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Characterization of Metal Ligand Mutants of Phenylalanine Hydroxylase: Insights into the Plasticity of a 2-Histidine-1-Carboxylate Triad[†]

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Abstract

The iron atom in the nonheme iron monooxygenase phenylalanine hydroxylase is bound on one face by His285, His290, and Glu330. This arrangement of metal ligands is conserved in the other aromatic amino acid hydroxylases, tyrosine hydroxylase and tryptophan hydroxylase. A similar 2-His-1-carboxylate facial triad of two histidines and an acidic residue are the ligands to the iron in other nonheme iron enzymes, including the α -ketoglutarate dependent hydroxylases and the extradiol dioxygenases. Previous studies of the effects of conservative mutations of the iron ligands in tyrosine hydroxylase established that there is some plasticity in the nature of the ligands and that the three ligands differ in their sensitivity to mutagenesis. To determine the generality of this finding for enzymes containing a 2-His-1-carboxylate facial triad, the His285, His290, and Glu330 in rat phenylalanine hydroxylase were mutated to glutamine, glutamate, and histidine. All of the mutant proteins had low but measurable activities for tyrosine formation. In general, mutation of Glu330 had the greatest effect on activity and mutation of His290 the least. All of the mutations resulted in an excess of tetrahydropterin oxidized relative to tyrosine formation, with mutation of His285 having the greatest effect on the coupling of the two partial reactions. The H285Q enzyme had the highest activity as tetrahydropterin oxidase at 20% the wild-type value. All of the mutations greatly decreased the affinity for iron, with mutation of Glu330 the most deleterious. The results complement previous results with tyrosine hydroxylase in establishing the plasticity of the individual iron ligands in this enzyme family.

Phenylalanine hydroxylase (PheH)¹ is a tetrahydropterin-dependent nonheme enzyme that catalyzes the hydroxylation of phenylalanine to tyrosine, an important pathway for phenylalanine catabolism (Scheme 1) [1]. As a liver enzyme, PheH is critical for catabolizing excess phenylalanine in the diet. Consequently, a deficiency in PheH results in the debilitating disease phenylketonuria [2]. PheH belongs to the small family of aromatic amino acid hydroxylases, all of which use tetrahydrobiopterin as the source of electrons to hydroxylate the aromatic ring of an aromatic amino acid. Tyrosine hydroxylase (TyrH) and tryptophan

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¹Abbreviations: PheH, phenylalanine hydroxylase; 6-MePH₄, 6-methyltetrahydropterin; TyrH, tyrosine hydroxylase; TrpH, tryptophan hydroxylase.

hydroxylase (TrpH) are the other two members; these are the rate-limiting catalysts for catecholamine and serotonin biosynthesis, respectively. The three eukaryotic enzymes form homotetramers in solution [3–5]. Each monomer consists of three domains: a regulatory domain at the amino terminus, a catalytic domain and a tetramerization domain at the carboxyl terminus [6,7]. The catalytic domains are homologous and contain all the residues required for catalysis and the determination of substrate specificity [8], while the regulatory domains show low levels of sequence identity, consistent with their diverse regulatory mechanisms [7].

The similar structures of the catalytic domains [9–11] and a variety of mechanistic studies suggest that the three aromatic amino acid hydroxylases share a common catalytic mechanism [12]. For all three enzymes, hydroxylation of the physiological substrate occurs via an electrophilic aromatic substitution reaction with an activated oxygen species [13–15]. In the case of TyrH the latter has been shown to be an Fe(IV) species [16], consistent with the proposed involvement of an Fe(IV)O as the hydroxylating intermediate in all three enzymes [12]. The reactivities of the hydroxylating intermediates in all three enzymes are similar [17] and they have overlapping substrate specificities [18–20], supporting the proposal for a common Fe(IV)O intermediate. The iron atom in each is coordinated by two histidines and a glutamate [9–11,21,22]. This metal-binding arrangement has been named a “2-His-1-carboxylate facial triad” and is also found in a variety of nonheme iron enzymes with different three-dimensional structures, including the extradiol and Rieske dioxygenases, the α -ketoglutarate dependent hydroxylases, and isopenicillin N synthase [23]. The ubiquity of the 2-His-1-carboxylate facial triad suggests that it is ideally suited for formation of a high-valent iron-oxo hydroxylating intermediate [23].

The extent to which the 2-His-1-carboxylate facial triad is optimal for specific reactions should in principle be reflected in the tolerance to substitution of the three amino acid residues in the triad in the different families of enzymes containing this motif. The metal sites of TyrH and PheH are shown in Figure 1. Mutagenesis of either histidine in TyrH to alanine [22] or in PheH to serine [21] is reported to give inactive enzyme. However, in the case of TyrH, less drastic mutations give more interesting results [24]. Both the H336E and H336Q enzymes retain significant activity at tyrosine hydroxylation. H331E TyrH is unable to hydroxylate tyrosine, but is able to catalyze tetrahydropterin oxidation in the absence of tyrosine. Finally, both E376Q and E376H TyrH retain low activity for tyrosine hydroxylation. This very limited series of results suggests that the different ligands in the 2-His-1-carboxylate triad differ in their plasticity. As a test of this hypothesis for the aromatic amino acid hydroxylases in general, the effects of mutating the iron ligands in PheH to histidine, glutamate or glutamine have now been determined.

EXPERIMENTAL PROCEDURES

Materials

Leupeptin and pepstatin A were from Peptides Institute, Inc. (Osaka, Japan). Glycerol was from EM Science. Hepes was purchased from USB (Cleveland, Ohio). Tetrahydrofuran, 2, 3-naphthalene dicarboxaldehyde and dihydropteridine reductase (DHPR) were from Sigma. Restriction and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA) and Promega (Madison, WI). Tween-80 and 6-methyltetrahydropterin (6-MePH₄) were from Calbiochem (San Diego, California). Phenyl-Sepharose CL-4B was purchased from Amersham Biosciences (Sweden). Catalase was from Roche Pharmaceuticals (Nutley, N.J.).

Mutagenesis

The wild type rat phenylalanine hydroxylase cDNA-containing plasmid pERPH5 was described previously [8]. This plasmid was used as a template to construct plasmids for expression of PheH mutants. Site-directed mutagenesis was carried out with the Stratagene QuikChange Kit using *pfu* DNA polymerase. The mutagenic oligonucleotides were from Integrated DNA Technologies, Inc. The sequences of the oligonucleotides and the corresponding new restriction cleavage sites created are given in Table 1. QIAfilter plasmid midi prep kits and QIAprep Spin miniprep kits were used for purifying plasmids, which were used to transfect OmniMax competent cells from Invitrogen (Carlsbad, California). Mutant plasmids were sent to the Gene Technology Laboratory of Texas A&M University for DNA sequencing to confirm the mutations.

Bacterial cell growth and protein purification

Mutant and wild-type PheH were expressed in C41(DE3) *E. coli* and purified on a Phenyl-Sepharose CL-4B column according to the protocol of Daubner et al. [8], except for the use of 50 mM Hepes and 15% glycerol instead of 50 mM Tris-HCl and 10% glycerol in the elution buffer. In addition, 50 μ M EDTA was added to the elution buffer. Cells expressing wild-type PheH were grown at 37 °C, while cells expressing the mutant enzymes were grown at 22 °C after induction. The purified proteins were concentrated and stored in 50 mM Hepes, 15% glycerol, 1 μ M leupeptin and 1 μ M pepstatin A, pH 7.0, at -80 °C.

Assays

For wild type PheH, the formation of tyrosine from phenylalanine was determined by monitoring the absorbance increase at 275 nm [25]. For the mutant enzymes, a more sensitive HPLC assay [18,26] was used. The standard conditions were 5 mM dithiothreitol, 50 mM Hepes, pH 7.0, at 25 °C, and varied amounts of ferrous ammonium sulfate, phenylalanine and 6-MePH₄ as indicated in figure and table legends. The total volume of each assay was 100 μ l. The assays were quenched after 1 min and derivatized with 2, 3-naphthalene dicarboxaldehyde before being injected onto a 2.1 $\times$ 150 mm Waters Nova-Pak C18 column with fluorescence detection.

A coupled assay with dihydropteridine reductase and NADH was used to measure tetrahydropterin oxidation [8]. The standard condition was 30 mM Hepes, pH 7.0, 250 μ M NADH, 200 μ M 6-MePH₄, 4 mM phenylalanine, 0.2 mM ferrous ammonium sulfate, 0.1 mg/ml catalase, and 0.1 unit/ml dihydropteridine reductase at 25 °C. The total volume was 1 ml. The amount of 6-MePH₄ oxidation was determined by monitoring the decrease of absorbance at 340 nm and correcting for autoxidation of 6-MePH₄. In order to determine the ratio of phenylalanine hydroxylation to 6-MePH₄ oxidation, a 100 μ l aliquot was removed from the cuvette after 1 min and quenched with 100 μ l 10 mM NaCN, pH 9; 100 μ l 10 mM 2, 3-naphthalene dicarboxaldehyde in methanol; and 0.34 ml 10 mM sodium borate buffer, pH 9; and then analyzed by HPLC to measure tyrosine. Steady-state kinetic data were fit to the Michaelis-Menten equation using the program KaleidaGraph (Synergy Software, PA).

RESULTS

Expression and purification of PheH mutant proteins

The three iron ligands of PheH, His285, His290 and Glu330, were each replaced with glutamate, histidine or glutamine using site-directed mutagenesis. All the mutant enzymes were expressed at significantly higher levels in C41(DE3) *E. coli* than in BL21(DE3). A significant amount of protein was found in insoluble inclusion bodies for all of the mutant proteins; this could be minimized by growth at 22 °C. The yield of the mutant enzymes was about 1 mg of

purified protein per liter of bacterial culture, significantly less than the typical 20 mg/l of the wild type enzyme.

Kinetic characterization of PheH mutant proteins

The activities of all of the mutant proteins were significantly less than that of the wild-type enzyme. Still, by using a sensitive HPLC-based assay it was possible to determine the kinetic parameters of most of the mutant proteins. While PheH requires Fe(II) for activity, the affinity of the wild-type enzyme for iron is high enough that the enzyme is fully active even in the absence of added iron. However, the activities of all the mutant proteins are clearly iron-dependent (Figure 2). Mutation of Glu330 has the greatest effect on the value of K_{Fe} , the concentration of ferrous ion required for half-maximal activity, while the magnitude of the effects of mutating the two histidines differ with the individual mutation (Table 2). Even in the presence of saturating concentrations of Fe(II), the apparent V_{max} values for the H285E and E330Q enzymes decrease by 10^5 -fold from the wild-type value. Because of these very low activities, further kinetic characterization of these two enzymes was not attempted. The K_m values for phenylalanine and 6-MePH₄ for the remaining mutant enzymes are given in Table 2. Mutation of any of the iron ligands increases the K_{6MePH_4} value about one order of magnitude. The K_{phe} values of mutants similarly increase 3–16 fold. The K_{phe} value of H285Q PheH is the largest at about 15-fold the wild-type value. H290E and E330H PheH display more modest increases.

Hydroxylation stoichiometry of PheH mutant proteins

The activity of PheH can be determined by following the formation of tyrosine or of dihydropterin. For the wild-type enzyme these yield the same result, since phenylalanine hydroxylation is tightly coupled to tetrahydropterin oxidation. This need not be the case for mutant proteins, where unproductive breakdown of an iron-oxygen intermediate to release hydrogen peroxide can occur without hydroxylation of the amino acid. The relative stoichiometries of tyrosine formation and 6-MePH₄ oxidation for each of the enzymes were determined using a coupled assay with NADH-dependent dihydropteridine reductase to measure 6-MePH₄ oxidation and an HPLC assay to determine the amount of tyrosine formed in the same reaction mixture. All of the enzymes oxidized an excess of 6-MePH₄ relative to the amount of tyrosine formed (Table 3). The His285 mutant enzymes exhibited the greatest uncoupling of 6-MePH₄ oxidation and phenylalanine hydroxylation, while the two partial reactions were most tightly coupled with the His290 mutant enzymes. Knowledge of these stoichiometries made it possible to calculate the V_{max} value for 6-MePH₄ oxidation by each of the mutant enzymes by dividing the V_{max} value for tyrosine formation by the stoichiometry (Table 3).² Based on the ability to catalyze 6-MePH₄ oxidation, the H285Q enzyme is only 5-fold less active than the wild-type enzyme, while the H285E enzyme is another 100-fold less active. In contrast, the 6-MePH₄ oxidation activities of the other four mutant enzymes are comparable at two orders of magnitude less than the wild-type value.

DISCUSSION

The conservative mutations of the iron ligands in PheH described here affect virtually all aspects of catalysis. Mutagenesis of the metal ligands appears to have effects on protein stability in addition to the effects on catalysis. All of the mutant proteins described here readily form

²It is possible to determine the V_{max} value for 6-MePH₄ oxidation directly by varying the concentration of 6-MePH₄ at a saturating concentration of phenylalanine using the NADH-linked assay. However, the autooxidation of 6-MePH₄ results in a significant background oxidation of NADH in this assay. With wild-type PheH this is not a problem, but the very high K_{6MePH_4} and K_{Fe} values of the mutant enzymes require using concentrations of 6-MePH₄ and ferrous ammonium sulfate that result in very high rates of autooxidation, dramatically limiting the precision of the initial rate measurements. In contrast, the stoichiometry is independent of the substrate concentrations, allowing lower concentrations to be used in order to minimize background autooxidation.

inclusion bodies when expressed at 37 °C, in contrast to the wild-type enzyme, and yield lower amounts of active protein even when grown at lower temperatures. Moreover, attempts to obtain enzymes containing multiple mutations were not successful (results not shown). The lower yields of the mutant proteins are probably not due to the loss of a stabilizing effect of bound iron, since wild-type versions of the aromatic amino acid hydroxylases can readily be isolated in large amounts even when containing substoichiometric iron or in the apo form [20,27–29]. Instead it suggests that interactions of the side chains of the metal ligands with other residues in the protein are important for structural integrity. A corollary of this is that other residues in the protein play a significant role in properly positioning the metal ligands for metal binding and reactivity.

Still, the results do allow some general distinctions to be drawn about the plasticity of the individual residues, especially when combined with the results of similar mutations in TyrH [24]. The results also show that there are significant differences in the plasticity of these residues in the two enzymes. In combination, the two mutations of Glu330 in PheH have the greatest effects on the $V_{\max}$ value for 6-MePH₄ oxidation and tyrosine hydroxylation and intermediate effects on the coupling of these two partial reactions.³ These two mutations also have the greatest effects on iron affinity as measured by the dependence of activity on the iron concentration. A qualitatively similar result was obtained with TyrH, in that the Glu376 mutant enzymes were on average the least active. Both structural [30] and spectroscopic [31] studies of the iron site in PheH show that Glu330 is a monodentate ligand to the metal in the resting enzyme, but binds in bidentate fashion once both amino acid and tetrahydropterin are bound. However, the structure of TyrH with an amino acid bound has not been determined, so there is no direct evidence to date that the glutamate in that enzyme must be able to bind the iron with both carboxylate oxygens. The present results with PheH are consistent with the need for a negative charge at this position for iron binding and for the ability to act as a bidentate ligand for efficient formation of the Fe(IV)O intermediate and its reaction with the amino acid substrate in that enzyme. The similarities in the effects of mutating this residue in TyrH and PheH support a similar requirement for bidentate binding in TyrH.

Mutations of His290 yield the highest $V_{\max}$ values for tyrosine formation, with mutation to glutamate resulting in greater uncoupling of the two reactions. A similar result was found for TyrH, in that the H336Q enzyme was the most active mutant protein of those examined, although the activity in that case was much higher at one-third the activity of the wild-type enzyme. In general, mutation of His290 in PheH to glutamate has a much more deleterious effect than mutation to glutamine. The K_{Fe} value for the H290E enzyme is two orders of magnitude greater than that of H290Q PheH. This likely reflects the need for conservation of charge for iron binding. The greater effect of the H290E mutation on the K_m value for phenylalanine also likely reflects the detrimental introduction of a negative charge into the hydrophobic active site. In contrast, the coupling of 6-MePH₄ oxidation and phenylalanine hydroxylation is disrupted the least in these two mutant proteins, although substitution with glutamate again is more detrimental. This is consistent with proper positioning of the amino acid in the active site once it binds, despite the lower affinity.

The two mutations of His285 also have disparate effects. His285 plays two roles in the active site of PheH. In addition to being a metal ligand, the imidazole ring of His285 stacks on the side chain of the amino acid substrate. When the homologous residue in TyrH, His331, is mutated to glutamate, binding of the amino acid substrate is eliminated and the enzyme becomes a tetrahydropterin oxidase [24]. In that case it was not possible to characterize the effect of a mutation to glutamine. With PheH, the activity of the H285E enzyme at tyrosine

³For PheH hydroxylation is rate-limiting (Pavon, J. A., and Fitzpatrick, P. F., unpublished observations and [15]), so that changes in $V_{\max}$ reflect changes in rate constants for chemical steps rather than product release.

formation is too low to measure a K_{phe} value, but the tetrahydropterin oxidation activity does require the amino acid substrate. Thus, the dramatic loss of the need for binding of an amino acid substrate to activate the iron for reaction with oxygen is not seen when this residue is mutated in PheH. H285Q PheH enzyme has the highest K_{phe} value of the four mutant proteins with enough activity to measure this value, consistent with disrupted phenylalanine binding. Both of these mutant proteins exhibit very low coupling of 6-MePH₄ oxidation to tyrosine formation, again consistent with altered positioning of the amino acid substrate in the active site. The H285Q enzyme is quite active as a phenylalanine-dependent tetrahydropterin oxidase, while the H285E enzyme has activity comparable to that of the two His290 mutant proteins. Overall, mutation of either histidine residue is less disruptive of tetrahydropterin oxidation than is mutation of the glutamate, consistent with the critical importance of the change to bidentate binding of this residue for subsequent reactions.

The plasticity in the metal ligands in PheH is much less than is the case for TyrH [24]. In both enzymes, mutagenesis of the glutamate residue is generally more disruptive of catalysis than is mutation of either histidine. Replacement of either histidine with glutamate is more disruptive than replacement with glutamine, consistent with the more conservative nature of the latter change. Of the two histidines, His290 of PheH is less sensitive to mutagenesis than is His285; a similar situation was found for the homologous residue in TyrH. However, mutating His285 in PheH does have the same effect on the need for an amino acid substrate that mutation of the analogous mutation has in TyrH, establishing this effect as specific to TyrH instead of a reflection of the general plasticity of the iron ligands in this enzyme family. The iron ligands of TyrH and PheH are virtually superimposeable on one another (Figure 1) and the two enzyme share overlapping substrate specificities [8,32]. Indeed, mutating Asp425 in TyrH to valine converts it to an enzyme which is a better catalyst for converting phenylalanine to tyrosine than is wild-type PheH [32]. Asp425 does not interact directly with either substrate or with the iron in TyrH, so that the ability to convert the enzyme to PheH establishes that there is no difference in the reactivities of the iron site in the two enzymes. PheH has a much more restricted substrate range than does TyrH [8,33] and binds iron more tightly. These properties may reflect an active site that is optimized for iron binding and amino acid hydroxylation to a greater extent in PheH than in TyrH and that is consequently less tolerant of any disruption.

The α -ketoglutarate-dependent family of non-heme monooxygenases also contains a 2-His-1-carboxylate metal site, although the acidic residue is aspartate. The effects of mutating the metal ligands in a number of these enzymes have been determined, as summarized in Grzyska et al. [34]. In many cases residual activities of <1% were not measurable, limiting the conclusions one can draw. However, even with this caveat there are discernable differences in the sensitivities to substitution of the three metal ligands in this enzyme family. Using the numbering of TauD, mutation of His99 to other residues generally yields enzyme with no detectable activity.⁴ In TauD the H99E and H99Q enzymes have V_{max} values of about 0.5% the wild-type enzyme. His99 is coplanar with the α -ketoglutarate substrate in the active site, so that mutation of this residue would also be expected to disrupt substrate binding analogously to the effects of mutating His285 in PheH. In contrast, mutation of His255 in TauD and related enzymes yields enzyme with significant activity in a number of cases. For example, H255Q and H255E TauD have V_{max} values that are 89 and 36% the wild-type values. Finally, mutation of the aspartate residue to anything but glutamate generally yields enzyme with no reported activity. Thus, the general pattern for the α -ketoglutarate-dependent family of hydroxylases is similar to that with the pterin-dependent enzymes. The acidic residue in the 2-His-1-carboxylate facial triad is very sensitive to mutagenesis. Of the two histidine residues, the residue interacting directly with a substrate, either α -ketoglutarate or the aromatic amino acid,

⁴However, Galonic Fujimora et al. [35]. have reported that H99A TauD has substantial activity. The reason for this discrepancy is not clear.

is also not tolerant to replacement with other amino acids, while the other, His285 here and His255 in TauD, is quite plastic, tolerating replacement with glutamine or glutamate.

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Figure 1. Iron ligands in phenylalanine hydroxylase (magenta carbons) and tyrosine hydroxylase (gray carbons). The structures are from the pdb files 1PAH and 1TOH.

Figure 2. Iron dependence of tyrosine formation by phenylalanine hydroxylase mutant enzymes: A, E330H (open triangles), E330Q (filled triangles), and H285E (filled squares); B, H290Q (open circles), H285Q (open squares), and H290E (filled circles).

Scheme 1.

Table 1Oligonucleotides used to perform site-directed mutagenesis^a

mutation	Oligonucleotide sequence	New restriction site
H285E	5'- gaa cct gac ATA tgg GAG ctc ttg gg - 3'	NdeI
H285Q	5'-cct gac ata tgc CAG gaa ctc ttg gga cat- 3'	NdeI
H290E	5'-gc cat gaa ctc ttg gga GAG GTA cct ttg ttt tca g- 3'	Acc651
H290Q	5'-gaa cct GAT atc tgg cat gaa ctc ttc gga CAG gtg cct- 3'	EcoRV
E330H	5'-c tgg ttt act gtg CAC ttt ggg ctt tgc- 3'	ApalI
E330Q	5'-tgg ttt act gtg CAG ttt GGT CTC tgc aag gaa gga- 3'	BsaI

^aThe changes from the wild-type sequence are capitalized.

Table 2
Steady-state kinetic parameters of for phenylalanine hydroxylation by PheH mutant enzymes*

kinetic parameter	wt PheH ^a	H285E ^b	H285Q ^c	H290E ^d	H290Q ^b	E330H ^b	E330Q ^b
$V_{\max}$ (min ⁻¹)	399 ± 14	0.035 ± 0.003	2.0 ± 0.15	1.3 ± 0.4	4.3 ± 0.1	0.35 ± 0.07	0.035 ± 0.009
K_{Fe} (mM)	N/A	0.16 ± 0.06	0.60 ± 0.11	3.00 ± 1.4	0.03 ± 0.01	3.9 ± 1.5	3.2 ± 1.9
K_{6MePH_4} (mM)	0.05 ± 0.01	ND	0.82 ± 0.40	0.23 ± 0.04	0.38 ± 0.11	0.25 ± 0.074	ND
K_{phe} (mM)	0.43 ± 0.04	ND	6.9 ± 3.1	3.4 ± 2.1	1.2 ± 0.4	1.6 ± 0.4	ND

* Commonly used conditions: 5 mM dithiothreitol, 50 mM Hepes, pH 7.0, at 25°C.

^a 0.2 mM ferrous ammonium sulfate, 2 mM phenylalanine, 0.2 mM 6-MePH₄.

^b 4 mM ferrous ammonium sulfate, 5 mM phenylalanine, 2mM 6-MePH₄.

^c 2 mM ferrous ammonium sulfate, 12 mM phenylalanine, 2 mM 6-MePH₄.

^d 6 mM ferrous ammonium sulfate, 8mM phenylalanine, 2mM 6-MePH₄.

^e ND, not determined.

Table 3Coupling of phenylalanine hydroxylation to 6-MePH₄ oxidation for wild-type and mutant PheH enzymes*

Enzyme	Phenylalanine hydroxylation/6-MePH ₄ oxidation ^a	6-MePH ₄ oxidation V _{max} (min ⁻¹) ^b
Wild-type PheH	0.99 ± 0.05	403 ± 24
H285E	0.062 ± 0.009	0.68 ± 0.11
H285Q	0.031 ± 0.008	67 ± 16
H290E	0.15 ± 0.03	8.7 ± 2.6
H290Q	0.42 ± 0.06	10 ± 1.4
E330H	0.12 ± 0.03	1.8 ± 0.48
E330Q	0.079 ± 0.017	0.44 ± 0.15

* 30 mM Hepes, pH 7.0, 250 μM NADH, 200 μM 6-MePH₄, 4 mM Phenylalanine, 0.2 mM ferrous ammonium sulfate, 0.1 mg/ml catalase, 0.1 unit/ml dihydropteridine reductase.

^a Determined from the amount of 6-MePH₄ oxidized in 1 min as measured using an NADH-linked assay and the amount of tyrosine produced in the same sample in 1 min as measured by HPLC.

^b Calculated from the value for (phenylalanine hydroxylation/6-MePH₄ oxidation) and the V_{max} for phenylalanine hydroxylation.