Identification of iron ligands in tyrosine hydroxylase by mutagenesis of conserved histidiny residues

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Abstract

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine and other aromatic amino acids using a tetrahydropterin as the reducing substrate. The enzyme is a homotetramer; each monomer contains a single nonheme iron atom. Five histidine residues are conserved in all tyrosine hydroxylases that have been sequenced to date and in the related eukaryotic enzymes phenylalanine and tryptophan hydroxylase. Because histidine has been suggested as a ligand to the iron in these enzymes, mutant tyrosine hydroxylase proteins in which each of the conserved histidines had been mutated to glutamine or alanine were expressed in *Escherichia coli*. The H192Q, H247Q, and H317A mutant proteins contained iron in comparable amounts to the wild-type enzyme, about 0.6 atoms/subunit. In contrast, the H331 and H336 mutant proteins contained no iron. The first three mutant enzymes were active, with V_{max} values 39, 68, and 7% that of the wild-type enzyme, and slightly altered V/K_m values for both tyrosine and 6-methyltetrahydropterin. In contrast, the H331 and H336 mutant enzymes had no detectable activity. The EPR spectra of the H192Q and H247Q enzymes are indistinguishable from that of wild-type tyrosine hydroxylase, whereas that of the H317A enzyme indicated that the ligand field of the iron had been slightly perturbed. These results are consistent with H331 and H336 being ligands to the active site iron atom.

Keywords: active site; iron binding; mutagenesis; tyrosine hydroxylase

Tyrosine hydroxylase catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine (DOPA), the first and rate-limiting step in the catecholamine biosynthetic pathway (Weiner, 1979; Zigmond et al., 1989). The enzyme is a tetramer containing four identical subunits. The structural and catalytic properties of tyrosine hydroxylase are very similar to those of phenylalanine hydroxylase and tryptophan hydroxylase. All three are nonheme iron-containing enzymes that catalyze the hydroxylation of aromatic amino acids using tetrahydrobiopterin as the physiological reducing substrate. Comparison of their primary sequences indicates they are homologous over the C-terminal 300 amino acids. Studies with tyrosine hydroxylase have established that the catalytic domain containing all amino acid residues required for catalysis is located within this region (Daubner et al., 1993).

An outstanding issue in understanding the catalytic mechanism of these enzymes is the role of the nonheme iron atom and the structure of the iron site. The three-dimensional structure is not known for any of the pterin-dependent hydroxylases. Based on the effects of an enzyme-bound ferric atom on the re-

laxation times of nuclei in substrates bound to tyrosine hydroxylase, Martinez et al. (1993a, 1993b) concluded that the iron atom is 7 Å from the aromatic ring of the bound amino acid substrate and 4 Å from the C4a position of the bound tetrahydropterin substrate. The resonance Raman spectra of the catechol complexes of both phenylalanine and tyrosine hydroxylase are consistent with 2–3 histidines and 1–2 carboxylates as metal ligands (Andersson et al., 1988; Cox et al., 1988). EXAFS analysis of the bacterial phenylalanine hydroxylase with copper in the active site suggests that there are 1–3 histidine ligands to the metal (Blackburn et al., 1992). Site-directed mutagenesis has been done on two conserved histidine residues in liver and bacterial phenylalanine hydroxylase. In both cases, conversion of these residues to serine abolished the activity (Gibbs et al., 1993; Balasubramanian et al., 1994). Beyond that, no essential amino acid residues have been identified in these enzymes.

There are five histidine residues that are conserved in the eukaryotic pterin-dependent hydroxylases, located at amino acids 192, 247, 317, 331, and 336 in rat tyrosine hydroxylase. The two residues that have been mutated in phenylalanine hydroxylase correspond to H331 and H336 in rat tyrosine hydroxylase (Balasubramanian et al., 1994). To determine which of the conserved histidine residues in tyrosine hydroxylase are required for

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iron binding, a series of mutant proteins was constructed in which each of the five histidines was replaced with either an alanine or a glutamine. This paper describes the design, purification, and characterization of these proteins.

Results

Purification

Initially, mutant proteins were designed in which glutamine replaced the conserved histidine residues in rat tyrosine hydroxylase. Glutamine was chosen because of its potential to replace a hydrogen bond supplied by histidine without acting as an iron ligand. However, in every case, removing one of the conserved histidines produced a significant reduction in expression. To compensate for this, the mutated constructions were expressed at 30 °C rather than the standard 37 °C. Although this change in temperature does not affect the expression of the wild-type enzyme (results not shown), it resulted in a significant increase in the levels of most of the mutant proteins. All of the mutant proteins could be purified using the method developed for the wild-type enzyme (Fitzpatrick et al., 1990). The H192Q and H247Q proteins had significant activity (Table 1). However, replacement of histidine with glutamine at positions 317, 331, or 336 resulted in protein with very low or no activity and very low levels of expression. A second series of mutant proteins was then constructed in which alanine replaced histidine at positions 317, 331, and 336. In all cases, significant amounts of pure protein could be obtained. In contrast to the H317Q protein, the H317A protein had significant, although low, activity. The H331A and H336A proteins still had no detectable tyrosine hydroxylase activity. The levels of expression and specific activities of all the mutant proteins are summarized in Table 1.

In order to determine whether loss of activity correlated with loss of iron binding, the iron contents of the mutant proteins that could be obtained in significant amounts were determined. In our hands, purified recombinant rat tyrosine hydroxylase contains 0.5–1 iron atom per subunit. The H192Q, H247Q, and H317A proteins contained iron at levels comparable to the wild-type enzyme (Table 1), consistent with iron binding being unaffected by loss of these residues. In contrast, neither the H331

nor the H336 mutant proteins contained significant amounts of iron. This is consistent with the loss of activity in these two proteins being due to loss of an amino acid ligand to the iron.

EPR spectroscopy

EPR spectroscopy was used to further probe the ligand environment of the iron atom in the mutant proteins that contained iron. In our hands, the iron in purified recombinant wild-type tyrosine hydroxylase is in the ferric state when isolated. The EPR spectrum at liquid helium temperatures of the wild-type enzyme has features between $g = 10$ and $g = 2$, consistent with a spin 5/2 system (Fig. 1). The EPR spectra of the H192Q and H247Q enzymes are indistinguishable from that of the wild type, consistent with no change in the ligand field of the iron in these mutant proteins. In contrast, the spectrum of the H317A enzyme is perturbed, mainly in the feature near $g = 7$, suggesting that there is a slight change in the ligand field of the iron in this enzyme. Controls in which temperature and power were varied established that this difference was not simply an effect of temperature.

Determination of kinetic parameters

To further characterize the effects of the mutations on the catalytic activity of tyrosine hydroxylase, steady-state kinetic pa-

Fig. 1. EPR spectra of wild-type and mutant tyrosine hydroxylase. Samples contained approximately 60 μ M protein-bound iron in 50 mM HEPES, 10% glycerol, 100 mM KCl, pH 7.1. Spectra were recorded at 9.43 GHz with the following settings: field set, 2,530 G; field width, 5,000 G; microwave power, 0.2 mW; modulation amplitude, 25 G; receiver gain, 100,000; scan time, 167 s; time constant, 0.3 s; temperature, 5 K.

Table 1. Properties of tyrosine hydroxylase histidine mutants

Protein	Yield (mg) ^a	Specific activity ^b	Fe/subunit
Wild type	18	2.9	0.64
H192Q	54	0.66	0.53
H247Q	8.9	1.7	0.55
H317Q	0.85	0.016	0.07
H317A	48	0.21	0.62
H331Q	0.2	<0.01	ND ^c
H331A	3.4	<0.01	0.03
H336Q	1	<0.01	0.05
H336A	16	<0.01	<0.01

^a Yield of purified protein from 10 L of culture medium.

^b Units of specific activity were μ mol DOPA produced/min-mg.

^c Not determined.

rameters of the H192Q, H247Q, and H317A proteins were determined and compared to those of the wild type. Table 2 demonstrates that removal of any of these three histidine residues produces a drop in maximum velocity, consistent with the decreased specific activity of the purified proteins. In the case of the H247Q enzyme, the decrease in kinetic parameters was limited to a decrease in the V_{max} value, with no significant change in the V/K value for either tyrosine or 6-methyltetrahydropterin. The effects of the H192Q mutation were greater, with decreases of severalfold in both V/K values in addition to the sixfold decrease in the V_{max} value. The kinetic properties of the H317A enzyme were affected most drastically. In addition to a decrease in the V_{max} value to only 7% that of the wild-type enzyme, the K_{tyr} value decreased at least 10-fold, so that accurate K_{tyr} and V/K_{tyr} values could not be obtained.

Discussion

The data presented here are consistent with H331 and H336 as ligands to the iron atom in tyrosine hydroxylase and suggest that amino acids other than histidine supply the remaining ligands. Thus, mutagenesis of either H331 or H336 results in mutant proteins lacking all activity and containing no significant iron. For each of the other three conserved histidines, it was possible to construct at least one protein in which the histidine had been mutated to an amino acid that would not be expected to be an iron ligand. These findings are in agreement with the results obtained with phenylalanine hydroxylase, where the mutants corresponding to H331S and H336S in tyrosine hydroxylase were inactive (Gibbs et al., 1993; Balasubramanian et al., 1994). However, in that case no other histidines were examined.

The resonance Raman data of the catechol complexes of phenylalanine and tyrosine hydroxylase have been interpreted as evidence for iron ligation by three histidine residues, one carboxylate, and both oxygens of the catechol (Andersson et al., 1988; Cox et al., 1988). The data presented here limit the number of histidine ligands in these enzymes to two. If a carboxylate is indeed a third ligand, the identity of a fourth ligand must remain speculative. A reasonable possibility is a second carboxylate. The phenylalanine hydroxylase from *Chromobacterium violaceum* is isolated with copper instead of iron (Pember et al., 1986). EXAFS data for the copper site in that enzyme have been interpreted in favor of four ligands to the metal, 1–2.5 histidines and 1–3 neutral oxygen or nitrogen ligands (Blackburn et al.,

1992). However, the copper form of this enzyme may not be the active one, because the copper is reported to be removed without loss of activity (Carr & Benkovic, 1993). Still, the data presented here are consistent with these results.

The roles of the 192, 247, and 317 histidine residues are less clear. Atomic absorption analyses indicate that the loss of any of these three residues has little effect on the enzyme's iron content. It is also apparent that the 192 and 247 residues have little effect on the environment of the enzyme-bound iron, because substituting either of the histidines has no detectable effect on the EPR signal. Taken together, these results rule out these residues as candidates for iron ligands. Furthermore, the changes of less than sixfold in the kinetic parameters of the H192Q and H247Q enzymes suggest that these two residues are also not critical for catalytic activity. However, the kinetic data do suggest that H317 is important for conversion of tyrosine to DOPA. The V_{max} value for the H317A enzyme is only 7% that of the wild type, whereas the H317Q enzyme is even less active. The greatest effect is on the V_{max} value, although the very low K_{tyr} value only allows one to say that the V/K_{tyr} value is not greatly decreased. The steady-state kinetic mechanism for tyrosine hydroxylase is ordered, with 6-methyltetrahydropterin the first substrate to bind and tyrosine the last (Fitzpatrick, 1991a). Analyses with alternate substrates have indicated that the rate-limiting step in catalysis is probably formation of the hydroxylating intermediate (Fitzpatrick, 1991b). Because this is very likely to be an irreversible step, the rate of the actual hydroxylation step is reflected in the V_{max} value but not in the V/K_{tyr} value. Thus, the much greater effect in the V_{max} value than the V/K_{tyr} value for the H317Q mutant suggests that this mutation affects the rate of hydroxylation. The magnitude of the effect is consistent with removal of a general acid or base. The changes in the EPR spectrum of the H317A enzyme are consistent with a perturbation of the active site structure resulting in a slight change in the ligand field of the iron. Obviously, further mechanistic analysis of this mutant is required to clarify this point. Definitive understanding of whether the decreased catalytic activities caused by the substitutions at 192, 247, and 317 are due to an alteration in protein conformation or a disruption in the catalytic mechanism will require crystallographic data.

In conclusion, the data presented here provide strong evidence that H331 and H336 are ligands to the iron in tyrosine hydroxylase, and that no other histidines are metal ligands. The data also suggest that H317 plays a role in hydroxylation without being a metal ligand.

Table 2. Steady-state kinetic parameters for tyrosine hydroxylase histidine mutants^a

Parameter	Wild-type enzyme	H192Q	H247Q	H317A
V_{max} (min ⁻¹) ^b	180 ± 9.4	30 ± 1.0	83 ± 4.2	12 ± 1.5
K_{MPH4} (μM) ^c	51 ± 16	81 ± 3.1	37 ± 8.2	13 ± 4.7
V/K_{MPH4} (μM ⁻¹ min ⁻¹) ^c	2.5 ± 0.36	0.60 ± 0.088	2.3 ± 0.28	0.72 ± 0.19
K_{tyr} (μM) ^b	51 ± 6.1	23 ± 2.5	17 ± 3.2	1.3 ± 2.3
V/K_{tyr} (μM ⁻¹ min ⁻¹) ^b	3.6 ± 0.27	1.3 ± 0.10	5.0 ± 0.73	9.6 ± 16

^a Conditions: 50 mM MES, 14 mM β-mercaptoethanol, 10 μM ferrous ammonium sulfate, 75 μg/mL catalase, pH 6.5, 30 °C.

^b Determined at 420 μM 6-methyltetrahydropterin, varied tyrosine.

^c Determined at 150 μM tyrosine, varied 6-methyltetrahydropterin.

Table 3. Oligonucleotides used to perform site-directed mutageneses of histidines to glutamine or alanine

Mutation	Oligonucleotide sequence ^a	Restriction digest change
H192Q	5'-acctg gacca accgg gcttc-3'	Loss of <i>Ava</i> I site
H247Q	5'-ctatg ctacc caagc atgcc gggag-3'	Gain of <i>Bbu</i> I site
H317Q	5'-gtata tccgc caagc ttect caect atgc-3'	Gain of <i>Hind</i> III site
H317A	5'-cagta tatcc gcgcg gcctc tctac ct-3'	Gain of <i>Hin</i> P1 I site
H331Q	5'-actgc tgcca agagc tcttg ggacatg-3'	Gain of <i>Sac</i> I site
H331A	5'-gagcc ggact gctgc gctga gctct tggga catgt acc-3'	Gain of <i>Sac</i> I site
H336Q	5'-gctgt tggga caagt accca tgttg-3'	Gain of <i>Kpn</i> I site
H336A	5'-gagct gttgg gagcg gtacc catgttg-3'	Gain of <i>Kpn</i> I site

^a The codon that gives the altered amino acid is in bold. The gain or loss of a recognition site that allows different DNA restriction between wild-type and mutant is underlined.

Materials and methods

Materials

[3,5-³H]Tyrosine was purchased from Amersham Corp. and purified prior to use by the method of Ikeda et al. (1966). 6-Methyltetrahydropterin was synthesized as described previously (Fitzpatrick, 1989); the concentrations of stock solutions were determined in 2 M perchloric acid using an ϵ_{266} value of 17.8 mM⁻¹ cm⁻¹ (Shiman et al., 1971). Catalase was purchased from Boehringer-Mannheim. Optima grade nitric acid used in the atomic absorption experiments was purchased from Fisher.

Construction of vectors for the expression of chimeric mutants

Site-directed mutagenesis was carried out according to the protocol of Kunkel et al. (1987). Subcloning was performed by separating fragments of DNA on low melting point agarose gels, followed by melting the agarose slices and removing the DNA with BioRad Prep-a-Gene resin. The purified fragments of DNA were then recombined with T₄ DNA ligase according to Sambrook et al. (1989).

Plasmid pTH10 contains the cDNA for rat tyrosine hydroxylase inserted into the *Bam*H I site of pTZ18R, with a unique *Nco* I site (CCATGG), providing the code for methionine 1. Plasmid pTH10 was mutated to give altered amino acid residues concomitant with new restriction sites (or in the case of H192, loss of existing sites). The oligonucleotides, altered amino acids, and restriction sites are listed in Table 3. The new plasmids and pET3d were digested with *Nco* I and *Bam*H I; the linearized pET3d and the fragments from the mutated pTH10s that code for tyrosine hydroxylase were purified and recombined. Plasmids were screened for the correct construction by restriction enzyme analysis, and positive recombinants were confirmed by DNA sequencing. Each positive clone was introduced into competent *Escherichia coli* strain BL21(DE3).

Protein purification

Growth of *E. coli* containing the expression plasmid for wild-type rat tyrosine hydroxylase was at 37 °C with induction of en-

zyme expression by 0.4 mM IPTG as described previously (Daubner et al., 1992). The cells containing expression plasmids for the mutant proteins were grown at 30 °C instead of 37 °C and induced with 10 mM lactose. All the proteins were purified using the procedure previously described for the wild-type enzyme (Fitzpatrick et al., 1990). The purified proteins were then concentrated by precipitation with 50% ammonium sulfate. After centrifugation at 12,000 × *g* for 15 min at 4 °C, the proteins were dissolved in 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1. The samples were dialyzed extensively against this buffer and then stored at -70 °C until needed. Protein concentrations were determined spectrophotometrically using an $A_{280}^{1\%}$ value of 10.4 and a subunit molecular weight of 56,000.

Tyrosine hydroxylase activity was measured by following the release of tritium from 3,5-[³H]tyrosine (Fitzpatrick, 1989) in 50 mM MES, pH 6.5, at 32 °C during enzyme purification and at 30 °C for the determination of the kinetic perimeters. To determine kinetic parameters, kinetic data were fit to $v = V_{max} * S / (K_m + S)$ using the KinetAsyst software (IntelliKinetics, State College, Pennsylvania).

Iron measurements

A Perkin-Elmer 2380 spectrophotometer with a graphite furnace (HGA-400) was used to determine the iron content of the purified proteins. Proteins were first dissolved in 2 M nitric acid to solubilize the bound iron.

Spectroscopy

The EPR spectra were obtained using a Bruker ESR 300 system operating at 5.6 K using 0.2 mW microwave power. The measurements were performed on approximately 60 μM protein-bound iron using 4-mm-diameter tubes.

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