Characterization of the Active Site Iron in Tyrosine Hydroxylase

REDOX STATES OF THE IRON*

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Tyrosine hydroxylase is an iron-containing monooxygenase that uses a tetrahydropterin to catalyze the hydroxylation of tyrosine to dihydroxyphenylalanine in catecholamine biosynthesis. The role of the iron in this enzyme is not understood. Purification of recombinant rat tyrosine hydroxylase containing 0.5–0.7 iron atoms/subunit and lacking bound catecholamine has permitted studies of the redox states of the resting enzyme and the enzyme during catalysis. As isolated, the iron is in the ferric form. Dithionite or 6-methyltetrahydropterin can reduce the iron to the ferrous form. Reduction by 6-methyltetrahydropterin consumes 0.5 nmol/nmol of enzyme-bound iron, producing quinonoid 6-methyldihydropterin as the only detectable product. In the presence of oxygen, reoxidation to ferric iron occurs. During turnover the enzyme is in the ferrous form. However, a fraction is oxidized during turnover; this can be trapped by added catechol or by the dihydroxyphenylalanine formed during turnover.

Tyrosine hydroxylase catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA), the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters (1). This is one of a small family of tetrahydropterin-utilizing monooxygenases found in the central nervous system; the others are phenylalanine hydroxylase and tryptophan hydroxylase. The mechanisms of these enzymes are very poorly understood, and there are as yet no structures available. Tyrosine hydroxylase has been known for some time to contain 1 iron atom/subunit (2). Studies of the metal dependence of the catalytic activity have shown that ferrous iron is required; no other metal has been found to be catalytically active (3, 4). The iron atom is bound to amino acid side chains rather than a porphyrin ring; recently two of the metal ligands in rat tyrosine hydroxylase have been identified as histidines 331 and 336 (5). As yet, the role of the iron in tyrosine hydroxylase remains unclear. However, the lack of activity of iron-depleted enzyme (3, 4), or of enzyme in which a metal ligand has been modified by site-directed mutagenesis (5), is consistent with an essential role in catalysis. In addition, NMR measurements have shown that the amino acid substrate binds close to the iron, placing the metal in the active site (6).

Until relatively recently, studies of the metal site in tyrosine hydroxylase were hindered by the difficulties of obtaining sufficient amounts of purified enzyme for study. Within the last decade preparations from bovine adrenal medulla (7) and rat pheochromocytoma (8) have permitted physical studies. The enzyme from both sources contained 0.6–0.7 iron atoms/subunit. EPR spectroscopy showed that the iron was in the iron(III) state when isolated. Furthermore, the enzyme had a blue-green color due to the presence of tightly bound catecholamines interacting with the metal (8, 9). More recently, several laboratories have successfully expressed human or rat tyrosine hydroxylase in bacteria, providing access to significantly more material (4, 10, 11). Using the recombinant rat enzyme, Daubner et al. (10) showed that the catecholamine-bound enzyme was substantially inhibited, with less than 5% of the activity of the catecholamine-free enzyme. This suggested that the blue-green form of the enzyme isolated from nonrecombinant sources was not the catalytically active species. Several studies have been reported of metal binding to recombinant tyrosine hydroxylase (4, 12). However, the enzyme used in these was isolated as the apoenzyme and showed relatively low specific activity when reconstituted with ferrous iron. This has left unaddressed the question of the native form of the iron.

One of the outstanding questions in the understanding of tyrosine hydroxylase is the role of the iron. As noted, characterization of the iron site to date has involved an enzyme isolated in an inhibited or inactive form. This has made it difficult to evaluate the resting form of the enzyme in the absence of inhibitors. To alleviate this difficulty, we have focused on obtaining a highly active recombinant enzyme, which contains iron and lacks inhibitors. This has allowed us to characterize the active, resting form of the enzyme. The results of these studies are reported here.

EXPERIMENTAL PROCEDURES

[3,5-3H]Tyrosine was purchased from Amersham Corp. and purified before use by the method of Ikeda et al. (13). 6-Methyltetrahydropterin was synthesized as described previously (14, 15); stock solutions in 5 mM HCl were prepared on the day of use. 6-Methyl-7,8-dihydropterin was synthesized as described by Mager et al. (24). Quinonoid 6-methylidihydropterin was formed immediately before use by oxidation of 6-methyltetrahydropterin with bromine. 2,3-Dihydroxynaphthalene, sheep dihydropterin reductase, bathophenanthroline disulfonic acid, and DOPA were purchased from Sigma.

Rat tyrosine hydroxylase was expressed in Escherichia coli and purified essentially as described previously (10), with the following modifications. Ferrous ammonium sulfate was added to a final concentration of 100 μM to growing cells once they entered log phase. Buffers contained 50 mM diethylenetriaminopentaacetate instead of EDTA. The purified enzyme was concentrated by precipitation with 50% ammonium sulfate, pH 7.1; the pellet was dissolved in 50 mM Hepes, 100 mM KCl, 10% glycerol, pH 7.1, and dialyzed against a 300-fold excess of the same buffer for 9 h. To avoid loss of enzyme-bound iron, it was critical that the pH be kept above pH 7.0. The protein content of enzyme samples was determined using an A280 value of 10.4 (7). The specific
activity of tyrosine hydroxylase was determined using an assay based on the release of tritium from 3,5-[^3H]tyrosine as described previously (3). The specific activities of the enzyme used in this study varied from 2.5 to 3.5 μmol of DOPA produced per min per mg at pH 6.5 and 32 °C.

The iron content of the purified enzyme was determined after releasing the iron from the enzyme by adding 40 μl of 2.5 M nitric acid to 10 μl of protein. The resulting iron solution was diluted to 4 ml with water and 40 min at room temperature. After the addition of 200–450 μl of water, the protein was removed by centrifugation. A Perkin-Elmer model 2380 atomic absorption spectrophotometer equipped with a graphite furnace was then used to measure the iron content of this sample. All enzyme concentrations are given in terms of enzyme-bound iron.

The effect of iron chelators on the activity of tyrosine hydroxylase was determined using a coupled assay with dihydropterin reductase (16). This allowed turnover to be followed continuously at 340 nm. The assay contained 90 μl of 16% TMB (15). This allowed turnover to be followed continuously at 340 nm. The reaction was initiated by adding 0.6 ml of 16% TMB, 100 mM KCl, 10% glycerol, pH 7.1, 4 °C. The enzyme contained 0.5–0.65 atom of iron/monomer. The visible absorbance spectrum of the purified enzyme is shown in Fig. 1.

To determine the pterin products from reduction of the enzyme-bound iron, a reaction mixture containing 20 mM Tris acetate, pH 8.0, and various concentrations of tyrosine hydroxylase (0–30 μM) in a volume of 0.06 ml was incubated at 25 °C. The reaction was initiated by the addition of 6-methyltetrahydropterin by subtracting the rate in the absence of enzyme. Catalysis was quenched after 15–60 s by filtration through a 3,5-[3H]tyrosine as described previously (3). The specific activities of the enzyme used in this study varied from 2.5 to 3.5 μmol of DOPA produced per min per mg at pH 6.5 and 32 °C.

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HPLC was performed using a Waters 501 pump with a Waters 680 automated gradient controller, a Waters Lambda-Max 481 LC spectrophotometer, a Waters U6K injector, and a Waters 740 data module. Fluorescence experiments were performed on a SLM Instruments SLM 8000 fluorometer at 10 °C using 1-cm path length cuvettes. EPR spectra were obtained at 5–6 K at 9.43 GHz with a Bruker ESP300 system with an Oxford Instruments ER910A cryostat. Each EPR experiment utilized approximately 60 μM protein. Additions were made directly to the enzyme sample in 0.4-mm diameter EPR tubes.

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RESULTS

Resting State of the Iron in Tyrosine Hydroxylase—When recombinant rat tyrosine hydroxylase was purified from E. coli as described under "Experimental Procedures," the purified enzyme contained 0.5–0.65 atoms of iron/monomer. The visible absorbance spectrum of the purified enzyme is shown in Fig. 1.

The enzyme had a slight greenish tint, due to the broad absorbance extending from the ultraviolet to about 500 nm. The redox state of the iron in such preparations was examined using ferric and ferrous iron specific chelators. The addition of tyrosine hydroxylase to the iron(III) chelator DHN resulted in a large drop in the fluorescence of DHN (Fig. 2A). However, the addition of enzyme to the iron(II) specific chelator bathophenanthroline disulfonic acid did not produce a significant alteration in the fluorescence of that chelator (Fig. 2B). This suggested that all of the iron present in the purified enzyme was in the iron(III) form. To determine more directly the state of the active site iron, the EPR spectrum at 5 K was obtained. As shown in Fig. 3 (spectrum A), the enzyme possesses EPR features between 10 and 2 g, with a major derivative feature with an apparent g value of 4.2. These features establish that the enzyme contains high spin iron(III) with S = 5/2, consistent with the effects of the chelators.

Reduction of the Enzyme-bound Iron—Previous studies of the metal specificity of tyrosine hydroxylase have shown that the enzyme requires ferrous iron for activity (3). This suggested that the ferric iron present in the resting form must be reduced for activity. Addition of sodium dithionite resulted in a loss of the EPR signal, consistent with reduction of the iron. The reaction was slow, requiring the enzyme to be incubated on ice.
The spectra were measured at 5 K at 9.43 GHz with the following settings: field set, 2530 G; field width, 5000 G; microwave power, 0.2 milliwatt; modulation amplitude, 25 G; receiver gain, 100,000; scan time, 167 s; time constant, 0.3 s.

FIG. 3. Reduction of tyrosine hydroxylase by dithionite. A, EPR spectrum of tyrosine hydroxylase as isolated. The sample contained 55 μM enzyme in 50 mM Hepes, 10% glycerol, 100 mM KCl, pH 7.1. B, after the addition of 17 mM dithionite. The spectra were measured at 5 K on ice exposed to air. The reason for the less than complete return of activity has not been determined. A reasonable explanation is inactivation by the products of autoxidation of the sodium dithionite. Tyrosine hydroxylase is readily inactivated by hydrogen peroxide (23).

FIG. 4. Reduction of tyrosine hydroxylase by 6-methyltetrahydropterin. Tyrosine hydroxylase (49 μM) was placed in an EPR tube, anaerobic by passing argon through the tube for 2 h. Spectrum A was then taken. 6-Methyltetrahydropterin (560 μM, final concentration) was added under argon to obtain spectrum B.

FIG. 5. Oxidation of 6-methyltetrahydropterin (6-MPH, ●) and formation of quinonoid 6-methylidihydropterin (q6-MPH, ○) during reduction of tyrosine hydroxylase. Tyrosine hydroxylase (16.8 μM) was allowed to react with 34 μM 6-methyltetrahydropterin for the indicated times in 50 mM Tris acetate, pH 8.0, at 25°C. The concentrations of the different pterin species were then determined by HPLC as described under “Experimental Procedures.” The lines are fits of the data to a single exponential decay.

formed were determined as a function of the concentration of enzyme-bound iron, giving a stoichiometry of 0.54 ± 0.03 nmol of pterin/nmol of iron reduced (Fig. 6). This result is consistent with a stoichiometry of one electron transferred from the tetrahydropterin to the ferric iron during reduction.

Iron Redox States during Catalysis—In order to determine the predominant redox state of the iron during turnover, the enzyme was reduced with 6-methyltetrahydropterin anaerobically and then reacted with oxygen and tyrosine. The EPR spectra that were obtained are shown in Fig. 7. The first spectrum is that of the enzyme after reaction with 6-methyltetrahydropterin. After the loss of the high spin ferric EPR signal was complete, the sample was opened to the atmosphere and an air-saturated sample of tyrosine added. The sample was immediately frozen and the EPR spectrum obtained. As shown in Fig. 7 (spectrum B), the EPR spectrum was unchanged at the start of catalytic turnover. The sample was then warmed to 4°C, allowing turnover to proceed. After 15 min, the sample was again frozen and the EPR spectrum taken (Fig. 7, spectrum C). This spectrum was of a ferric species but different from that present before reduction. Integration of the spectrum showed that 50% of the signal present initially was present as the new oxidized species.

Catechols such as DOPA have been reported to form complexes with the tetrahydropterin-dependent hydroxylases (8, 9). Thus, a reasonable candidate for the new species formed after turnover was a DOPA-tyrosine hydroxylase complex. The
Tyrosine Hydroxylase Iron Site

**DISCUSSION**

The presence of 1 iron atom/subunit of tyrosine hydroxylase was first established by Hoeldtke and Kaufman (2) using proteolytically treated enzyme. This was extended by Haavik et al. (7), who reported that the intact bovine enzyme contained 0.6–0.8 iron atoms/subunit and showed by EPR spectroscopy that the iron was in the ferric form. These authors also reported for the first time that the enzymes from bovine (7) and rat (8) tissues were colored, with a broad absorbance band around 700 nm. This blue color was shown to be due to the presence of bound catecholamines in a charge-transfer interaction with the iron (8, 9). Thus, the enzyme from nonrecombinant sources was in the ferric form and contained a tightly bound inhibitor.

In the last few years, several research groups have expressed rat or human tyrosine hydroxylase in heterologous systems, making it possible to more readily carry out studies of the iron site. In the cases of expression of the rat enzyme in insect cells using a baculovirus vector (17) and of expression of the human enzyme in bacteria (4), the enzyme contained almost no iron. In both cases iron was the only metal that would restore catalytic activity.

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Fig. 6. Stoichiometry of 6-methyltetrahydropterin consumption and quinonoid 6-methylhdropterin formation during reduction of tyrosine hydroxylase. Data points represent the amount of 6-methyltetrahydropterin consumed (C) or quinonoid 6-methylhdropterin formed (●) at specific enzyme concentrations. Conditions were: 50 mM Tris acetate, pH 8.0, 37 μM 6-methyltetrahydropterin, and various concentrations of tyrosine hydroxylase in a volume of 0.06 mL at 25°C. Reactions were run as described under “Experimental Procedures.”

**Fig. 7.** EPR spectra of tyrosine hydroxylase during and after catalysis. Tyrosine hydroxylase (49 μM) was reduced anaerobically with 6-methyltetrahydropterin (final concentration, 560 μM), giving spectrum A. The sample was then thawed and opened to the atmosphere, and an aerobic sample of tyrosine was added to give a final concentration of 260 μM. The enzyme was frozen as soon as mixing was complete and spectrum B was taken. The sample was then thawed and left on ice for 15 min before taking spectrum C. The spectra were measured at 7 K with all other conditions as described for Fig. 3.

**Fig. 8.** Effect of DOPA binding on the EPR spectrum of tyrosine hydroxylase. The spectrum of tyrosine hydroxylase (77 μM in 50 mM Hepes, 10% glycerol, 100 mM KCl, pH 7.1) was taken before (A) and after (B) the addition of DOPA to a final concentration of 150 μM. The spectra were taken at 6 K; otherwise, the conditions were as described for Fig. 3.

**Fig. 9.** Inhibition of tyrosine hydroxylase activity by iron-specific chelators. The enzyme assay containing 90 μg/ml catalase, 190 μM NADH, 0.45 unit/ml sheep dihydropterin reductase, 200 μM phenylalanine, 10 μM 6-methyltetrahydropterin, 80 mM Hepes, 80 mM KCl, 8% glycerol, pH 7.1, was allowed to equilibrate at 25°C before tyrosine hydroxylase was added (final concentration, 0.16 μM). After 90 s bathophenanthroline disulfonic acid (B) or DHN (C) was added to give a final chelator concentration of 1.3 μM. Water replaced the chelator in the control assays (A). The decrease in absorption in blank assays lacking tyrosine hydroxylase was subtracted to correct for tetrahydropterin autoxidation.

The decrease in absorption continued so that almost complete inhibition had occurred 150 s after DHN was added.
activity (3, 4); with the rat enzyme only the ferrous form of the metal was effective (3). This was in contrast to the presence of ferric iron in the enzyme from nonrecombinant sources.

Studies of these enzymes have certainly provided a great deal of insight into the regulation of the enzyme, the identities of the iron ligands, and the metal binding specificity. However, the most heavily studied forms of tyrosine hydroxylase have been inhibited, in the case of the nonrecombinant enzymes, or isolated as the apoenzyme, in the case of the recombinant human enzyme. Consequently, analysis of the redox forms of the enzyme that predominate in the absence of inhibitors and during turnover have not been amenable to study with such preparations. Contributing to this is the lability of the iron in the absence of bound inhibitors. Isolation procedures that produce iron-loaded enzyme from bovine or rat tissues result in apoenzyme from bacterial sources in the absence of catecholamines (4). Dissociation constants for ferrous iron of 1–3 and 0.15 μM have been reported for the human (4) and rat (3) enzymes, respectively, consistent with relatively loose binding. We have been able to obtain preparations of recombinant rat tyrosine hydroxylase with iron contents of 0.5–0.65 atoms/subunit, comparable with the amounts obtained with nonrecombinant enzyme but lacking bound catecholamine. These preparations have allowed us to carry out the first studies with large amounts of active enzyme that contain iron. In the experiments described here we have focused on the redox states of the iron in the resting enzyme and during and after catalytic turnover.

The iron in the isolated enzyme is in the ferric form even in the absence of inhibitory catechols. This is clearly established by the results with the redox-specific iron chelators and by the spectral properties of the enzyme. Quenching of the fluorescence of DHN but not of bathophenanthroline is consistent with all of the iron being in the ferric form. The low temperature EPR spectrum is clearly that of high spin ferric iron. The light green color of the enzyme, due to absorbance out past 450 nm, resembles that seen with the iron-containing enzymes lipoygenase and superoxide dismutase (18, 19). This absorbance is likely to be due to histidine to Fe(III) charge-transfer interactions. Consistent with this conclusion, two of the ligands of the iron in tyrosine hydroxylase have been identified as His-331 and His-336 (5).

As noted above, metal replacement studies have implicated ferrous iron as the active species in tyrosine hydroxylase. This suggests that the iron must be reduced during or before the catalytic cycle. As shown here, either dithionite or 6-methyltetrahydropterin can supply the electrons for the reduction. The physiological reduc tant is most likely tetrahydrobiopterin, given the ready reduction of the iron by 6-methyltetrahydropterin. The reduction by 6-methyltetrahydropterin is quite rapid. If one assumes a second order kinetic mechanism for reduction, a rate constant for reduction of 2 m M⁻¹ s⁻¹ can be calculated from the data in Fig. 5. Previous studies of the rate of DOPA formation were unable to detect any lag in turnover (20). At the standard concentration of 500 μM 6-methyltetrahydropterin used in enzyme assays, the half-life for reduction of the iron by the pterin can be estimated as 0.7 s, consistent with the lack of a detectable lag. The stoichiometry of the reduction requires that oxidation of one molecule of tetrahydropterin to the p-quinonoid form results in reduction of two active site iron atoms. The data presented here do not address the intermediates involved or the fate of pterin radical that must be generated. This result suggests that two active sites may be relatively close together in the tyrosine hydroxylase tetramer.

Once reduced, the predominant form of the enzyme during turnover is EPR-silent. Previous studies with alternate substrates have indicated that the rate-limiting step in turnover is formation of the hydroxylating intermediate (16). Once this forms, the reaction with the amino acid substrate is rapid, so that the hydroxylating intermediate is unlikely to accumulate to detectable levels. Previous studies have also shown that product release is rapid (20). Thus, free enzyme and enzyme-substrate complexes are likely to predominate during turnover. If so, the EPR-silent form of the enzyme present during turnover contains iron in the ferrous state. There appears to be no requirement for cycling through the ferric form during a single turnover. Consistent with this interpretation, the addition of a ferric iron chelator such as DHN initially has no effect on the catalytic rate, while the ferrous iron chelator bathophenanthroline behaves as a competitive inhibitor, binding to the ferrous enzyme.

During turnover, a small fraction of the enzyme is oxidized to the ferric form. The most straightforward mechanism for this is a reaction with molecular oxygen. Simply allowing the ferrous form of the enzyme to stand open to the atmosphere results in the eventual oxidation of all the iron to the ferric form. The time-dependent inhibition by DHN during turnover can be explained by reaction of the chelator with the small fraction of ferric enzyme that forms. The binding of DHN to the ferric form is extremely tight, with a $K_d$ value of 14 nM under the conditions of these experiments. Consequently, the inhibition is essentially irreversible and the activity in the presence of DHN continuously decreases to zero.

The product DOPA can similarly react with the oxidized enzyme to form the enzyme-DOPA complex, as shown in Fig. 7. DOPA also binds very tightly to the ferric iron in tyrosine hydroxylase, with a $K_d$ value of 53 nM. Unlike the reaction with DHN, inhibition by DOPA is likely to have physiological relevance due to the regulatory properties of tyrosine hydroxylase. The enzyme is regulated post-translationally by phosphorylation of several serine residues in a regulatory domain (21, 22). Only in the case of phosphorylation of serine 40 by cAMP-dependent protein kinase have the effects of phosphorylation been studied in any detail. Daubner et al. (10) showed that phosphorylation at serine 40 activated tyrosine hydroxylase by relieving the enzyme from the inhibitory effects of bound catecholamines. Based on these results they proposed that phosphorylation at serine 40 and inhibition by catecholamines act in concert to regulate the activity of tyrosine hydroxylase. Newly synthesized enzyme would be free of bound catecholamines. However, as levels of DOPA and other catecholamines rise in the nerve cell, the enzyme would increas-

\[ \text{DOPA, H}_2\text{OH} \rightarrow \text{PH}_4\text{O}_2\text{tyr} \]

\[ \text{EFe(II)PH}_4\text{O}_2\text{tyr} \rightarrow \text{EFe(III)DOPA} \]

\[ \text{EFe(III)DOPA} \]
ingly be trapped as the ferric form by a catecholamine, decreasing the rate of catecholamine biosynthesis. Phosphorylation of serine 40 by cAMP-dependent protein kinase reverses the inhibition by catecholamines, resulting in a large increase in the rate of biosynthesis of DOPA and the other catecholamines. The data described here are fully consistent with such a regulatory balance between phosphorylation and catecholamine inhibition.

In conclusion, the redox states of the iron in tyrosine hydroxylase described here can be summarized by the mechanism in Scheme 1. The ferrous form of the enzyme is the active species. This binds the substrates and passes through the catalytic cycle, ending still in the ferrous form. The ferrous iron can be oxidized to the ferric form by molecular oxygen. A tetrahydropterin can rapidly reduce the metal back to the ferric form, regenerating the active enzyme. Alternatively, the ferric form can react with a catechol such as DOPA or dopamine, forming an inactive complex. Reactivation of this latter complex requires phosphorylation of the enzyme.

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