the effects of serine phosphorylation. The results presented here allow evaluation of these approaches in the case of TyrH.

Glutamate is isosteric with a phosphorylated serine, so that it would be expected to be a better mimic than aspartate. This is clearly the case when the affinity of dopamine for TyrH is considered. The K_a value and the individual association and dissociation rate constants for the S40E enzyme are all within a factor of 2 of the phosphorylated enzyme. Glutamate can thus functionally replace serine phosphate in TyrH. This is in contrast to the results of similar mutageneses with glycogen phosphorylase and isocitrate synthase, where the S14E enzymes do not show the full magnitude of the changes seen in the phosphorylated enzymes (*31*, *32*). Aspartate should be less effective than glutamate based simply on steric considerations, and this expectation is met. When dopamine binding is analyzed, aspartate is not as good as glutamate in replacing serine phosphate in TyrH. The K_a value of the S40D enzyme for this catecholamine is an order of magnitude greater than the corresponding value for the phosphorylated enzyme.

The changes in DOPA binding upon replacement of Ser40 with an acidic residue are qualitatively similar to those seen with dopamine. The ability of the S40E mutation to mimic the effect of phosphorylation on DOPA binding is not quite as good as with dopamine, although the values are still within 3-fold. In addition, the S40D enzyme shows a greater decrease in affinity for DOPA than does S40E TyrH, in contrast to the case with dopamine. These differences may be due to the effect of the carboxylate in DOPA upon the charge density in the binding site. In the case of isocitrate dehydrogenase, the relative effects of substitution with aspartate versus glutamate similarly depend on the identity of the ligand, in that there are different effects on the V_{max} value depending upon whether isocitrate or malate is used (*32*). However, because of the relatively small degree of activation seen with DOPA as the ligand compared to the effect with dopamine even with the phosphorylated enzyme, caution should be used in drawing conclusions from the DOPA data about the relative abilities of aspartate and glutamate to mimic serine phosphate. In contrast, the much larger dynamic range which dopamine binding affords should reflect the relative effects of replacement with these two amino acids.

The data for the S40E enzyme demonstrate that it should be useful in studies of the effect of phosphorylation on other properties of TyrH. In contrast, the results with the S40A enzyme demonstrate that replacement of Ser40 with alanine generates a less accurate model for the unphosphorylated enzyme. The K_a value for dopamine is an order of magnitude lower in the S40A enzyme than in the unphosphorylated wild-type enzyme. Even the binding of DOPA shows a significant effect of this replacement; this is most readily seen in the effect on the k_{off} value for DOPA. A similar activating effect occurs with the S40V enzyme. The activation seen with these two enzymes is likely due to the loss of the serine hydroxyl. This conclusion is supported by the properties of the S40T enzyme, which are very close to those of the wild-type enzyme. Since valine and threonine are isosteric, differences in the effects of replacement with the two residues are unlikely to be due to steric effects.

The activation seen with the S40A and S40V enzymes may explain the results of Wu et al. (*33*, *34*). These authors studied the effects of mutagenesis of Ser40 on DOPA production in AtT-20 cells. They found that cells expressing the S40L enzyme showed a higher level of DOPA production than cells expressing the wild-type enzyme and concluded that the mutant enzyme is in an activated state comparable to the phosphorylated enzyme (*34*). The S40L enzyme would be expected to have properties very similar to those of the S40V enzyme described here and thus would behave similarly to the partially phosphorylated enzyme.

The results with the S40T, S40A, and S40V enzymes provide insight into the structural basis for the effect of phosphorylation. Limited proteolysis has previously been used as a probe of the conformational changes which occur upon phosphorylation of Ser40 and upon dopamine binding to TyrH (*12*). The region of TyrH from amino acid residues 33−50 is extremely sensitive to trypsinolysis. Dopamine decreases the sensitivity to proteolysis, suggesting that this region is less exposed to solvent when dopamine is bound. In contrast, phosphorylation results in a comparable increase in the protease sensitivity of this region. The activating effect upon replacement of serine with either alanine or valine suggests that the serine hydroxyl contributes to the maintenance of the inhibited conformation of TyrH, presumably via a hydrogen bond. This interaction would clearly be disrupted by phosphorylation of Ser40. It would also be disrupted by loss of the hydroxyl. The effect is less than that of phosphorylation because the latter modification also introduces negative charge into this area. In the few cases where the effects of phosphorylation on the structure of an enzyme have been determined, a common observation is that the phosphate forms an ionic interaction with an arginine residue (*35*). Such an interaction could be mimicked by a glutamate residue but not by alanine or valine.

In conclusion, the characterizations of TyrH Ser40 mutant proteins described here indicate that the activation upon phosphorylation of this residue is due to both the introduction of negative charge and the loss of the serine hydroxyl. The properties of the glutamate and threonine mutant proteins establish that they will be useful as mimics of the phosphorylated and unphosphorylated forms, respectively, of TyrH, while the alanine enzyme will not.

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