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Kinetics of regulatory serine variants of tyrosine hydroxylase with cyclic AMP-dependent protein kinase and extracellular signal-regulated protein kinase 2

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Abstract

Rat tyrosine hydroxylase is phosphorylated at four serine residues, at positions 8, 19, 31, and 40 in its amino terminal regulatory domain by multiple protein kinases. Cyclic AMP-dependent protein kinase phosphorylates S40, which results in alleviation of inhibition by dopamine. Extracellular signal-regulated protein kinase 2 phosphorylates S8 and S31. Site-directed serine-to-glutamate mutations were introduced into tyrosine hydroxylase to mimic prior phosphorylation of the regulatory serines; these proteins were used as substrates for cAMP-dependent kinase and extracellular signal-regulated kinase 2. The activity of cAMP-dependent kinase was unaffected by the substitution of serines 8, 19 or 31 with glutamate and the activity of extracellular signal-regulated kinase 2 was unaffected by substitution of serines 19 or 40 with glutamate. Cyclic AMP-dependent kinase was less active in phosphorylating S40 if dopamine was bound to tyrosine hydroxylase, but extracellular signal-regulated kinase 2 phosphorylation at S31 was unaffected by the presence of dopamine.

Keywords

Tyrosine hydroxylase; cAMP-dependent protein kinase; Extracellular signal-regulated kinase 2; Protein phosphorylation; Feedback inhibition; Catecholamine synthesis

1. Introduction

Tyrosine hydroxylase (TyrH[‡], E. C. 1.14.16.2) is an iron-containing monooxygenase that catalyzes the formation of 3,4-dihydroxyphenylalanine (DOPA) from tyrosine, utilizing oxygen and tetrahydrobiopterin. The reaction is shown in Scheme 1. TyrH is the rate-limiting enzyme in the biosynthesis of dopamine, epinephrine and norepinephrine. Because of its role in determining the levels of the catecholamine neurotransmitters it is highly regulated. Post-translationally TyrH activity is modulated by feedback inhibition by catecholamines and by enzyme phosphorylation [1].

There are four serines in rat tyrosine hydroxylase that become phosphorylated both in vivo and in vitro, at positions 8, 19, 31 and 40 [2–4]. The fact that most vertebrate tyrosine hydroxylases sequenced to this time (fish are the exception) have these sites of phosphorylation [5] indicates that each modification site is of regulatory importance. The other two enzymes in the family of aromatic amino acid hydroxylases, phenylalanine hydroxylase (E. C. 1.14.16.1) and tryptophan hydroxylase (E. C. 1.14.16.4), are also regulated by phosphorylation [1]. The other two enzymes are also the rate-limiting enzymes of important metabolic pathways; PheH, phenylalanine catabolism, and TrpH, serotonin synthesis. All three enzymes consist of a

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catalytic core and an amino terminal regulatory (R) domain [1]. The R domains are of different sizes and are at most 15% similar to each other, but each one contains a serine residue that is phosphorylated by cAMP-dependent protein kinase (PKA). Therefore, all three enzymes evolved from an ancient aromatic amino acid hydroxylase into one finely designed for the regulation of a critical metabolic pathway, by the recruitment and mutation of separate DNAs that code for phosphorylated regulatory domains. Only TyrH has multiple phosphorylation sites in its regulatory domain; presumably the regulation of catecholamine synthesis benefits from several different phosphorylation scenarios.

Particular protein kinases can be assigned to particular serine residues of TyrH. Purified PKA will phosphorylate S40 specifically [6] and agents that increase cAMP levels in cells increase the level of phosphorylation of S40 [2,6,7]. Purified TyrH can be phosphorylated at S19 by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), however, CaMKII also phosphorylates S40 [6,8-10]. In vivo, calcium or agents that increase the levels of intracellular calcium increase the level of phosphorylation of S19 [2,3,11,12] although additional residues are phosphorylated under these conditions [11-13]. In vitro, S31 can be phosphorylated by the MAP kinases ERK1 and ERK 2 [10,14,15]. In cells, phorbol esters and nerve growth factor increase both the activities of ERK1 and ERK2 and the level of phosphorylation of S31 [2,3,7,11,14]. Purified TyrH can also be phosphorylated in vitro at S8 by ERK2, but more slowly than at S31 [16]. S8 is thought to be phosphorylated by a cyclin-dependent kinase in vivo [17]; only phosphatase inhibitors have been found to alter the level of phosphorylation of this residue [2]. This position in the human enzyme has a threonine residue and nothing is known about whether it becomes phosphorylated.

While the sites of modification have been located and at least two kinases for every serine identified, the significance of the multiple phosphorylation sites of TyrH is not yet understood. Considering each site in turn, starting with S8, no in vivo experiment has been reported in which S8 phosphorylation leads to activated TyrH or increased DOPA levels. Furthermore, no in vitro experiment has shown that TyrHpS8 has altered kinetic properties. For S19, phosphorylation itself is not reported to activate TyrH but instead to allow the binding of brain 14-3-3 proteins. This binding by 14-3-3 proteins is reported to activate TyrH 1.5- to 2-fold [18,19]. As for S31, recent work showed that increased DOPA levels are closely linked to ERK phosphorylation at S31 [20]. Moreover, TyrH purified from neural tissues has been reported to be modestly activated (about 1.7-fold) after incubation with ERK2 [14]. However, an effect on the kinetic properties of TyrH has not been discovered for S31 phosphorylation.

Early in vivo experiments suggested strongly that phosphorylation at S40 in response to increased cAMP levels was the major mode of increasing DOPA levels [21,22]. The mechanism of activation by S40 phosphorylation is known. TyrH phosphorylated at S40 (TyrHpS40), as well as the site-directed variant TyrHS40E which mimics TyrHpS40, are resistant to inhibition by dopamine and the other catecholamines [23-25]. Scheme 2 presents the mechanism of the interrelationship between S40 phosphorylation and catecholamine inhibition. TyrH is inhibited reversibly by dopamine and the other catecholamines, whether phosphorylated or not, but if phosphorylated by PKA at S40, the equilibrium lies toward the catecholamine-free enzyme form. This effect is primarily due to the 500-fold faster release of catecholamine from TyrHpS40 [25,26].

Prior to recombinant DNA technology, interpretation of experiments on the activities of TyrHpS8, TyrHpS19 and TyrHpS31 was complicated by the difficulty of obtaining TyrH stoichiometrically phosphorylated at a single site. This complication was partly due to the lack of specificity of the protein kinases, and perhaps partly to the presence of endogenous catecholamines. For example, an in vitro experiment on the effect of TyrH phosphorylation by ERK2 can be complicated by TyrH that is once-phosphorylated at S8, once-phosphorylated at

S31, phosphorylated at both, and totally unphosphorylated. To circumvent this difficulty and determine whether the intrinsic properties of TyrH are altered by phosphorylation, we purified and studied variants of TyrH with a glutamate residue substituted for S8, S19, or S31. The glutamate residues mimic phosphorylated serine residues; glutamate is isosteric with serine-phosphate and is therefore a good first choice for substitution. The variant TyrHS40E is a better mimic of TyrHpS40 than is TyrHS40D [27]. The TyrHS8E, -S19E, and S31E enzymes have modestly enhanced stability (a 1.8-fold decrease in decay rate upon exposure to higher temperature) over wild-type TyrH but they show no alterations in steady-state kinetic behavior or catecholamine binding [28].

Thus, the physiological relevance of the multisite phosphorylation of TyrH remains uncertain. The fact that the glutamate-substituted variants TyrHS8E, -S19E, and -S31E do not display any dramatically altered activity suggests that the effect of TyrH phosphorylation at positions 8, 19, and 31 may involve other macromolecules or effectors. One possibility is that the kinases are stimulated or inhibited by negative charges at the positions of particular serine residues. The present report documents steady-state kinetic analysis of PKA and ERK2 with TyrH and variants of TyrH that contain glutamate in place of the regulatory serines. Using relative V/K values for the kinases as indicators of their substrate specificities for TyrH, deductions are made for the effects of negative charges at the positions of the regulatory serine residues.

2. Materials and methods

Custom oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were from New England Biolabs Inc. (Beverly, MA). *Pfu* DNA polymerase was obtained from Stratagene USA (La Jolla, CA). DNA sequencing was performed using the BigDye kit of ABI (Foster City, CA). Plasmids were purified using kits from Qiagen Inc. (Valencia, CA). 6-Methyltetrahydropterin (6-MPH₄) was purchased from B. Schircks Laboratories (Jona, Switzerland). Leupeptin and pepstatin A were obtained from Peptides International (Louisville, KY). Catalase was obtained from Roche (Gaithersburg, MD). Distilled glycerol was from Invitrogen (Carlsbad, CA). *E. coli* strain BL21 starTM (DE3) from Invitrogen was used for protein expression and XL-10-GoldTM, used for DNA preparations and cloning, was from Stratagene. Dopamine was from Sigma-Aldrich Corp. (St. Louis, MO). Heparin-Sepharose CL-6B was purchased from Amersham-Pharmacia Biotech Inc. (Piscataway, NJ, now GE Healthcare). Nickel NTA HisBind[®] resin was from Novagen Corp (Madison, WI).

2.1. Expression and purification of recombinant proteins

Plasmids for expression of S8A, S8E, S19E, S31A, S31E, and S40E proteins have been previously described [16,23,27,28]. Briefly, the Stratagene QuikChange method was employed to introduce site-directed alterations into pETYH8, a pET23d vector that contains the rat TyrH cDNA, and the coding regions of all plasmids were sequenced to check for unintended mutations. For expression, BL21(DE3) starTM *E. coli* cells were utilized, and expression and enzyme purification were carried out as previously described for the wild-type enzyme [27, 29]. The concentrations of wild-type TyrH and variants were determined using a value of A_{280} of 1.04 [30]. All of the tyrosine hydroxylases were reconstituted with iron and the amount of iron present was determined by atomic absorption spectroscopy as previously described [27,31].

The plasmid for expression of an active 6-histidine-tagged ERK2, pBB131, was kindly provided by Dr. Melanie Cobb of UT-Southwestern; the enzyme was expressed and purified essentially as described previously [32]. The catalytic subunit of PKA was purified from beef heart using the protocol of Flockhart and Corbin [33].

2.2. Phosphorylation of wild-type and variant TyrH by PKA

To measure rates of phosphorylation of TyrH by PKA, a modification of the protocol of Roskoski was used [34]. PKA (2 nM) was incubated with 0.5–30 μM TyrH for 1 min at 30 °C in a 35 μl reaction containing 700 μM ATP, 5 $\mu\text{Ci/pmol}$ [γ - ^{32}P]ATP, 10% glycerol, 10 mM MgCl_2 , 50 mM HEPES, pH 7.0. The reaction was quenched by removal of 25 μl to Whatman phosphocellulose filter paper P-81. The filters were washed three times in 75 mM H_3PO_4 , dehydrated by swirling in acetone for 5 min, and finally air-dried. The amount of ^{32}P incorporated into TyrH was determined by scintillation counting. To determine whether TyrH-bound dopamine affects the phosphorylation of TyrH by PKA, the same protocol was followed except that the TyrH was first incubated with a 1.5-fold molar excess of dopamine for 15 min, followed by assays containing 0.5–30 μM TyrH and 45 μM dopamine.

2.3. Phosphorylation of wild-type and variant TyrH by ERK2

To measure the initial rate of phosphorylation of TyrH, 0.3 μM ERK2 was incubated with 5–50 μM TyrH for 5 min in 0.8 mM ATP, 3 $\mu\text{Ci/pmol}$ [γ - ^{32}P]ATP, 10 mM MgCl_2 , 1 mM DTT, 20 mM HEPES, pH 7.3, at 30 °C [32]. At time points aliquots (100 μl) were removed and mixed with 4 μl of 15 mg/ml bovine serum albumin and 100 μl of 10% trichloroacetic acid (TCA). The samples were incubated for at least 15 min at 4 °C and then centrifuged for 10 min at 32,900 g. The pellets were washed twice with 200 μl 5% TCA and finally resuspended in 100 μl of 1 M NaOH. The amount of ^{32}P incorporated into TyrH was measured using liquid scintillation counting. For the initial rate of phosphorylation in the presence of dopamine, 50 μM TyrH was preincubated with a 1.5-fold molar excess of dopamine for 20 min at 4 °C, followed by assays containing 5 μM TyrH and 10 μM dopamine.

All steady-state kinetic data were fit directly to the Michaelis–Menten equation by non-linear regression using the program KaleidaGraph (Synergy Software, Reading PA).

3. Results

Steady-state kinetic parameters for phosphorylation of TyrH by PKA and ERK2 were determined. In each case, initial rates of phosphorylation were measured with respect to wild-type TyrH concentration. For PKA the plot of v vs. $[\text{TyrH}]$ was the typical hyperbola of Michaelis–Menten kinetics, showing enzyme saturation with substrate (results not shown). Therefore, for PKA, V_{max} and K_{M} values could be obtained. The V/K value for wild-type TyrH is $120 \pm 7 \text{ min}^{-1}$. For ERK2, very high K_{M} values ($>250 \mu\text{M}$) for TyrH made it impossible to attain saturation conditions. Straight lines were obtained for plots of v vs. $[\text{TyrH}]$, from which V/K values can be determined [35]. The ERK2 V/K value for wild-type TyrH is $0.52 \pm 0.006 \text{ min}^{-1}$.

The K_{M} , V_{max} and V/K values of PKA for TyrH and all the serine-to-glutamate variants were easily measured. The K_{M} values for all the TyrH forms are in the micromolar range, consistent with the previously reported K_{M} value for human TyrH [24]. All the variant enzymes are relatively similar substrates as judged by V/K values (Fig. 1). These data show that a negative charge at any of the other serine positions does not vastly affect the rate of phosphorylation of S40 by PKA.

Previous work has shown that dopamine forms a tight complex with TyrH, giving a long-wavelength charge-transfer complex with the enzyme-bound iron [26]. The dopamine binding site can be pinpointed to the region between positions 33 and 50, because rates of trypsinolysis have shown that dopamine alters the enzyme conformation in this region [36]. It has previously been reported that the activity of PKA with TyrH is decreased by dopamine [23,24]. Therefore, the PKA V/K values for TyrH variants were measured after preincubation of TyrH with dopamine. TyrH was preincubated with a slight excess of dopamine to ensure complete

complex formation, and dopamine was included in assay reaction mixtures to maintain the complex. All the TyrH variants were poorer substrates for PKA if complexed with dopamine (Fig. 1). The decreases in the V/K values are 2- to 5-fold.

ERK2 phosphorylates TyrH at two positions, S8 and S31 [16]. Initial rates of phosphorylation of TyrH and the TyrH variants by ERK2 were determined under conditions that would give maximum phosphorylation of wild-type TyrH (1.7 ± 0.08 moles of phosphate per mol of TyrH) at long reaction times. As with wild-type TyrH, initial rates vs. [TyrH] gave straight lines for all the variants rather than hyperbolae. Because K_M values were too high to achieve saturation, V/K values were obtained from the slopes of the lines and appear in Table 1. The 10-fold higher V/K value for TyrHS8E over that for TyrHS31E confirms that TyrHS8E is a 10-fold better substrate than TyrHS31E. ERK2 has similar V/K values for TyrHS31A and -S31E, confirming that loss of S31 renders TyrH a much poorer substrate. Because of the 10-fold preference of ERK2 for S31, phosphorylation of TyrH and the S19 and S40 variants by ERK2 probably occurs for the most part at S31. The V/K values of ERK2 for TyrHS19E and TyrHS40E are very similar to that for wild-type TyrH. In rare cases, non-hyperbolic plots of velocity vs. substrate concentration can be indicative that the enzyme has a low-affinity second substrate site [37,38]. As this is the first report describing the steady-state kinetic properties of purified ERK2 with TyrH, this scenario cannot be absolutely ruled out. However, the V/K values determined here, from the slopes of velocity vs. substrate concentration plots, did not change upon site-directed mutagenesis except in the cases where the serine residue of preference was removed, arguing that the values are indicative of ERK2 substrate specificity.

As noted above, phosphorylation of TyrH by PKA proceeds more slowly in the presence of dopamine, presumably due to a conformational change upon dopamine binding. To see if this conformational change affects the substrate specificity of ERK2 as it does for PKA, the effect of dopamine on phosphorylation of TyrH by ERK2 was studied. Fig. 2 shows the V/K values for ERK2 for the glutamate TyrH variants in the absence and presence of dopamine. No substantial differences were observed. This shows that bound dopamine does not alter the substrate specificity of ERK2 for the various phosphorylated forms of TyrH.

4. Discussion

The present report was undertaken to examine the role prior phosphorylation plays in the reactivity of two protein kinases for TyrH. The project's goal was to separate each kinase's response to prior phosphorylation, and the strategy was to use glutamate substitution to mimic phosphorylation. This had been done previously with TyrHS40E to discover the result of S40 phosphorylation [23]. Glutamate or aspartate substitution for phosphorylated serines or threonines has been very widely used to determine the effects of phosphorylation [39-45]. Each glutamate-substituted TyrH in the present project mimics a TyrH stoichiometrically phosphorylated at a single site. Because ERK2 phosphorylates TyrH at two sites, S8 and S31, additional variants of TyrH were designed to limit phosphorylation to only one serine residue. The alanine-substituted forms of TyrH filled this requirement. It was found that (1) PKA activity for TyrH S40 is relatively unaltered when S8, S19, or S31E are replaced by glutamate residues; (2) ERK2 activity for TyrH S31 is relatively unaltered when S8, S19, or S40 are replaced with glutamate residues; and (3) dopamine binding to TyrH significantly affects PKA but not ERK2 activity toward TyrH, regardless of identity of the amino acid in positions 8, 19, 31, or 40.

The steady-state kinetic analysis of PKA and ERK2 described here provided V/K values for the TyrH variants. Relative V/K values are generally accepted to describe substrate specificity, so a higher V/K value for one of the variants would suggest that that kinase was affected

positively by prior phosphorylation by another kinase, and a lower V/K value would suggest that that kinase was inhibited by prior phosphorylation.

The data in Fig. 1 show clearly that negative charges at positions 8, 19, and 31 have little effect on the V/K values for PKA. Therefore, PKA-catalyzed phosphorylation at S40 is not affected by a negative charge at positions 8, 19, or 31. Because glutamate and phosphoserine are similar in charge and in size, TyrH phosphorylated at S8, S19 or S31 is probably unaffected as a substrate for PKA.

The only significant change for PKA is a drop in the V/K value for TyrH in the presence of dopamine. This finding is consistent with several reports in the literature of wild-type TyrH being less easily phosphorylated by PKA in the presence of dopamine [23,24]. Conversely, TyrH binds dopamine less tightly if it is phosphorylated at S40 [26]. This argues that a conformational change occurs in the R domain of TyrH upon dopamine binding which protects S40 from phosphorylation, and a conformational change that occurs upon S40 phosphorylation prevents dopamine binding. Such a conformational change has been detected previously by trypsin digest [36].

In the case of ERK2, not all changes in the V/K value for a glutamate-substituted TyrH variant signify a change in substrate specificity due to previous phosphorylation. Some changes are due to the loss of one of the two serine residues phosphorylated by ERK2. Because of this, alterations in the V/K value for ERK2 for TyrHS8E and TyrHS31E are predominantly due to the preference of ERK2 for S31. This is manifested in the 10-fold lower V/K value for TyrHS31E and the slightly lower V/K value for TyrHS8E. However, there remains the possibility that prior phosphorylation at S8 or S31 affects ERK2 phosphorylation of the other site. The comparison of alanine-substituted variants to glutamate variants can be used to detect the effect of prior phosphorylation. If S8 phosphorylation were to stimulate ERK2 activity at S31, the V/K value for TyrHS8E would be greater than that for TyrHS8A. However, the relative V/K values for the alanine and glutamate-substituted variants, presented in Fig. 3, demonstrate clearly that negative charges at positions 8 and 31 do not affect ERK2 activity with TyrH.

The V/K values for TyrHS19E and TyrHS40E show further that ERK2 is not greatly affected by introduction of a negative charge at S19 or S40. The V/K value for TyrHS40E is increased by 15% over wild-type TyrH. TyrHS19E is a mimic of TyrH that has been phosphorylated by CaMKII. The V/K value for TyrHS19E is 12% lower than the value for wild-type. These are but slight alterations. Since CaMKII (as well as MAPKAPK-2) phosphorylates both positions S19 and S40, TyrHS19E/S40E was also tested. The V/K value for this double variant was not changed from the value for wild-type TyrH. This is highly indicative that TyrH phosphorylated by PKA, CaMKII or MAPKAPK-2 is no better or worse a substrate for ERK2 than is wild-type TyrH. These results are consistent with the finding that specificity of substrate binding to ERK2 is determined at a docking site far removed from the residue to be phosphorylated [46].

ERK2 does not discriminate between TyrH bound to dopamine or free of dopamine as markedly as PKA. For TyrHS8E, the V/K value in the presence of dopamine is 30% higher than the value in the absence of dopamine, and for TyrHS19E, 23%. However, in consideration of the magnitude of the error for those values, the changes may be as small as 7.5% and 15%. In contrast, the V/K values for PKA decreased 22% to 62% in the presence of dopamine. This is a striking difference between the two kinases. Again, the smaller changes for ERK2 are consistent with ERK2 binding to a distal docking site. A distant site is less likely to be affected by dopamine binding between positions 33 and 50, the region identified by trypsinolysis as the dopamine-binding location [36]. There must be a conformational change that occurs upon S31 phosphorylation, since limited V8 proteolysis of TyrHS31E produces a conformational change

that is not observed in TyrHS40E [28]. This is consistent with a model of two conformational changes affecting the R domain, one upon phosphorylation of S31 and one upon dopamine binding. Glutamate substitution for S19 or S31 does not affect dopamine binding [28]. This also indicates that S40 phosphorylation and dopamine binding are involved in a conformational change that does not affect S19 or S31.

Summarizing, neither PKA nor ERK2 activity for phosphorylation of TyrH is affected by pre-existing negative charges at the positions of the regulatory serines. Phosphorylation of S40 by PKA is regulated by bound dopamine, but phosphorylation of S31 by ERK2 is much less so. These findings make a case for two different conformational changes upon TyrH phosphorylation. The first, at S40, is intricately involved with dopamine feedback inhibition. The second occurs when S31 is phosphorylated. The question still remains as to what is the result on TyrH activity, if any, of that second conformational change. Hierarchical phosphorylation does not appear to be a factor in the activities of two important kinases that regulate TyrH, PKA and ERK2.

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Abbreviations

TyrH, tyrosine hydroxylase
 PKA, cAMP-dependent protein kinase
 CaMKII, Ca²⁺/calmodulin-dependent protein kinase
 ERK2, extracellular signal-regulated kinase 2
 MAPKAPK-2, mitogen-activated-protein-kinase-activated protein-kinase 2
 Cdk5, cyclin-dependent kinase 5
 TCA, trichloroacetic acid
 TyrHpS8, tyrosine hydroxylase phosphorylated at S8
 TyrHpS19, tyrosine hydroxylase phosphorylated at S19
 TyrHpS31, tyrosine hydroxylase phosphorylated at S31
 TyrHpS40, tyrosine hydroxylase phosphorylated at S40
 R domains, regulatory domains
 PH₄, tetrahydropterin
 PH₃OH, C4a-hydroxypterin

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Scheme 1.

Scheme 2.

Fig. 1.

V/K values for PKA for TyrH and TyrH regulatory serine variants in the presence and absence of dopamine. Gray bars represent dopamine-free reactions, and white bars are the V/K value in the presence of dopamine.

Fig. 2.

V/K values for ERK2 for TyrH and TyrH regulatory serine variants in the presence and absence of dopamine. Gray bars represent dopamine-free reactions, and white bars represent the V/K values in the presence of dopamine.

Fig. 3. Comparison of V/K values for ERK2 for TyrHS8A and -S8E, and TyrHS31A and -31E. Gray bars represent the alanine-substituted variants and white bars the glutamate-substituted variants.

Table 1
Steady-state kinetic parameters for wild-type and mutants of TyrH as substrates for ERK2

Substrate	V/K ($\text{min}^{-1} \mu\text{M}^{-1}$)
Wild-type TyrH	0.520 ± 0.006
TyrHS8A ^a	0.550 ± 0.008
TyrHS8E	0.500 ± 0.006
TyrHS19E	0.460 ± 0.004
TyrHS31A ^a	0.060 ± 0.004
TyrHS31E	0.050 ± 0.002
TyrHS40E	0.60 ± 0.07
TyrHS19E/S40E	0.50 ± 0.13

Conditions: 5 to 50 μM TyrH and 0.3 μM ERK2 in 20 mM HEPES, 800 μM ATP, 10 mM MgCl_2 pH 7.3. Assays were carried out for 5 min at 30 °C.

^aFrom reference [16].