The Accessibility of Iron at the Active Site of Recombinant Human Phenylalanine Hydroxylase to Water As Studied by \textsuperscript{1}H NMR Paramagnetic Relaxation

EFFECT OF L-PHE AND COMPARISON WITH THE RAT ENZYME\textsuperscript{*}

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The high-spin ($S = 5/2$) Fe(III) ion at the active site of recombinant human phenylalanine hydroxylase (PAH) has a paramagnetic effect on the longitudinal relaxation rate of water protons. This effect is proportional to the concentration of enzyme, with a paramagnetic molar-relaxivity value at 400 MHz and 25°C of 1.3 ($\pm 0.03$) $\times 10^3$ s$^{-1}$ M$^{-1}$. The value of the Arrhenius activation energy ($E_a$) for the relaxation rate was $-14.4 \pm 1.1$ kJ/mol for the resting enzyme, indicating a fast exchange of water protons in the paramagnetic environment. The frequency dependence of the relaxation rate also supported this hypothesis. Thus, the recombinant human PAH appears to have a more solvent-accessible catalytic iron than the rat enzyme, in which the water coordinated to the metal is slowly exchanging with the solvent. These findings may be related to the level of basal activity before activation for these enzymes, which is higher for human than for rat PAH. In the presence of saturating (5 mM) concentrations of the substrate L-Phe, the paramagnetic molar relaxivity for human PAH decreased to 0.72 ($\pm 0.05$) $\times 10^3$ s$^{-1}$ M$^{-1}$ with no significant change in the $E_a$. Effective correlation times ($\tau_C$) of 1.8 ($\pm 0.3$) $\times 10^{-10}$ and 1.25 ($\pm 0.2$) $\times 10^{-10}$ s$^{-1}$ were calculated for the enzyme and the enzyme-substrate complex, respectively, and most likely represent the electron spin relaxation rate ($\tau_\theta$) for Fe(III) in each case. Together with the paramagnetic molar-relaxivity values, the $\tau_C$ values were used to estimate Fe(III)-water distances. It seems that at least one of the three water molecules coordinated to the iron in the resting rat and human enzymes is displaced from coordination on the binding of L-Phe at the active site.

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Mammalian phenylalanine hydroxylase (PAH, EC 1.14.16.1)

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\textsuperscript{1} The abbreviations used are: PAH, phenylalanine hydroxylase; $C_o$, subunit concentration; $C_{Fe}$, iron concentration; $E_a$, activation energy; BH$_4$, (6R)-L-erythro-tetrahydrobiopterin; $T_{1\text{ex}}^{-1}$, dipolar correlation time; $T_{1\text{ip}}^{-1}$, paramagnetic contribution to the longitudinal relaxation rate; $T_{1\text{ORBS}}^{-1}$, observed water proton longitudinal relaxation rate; $T_{1\text{mag}}^{-1}$, outer-sphere contribution to the relaxation rate; $\tau_C$, dipolar correlation time; $\tau_\theta$, exchange lifetime; $\tau_{\text{es}}$, electron spin relaxation rate; $T_{1\text{ph}}$ and $T_{1\text{ph}}$, proton and electron Larmor frequencies, respectively.

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seems to be in agreement with the properties of PAH from human liver (16, 17).

By using 1H NMR paramagnetic relaxation of the water protons, we have shown earlier that the Fe(III) ion at the active site of resting rat PAH contains coordinated water and that following the binding of both L-Phe and L-noradrenaline at least one water molecule is displaced from coordination (18). In this study we have measured the paramagnetic effect of the ferric site in recombinant human PAH on the water protons in the absence and the presence of the substrate l-Phe. The differences encountered between the accessibility of the solvent to the iron in the rat and the human enzymes are interpreted based on the different degree of activity in the resting enzymes.

EXPERIMENTAL PROCEDURES

Materials—l-Phenylalanine and bathophenanthroline disulfonic acid were from Sigma and 2H2O (99.8%) from Aldrich. Expression of recombinant human PAH in Escherichia coli (TBE) as fusion protein with maltose-binding protein, purification of the fusion proteins by affinity chromatography on amylose resin followed by high-performance size exclusion chromatography, cleavage by factor Xa or enterokinase, and further purification of the hydroxylase was performed as described (7). Rat PAH was isolated from rat liver by the method (procedure II D) of Shimam et al. (19). The enzyme activity was measured by determination of l-Tyr formed by high pressure liquid chromatography and fluorometric detection as described (7). The concentration of purified PAH (both rat and human) was estimated using an absorption coefficient at 280 nm of 1.0 cm−1 for 1 mg/ml (7, 19).

Metal Analyses—The metal content of the enzyme preparations was determined by a Perkin-Elmer model 402 atomic absorption spectrophotometer equipped with a graphite furnace (type HGA-76B from Perkin-Elmer).

Preparation of Iron-free Apoenzymes—The iron was extracted from the isolated rat and human holoenzymes by a modification of the method of Gottschall et al. (20) using 1 mM bathophenanthrolone, 1 mM L-Phe, 0.5 mM BH4, and 1 mM dithiothreitol. The formation of the Fe(II)-bathophenanthroline complex was followed by measuring the increase in absorbance at 535 nm. The concentration of the isolated iron was calculated from the molar extinction coefficient for the Fe(II)-bathophenanthroline complex (ε355 = 22,000 M−1 cm−1) (21). The enzyme was separated from the chelating agent and other low molecular weight compounds by gel filtration on a G-25 Sephadex column (1.5–20 cm) equilibrated in 20 mM Na-Hepes, 0.2 mM NaCl, pH 7.0, and 1 mM L-Phe, included to stabilize the apoenzyme. The catalytically inactive apoenzyme was concentrated in Centriplus 30 concentrators (Amicon) and further purified of the hydroxylase was performed as described (18). The enzyme was concentrated in Centriplus 30 concentrators (Amicon, MA).

NMR Measurements—Longitudinal relaxation rates of the residual water signal (HDO) were measured on enzyme samples prepared by 3–4 cycles of 20-fold concentration and dilution in H2O, containing 20 mM potassium phosphate of pH 7.2 (pH value determined with an Ingold electrode and representing the uncorrected value in 2H2O) and 0.2 mM KCl, using Centrion 30 microconcentrators (Amicon). The NMR spectra were recorded on a Bruker DMX-400 and in experiments with variable field, measurements at 250 and 600 MHz were made on Bruker AM-250 and Bruker DRX-600 spectrometers, respectively. The longitudinal relaxation times (T1obs) were measured at the indicated probe temperatures by using a standard inversion-recovery sequence, with acquisition parameters including 16K data points, four transients per time increment and recycle delay (≥5 × T1). In the titration experiments, the enzyme solution was allowed to equilibrate at the indicated concentrations of L-Phe in the NMR tubes for 5 min at room temperature, before measurements. Titrations were performed by adding microliter amounts of concentrated solutions of L-Phe. Final dilution of the samples was ≤2.5%.

Theoretical Details—The longitudinal paramagnetic relaxation time of the bound water proton (T1M) is described by the dipolar term of the Solomon-Bloembergen equation (22, 23), i.e.

\[ T_{1M}^{-1} = 2 \gamma_i \gamma_p B_0^2 S(S+1) \left( \frac{3 \zeta c}{1 + \omega_n^2 \zeta c} + \frac{7 \zeta c}{1 + \omega_e^2 \zeta c} \right) \]

where \( \gamma_i \) is the nuclear gyromagnetic ratio, \( \gamma_p \) is the electronic g factor (isotropic splitting factor), \( \beta \) is the Bohr magneton, \( S \) is the electronic spin at the ground state of the paramagnetic ion (24), \( r \) is the metal-proton internuclear distance, \( \omega_n \) and \( \omega_e \) are the nuclear and electron Larmor precession frequencies, respectively, and \( \zeta \), the effective dipolar correlation time, which describes the molecular events which modulate the electron–nuclear dipolar coupling and can be calculated from the frequency dependence of the longitudinal paramagnetic relaxation (25).

The observed longitudinal relaxation rate of water protons in a protein solution (T1obs−1) is equal to TIP−1 + TIP−1, where TIP−1 is the longitudinal relaxation rate of water protons due to the paramagnetic ion and TIP−1 is the diamagnetic contribution of the protein due to the effects of protein residues–solvent interactions. TIP−1 was estimated using sodium dithionite-treated enzyme, in which the iron is fully reduced (26), or using apoenzyme forms without iron. TIP−1 values for iron after proteins, normalized to a subunit concentration (C) of 1 mM are expressed as paramagnetic molar relaxivity (TIP−1C−1), which is related to T1M by the following expression (27):

\[ T_{1P}^{-1} = \frac{1}{55.6} \left( T_{1M} + \gamma_p M^2 + (T_{0.5})^{-1} \right) \]

where \( q \) is the number of water ligands that are coordinated to the paramagnetic ion (i.e. in the first coordination sphere) and \( T_{0.5}^{-1} \) is the outer sphere contribution to the relaxation rate. T1M−1 is usually small in paramagnetic systems with coordinated water. The effect of varying temperature and frequency on TIP−1 was used to determine the predominant contributions (T1M, \( \gamma_p M^2 \) or \( T_{0.5} \)) to the observed relaxation rate (25). Further theoretical considerations relevant for this paper have been described (18).

RESULTS AND DISCUSSION

The Enzyme Preparations—The specific activities of the isolated recombinant human and rat liver PAH preparations with 1 mM l-Phe and 500 μM BH4 in the presence and the absence of prior incubation (5 min, 25 °C) with 1 mM l-Phe are shown in Table 1. Although the activity of the rat liver holoenzyme was not stimulated by addition of ferrous ammonium sulfate (100 μM) in the assay mixture, the activity of the recombinant human PAH increased by about 10% in the presence of ferrous ions, indicating the presence of some iron-free apoenzyme in the preparations. Thus, as determined by atomic absorption spectroscopy the preparations of rat and human PAH used in this study contained 0.98 ± 0.03 and 0.48 ± 0.05 atom of iron/mol subunit, respectively. By measuring the formation of the Fe(II)-bathophenanthroline complex at 535 nm, it was determined that a maximal amount of about 0.5 atom of iron/mol subunit was extracted from both the rat and human enzymes after a 30–45-min incubation period with the chelator at reducing conditions (see “Experimental Procedures”). After iron extraction, the resulting proteins were devoid of catalytic activity and were referred to as apoenzymes (Table 1). However, as shown by atomic absorption spectroscopy measurements, although no remaining iron was present in the apoenzyme forms of the recombinant human PAH, the apoenzyme of the rat liver enzyme contained 0.4–0.5 atom of iron/mol subunit. This population of inactive iron-containing PAH has previously been found not to be reduced by the tetraphydroner in cofactor and not to participate in catalysis (4, 5, 26). After reconstitution of the holoenzyme from the apoenzyme by incubation with 0.1 mM ferrous ammonium sulfate, full activity was recovered.

Effect of Recombinant Human Phenylalanine Hydroxylase on the Water Proton Relaxation Rates—T1obs−1 values were measured at 400 MHz on the bulk residual water signal (HDO).

| Enzyme Sample | Specific activity |
|---------------|------------------|
|                | Not activated     | Activated with l-Phe |
| Human PAH, holoenzyme | 510 1700 | fold |
| Human PAH, apoenzyme | ND* ND* |
| Rat PAH, holoenzyme | 223 3800 | 17 |
| Rat PAH, apoenzyme | ND 10 |

* ND, not detectable.
(4.8 ppm) at 295 K in deuterated samples of recombinant human PAH at various concentrations (up to 110 μM enzyme subunit), in the absence and presence of 5 mM L-Phe (Fig. 1). In this concentration range we found no significant diamagnetic contribution of the protein to the relaxation rate, measured either with the iron-free human apoenzyme or with the dithionite reduced enzyme (Fig. 1). The large effect of the enzyme as isolated on T₁OBS (1.31(± 0.03)×10⁻²) s⁻¹ indicates that the high-spin Fe(III) (S = 1/2) site in the recombinant human PAH is accessible to exchangeable water molecules, as was previously found for the rat liver enzyme (18). Accordingly, the x-ray structure of the catalytic domain of human PAH has shown that the ferric iron is six-coordinated to His-285, His-290, Glu-330 and to three water molecules, referred to as Wat (1), Wat (2), and Wat (3) (12). As shown by magnetic circular dichroism and x-ray absorption spectroscopy, the iron sites for both the resting ferric (inactive) and ferrous (active) forms in the rat enzyme also seem to be six-coordinate distorted octahedral and substrate binding results in geometric and electronic structural changes at the iron center (28).

Although the paramagnetic molar relaxivity (T₁P⁻¹Cₐ⁻¹) at 298 K is higher for the rat than for the human enzymes, the values become similar when they are normalized to molar concentration of iron (T₁P⁻¹Cₐ⁻¹) (Table II). The paramagnetic molar relaxivity of human PAH was found to decrease about 2-fold when 5 mM L-Phe was added (Table II), indicating either occlusion or displacement of the coordinated water molecules. The effect of L-Phe binding to the human enzyme on 1/T₁P was studied in more detail as a function of ligand concentration (Fig. 2). The titration curve was found to be hyperbolic, unlike the curves obtained on titration of rat liver PAH either with L-Phe or L-noradrenaline, which are three-phasic and nonhyperbolic (18). The three-phasic curves have been interpreted as being the result of the change from a system in which water is coordinated to Fe(III) at the active site and slowly exchanging with the bulk water, to a system in which water is fast exchanging at a site close to the iron, but not coordinated (18). In the case of the recombinant human PAH the hyperbolic titration curve (Fig. 2) seems to indicate the absence of exchange limitations in the enzyme as isolated, as well as in the L-Phe-enzyme complex.

**Temperature and Field Dependence of Water Proton Relaxation Rates**—The effect of temperature (16–40 °C) on the paramagnetic molar relaxivity of water protons is shown in Fig. 3. The Arrhenius activation energy (Eₐ) for the paramagnetic contribution to the relaxation, effective correlation times (τₑ), and estimated water-iron distances (r) for the rat and the recombinant human PAH in the absence and the presence of L-Phe

| [hPAH subunit] (μM) | Human PAH | Rat PAH* |
|---------------------|-----------|---------|
|                     | No L-Phe  | +5 mM L-Phe | No L-Phe  | +5 mM L-Phe |
| T₁OBS⁻¹(Cₐ⁻¹) (s⁻¹ M⁻¹) | 1.3 (±0.03)×10⁻² | 0.7 (±0.05)×10⁻² | 2.2 (±0.05)×10⁻² | 1.3 (±0.05)×10⁻² |
| T₁P⁻¹(Cₐ⁻¹) (s⁻¹ M⁻¹) | 2.7 (±0.07)×10⁻² | 1.5 (±0.2)×10⁻² | 2.3 (±0.05)×10⁻² | 1.4 (±0.05)×10⁻² |
| Eₐ (kJ/mol) | −14.4 ± 1.1 | −17.9 ± 0.4 | 11.3 ± 0.8 | −1.5 ± 0.2 |
| τₑ (ns) | 1.8 (±0.3)×10⁻¹ | 1.2 (±0.2)×10⁻¹ | ND | 4.2 (±0.5)×10⁻¹ |
| r(A) | 3.0 | 3.3 | <3.5 | 3.9 |

* Data from Martínez et al. (18).

* For q = 1, see text.
protein may impose hindrances to the free exchange of the coordinated water. This limitation was abolished in the L-Phe-activated enzyme \( (E_a = 1.5 \pm 0.2 \text{ kJ/mol}) \) (Table II).

The paramagnetic contribution to the relaxation time \( (T_{1P}) \) for solutions of human PAH was measured at 295 K and three different Larmor frequencies, i.e. 250, 400, and 600 MHz, and found to be frequency-dependent both in the presence and the absence of 5 mM L-Phe (data not shown), also consistent with a fast exchange condition of water protons in the paramagnetic environment (25). A linear fit of the \( T_{1P}^{-1} \) values at the three frequencies was used to calculate the effective dipolar correlation time \( (\tau_C, \text{Eq. 1}) \) under the assumption that it is constant in this frequency range (25, 30) (Table II). The \( \tau_C \) values thus obtained for both the human enzyme \( (1.8 \pm 0.3 \times 10^{-10} \text{ s}) \) and its enzyme-substrate complex \( (1.25 \pm 0.2 \times 10^{-10} \text{ s}) \) seem to be dominated by \( \tau_p \), the electron-spin relaxation time for the high-spin Fe(III) center (18, 25, 27). For the rat PAH a frequency dependence of the relaxation rates was only observed for the enzyme-substrate complex, yielding a \( \tau_C \) value of 4.2 \( (\pm 0.5) \times 10^{-10} \text{ s} \) (18). These \( \tau_C \) values should be considered as an approximation, because they were calculated from measurements at high field (\( \approx 100 \text{ MHz} \)), where dispersion of magnetization may occur (24).

Estimation of the Fe(III)-Water Proton Internuclear Distances in Recombinant Human Phenylalanine Hydroxylase and the Enzyme-Substrate Complex—For nonexchange-limited processes, the paramagnetic relaxation rate of the water proton resonance is dependent on the distance between the exchangeable water molecules and the ferric iron. Distances can be estimated from the paramagnetic molar relaxivity values (Table II) using the value of \( \tau_C \) (see Refs. 24 and 25, and Equation 1). Assuming that the paramagnetic contribution to the relaxation rate of the bulk water is mainly because of the exchange of one of the water molecules coordinated to the paramagnetic

\[ \text{FIG. 2. Effect of L-Phe concentration on the paramagnetic longitudinal relaxation of the residual water proton resonance in solutions of recombinant human PAH. The enzyme samples (100 \( \mu \text{M} \) subunit) were allowed to equilibrate with the indicated concentrations of substrate for 5 min. Measurements were made at 400 MHz and 295 K, pH 7.2.} \]

\[ \text{FIG. 3. Temperature dependence of the paramagnetic molar relaxivity. Measurements were made at 400 MHz in solutions (pH 7.2) of human PAH (73 \( \mu \text{M} \) subunit) in the absence (●) and the presence of 5 mM L-Phe (○). The slopes of linear regression lines yield activation energies \( (E_a) \) of \(-14.4\) and \(-17.9 \text{ kJ/mol} \), respectively.} \]
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ion (q = 1, Eq. 2), $T_{1M}$ is calculated to be $6.6 \times 10^{-6}$ s and the estimated distance ($r$) between the iron and the water protons (averaged distance for the two protons) is $3.0 \pm 0.3$ Å (Eq. 1). Assuming that two (q = 2) or three (q = 3) of the water molecules coordinated to the Fe(III) (12) contribute equally to the relaxation of the bulk water $r$ increases to $3.4 \pm 0.4$ Å and $3.7 \pm 0.4$ Å, respectively. Thus, our data fit best with the enhancement of solvent bulk water proton relaxation rates being due to one or two of the coordinated water molecules transferring the paramagnetic effects to the bulk water through exchange. These water molecules are most likely Wat (1) and Wat (2), the most mobile of the three coordinated water molecules to the Fe(III) in the crystal structure of the catalytic domain (12). Although Wat (1) has a temperature factor that is slightly higher than that of Wat (2) (48.7 Å$^2$ versus 35.8 Å$^2$), it is hydrogen bonded (2.8 Å) to the hydroxyl group in the phenolic ring of Tyr-325, whereas Wat (2) is not stabilized by any additional interaction with the protein. Moreover, Wat (3) is hydrogen bonded (2.7 Å) to Glu-286 and has a low temperature factor (18.7 Å$^2$) and probably contributes little to the transfer of paramagnetic effect to the bulk water.

The estimated water protons-Fe(III) distances increased by 13% on incubation of the enzyme with l-Phe, regardless of the value of $q$, indicating a displacement of, at least, one of the coordinated water molecules, as earlier found for rat PAH in the presence of l-Phe (18) and for other systems in which bound water is known to be displaced upon binding of ligands (27, 31, 32). However, to date there is no crystal structure of complexes of the enzyme with l-Phe, and a possible displacement of water on binding of the substrate has not been proved. The binding of l-Phe to either the rat or the human PAH does not affect the spin state of the Fe(III) (26) and the decrease in paramagnetic molar relaxivity upon binding cannot be because of high-spin to low-spin transition of the iron.

Most groups working with PAH interpret the activation of PAH by l-Phe as the result of its cooperative binding at an allosteric site, which is physically distant to the binding site of l-Phe at the active site (1, 2). We have, however, interpreted the activation as the result of the cooperative binding of l-Phe at the active site (18, 33) because: l-noradrenaline, a ligand with positive cooperativity to rat PAH and induces conformational changes similar to l-Phe (18, 26, 33). Thus, displacement of water from the active site Fe could be because of the rearrangement of the coordination geometry of the metal upon substrate binding and enzyme activation. Although the local effects related to water displacement seem to be similar for rat and human PAH, larger conformational effects seem to be induced on the rat enzyme by activation with l-Phe increasing the limited accessibility of the active site, a limitation that is not observed in the human enzyme.

In conclusion, the results presented here support that the observed limitation to the exchange of the coordinated water in PAH isolated from the rat liver is not detected for the human enzyme. This may be related to the state of activation, which is higher in human than in rat PAH (1). These results have important implications for understanding the structural and regulatory differences between the hydroxylases from both sources, as well as the phenylalanine homeostasis in man (1). Moreover, we have further shown that the binding of l-Phe and activation of both mouse and rat enzyme is accompanied by the displacement of at least one water molecule from coordination to the iron.

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