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

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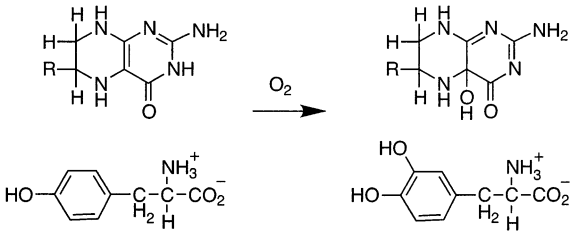
Received November 11, 2002; Revised Manuscript Received January 9, 2003

ABSTRACT: The amino acid ligands to the active site iron in the aromatic amino acid hydroxylase tyrosine hydroxylase are two histidines and a glutamate. This 2-histidine-1-carboxylate motif has been found in a number of other metalloenzymes which catalyze a variety of oxygenase reactions. As a probe of the plasticity of this metal binding site, each of the ligands in TyrH has been mutated to glutamine, glutamate, or histidine. The H336E and H336Q enzymes show dramatic decreases in iron affinity but retain substantial activity for both tyrosine hydroxylation and tetrahydropterin oxidation. The H331E enzyme shows a lesser decrease in iron affinity and is unable to hydroxylate tyrosine. Instead, this enzyme oxidizes tetrahydropterin in the absence of added tyrosine. The E376H enzyme has no significant activity, while the E376Q enzyme hydroxylates tyrosine at about 0.4% the wild-type rate. When dopamine is bound to either the H336Q or H331E enzymes, the position of the long wavelength charge-transfer absorbance band is consistent with the change in the metal ligand. In contrast, the H336E enzyme does not form a stable binary complex with dopamine, while the E376H and E376Q enzymes catalyze dopamine oxidation.

Tyrosine hydroxylase (TyrH)[1] is a tetrahydropterin-dependent monooxygenase that catalyzes the rate-determining step in the biosynthesis of catecholamines, the hydroxylation of tyrosine to L-dihydroxyphenylalanine (DOPA) (*1*). The enzyme utilizes tyrosine, a tetrahydropterin, and molecular oxygen as substrates; the reaction products are DOPA and a 4a-hydroxytetrahydropterin (Scheme 1). Phenylalanine hydroxylase (PheH) and tryptophan hydroxylase, the two other tetrahydropterin-dependent enzymes that belong to this family, have catalytic domains that are homologous to that of TyrH (*1*, *2*). All three enzymes contain one iron atom per monomer, which is essential for hydroxylation of the amino acid substrate (*3*−*5*). The protein ligands to the iron in both tyrosine hydroxylase and phenylalanine hydroxylase have been identified by site-directed mutagenesis and direct structure determination as two histidines and a glutamate (*6*−*9*). The arrangement of iron ligands in TyrH has also been found in a number of other enzymes and has been termed a 2-His-1-carboxylate facial triad by Que (*10*).

The increasing number of enzymes which contain the 2-histidine-1-carboxylate triad suggests that this arrangement of metal ligands provides unique advantages to iron-dependent oxygenases. Complete understanding of the specific properties of this set of ligands which contribute to its differential reactivities will require a combination of biochemical and biomimetic studies of the different enzyme classes. The present report describes the effects of conservative mutagenesis of the iron ligands in TyrH on catalysis, iron binding, and binding of substrates and inhibitors. The results should provide a reference point for further structural studies of the pterin-dependent enzymes and for similar studies of other enzymes sharing this iron-binding motif.

Scheme 1



EXPERIMENTAL PROCEDURES

Materials. Custom oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer by the Gene Technology Laboratory of Texas A&M University. Restriction endonucleases were from New England Biolabs Inc. Pfu DNA polymerase was obtained from Stratagene USA. Plasmids were purified using kits from Qiagen Inc. 6-Methyltetrahydropterin was purchased from Schircks Laboratories. Leupeptin, pepstatin, and catalase were from Boehringer Mannheim Corp. Sheep dihydropteridine reductase, L-tyrosine, dopamine, NADH, and cytochrome *c* were from Sigma Chemical Co. Heparin-Sepharose was from Pharmacia Biotech Inc. *o*-Phthaldehyde and 3-mercaptopropionic acid

† This research was supported in part by NIH Grants R01 GM47291 (to PFF), F32 GM20116 (HRE), and T32 GM08523 (ER) and an Office of Naval Research Interns in Biomolecular Sciences Summer Research Program (OJW).

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[1] Abbrevations: TyrH, tyrosine hydroxylase; PheH, phenylalanine hydroxylase; 6-MePH$_4$, 6-methyltetrahydropterin.

10.1021/bi0271493 CCC: $25.00
Published on Web 01/29/2003

Table 1: Oligonucleotides Used to Perform Site-Directed Mutagenesis[a]

mutation	oligonucleotide sequence	restriction digest change
H331E	5′-gcc gga ctg ctg cGA Aga gct gtt ggg ac-3′	loss of SapI site
H336E	5′-gag ctg ttg gga GAG gta ccc atg ttg gc-3′	gain of a Kpn1 site
E376H	5′-ctg gtt cac tgt gCA Ctt cgg gct atg-3′	loss of an EcoRI site
E376Q	5′-ctg gtt cac tgt gCA Att cgg gct atg-3′	loss of an EcoRI site

[a] The changes from the wild-type sequence are capitalized.

were from Aldrich Chemical Co. Superoxide dismutase was from Worthington Biochem. Co.

Vectors for Enzyme Expression. The preparation of pETYHH331A, pETYHE331Q, pETYHE336A, and pETYHH336Q has been previously described (*7*). Construction of the pETYHH331E, pETYHH336E, pETYHE376H, and pETYHE376Q plasmids was done with the Stratagene QuikChange Kit using Pfu DNA polymerase and plasmid pETYH8 as the template (*11*). pETYHE376H was similarly used as a template for construction of pETYHH331E/E376H and pETYHH336E/E376H. Mutated plasmids were detected by electrophoretic analysis of restriction digests. The mutagenic oligonucleotides and the restriction enzymes used to screen for mutations are summarized in Table 1. In all cases, the entire coding region was sequenced to confirm the desired mutation.

Bacterial Cell Growth and Protein Purification. Bacterial cells expressing wild-type rat TyrH were grown as previously described (*12*). Mutant proteins were obtained by introducing the appropriate plasmids into competent E. coli BL21(DE3) cells. Cell growth and protein purification were as previously described for other TyrH mutant proteins (*13*) with the following modifications. After induction with IPTG, cells were grown at 20 °C for 14−18 h. Nucleic acid precipitation was done with 2% (w/v) streptomycin sulfate. The H331Q and the H336E/E376H mutant proteins could not be expressed, even at these lower temperatures; consequently, these proteins were not characterized. Purified proteins were stored in 50 mM HEPES, 10% glycerol, 100 mM KCl, pH 7, at −80 °C.

Assays. The iron contents of all enzymes were determined by atomic absorbance spectroscopy as previously described (*14*), except that all samples and standards were prepared in a final concentration of 0.25 M nitric acid. Enzyme concentrations were determined from their absorbance at 280 nm (*15*). Two assays were used to measure the rate of DOPA formation from tyrosine. In the case of enzymes with moderate activity (>5% wild type), a colorimetric end point assay was used as previously described (*11*). Standard assay conditions were 400 μM 6-MePH$_4$, 170 μg/mL catalase, 1.7 mM dithiothreitol, 250 μM tyrosine, 10−300 μM ferrous ammonium sulfate as indicated in the figure and table legends, and 70 mM HEPES/NaOH, pH 7, 25 °C. For enzymes with lower activity, an HPLC assay was used. The assay conditions were the same as for the colorimetric assay. Reactions were run for 2−5 min and quenched by the addition of concentrated sodium borate, pH 9.5, to give a final concentration of 73 mM. The amount of DOPA produced was measured by HPLC analysis of the *o*-phthaldehyde derivative; the conditions for this reaction were as described previously (*16*). The HPLC elution conditions were 3 min of 100% 12.5 mM sodium phosphate, 0.5% trifluoroacetic acid, pH 7.0 (solvent A), a 3 min linear increase to 20% acetonitrile/80% A, and a final 19 min linear increase to 40% acetonitrile/60% A at 0.2 mL/min, using a 2 × 150 mm Waters NovaPak C18 column. The modified amino acids were detected using a Waters Model 470 fluorescence detector, with an excitation wavelength of 344 nm and an emission wavelength of 450 nm. Under these conditions, the elution time for DOPA is 21.5 min. The rate of tetrahydropterin oxidation was determined under similar assay conditions, coupling dihydropterin formation to NADH consumption with dihydropteridine reductase and measuring the decrease in absorbance at 340 nm, as previously described (*17*). Standard assay conditions were 0.2−0.4 units/mL sheep dihydropteridine reductase, 100 μM 6-MePH$_4$, 170 μg/mL catalase, 250 μM tyrosine, 100 μM NADH, 130 μM ferrous ammonium sulfate, 70 mM HEPES/NaOH, pH 7, 25 °C. When the concentration of either the metal or a substrate was varied, steady state kinetic data were fit directly to the Michaelis−Menten equation using the program Kaleidagraph (Adelbeck Software, Reading, PA).

To obtain the enzyme-dopamine complex, proteins (200 μM) were incubated with a stoichiometric amount of dopamine for a minimum of 10 min in 50 mM HEPES/NaOH, 10−30%glycerol, pH 7, at 20 °C. For those proteins which contained a substoichiometric amount of iron (H336Q, H336E, E376Q, and H331E/E376H), ferrous ammonium sulfate was then added in 10 μM aliquots to yield a stoichiometric equivalent of iron. The same complex could be formed with the H336Q enzyme (200 μM) if iron was added first to a final concentration of 250 μM. Catalase (0.125 mg/mL) was also added in the cases of the E376H and E376Q enzymes. After each addition, the absorbance spectrum was monitored from 250 to 800 nm until no further changes were seen.

RESULTS

Iron Content of Mutant Proteins. The three protein ligands to the iron in TyrH are His331, His336, and Glu376. When either histidine residue is mutated to alanine or glutamine, the mutant protein is essentially iron free and lacks significant activity in a standard tyrosine hydroxylase assay (*7*); this result previously allowed identification of these residues as iron ligands prior to the availability of a three-dimensional structure. As a more extensive probe of the plasticity of the 2-histidine-1 carboxylate triad in this enzyme, each of the three residues was individually replaced with glutamate, glutamine, or histidine; all but the H331Q enzyme were successfully expressed and purified. In addition, the expression constructs for both the His331E/E376H and His336E/E376H enzymes were made, but only the former enzyme could be expressed successfully.

The effects of the mutations on the iron contents of the individual proteins are shown in Table 2. The iron content is not significantly altered by the mutation of Glu376 to

Table 2: Iron Content and Relative Catalytic Activities of TyrH Mutant Proteins

enzyme	fe:monomer	pterin oxidation (%)[a]	tyrosine hydroxylation (%)[b]
H331E	1.2 ± 0.2	2.4 ± 0.4	<0.002
H336Q	0.050 ± 0.003	11.9 ± 1.3	3.7 ± 0.5
H336E	0.51 ± 0.13	6.3 ± 1.5	0.78 ± 0.17
E376Q	0.83 ± 0.13	0.4 ± 0.1	0.39 ± 0.07
E376H	1.2 ± 0.3	<0.4	0.0027 ± 0.0011
H331E/E376H	0.21 ± 0.08	<0.4	<0.002

[a] Percentage of wild-type value of 2.5 s^{-1} (*11*). Conditions were 0.2−0.4 units/mL sheep dihydropteridine reductase, 100 μM 6-methyltetrahydropterin, 170 μg/mL catalase, 250 μM tyrosine, 100 μM NADH, 130 μM ferrous ammonium sulfate, 70 mM HEPES/NaOH, pH 7, 25 °C. [b] Percentage of wild-type value. Conditions were 170 μg/mL catalase, 1.7 mM dithiothreitol, 400 μM 6-methyltetrahydropterin, 250 μM tyrosine, 130 μM ferrous ammonium sulfate, and 70 mM HEPES/NaOH, pH 7, 25 °C. Rates were determined by HPLC analysis, with the exception of H336Q, which was assayed using a colorimetric assay as described in Experimental Procedures.

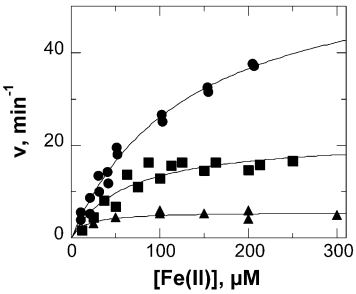


FIGURE 1: Iron dependence of activity of TyrH mutants: H336Q (circles); H336E (squares); H331E (triangles). The lines were obtained by fitting the Michaelis−Menten equation to the data. For the H336Q, rates of tyrosine hydroxylation were measured using the colorimetric assay described in Experimental Procedures. For the H336E and H331E enzymes, the rate of pterin oxidation was measured as described in Experimental Procedures.

histidine or glutamine or by the mutation of His331 to glutamate. In contrast, mutation of His336 decreases the iron content, with the H336Q enzyme being effectively iron-free. Although both the H331E and the E376H enzyme have normal levels of iron, the double mutant H331E/E376H contains significantly less iron.

Catalytic Activity. None of the recombinant enzymes had significant activity in a standard tyrosine hydroxylation assay (results not shown). However, the low iron contents of some of the mutant proteins raised the possibility that the lack of activity was due to a lack of iron. Consequently, the abilities of the mutant enzymes to catalyze tyrosine hydroxylation were determined in the presence of 130 μM Fe(II).[2] To extend the sensitivity of our measurements, an HPLC-based assay was used for mutants having less than 5% residual activity in tyrosine hydroxylation. The ability of these enzymes to catalyze tetrahydropterin oxidation was also measured, since a number of active site mutants of TyrH have been described in which the effect on the ability to catalyze tyrosine hydroxylation is much greater than the effect on the ability to catalyze tetrahydropterin oxidation (*11*, *13*, *18*). The results of these assays are given in Table 2. Both enzymes in which His336 had been mutated had substantial activity as tyrosine-dependent tetrahydropterin oxidases in the presence of high concentrations of iron. The H336Q enzyme also was able to hydroxylate tyrosine at a substantial rate, although less than the rate of pterin oxidation. Thus, one effect of this mutation is to partially uncouple tetrahydropterin oxidation from tyrosine hydroxylation, so that a fraction of reducing equivalents is consumed unproductively. Replacement of His336 with glutamate has a more drastic effect on the coupling of pterin oxidation and amino acid hydroxylation than does replacement with glutamine. Replacement of His331 with glutamate results in an enzyme with some ability to oxidize pterin and a decrease in the ability to hydroxylate tyrosine of at least 50 000-fold. Thus, this mutation partially and probably completely uncouples pterin oxidation and tyrosine hydroxylation. Both mutations of Glu376 greatly decrease the catalytic activity. In the case of the E376Q enzyme, there is low but detectable activity in the pterin oxidation assay. This activity is identical to the activity found in the tyrosine hydroxylation assay, so that these two reactions do not appear to be uncoupled by this mutation. It is not possible to ascertain the relative stoichiometry of tetrahydropterin oxidation and tyrosine hydroxylation for the E376H enzyme, since the pterin oxidation activity is below our detection limits. However, the ability to hydroxylate tyrosine is down 37 000-fold and is very close to the limit of our ability to detect such activity; this is consistent with our inability to detect catalysis of pterin oxidation. The double mutant H331E/E376H was also analyzed to determine if either single mutation could be rescued by a complementary change in another ligand; no activity could be detected in either assay. As noted above, the other such double mutant, H331E/E376H, could not be expressed.

Iron Dependence. As a more quantitative measure of the effects of the mutations on the iron affinities, the dependence on iron concentration of the activities was determined for the three enzymes with significant activity, H336E, H336Q, and H331E. The results are illustrated in Figure 1. The concentrations of Fe(II) in assays at which half-maximal activity is found, the K_{Fe} value, for these enzymes are given in Table 3. The values are consistent with the iron contents of the purified enzymes (Table 2). The apparent affinity of the H336Q enzyme for iron is down 1000-fold from the wild type value. The H336E mutation has a slightly less drastic effect on iron binding, while the K_{Fe} value for the H331E enzyme is increased about 100-fold.

Steady-State Kinetics. The effects of both His336 mutations on steady-state kinetic parameters were determined using the tyrosine hydroxylation assay. The results are given in Table 3. With both mutant proteins, the K_m values for both tyrosine and 6-MePH_4 increase severalfold. The V_{max} value for the H336Q enzyme is down only 3-fold from the wild type value. Since the rate of pterin oxidation by this enzyme is more than twice the rate of tyrosine hydroxylation, the replacement of His336 with glutamine has only a small effect on the ability of the protein to catalyze pterin oxidation. The H331E enzyme lacks any detectable activity at tyrosine

[2] The lower limit for detection of activity in the pterin oxidation assay is determined by the background due to the autoxidation of 6-methyltetrahydropterin. This increases with increasing iron, limiting the amount of iron that can be added to this assay and setting a lower limit for the amount of activity that can be detected. The HPLC-based assay for dihydroxyphenylalanine is much more sensitive than the coupled assay used for pterin oxidation, allowing much lower activities to be detected.

Table 3: Steady-State Kinetic Parameters of Tyrosine Hydroxylase Mutant Proteins[a]

kinetic parameter	wild-type TyrH[b]	H336Q[c]	H336E[d]	H331E
V_{max} (min^{-1})	180 ± 9.4	61.3 ± 4.4[e]	1.33 ± 0.025	0
K_{tyr} (μM)	51 ± 6.1	311 ± 37	330 ± 23	−
K_{MPH4} (μM)	51 ± 16	409 ± 53	190 ± 109	339 ± 96[g]
K_{Fe} (μM)	0.15[f]	135 ± 18	62 ± 20	16 ± 7[g]

[a] Conditions: 125 μg/mL catalase, 1 mM dithiothreitol, 50 mM Hepes/NaOH, pH 7.1 at 25 °C. [b] The concentrations of the nonvaried substrates were tyrosine (250 μM), 6-$MePH_4$ (1 mM), and ferrous ammonium sulfate (10 μM). [c] The concentrations of the nonvaried substrates were tyrosine (600 μM), 6-$MePH_4$ (1 mM), and ferrous ammonium sulfate (300 μM). [d] The concentrations of the nonvaried substrates were tyrosine (1 mM), 6-$MePH_4$ (1 mM), and ferrous ammonium sulfate (300 μM). [e] At saturating concentrations of Fe(II), from the data of Figure 1. [f] From ref *4*. [g] Determined from the rate of 6$MePH_4$ oxidation.

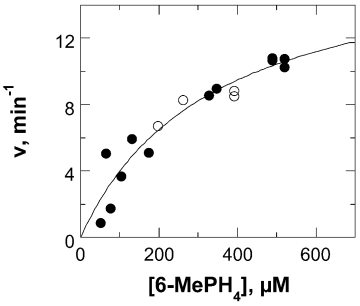


FIGURE 2: Effect of 6-methyltetrahydropterin and tyrosine on the rate of pterin oxidation by H331E tyrosine hydroxylase. The rate of oxidation of 6-$MePH_4$ was measured in the presence (filled circles) and absence (open circles) of 250 μM tyrosine with 0.2−0.4 units/mL sheep dihydropteridine reductase, 170 μg/mL catalase, 100 μM NADH, 130 μM ferrous ammonium sulfate, 70 mM HEPES/NaOH, pH 7, at 25 °C. The line is a fit of the data to the Michaelis−Menten equation.

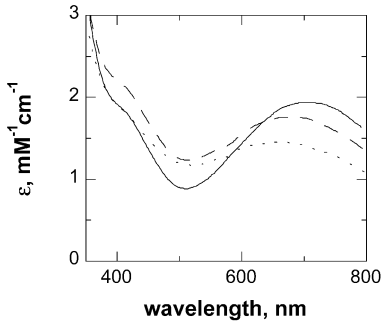


FIGURE 3: Absorbance spectra of complexes of dopamine-bound TyrH (solid line) and H336Q (dashed line) and H331E (dotted line) proteins.

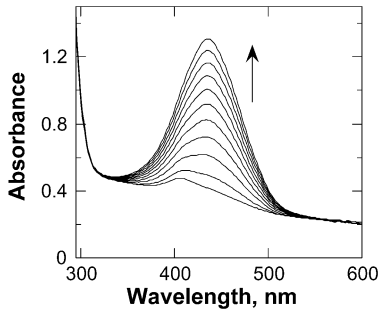


FIGURE 4: Reaction of dopamine with E376H TyrH. The enzyme (25 μM) was incubated with 290 μM dopamine in 50 mM HEPES/NaOH, 100 mM KCl, 0.125 mg/mL catalase, 25% glycerol, pH 7, at 20 °C. Spectra were taken at 2 min intervals from 0 to 10 min.

hydroxylation, so the steady-state kinetic parameters of this enzyme were determined using the pterin oxidation assay. Varying the concentration of 6-$MePH_4$ gives typical Michaelis Menten kinetics (Figure 2), with a V_{max} value for pterin oxidation of 17.5 ± 2.5 min^{-1}. Remarkably, the rate of oxidation of 6-$MePH_4$ was independent of the concentration or even the presence of tyrosine (Figure 2). Thus, the H331E mutation converts TyrH into a tetrahydropterin oxidase.

Dopamine Binding. The ferric form of wild-type TyrH will bind dopamine with a K_d value of about 5 nM, resulting in a ligand-to-metal charge-transfer band at about 700 nm (*19*, *20*). Model studies have shown the λ_{max} of this band is sensitive to the charge and orientation of the metal ligands (*21*). Therefore, the position of this band was used as a probe of the metal ligands in the mutant enzymes. The complex obtained with the H331E protein has a λ_{max} at about 660 nm (Figure 3). This is blue shifted from the wild-type absorbance band, consistent with the expected reduction in the Lewis acidity of the iron upon replacement of an imidazole ligand with a carboxylate. The H336Q enzyme has a maximum absorbance at about 680 nm (Figure 3), intermediate between the wild-type enzyme and H331E TyrH. Ligation of the glutamate amide moiety is expected to involve the amide oxygen (*22*). The blue shift of the dopamine complex is consistent with some anionic character to this oxygen. In studies of biomimetic models for nonheme iron proteins, Mandal and Que (*23*) previously found that an amide ligated to Fe(III) behaves more like a carboxylate than a neutral amide, consistent with the absorbance maximum of the H336Q enzyme. No long-wavelength absorbing species was seen in the visible absorbance spectra of the H336E and H331E/E376H proteins in the presence of dopamine concentrations up to 500 μM. Instead, a species with maximal absorbance at 580 nm was formed. An identical species formed when dopamine was added to a solution of ferrous ammonium sulfate in the absence of enzyme, suggesting that this is an iron−catecholate complex rather than an enzyme-bound species.

Incubation of the E376H or E376Q enzyme with dopamine, rather than leading to formation of a stable complex, resulted in a time-dependent accumulation of a new species with a λ_{max} at 435 nm at pH 7 (Figure 4). The formation of this yellow species was much slower than the formation of the blue complex seen with the wild type enzyme. The colored compound could be separated from the enzyme by passage down a gel filtration column, demonstrating that it is not bound tightly to the enzyme. When the pH was decreased to below 6, the λ_{max} value shifted to 350 nm, with a decrease in the extinction coefficient, consistent with a pK_a value between 5 and 6. When the experiment was done under anaerobic conditions, no formation of the yellow compound was seen, suggesting that the reaction requires oxygen (data not shown). No increase in visible absorbance was seen upon the addition of dopamine if 370 μM 6-methyltetrahydropterin was added first. Addition of a tetrahydropterin to the ferric enzyme reduces the iron to the ferrous form (*14*); thus, the reaction appears to require Fe(III). Both the rate of the reaction and magnitude of the final absorbance at 435 nm

increased in the presence of 0.125 mg/mL catalase and decreased in the presence of superoxide dismutase or cytochrome *c*. These results suggest that the yellow compound is formed in a redox reaction involving an initial reaction between the Fe(III) in the TyrH active site and dopamine. While it proved possible to separate the chromophore from the enzyme, all efforts to isolate a pure compound for further analysis were unsuccessful.

DISCUSSION

The 2-histidine-1-carboxylate triad has been found in an increasing number of iron enzymes in the last several years, suggesting that this arrangement of metal ligands is especially effective or especially versatile. There are several different families of such enzymes (*10*): the pterin-dependent aromatic amino acid hydroxylases PheH, TyrH, and tryptophan hydroxylase; several enzymes in β-lactam biosynthesis, including isopenicillin N synthase and the α-ketoglutarate-dependent dioxygenases clavaminic acid synthase and taurine dioxygenase; the extradiol cleaving catechol dioxygenases; and the aromatic dioxygenases such as naphthalene and toluene dioxygenase. The three-dimensional structures of these enzymes are quite distinct but all share a common metal-binding motif with two histidines and one carboxylic amino acid. In all cases which have been described to date, replacement of a ligand to the metal with alanine results in inactive enzyme (*24*−*28*), similar to the case with TyrH (*7*) and PheH (*6*). To our knowledge, the only previous report of residual activity upon mutagenesis of any of the residues is the report by Khaleei et al. (*25*) that the H145Q and H280Q mutants of clavaminate synthase have low (<3%) residual activity. While no further characterization of these proteins has been described, it does suggest that the results we have found with TyrH may be relevant to other families of enzymes with this arrangement of metal ligands.

Heme-dependent enzymes provide another mechanistically relevant family of enzymes. Peroxidases contain histidine as the axial ligand, and it has proved possible to replace that ligand in cytochrome *c* peroxidase with other amino acids. Mutation of His175 of cytochrome *c* peroxidase to glutamine has no significant effect on the k_{cat} value of the enzyme and increases the K_m value for H_2O_2 by an order of magnitude (*29*). The structure of the mutant enzyme is the same as that of the wild type, with the His175 N3 replaced by the amide oxygen of the glutamine (*29*). The H175E enzyme also has the same structure as the wild-type enzyme, with a severalfold greater k_{cat} value (*30*). Critically, in both the H175E and H175Q enzymes, the rate of formation of compound I, the Fe(IV)═O/porphyrin cation radical, is comparable to that of the wild-type enzyme, but its rate of decay is much greater (*30*). These results provide some precedent for retention of activity upon replacement of the histidine distal to the oxygen site with a glutamine.

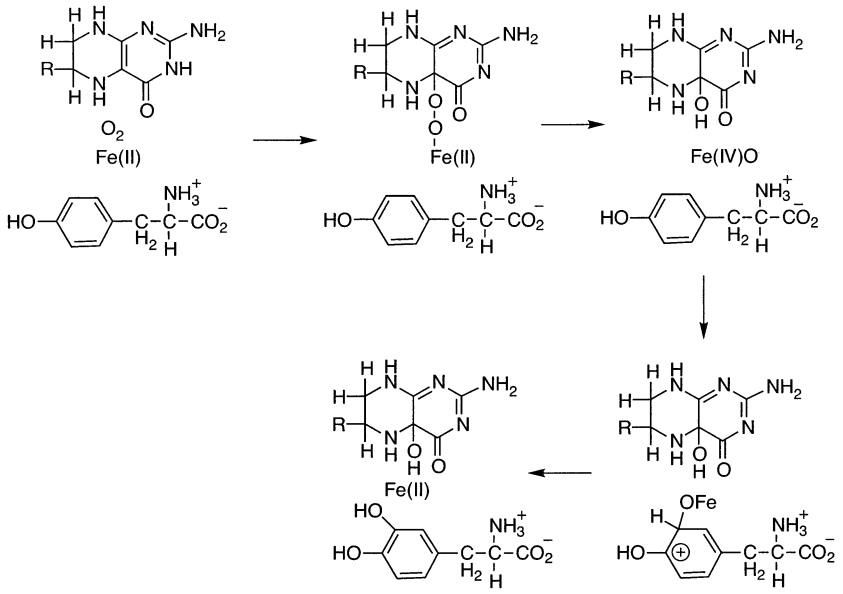
The results described here with the H336Q enzyme are similar to those of the cytochrome *c* peroxidase H175Q enzyme. This TyrH mutant retains substantial ability to catalyze tyrosine hydroxylation and an almost complete ability to catalyze tetrahydropterin oxidation. The major defects are decreased affinity for substrates and iron and some uncoupling of pterin oxidation and amino acid hydroxylation. The hydroxylating intermediate in TyrH is probably an Fe(IV)═O species (*13*) with properties similar to that of the hydroxylating intermediate in cytochrome P450 (*31*), an Fe(IV)═O/porphyrin radical cation similar to compound I of peroxidases (*32*, *33*). The rate-limiting step in the TyrH reaction occurs in the formation of this hydroxylating intermediate (*34*). The retention of pterin oxidase activity in the H336Q enzyme suggests that early events in the catalytic pathway are relatively unaffected by the mutation, but instead the mutation destabilizes the hydroxylating intermediate.

The site on the iron at which oxygen binds TyrH is not established. In the enzyme−pterin−amino acid Fe(II) complex of PheH, the iron is in a distorted square pyramidal geometry (*35*) with His285 (homologous to TyrH His331), a bidentate glutamate, and a water molecule as the equatorial ligands (*36*). On the basis of modeling of oxygen into the active site, Andersen et al. (*36*) have suggested that oxygen binds in place of the water molecule. This would place the oxygen molecule between the iron atom and the C4a position of the pterin, with His290 approximately opposite the oxygen on the other side of the iron atom. Such an arrangement would make His336 in TyrH most analogous to the axial histidine in cyt_c peroxidase, consistent with the comparable effects of the histidine to glutamine mutations in the two enzymes.

The effect of mutagenesis of His331 on the activity of TyrH is much greater than is the case for His336. The His331Q enzyme expresses very poorly (*7*), suggesting that it is unstable, while the H331E enzyme has no detectable tyrosine hydroxylation ability. This enzyme does have some activity as a tetrahydropterin oxidase. More remarkably, this activity does not require the presence of tyrosine. The PheH−pterin−amino acid Fe(II) structure shows that this histidine forms part of the binding site for the amino acid substrate, with the aromatic ring of the substrate stacking against the imidazole ring of the histidine. Thus, the His331Q mutation has removed much of the binding site for the amino acid substrate. In the case of the wild-type enzyme, binding of the amino acid substrate to the enzyme−pterin complex causes a significant conformational change (*35*, *36*). This conformational change is required before oxygen will react with the bound tetrahydropterin to initiate the catalytic cycle (*34*). The tyrosine-independent tetrahydropterin oxidase activity of H331E TyrH suggests that this conformational change may occur even in the absence of the amino acid substrate in the mutant enzyme.

Mutagenesis of Glu376 has the greatest effect on catalysis, in that both mutants have essentially no detectable activity at either tyrosine hydroxylation or tetrahydropterin oxidation. While the carboxylate of this residue in both TyrH and PheH binds the iron in monodentate fashion in the free enzyme and in the binary complex with tetrahydrobiopterin (*37*, *38*), the interaction becomes bidentate in the ternary complex (*36*). Such an interaction is clearly not possible with an imidazole, so that the lack of activity in the E376H enzyme is consistent with the structural data. In contrast, the E376Q enzyme has measurable, if very low, activity. The amide is clearly less likely to coordinate in the bidentate fashion seen in the structure of the PheH ternary complex. If the amide remains monodentate throughout the catalytic cycle, presumably with a water molecule remaining bound, the residual activity provides an approximation of the gain in activity upon

Scheme 2



bidentate coordination. Alternatively, a very small fraction of the protein may have the amide coordinating the iron through both the deprotonated nitrogen and the oxygen.

The properties of the dopamine complexes of the mutant proteins provide further insights into the effects of the mutations. While the Fe(III)−catecholamine complex is not an intermediate in catalysis, it is critical for the regulatory properties of TyrH (*20*). No structure is available of TyrH with dopamine bound, but structures are available of the catalytic domain of PheH with different catecholamines bound (*39*). In each of the structures, the catechol oxygens bind the metal in bidentate fashion, consistent with the conclusions drawn from spectroscopic studies of both enzymes (*21*, *40*). In the PheH structures, the catechol oxygens replace the water molecules opposite the two histidines; given the homology of PheH and TyrH, it is likely that an identical arrangement is found in TyrH. Despite this apparent similarity of the roles of the two histidines in such a complex, the effects of mutating His331 and His336 on catecholamine binding are distinctly different. In the case of His336, replacement of histidine with glutamine results in an enzyme which still binds dopamine with the characteristic charge transfer absorbance band. In this case the absorbance band is shifted to slightly higher energies. While replacement of an imidazole with an amide formally results in the replacement of one neutral ligand with another (*23*), in Fe(III) complexes there is some anionic character to the amide oxygen (*23*). Since the position of the absorbance band is sensitive to the identity of the ligands, with replacement of a neutral nitrogen with a partially negative oxygen shifting the band to higher energies (*21*), the absorbance maximum of the dopamine complex of the H336Q enzyme is fully consistent with replacement of a histidine with a glutamine. It was also possible to form a dopamine complex of the H331E enzyme. The charge-transfer band of this complex absorbs at higher energy than that of either the wild-type enzyme or the H336Q enzyme, consistent with the explicit negative charge on the carboxylate.

In contrast to the stability of the dopamine complex of the H331E and H336Q enzymes, addition of dopamine to the H336E enzyme resulted in the loss of the metal. Catechols bind to the iron in both TyrH and PheH as dianions (*39*, *40*). In structures of PheH with catechols bound, the oxygen at C4 of the catechol is opposite His285 (His331 in TyrH); this histidine is within hydrogen bonding distance of the phenolic oxygen of Tyr325 (Tyr371 in TyrH) and a carboxylate oxygen of the metal ligand Glu330 (Glu376) (*39*). These interactions could compensate in part for the increased negative charge on the metal ligand upon introduction of a negative glutamate in place of the neutral histidine. In contrast, no amino acid residue is within hydrogen bonding distance of the catechol C3 oxygen to ameliorate the increased negative charge density required to form the catechol complex. As a result, binding of dopamine to the H336E enzyme results in loss of the iron.

The two Glu376 mutants also stand out in their reactions with dopamine. In both cases a stable complex is not formed. Instead, there is the formation of a new chromophore. While the product of this reaction has not been identified, the results are most consistent with it being an oxidized form of dopamine. The reaction requires Fe(III) and oxygen, is inhibited by superoxide dismutase and cytochrome *c*, and is potentiated by catalase. This is consistent with initial formation of a dopamine−iron chelate in the active site, as occurs with the wild type enzyme and the H336Q and H331E enzymes. In the case of the Glu376 mutants, this species is not stable but undergoes one-electron transfer from dopamine to Fe(III) to form Fe(II) and dopamine semiquinone. Such a reaction has been shown to occur readily with free iron (*41*), with the neurotoxin 6-hydroxydopamine quinone as one of the products (*42*). Release of the semiquinone from the enzyme would then be followed by its reaction with oxygen or disproportionation to form the final product(s). Thus, the effect of mutagenesis of Glu376 is to destabilize the enzyme−dopamine complex. This is consistent with an increased redox potential at the iron due to the loss of the carboxylate ligand. The 430 nm species is not dopamine quinone, the two electron oxidized product, since this has an absorbance maximum at 390 nm (*43*), or the further oxidation product dopaminochrome, which absorbs at 480

nm. Dopamine oxidation products produced in neurons containing TyrH have been implicated in neurodegenerative diseases such as Parkinson's disease (*44*, *45*). It is likely that the structure of TyrH has evolved to minimize redox reactions of the active site iron with catecholamines. On the basis of the results of the mutageneses described here, Glu376 plays a major role in modulating this reactivity.

In several cases, mutation of a metal ligand dramatically decreases the affinity of TyrH for iron. On the basis of the ability to reconstitute the enzyme with iron, which results in Fe(III) enzyme irrespective of the initial oxidation state of the iron, mutation of His336 has the most drastic effect. Mutation of this residue also dramatically reduces the affinity for Fe(II), on the basis of the concentration dependence of the activity shown in Figure 1. The concentration of Fe(II) required for half-maximal activity increases by 3 orders of magnitude with both the H336Q and H336E enzymes. This is far more than the effect on catalytic activity seen with the H336Q enzyme.

The present understanding of the catalytic mechanism of TyrH and the other pterin-dependent hydroxylases is shown in Scheme 2 (*46*). This mechanism has no explicit role for the iron in the initial reaction between tetrahydropterin and oxygen. If the iron is truly not involved in this partial reaction, there is the possibility that proteins lacking iron would still be able to catalyze tetrahydropterin oxidation. The decreased affinities of the H331E, H336E, and H336Q enzymes for iron obviates a common problem in demonstrating a metal requirement for enzymatic activity, the presence of contaminating metal at concentrations comparable to the concentrations of enzyme used in assays, ~0.1 μM. With all three mutant proteins, the ability to catalyze tetrahydropterin oxidation was dependent upon Fe(II). The dependence of both pterin oxidation and tyrosine hydroxylation activities on the presence of ferrous iron is consistent with a requirement for the metal for both partial reactions.

The results presented here provide insight into the properties of the 2-histidine-1-carboxylate triad which are critical for catalysis by the pterin-dependent aromatic amino acid hydroxylases. Most straightforward is the optimization of iron affinity with these three ligands. However, other arrangements of ligands are found in enzymes which bind iron at least as tightly. The requirement for a glutamate residue can be attributed in part to the need for a ligand capable of both mono and bidentate binding. The imidazoles provided by the two histidines cannot be replaced by more electronegative amides or carboxylates without destabilizing the hydroxylating intermediate. Whether these results are general for other iron enzymes which also utilize the 2-histidine-1-carboxylate triad will require that similar analyses be carried out with those enzymes. Comparison of those results with the results of the present studies should result in a much better understanding of the molecular basis for the ubiquity of this motif and the factors responsible for its properties in individual systems.

ACKNOWLEDGMENT

The assistance of Edith Osborne in the initial characterization of the H331E enzyme is gratefully acknowledged. We thank Dr. Michael Valley for helpful comments on the manuscript.

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BI0271493