The Effect of Substrate, Dihydrobiopterin, and Dopamine on the EPR Spectroscopic Properties and the Midpoint Potential of the Catalytic Iron in Recombinant Human Phenylalanine Hydroxylase*

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Phenylalanine hydroxylase (PAH) is a tetrahydrobiopterin (BH$_4$) and non-heme iron-dependent enzyme that hydroxylates L-Phe to L-Tyr. The paramagnetic ferric iron at the active site of recombinant human PAH (hPAH) and its midpoint potential at pH 7.25 (Em) was measured upon the simultaneous binding of both L-Phe, BH$_4$, and dopamine. All three ligands induced a decrease in the Em value comparable with that of the ligand-free form (BH$_4$) and of dopamine. All three ligands induced a decrease in the Em value of the iron to +123 ± 4 mV (L-Phe), +110 ± 20 mV (BH$_4$), and −8 ± 9 mV (dopamine). On the basis of these data we have calculated that the binding affinities of L-Phe, BH$_4$, and dopamine decrease by 28, 47-, and 5040-fold, respectively, for the reduced ferrous form of the enzyme, with respect to the ferric form. Interestingly, an Em value comparable with that of the ligand-free, resting form of wt-hPAH, i.e. +191 ± 11 mV, was measured upon the simultaneous binding of both L-Phe and BH$_4$, representing an inactive model for the iron environment under turnover conditions. Our findings provide new information on the redox properties of the active site iron relevant for the understanding of the reductive activation of the enzyme and the catalytic mechanism.

Phenylalanine hydroxylase (PAH), phenylalanine 4-monoxygenase, EC 1.14.16.1) is a tetrahydrobiopterin (BH$_4$) and non-heme iron-dependent enzyme that hydroxylates L-Phe to L-Tyr using dioxygen. PAH is found mainly in the liver, and mutations in human PAH (hPAH) result in a dysfunction associated with the autosomal recessive disorder phenylketonuria, which is the most prevalent inborn error of amino acid metabolism. In recent years, the understanding of the crystal structure of the ligand-free PAH from human (1, 2) and rat (3) and of the homologous enzyme tyrosine hydroxylase (TH) (4) have been made in the elucidation of the crystal structure of the ligand-free PAH from human (1, 2) and rat (3) and of the homologous enzyme tyrosine hydroxylase (TH) (4). PAH and TH are structurally and functionally closely related enzymes containing a 2-His-1-carboxylate facial triad motif (5, 6) anchoring the iron cofactor and molecular docking (9). These structural studies support the proposal that an iron-peroxo-tetrahydropterin complex forms during the catalytic cycle (10), and may either act as the hydroxylating intermediate itself or be the precursor of a ferryl oxo intermediate capable of aromatic hydroxylation (11, 12). Thus, L-Phe seems to bind to the second coordination sphere of the iron with a distance between the hydroxylation sites (C4a of the tetrahydropterin and C4 in L-Phe) of 6.3 Å, which is adequate for the intercalation of iron-coordinated molecular oxygen (9). Moreover, the crystal structure of hPAH complexed with diverse catecholamines has revealed that the inhibitors bind to the iron by bidentate coordination through the catechol hydroxyl groups (13), as observed earlier by resonance Raman spectroscopy of PAH and TH (14, 15).

Although these recent structural studies have provided further insight into the function of the iron and the pterin in the catalytic reaction of the aromatic amino acid hydroxylases, little is yet known about the details of electron transfer reactions and the catalytic mechanism. It seems clear that no product or intermediate is released prior to the binding of all substrates, and the first observable product of the pterin is a 4a-hydroxytetrahydropterin, which is dehydrated to quinonoid-dihydrobiopterin (q-BH$_2$) either spontaneously or in a reaction.
action catalyzed by pterin 4a-carbinolamine dehydratase (16, 17). PAH isolated from rat liver and recombinant rat PAH contain the catalytic iron in the ferrie high spin (S = 5/2) state (18–21). In the catalytic reaction Fe(III) is reduced to Fe(II) by BH₄⁻, termed “reductive activation” of the enzyme (22), and in vitro this reduction is an obligate step that occurs in the pre-steady state period (18). Some experimental evidence has been presented in favor of Fe(II) during subsequent turnovers (16), but this has not been proven, and a redox cycling from the Fe(II) to Fe(III) and even Fe(IV) has alternatively been proposed (16, 23). The reductive activation produces ätzlich (22) and, although some controversy exists in the early literature about the number of electron equivalents consumed in this reduction and the requirement for dioxygen, 1.2 ± 0.1 pterin-derived electrons seem to be consumed per Fe(III) site, i.e. about 1 reduced tetrahydropterin/2 Fe(III)-PAH subunits, under either aerobic or anaerobic conditions (16, 24). 

Both BH₄⁻ and the substrate L-Phe also have important regulatory functions, which seem to be of physiological significance (25); inhibitory catecholamines regulate the activity of TH in an interplay with phosphorylation (26–28). The conformational changes induced by the substrate, the natural pterin cofactor (and its inactive analogue), and the catecholamine inhibitors have been studied at the level of the tertiary and quaternary structure of both PAH and TH (25, 29, 30). However, it is not known to what extent the binding of substrate, cofactor, and catecholamines at the active site affects the coordination environment of the catalytic iron and its reactivity.

Earlier EPR spectroscopic studies on rat PAH have revealed that the coordination geometry of the ferric iron depends on the buffer ions and the presence of ligands coordinating at the first and the second coordination sphere (20, 21). Although the enzyme isolated from rat liver seems to contain a stoichiometric amount of iron per enzyme subunit, not all of the iron has been found to be catalytically active (18–20). Moreover, the same proportion of iron that coordinates to catecholamines is reduced by the tetrahydropterin cofactor and participates in the catalysis (20).

In the present study we have further characterized the X-band EPR spectroscopic properties of both the tetrameric wild-type and a dimeric truncated form of human PAH corresponding to the catalytic domain, as well as the effect of the substrate L-Phe, reduced and oxidized pterin cofactor, and the inhibitor dopamine. We also report for the first time the midpoint potential of the iron in the wild-type human PAH as isolated and its modulation upon the binding of substrate, oxidized pterin cofactor (BH₄⁻), and dopamine. 

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of the Wild-type and Truncated Form of hPAH—Expression in Escherichia coli (TB1 cells) of human wild-type PAH (wt-hPAH) and the truncated hPAH (Gly103-Gln428), i.e. ΔN102/ΔC24-hPAH as fusion proteins with maltose-binding protein, purification of the fusion proteins by affinity chromatography on amylose resin, and their cleavage by the restriction protease factor Xa (New England Biolabs) were performed as described (31, 32). The tetrameric form of wt-hPAH and the dimeric hPAH (Gly103-Gln428), corresponding to the catalytic domain, were separated from aggregated/higher oligomeric forms and from maltose-binding protein and factor Xa by size exclusion chromatography on HiLoad Superdex 200 HR prepacked column (1.6 × 60 cm) from Amersham Pharmacia Biotech (32). Protein concentration was estimated spectrophotometrically using the absorption coefficients A₂₈₀ nm (1 mg mL⁻¹) = 1.0 for wt-hPAH and 0.9 for hPAH (Gly103-Gln428).**

**Preparation of Samples for EPR Spectroscopy—**Enzyme samples initially were prepared in 20 mM NaHepes, 0.2 mM NaCl, pH 7.0. The iron content of the enzyme samples, measured by atomic absorption spectroscopy, was as previously reported (1.8–2.0 atoms Fe/ tetramer for wt-hPAH and 0.8–0.9 atoms Fe/dimer for hPAH (Gly103-Gln428)) (32). In the experiments for the initial characterization of the iron center and the effect of ligands on the X-band EPR spectrum of hPAH, enzyme samples of each wt-hPAH or hPAH (Gly103-Gln428) were prepared in 20 mM NaHepes, 0.2 mM NaCl, pH 7.0. Additions and incubations (5 min, pH 7.0, 25 °C) of either of the following compounds: l-Phe, dopamine, l-erythro-5,6,7,8-tetrahydrobiopoterin (BH₄⁻, Dr. B. Schircks Laboratories) and 6-methyl-5,6,7,8-tetrahydropterin (BH₂, Dr. B. Schircks Laboratories) in the presence of dithiothreitol (DTT), were performed in the EPR tubes prior to freezing the samples. We have previously shown that DTT alone does not reduce the Fe(III) in rat PAH in the presence of dioxygen (20).

For the EPR-monitored redox titrations the samples were prepared in 50 mM Mops buffer, 0.2 mM KCl, pH 7.25, and the final enzyme (wt-hPAH) concentration was 100–150 μM subunit. Nonphysiologically bound Cu(II), giving rise to characteristic EPR signals around g = 2.0–2.3, was removed by incubation of the enzyme with 5 mM EDTA followed by three cycles of dilution and concentration in EDTA-free Mops buffer using Centricron 30 microconcentrators (Amicon). This treatment did not result in any significant change in the shape or intensity of the Fe(III) signal around g = 4.3.

**EPR Spectroscopy and Redox Titrations—**A first series of EPR analysis was performed on the recombinant hPAH in the absence and presence of ligands at 9.64 GHz microwave frequency on a Bruker ESP300E equipped with an Oxford Instruments cryostat 900 at 3.6 K. Other EPR parameters are given in the figure legends. The EPR spectra were smoothed with the polynomial filter (n = 15) provided in the program package Bruker ESP300E (Bruker). The EPR spectra were baseline corrected. The baseline correction and simulation of the Fe(III) EPR was performed by comparing the double integral of the spectra of hPAH with the double integral of a 500 μM transferrin standard at several temperatures (which excludes an error that might be introduced by very different D values). In the cases in which quantification was performed using simulated spectra, similar results were obtained.

Room temperature potentiometric titrations for subsequent EPR monitoring were performed in a 2-mL anaerobic cell under purified argon. The bulk potential of the stirred solution was measured using a platinum wire electrode with respect to the potential of a Radiometer REFP201 Ag/AgCl reference electrode. Reported potentials were all expressed relative to the normal hydrogen electrode. 100 μM subunit hPAH in 50 mM Mops, 0.2 mM KCl, pH 7.25 was poised at various potentials in the presence of 100 mM each of the following mediators: N,N,N',N'-tetramethyl-2,6-dichlorophenolindophenol, phenazine ethosulfate, methylene blue, resorufin, indigosulfonate, phenoasfranin, safranin O, neutral red, benzyl viologen, and methyl viologen. Sodium dithionite and K₃Fe(CN)₆ were used as reductant and oxidant, respectively. Redox equilibrium was obtained as judged by the attainment of a stable solution potential within a few minutes after the addition of the titrant to the reaction mixture. The samples were transferred anaerobically to EPR tubes and directly frozen in liquid nitrogen. Midpoint potentials (Eₚ) were obtained from the experimental data points with a least squares fit to the Nernst equation (n = 1). In addition to the titration of the ligand free enzyme, titrations in the presence of either l-Phe (5 mM), BH₂ (5 mM), both l-Phe (5 mM) and BH₂ (5 mM), or dopamine (1 mM) were also performed. The EPR-monitored titrations of hPAH samples were performed at a microwave frequency of 9.41 GHz and a microwave power of 8 mW, with a 10-Gauss (1.0 mT) modulation amplitude and a modulation frequency of 100 kHz at 29 K. These temperature values were used to optimize the signal-to-noise ratio under nonsaturating conditions. Simulation of the spectra was performed as described (33).

**RESULTS**

**EPR Spectra of Recombinant Human Phenylalanine Hydroxylase—**Tris has been found to be an inhibitor of the enzyme, competitive to the pterin cofactor (22), and our previous EPR spectroscopic studies on rat PAH demonstrated that Tris, in its base form, induces changes in the active site iron (20). Consequently, we have used Hepes and Mops buffers in the present EPR studies. Recombinant wt-hPAH revealed a low temperature (3.6 K) EPR spectrum typical for high spin (S = 5/2) Fe(III) (Fig. 1, spectrum A). The spectrum is dominated by a resonance centered around g = 4.3, with an accompanying broad signal with g value of ~9.7, which is characteristic of ferric iron in a rhombic environment, with an E/D value of ~1/3. Some minor iron species showing weak signals with g values spread from 9.7 to 7.0 and 5.3 to 4.3 with various E/D values between 0.05
and 0.33 were also present, indicating some microheterogeneity in the coordination geometry of the enzyme-bound iron. Nevertheless, the iron environment appears to be more homogenous in the recombinant hPAH than previously observed for the hepatic rat and bovine enzymes, which have been isolated through procedures including preincubation of the crude extracts with ferrous ions and DTT (34). Those PAH preparations showed a split of the major resonance at g = 4.3 and a higher proportion of iron in a less rhombic environment (gmax values in the range of 7 to 6 and E/D < 0.05) (20).

Reduction of High Spin (S = 5/2) Fe(III) by Tetrahydrobiopterin and the Effect of l-Phe—Quantification of the Fe(III) EPR in wild-type hPAH gave numbers that were in reasonable agreement with the total iron content measured by atomic absorption spectroscopy, i.e. 0.45–0.52 atoms iron/subunit for recombinant wt-hPAH (32). This value is also similar to the catalytically active iron measured in rat PAH (19, 21, 35). Thus, although the isolated rat and bovine liver enzyme preparations contain about 1 atom iron/subunit, only a fraction of it has been found to be reduced by the cofactor and thus to participate in catalysis (19, 20).

The effect of preincubation of the samples with either l-Phe or 6-MPH₄ as well as with both compounds simultaneously (turnover conditions) on the EPR spectrum of wt-hPAH is shown in Fig. 1. For a better estimation of the amount of iron that is not in the completely rhombic environment (E/D = 1/3, g = 4.3, resonance at 160 mT), the corresponding zero line for each spectrum is shown. The area enclosed by the recorded spectra between 70 and 100 mT and this zero line correlates with iron in a less rhombic environment. A comparison of these areas in spectra 1A and 1B shows that preincubation of wt-hPAH (spectrum A) with the substrate l-Phe (spectrum B) results in a decrease of these signals. Moreover, a concomitant increase of the intensity of the major signal at g = 4.3 is observed. Quantification, performed by double integration of the spectra shown in 1A and 1B between 60 and 240 mT reveals that the total intensity of both spectra is identical within the error range of the method (10%). By contrast, when the reducing cofactor analogue 6-MPH₄ is added to the resting form of the enzyme, the amount of ferric iron decreases to 35%, as judged by the decrease of the double integral of the major signal at g = 4.3 (Fig. 1, spectrum C). This reduction is slightly enhanced (only 28% of the iron is in the ferric state) if 6-MPH₄ is added to the enzyme preincubated with l-Phe, i.e., at turnover conditions (Fig. 1, spectrum D).

Effect of Truncation of the Enzyme and the Addition of Oxidized Cofactor Analogue (BH₂) on the EPR Spectrum—The deletion of the N-terminal regulatory and C-terminal tetramerization domains of hPAH results in an activated (about 3-fold) dimeric hPAH(Gly₁⁰³-Gln₄²₈) form that does not show any significant further activation by prior incubation with l-Phe, and contrary to the full-length wt-hPAH, it does not bind l-Phe with positive cooperativity (32). As seen by the crystal structures of this truncated form (1) and of rat PAH containing the catalytic and regulatory domains (3), the negative effect exerted by the regulatory domain on the catalytic activity is not accompanied by significant structural changes around the 6-coordinating iron site. Accordingly, no significant differences were observed in the main features of the X-band EPR spectra of the full-length and truncated hPAH forms either in the absence or presence of l-Phe (Fig. 1, spectra A and B and Fig. 2, spectra A and B). However, there seem to be differences in the coordination environment between the iron in the full-length and the isolated catalytic domain as indicated by the decreased P₀ values for the microwave power saturation behavior of the truncated PAH (Table I), which may indicate a more homogenous iron coordination environment for this form. The oxidized cofactor analogue BH₂ inhibits both wt-hPAH and hPAH(Gly₁⁰³-Gln₄²₈) by a competitive type of inhibition versus the natural pterin cofactor BH₄, with a Kᵢ of 120 μM for wt-hPAH and 100 μM for the truncated form (9). The structure of the complex between hPAH(Gly₁⁰³-Gln₄²₈) and BH₂ has recently been solved both by NMR spectroscopy and docking in the ligand-free crystal structure of the enzyme (9) and by x-ray crystallography (8). We have examined here the effect of BH₂ on the EPR spectrum of this double truncated form. The addition of BH₂ at concentrations of 500 μM results in the appearance of a less rhombic type of high spin ferric species with g values of 7.4, 4.3, and (1.8) from the lowest and 5.8, (1.7), and (1.5) from the middle Kramer’s doublet with E/D = 0.07 (Fig. 2, spectrum C). The g values below 2 (in parentheses) can be estimated only from rhombograms, as any g or D strain has severe broadening effects in these g value regions. It seems that spectrum 2C consists of two major species, i.e., one with E/D = 0.07 and one with E/D = 0.33, as well as some minor species with intermediate E/D values that distort the signal at g value = 5.8.

Determination of the Midpoint Potential (Eₘₐₓ) of the Catalytic Iron in hPAH and the Effects of Ligand Binding at the Active Site—Studies intended to determine the Eₘₐₓ(Fe(III)/Fe(II)) for the active site iron in hPAH by direct electrochemistry using activated glassy carbon as the working electrode were unsuccessful with all the promoters used. Thus, EPR-monitored redox titrations of the iron in wt-hPAH (100–120 μM enzyme subunit in Mops buffer at pH 7.25) were performed in the absence of ligands and in the presence of l-Phe (Fig. 3, spectrum A), BH₂ (Fig. 3, spectrum B), and l-Phe and BH₂ simul-
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The values of the estimated midpoint potential \( E_{m} \) in all the samples was reduced in the range of 200 to +300 mV. The apparent \( E_{m} \) value of the active site iron, an important novel finding in the present study.

The low temperature (4 K) X-band EPR spectra obtained for recombinant hPAH, both the wt-hPAH and its N- and C-terminal truncated form (catalytic domain), are very similar to those obtained previously for the hepatic rat and bovine PAH when the enzyme samples are prepared in buffers that do not interact with the active site non-heme iron, i.e. potassium phosphate, NaHepes, and Mops (20, 21). However, an important difference was observed between the isolated hepatic and the recombinant enzyme forms. Whereas isolated rat liver PAH seems to contain more than 50% of the iron that does not participate in catalysis (18–20), in hPAH the major signal at \( g = 4.3 \), characteristic of high spin (\( S = 5/2 \)), ferric iron in a rhombic coordination geometry, is largely reduced (to almost a quarter of the intensity in the enzyme as isolated) in the presence of both L-Phe and tetrahydropterin (turnover conditions) (Fig. 1D).

The apparent \( E_{m} \) at pH 7.25 for the catalytic iron was measured both in the absence and presence of substrate, pterin cofactor analogue, and catecholamine inhibitor, all compounds binding at the active site. Previously, the \( E_{m} \) value of the natural cofactor, i.e. \( q\)-BH2/BH4 couple, has been determined to be +174 mV at pH 7 (40), and the literature values vary from +140 to +184 mV for cofactor analogues with various substituents at the 6-position (40–42). Thus, the \( E_{m} \) value of +207 ± 10 mV for the catalytic iron in the resting enzyme (Table II) is in agreement with a thermodynamically feasible electron transfer from the reduced cofactor to the iron site, explaining the observed reductive activation of the enzyme by the tetrahydropterin cofactors (22).

As seen from the EPR spectrum of the resting form of wt-hPAH, with a major broad peak around \( g = 4.3 \) and minor features with \( g \) values stretching from 9.7 to 4.5, the coordination geometry of the active site iron seems to be rather flexible. This conclusion is further supported by the crystal structure showing three water molecules in the first coordination sphere of the iron (1, 3). A sharpening of the \( g = 4.3 \) signal and an increase of the \( P_{2} \) value for the microwave power saturation behavior was observed in the presence of L-Phe, indicating a change in coordination environment of the active site iron in the substrate-activated enzyme form. This effect is in good agreement with our previous finding that the binding of L-Phe at the active site is accompanied by a dissociation of one of the ferric iron-coordinating water molecules (43, 44). The recent structural study on the ternary PAH-Fe(III)-L-Phe-BH2 com-
Table I

Microwave power saturation ($P_{1/2}$) of the iron signal at $g = 4.3$ of different forms of hPAH at different temperatures

| Sample                                      | $P_{1/2}$ mW |
|---------------------------------------------|--------------|
| wt-hPAH                                     | 0.92 ± 0.14  |
| hPAH(Gly$^{103}$-Gln$^{428}$)              | 0.90 ± 0.13  |
| hPAH(Gly$^{103}$-Gln$^{428}$) + 1 mM l-Phe | 1.3 ± 0.1    |

TABLE II

Midpoint redox potential $E_m$ (Fe(III)/Fe(II)) at pH 7.25 in samples of wt-hPAH in the absence or presence of l-Phe (5 mM), BH$_2$ (5 mM), and dopamine (1 mM), and apparent $K_d$ values for ligand binding to the enzyme

| Sample                              | $E_m$ mV | $K_d$ox$^a$ mm | $K_d$red$^a$ mm |
|-------------------------------------|----------|---------------|----------------|
| wt-hPAH                             |          |               |                |
| wt-hPAH + L-Phe                      |          |               |                |
| wt-hPAH + BH$_2$                    |          |               |                |
| wt-hPAH + L-Phe + BH$_2$            |          |               |                |
| wt-hPAH + dopamine                  |          |               |                |
| q-BH$_2$/BH$_4$ couple              | +207 ± 10$^d$ | 0.10$^e$ | 2.79 |
| wt-hPAH                             | +123 ± 4$^d$ | 0.01$^f$ | 0.47 |

**Fig. 3.** EPR spectra of the samples used in the redox titrations. The samples contained wt-hPAH (100 μM subunit) in 50 mM Mops buffer, 0.2 M KCl, pH 7.25, with 5 mM L-Phe ($A$), 5 mM BH$_4$ ($B$), 5 mM L-Phe and 5 mM BH$_2$ ($C$), or 1 mM dopamine ($D$). The spectra were taken at a microwave frequency of 9.41 GHz and a microwave power of 8 mW, with a 1 mT modulation amplitude and a modulation frequency of 100 kHz at 29 K.

**Fig. 4.** EPR monitored redox titrations. The samples shown are: wt-hPAH (100 μM subunit) in 50 mM Mops buffer, 0.2 M KCl, pH 7.25, in the absence of ligands (■), with 5 mM L-Phe (▲), 5 mM BH$_4$ (□), 5 mM L-Phe and 5 mM BH$_2$ (○), and 1 mM dopamine (●). See also Table II and the legend to Fig. 3.

Potential were encountered on addition of the tetrahydrobiopterin cofactor analogue BH$_2$ and the inhibitor dopamine. Thus, a less rhombic type of signal is observed in the presence of these inhibitors ($g_{max}$ values of 7.4 and 7.0 for BH$_2$ and dopamine, respectively) (Figs. 2C and 3) indicating a decrease of the $E/D$ value (from 0.33 to −0.07 and 0.05, respectively) and a change in the coordination geometry of the iron. These spectroscopic changes were also accompanied by a decrease of the $E_m$ value of the iron to numbers, which are significantly lower than those of the pterin cofactor redox couple (Table II). Notably for the hPAH-dopamine complex it is clear that reduction of the Fe(III) by the cofactor is not possible to achieve because of a −200 mV negative shift of the $E_m$ value, and this effect could be of physiological significance in the case of TH, which is regulated by feedback inhibition by catecholamines and phosphorylation (12, 26, 27). The inhibition of PAH by catecholamines appears to be competitive with respect to the tetrahydropterin cofactors (45). Paramagnetic relaxation NMR experiments have shown that catecholamines do not compete for the cofactor binding site in hPAH and that both noradrenaline and dopamine can be bound simultaneously with the cofactor analogue BH$_2$ (9). Thus, the apparent competitive type of inhibition by catecholamines versus the pterin cofactor may rather be due to changes in the ligand field geometry of the active site iron as a result of formation of the tight bidentate catecholate-Fe(III) complex (13), lowering the $E_m$ value and thus stabilizing the ferric state. The −200-mV negative shift of the midpoint potential on dopamine binding also explains the experimental finding that recombinant human TH reconstituted with Fe(II) is rapidly oxidized upon the addition of catecholamines forming a ferric blue-green complex (46). Moreover, based on the large decrease of $E_m$ upon dopamine binding, we estimated that the $K_d$ value for the binding of this inhibitor to the reduced enzyme was increased by about 5040-fold (Table II).
for these discrepancies are not clear, although alternative explanations have been proposed (8). The less rhombic type of signals, which appear in the EPR spectra of both the truncated form hPAH(Gly103-Gln428) and the wt-hPAH in the presence of BH$_2$, are compatible with a coordination of BH$_2$ to the iron as indicated by the solution structure (9). Nevertheless, the interaction of the pterin ring with an iron-coordinated water molecule might well result in a loss of flexibility and a change of the water-iron bonding distance and thereby in a less rhombic coordination geometry. Regardless of the detailed interactions between the pterin ring and the iron, it seems clear that BH$_2$ changes the coordination geometry of the metal and decreases its midpoint potential (Table II). However, when bound simultaneously at opposite sides in the coordination environment of the iron in the ternary PAH-Fe(III)-t.-Phe-BH$_2$ complex (9) (Fig. 5), t.-Phe and BH$_2$ may exert compensatory changes in the electronic environment of the iron, in agreement with the finding that the midpoint potential in the ternary complex is more similar to that in the resting unbound enzyme than in the binary complexes (Table II). The t.-Phe induced conformational change at the protein level is well documented on the basis of intrinsic tryptophan fluorescence spectroscopy, limited proteolysis, and dynamic light scattering (reviewed in Refs. 16 and 17). Furthermore, it has been shown that in the double truncated form of hPAH, BH$_2$ binds by an induced fit mechanism, involving a conformational change in the protein at the active site (8) as well as in the dihydroxypropyl side-chain of the cofactor (9). All of the structural and kinetic data obtained so far are compatible with the proposed four-state conformational model (51); i.e. a resting state, a t.-Phe activated state, a BH$_4$/BH$_2$ inhibited state, and a state of catalytic turnover. In this model the resting state and the turnover state were found to be very similar by different indirect conformational probes.

The inactive ternary PAH-Fe(III)-t.-Phe-BH$_2$ complex seems to provide a good model for the coordination environment of the iron under turnover conditions. Although the midpoint potential for the BH$_4$/q-BH$_2$ couple may change when the cofactor binds to the enzyme, the +81 mV increase in $E_m$ obtained when both ligands are added simultaneously with respect to that obtained in the presence of BH$_2$ alone is in agreement with a thermodynamically favorable electron transfer from the cofactor to the iron at turnover conditions. As seen in Fig. 1, a larger reduction is obtained with 6-MPH$_4$ when wt-hPAH is also complexed with t.-Phe (Fig. 1, spectrum D; turnover conditions) than in the absence of the substrate (spectrum C). Accordingly, although the affinity for tetrahydropterin cofactors is decreased for the t.-Phe-activated enzyme (50), it has been reported previously that the reduction seems to be facilitated by the presence of t.-Phe (24). This modulation of the $E_m$ value upon ligand binding also agrees with a type of mechanism for PAH involving the formation of a complex of all the reactants prior to catalysis. Thus, only when both the substrate and the tetrahydropterin cofactor are simultaneously bound to ferrous PAH, dioxygen may bind and be activated at the open coordination position, as indicated by magnetic circular dichroism spectroscopic studies with the cofactor analogue 5-deaza-6-methyltetrahydropterin (52). Moreover, the $K_m$ values obtained for t.-Phe and BH$_2$ with the ferrous form of PAH (25) resemble the $K_{pox}$ (Table II), indicating that the decrease in affinity estimated for the independent binding of substrate and cofactor analogue with respect to the binding to the oxidized form of the enzyme is reversed when the ligands bind forming the ternary complex. The midpoint reduction potential of the heme iron in the BH$_4$-dependent enzyme nitric-oxide synthase has also been shown to be modulated similarly by substrate and active site inhibitors (37). However, the absolute values for the midpoint
potentials determined for this enzyme are about 400–500 mV lower than those determined in this work for hPAH, reflecting the different roles of BH4 in the reactions. The $E_m$ values measured for nitric-oxide synthase have been found adequate for a thermodynamically feasible reduction of the heme by the flavin cofactors (53), and neither 4-hydroxytetrahydropterin nor $q$-BH$_2$ has been detected in the reaction of this enzyme. Recently, a protonated trihydrobiopterin radical (54) has been shown to be formed in the redox cycling of BH$_4$ in nitric-oxide synthase. The different roles of BH$_4$ in the reactions. The potentials determined for this enzyme are about 400–500 mV lower than those determined in this work for hPAH, reflecting the different roles of BH$_4$ in the reactions. The $E_m$ values measured for nitric-oxide synthase have been found adequate for a thermodynamically feasible reduction of the heme by the flavin cofactors (53), and neither 4-hydroxytetrahydropterin nor $q$-BH$_2$ has been detected in the reaction of this enzyme. Recently, a protonated trihydrobiopterin radical (54) has been shown to be formed in the redox cycling of BH$_4$ in nitric-oxide synthase (55, 56). Formation of such a radical has been proposed but not detected for any of the aromatic amino acid hydroxylases (12). Although the details of the electron transfer reactions in the catalytic process of the aromatic amino acid hydroxylases are not yet clear (16), our present findings represent new information on the redox properties of the active site iron that is relevant for our understanding of their catalytic mechanism.

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The Effect of Substrate, Dihydrobiopterin, and Dopamine on the EPR Spectroscopic Properties and the Midpoint Potential of the Catalytic Iron in Recombinant Human Phenylalanine Hydroxylase

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