Proton NMR Studies on the Conformation of the Pterin Cofactor Bound at the Active Site of Recombinant Human Tyrosine Hydroxylase

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Summary

The binding of *L-erythro*-7,8-dihydrobiopterin (BH₂) in the presence of L-Phe to recombinant human tyrosine hydroxylase was studied by ¹H-NMR spectroscopy. The distances (± 1.3 Å) from the active site metal were estimated to be 5.3-6.4 Å for observable protons of BH₂ and 7.0-7.9 Å for L-Phe protons. Due to the lack of constraints from the pyrimidine ring in BH₂, two families of conformers were computed, with an average distance from the C4a in the pterin ring to the iron of 3.7 Å (family A) and 8.8 Å (family B). The conformers for 6-methyl-tetrahydropterin bound to the enzyme at near anaerobic conditions in the absence of L-Phe were also grouped into two families, with C4a-metal distances of 3.0-4.0 Å (A) and 7.0-7.5 Å (B). Based on the estimated conformation of the enzyme bound 5,6,7,8-tetrahydro-3-methylpterin, family A of conformers was selected for both oxidized and reduced pterins. Our results indicate that the pterin does not coordinate to the iron, which seems to be involved both in the binding and activation of O₂ and in the C-O bond formation in the aromatic ring of the substrate.

Key words : Tyrosine hydroxylase, (6*R*)-tetrahydrobiopterin, 7,8-dihydrobiopterin, Transferred NOESY, Paramagnetic NMR, Reaction mechanism

Introduction

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a non-heme iron and tetrahydrobiopterin-dependent enzyme which catalyzes the hydroxylation of L-tyrosine (L-Tyr) to L-3,4-dihydroxyphenylalanine (L-

DOPA), the rate-limiting step in the biosynthesis of catecholamines (1,2). The enzyme also catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-Tyr (3). The human enzyme exists as four isoforms (TH1-TH4) generated by alternative splicing events, and TH1 is the predominant form in the adrenal medulla and in the *locus coeruleus* and *substantia nigra* of the brain (4). TH1 is expressed in *Escherichia coli* as an homotetramer (55.9 kDa per subunit) and is purified as an apoenzyme which is activated (up to a 40-fold) by incorporation of a stoichiometric amount of Fe(II) (5). The enzyme is also competitively inhibited by other divalent metal ions, i.e. Zn(II), Co(II) and Ni(II) which bind to

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Abbreviations

BH₂, *L-erythro*-7,8-dihydrobiopterin; BH₄, (6*R*)-*L-erythro*-5,6,7,8-tetrahydrobiopterin; TH, tyrosine hydroxylase; TH1, human tyrosine hydroxylase, isoform 1; L-Phe, L-phenylalanine; 6-MPH₄, 6-methyl-tetrahydropterin; NOESY, nuclear Overhauser effect spectroscopy; TPPI, time proportional phase incrementation; TRNOESY, transferred nuclear Overhauser effect spectroscopy.

the same site as Fe(II) (3,6). Although Fe(II) is an essential component of the active enzyme (6,7), its mechanistic role in the hydroxylation reaction is not established. The identity of the putative hydroxylating species is also unknown, but the detection of a 4a-hydroxy-tetrahydropterin intermediate, released from the enzyme during catalysis (7-9), has suggested that a 4a-hydroperoxytetrahydropterin is likely to be formed in the oxygen-activation step. This hydroperoxy compound could serve either as the direct hydroxylating species through electrophilic addition to the aromatic ring of the substrate or as an intermediate to its formation (7,10). The crystal structure of a truncated form of tyrosine hydroxylase has been recently determined (11), which in combination with the elucidation of the conformation of the bound substrates will at last allow to establish the catalytic mechanism of the enzyme.

In a previous work (3) the conformation of the substrate (L-Phe) and its proximity to the active site metal was determined by NMR using TH1 reconstituted with Co(II) or Zn(II). It was shown that the aromatic ring was placed at the second coordination sphere of the metal and that Fe-bound oxy or peroxy species may approach molecular contact with the C3/C4 atoms of the ring. Preliminary data have also been obtained for the binding of the cofactor analogue 6-methyl-tetrahydropterin (6-MPH₄) (12) to Co(II)-reconstituted TH1 in the absence of amino acid. In the present study the conformation of the oxidized form of the natural pterin cofactor L-erythro-7,8-dihydrobiopterin (BH₂) and its proximity to the active site metal in the presence of L-Phe, forming the catalytically inactive ternary complex [TH1-metal · BH₂ · L-Phe], was determined based on metal-proton distances measured by the paramagnetic probe-T₁ method (3,13) supplemented by NOE distance constraints, measured by two-dimensional transferred NOESY (TNOESY) (14,15).

Materials and Methods

Materials

Before use, buffers and solutions of L-Phe (Sigma Chemical Co., St. Louis, MO), L-erythro-7,8-dihydrobiopterin (BH₂) (Dr. B. Schircks Laboratories, Jona, Switzerland) and 5,6,7,8-tetrahydro-3-methylpterin (see below) were passed through Chelex-100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA). ²H₂O (99.96%), free from paramagnetic impurities, was from Aldrich

(Aldrich Chemical Company, Inc., Milwaukee, WI). Recombinant human tyrosine hydroxylase isoform 1 (TH1) was expressed in *E. coli* and purified to homogeneity as previously described (5). The concentration of the protein was determined by the absorbance at 280 nm ($\epsilon^{1\%} = 10.4 \text{ cm}^{-1}$) at pH 7.0 (5). The purified preparations of TH1 contained about 0.02 iron/subunit, as determined by atomic absorption spectroscopy and were considered to represent the apoenzyme (apo-TH1).

Synthesis of 5,6,7,8-tetrahydro-3-methylpterin

A solution of 3-methylpterin (1.8 g, 0.01 mol), prepared as described (16) in trifluoroacetic acid (40 ml) was reduced catalytically with hydrogen in a shaking apparatus in the presence of PtO₂ (0.2 g) as catalyst. The theoretical uptake of 2 equivalents of hydrogen (450 ml) was complete after 6 h. The solution was filtered under nitrogen to remove the catalyst. The brownish filtrate was concentrated in vacuum to a small volume and then treated with a saturated solution of methanolic HCl. A solid precipitated, the yield of which can be increased by addition of little ether. The precipitate was filtered off and dried over KOH in a desiccator to give 1.71 g (63%) of a brownish crystalline powder. UV (0.1 N HCl) λ_{max} 264 (ϵ 13764). ¹H-NMR (D₆-DMSO): 8.0 (bs, 1H, H-N(8)); 4.2 (t, 2H, H₂C(6)); 3.5 (t, 2H, H₂C(7)); 3.3 (s, 3H, Me(3)). Anal. Calc. for C₇H₁₁N₅O × 2 HCl × H₂O (271.1): C 31.02, H 5.58, N 25.82; found: C 31.07, H 5.37, N 25.58. The purity of the 5,6,7,8-tetrahydro-3-methylpterin was established both by ¹H-NMR and by HPLC and UV detection as described (12). No other pterin in addition to the 5,6,7,8-tetrahydro-3-methylpterin was detected.

Tyrosine hydroxylase assay

TH activity was measured as described (17), with 25 μL [3,5-³H]-tyrosine, 0.5 mM (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄), 0.5 mg/ml catalase, 5 mM dithiothreitol, and 20 μM ferrous ammonium sulfate in 100 mM NaHepes, pH 7.0. The specific activity of purified TH1 was found to be $500 \pm 6 \text{ nmol DOPA min}^{-1} \text{ mg}^{-1}$ and the enzyme retained about 90% of its activity after prolonged NMR data acquisition (up to 24 h at 20°C, see below).

NMR measurements

TH1 samples were initially prepared in 20 mM NaHepes, pH 7.5 and 0.15 M NaCl. The sam-

ples were then subjected to several cycles of dilution/concentration in an Amicon Centricon 30 microconcentrator in order to exchange the buffer to 0.1 M deuterated K-phosphate, 0.1 M KCl, pH* 7.4. The pH* of the samples, determined with an Ingold electrode, represents the measured value in $^2\text{H}_2\text{O}$.

The paramagnetic effects of the enzyme-bound Co(II) on the longitudinal ($1/T_1$) (and the transverse ($1/T_2$)) relaxation rates of proton resonances of enzyme-bound ligands were measured on a Bruker AM 400-MHz spectrometer at a probe temperature of 20°C. The samples (0.5 ml) contained 0.05–0.1 mM TH1, 5 mM BH₂ (or 5 mM 5,6,7,8-tetrahydro-3-methylpterin), 0.1 M KCl and 0.1 M deuterated K-phosphate buffer, pH* 7.4. After 5 min incubation at 20°C, 5 mM L-Phe was added (except for measurements with 5,6,7,8-tetrahydro-3-methylpterin). Then, successive aliquots of CoCl₂ (up to 50 μM) were added and T_1 -values were measured by the inversion recovery method, with acquisition parameters including 16 K data points, spectral width 3597 Hz and 8 transients per time increment with a 10-s delay ($>5 T_1$). The T_2 -values of the H7 proton resonance of BH₂ were determined from the width of this resonance at half-height ($\Delta\nu_{1/2}$), where $1/T_2 = \pi \cdot \Delta\nu_{1/2}$. The paramagnetic effects of the enzyme-bound Co(II) on $1/T_1$ and $1/T_2$ were analyzed by plotting the increase in the relaxation rates as a function of the concentration of added Co(II) (5). The normalized paramagnetic contributions to the longitudinal ($1/fT_{1p}$) and transverse ($1/fT_{2p}$) relaxation rates were calculated using a K_1 -value of 70 μM and K_m -value of 85 μM for the binding of BH₂ and L-Phe, respectively (3). TH1 (0.1 mM subunit) was calculated to be $>98\%$ saturated with BH₂ and L-Phe (added at 5 mM) for determining the normalized relaxation rates. For the binding of 5,6,7,8-tetrahydro-3-methylpterin (added at 5 mM), with a K_m -value of 685 μM (see Results), TH1 (50 μM subunit) was calculated to be 94% saturated. The $1/fT_{1p}$ -values, together with the effective correlation time for the electron-nuclear dipolar interaction ($\tau_c = 1.8 \pm 0.1$ ps), determined earlier for the [TH1-Co(II) · BH₂ · L-Phe] complex (3), were used to calculate the metal-proton distances based on the general theory (13). Errors in the absolute distances include an approx. 15% contribution from the C -value (895 ± 125) arising from the g tensor for high-spin Co(II) (13) and an approx. 5% contribution due to errors in $1/fT_{1p}$ and τ_c . However, errors in the relative dis-

tances are practically limited to 3% (3).

Transferred NOEs from the bound to the free state of both pterin and amino acid substrates were measured from NOESY spectra on a Bruker AM 400-MHz spectrometer at 20°C. The samples (0.5 ml) contained 0.3 mM TH1, 5 mM BH₂, 0.3 mM ZnCl₂, 0.1 M KCl and 0.1 M deuterated K-phosphate, pH* 7.4. After 5 min incubation at 20°C, 5 mM L-Phe was added. A similar fraction of bound BH₂ and L-Phe ($p_B \approx 0.06$) was estimated at these concentrations of enzyme and ligands and this p_B -value was found to be optimal to obtain cross-peak intensities significantly above the noise level and to reduce spin diffusion effects (14). NOESY spectra were acquired at different mixing times (50, 100, 150, 250 ms), in the time-proportional phase-sensitive mode using TPPI for quadrature detection in ω_1 and with suppression of the residual water signal. Acquisition parameters included 3-s relaxation delay, sweep width of 3597 Hz and 16 scans per t_1 -value. The 2D-raw data sets of 512×2048 (TPPI) time domain data points (t_1, t_2) were processed on a MicroVax 2000 using the FT-NMR processing software (Harc Research, Inc.). Processing parameters included zero-filling in t_1 , sine-bell apodization shifted by $\pi/4$ in both directions and a 3rd order polynomial baseline correction after Fourier transformation. Matrices of 1024×1024 points real data were then obtained.

Both BH₂ and L-Phe were shown to be in fast exchange (see ref. 3 and Results) between the enzyme-bound and free states (on the chemical shift time scale) and the average signals in the NMR spectra represent

$$\delta_{\text{OBS}} = p_F \delta_F + p_B \delta_B \quad (1)$$

where δ_{OBS} is the observed chemical shift, δ_F and δ_B , the chemical shift for the signals of the free and bound ligand, respectively, and p_F and p_B , the fraction of free and bound ligand, respectively. Since p_B is small (≈ 0.06), both δ_{OBS} and the observed line width are dominated by the free ligand concentration. For a system in fast chemical exchange in which $W_{ijF} \ll W_{ijB}$ (cross-relaxation rates between spins i and j in the free and bound state, respectively), the transferred NOE cross-peak intensities (a_{ij}) at mixing time (τ_m) are given by

$$a_{ij}(\tau_m) \approx - (p_B W_{ijB}) \tau_m \quad (2)$$

with $W_{ijB} \propto 1/r_{ijB}^6$ and r being the distance between spins i and j in the bound state (18,19).

Intensities of transferred NOE cross-peaks were

measured by volume integration, and interproton distances were calculated at each mixing time using as a reference the distance of 2.46 ± 0.02 Å between the H2,6 and the H3,5 protons of L-Phe as measured by neutron diffraction (20) and extrapolation to zero mixing time to minimize spin-diffusion effects. Distances from aliphatic to degenerate pairs of aromatic protons in L-Phe were treated as single proton-proton interactions.

Molecular modeling

Conformation analyses were performed on an Indy workstation (Silicon Graphics, Inc.) using the programs Insight II and NMRchitect (Biosym Technologies, San Diego, CA). A series of conformers were generated from metal-proton and NOE distance constraints supplemented by chirality constraints by the DGII software package. Lower and upper limits for the metal-proton distances were set by considering the experimental error for relative distances to the metal (about 3%, see above), while the error limits for interproton distances (r_{ij}) were set to ± 0.2 Å if $r_{ij} \leq 3.0$ Å, to ± 0.3 Å if $3.0 \text{ Å} \leq r_{ij} \leq 4.0$ Å and to 0.4 Å if $r_{ij} \geq 4.0$ Å. Degenerated protons were treated considering an average distance from their center of mass. Calculations included triangle bound smoothing and embedding in four dimensions using metrized distance matrices (21,22). The embedded set of coordinates were refined using simulated annealing optimization followed by a conjugate gradient minimization of the error function (22) to obtain the converged distance geometry based structures.

Results

In order to determine the conformation of the pterin and the amino acid substrate (L-Phe) when simultaneously bound to Co(II)-reconstituted TH1 the oxidized form of the natural cofactor, L-erythro-7,8-dihydrobiopterin (BH₂), was used as a catalytically inactive analogue. Reduced pterin cofactors could not be used simultaneously with L-Phe due to the presence of some residual iron (trace amounts) in apo-TH1 (see Materials and Methods), which results in some catalytic turnover. BH₂ is stable at neutral pH and binds to the enzyme as a competitive inhibitor *versus* BH₄ (3). The advantages of using L-Phe instead of L-Tyr as the amino acid substrate have already been discussed (3).

Paramagnetic effects of enzyme-bound Co(II) on the relaxation rates of the proton resonances of BH₂ and L-Phe

In the 400-MHz ¹H-NMR spectrum of a mixture of BH₂ and L-Phe in the presence of apo-TH1, proton signals from the pterin and the amino acid are observed beyond the protein envelope and, except for the overlapping H α proton of L-Phe and the H2' from BH₂, the proton signals are well separated (Figure 1). We have previously shown that both Co(II) and Zn(II) bind to apo-TH1 with high affinity competitive to Fe(II) ($K_d \leq 1$ μ M) (5). Moreover, the binding of both Co(II) and Zn(II) was shown to be independent and mutually exclusive at the Fe(II)-binding site (3). On addition of Co(II) (up to 50 μ M) to the apoenzyme in the presence of BH₂ and L-Phe forming a catalytically inactive complex [TH1-Co(II) · BH₂ · Phe], a decrease in $1/T_1$ and $1/T_2$ of the proton resonances of both ligands was observed. By contrast, reconstitution of apo-TH1 with diamagnetic Zn(II) had no effect on the relaxation rates of the ligand resonances (data not shown). Dopamine has been shown to bind to TH1 competitively to the pterin cofactor (23); after addition of 0.2 mM dopamine to the samples the paramagnetic contributions to the relaxation of BH₂ protons was eliminated, indicating a total displacement of BH₂ and that the enzyme-bound Co(II) has no paramagnetic effect on free BH₂. Total displacement of BH₂ from the

Table 1. Calculated distances from Co(II) to observable protons of BH₂ in the [TH1-Co(II) · BH₂ · Phe] complex

nucleus	$\delta(\text{ppm})^a$	$1/fT_{1\rho}(\text{s}^{-1})$	$r(\text{\AA})^b$
H7 ^c	4.17	37	6.2 ± 1.1
H1'	4.08	125	5.3 ± 1.0
CH ₃	1.20	43.4	6.4 ± 1.3

^aFrom external sodium 3-(trimethylsilyl)-propionate.

^bTotal errors in the absolute distances r , include contributions of 6% from errors in $1/fT_{1\rho}$ and τ_c , and contributions of 14% from the assumption that the value of g for high-spin Co(II) is 4 ± 2 (15).

^cAverage position of the two H7 protons.

ternary complex was also observed after addition of 500 μM BH₄. Thus, the outer-sphere contribution to the relaxation rates of BH₂ in the complex [TH1-Co(II) · BH₂ · Phe] was calculated to be equal to zero.

The $1/fT_{1\rho}$ -values for BH₂ protons and the distances from the paramagnetic center to these protons (except for the H2' proton whose signal overlaps with that of the H α proton of L-Phe) were then calculated (see Materials and Methods and ref. 3) (Table 1). The linearity of the paramagnetic effects with the Co(II) concentration (data not shown and ref. 3) and the inequality of the $1/fT_{1\rho}$ values for the different protons (Table 1) argue against significant contributions of spin diffusion to the relaxation rates. The $1/fT_{2\rho}$ -value for the H7 proton of BH₂ was found to be $1664 \pm 97 \text{ s}^{-1}$, an order of magnitude greater than its $1/fT_{1\rho}$ -value, indicating that the lifetime of the complex (τ_M) contributes little to the $T_{1\rho}$ -values and can be excluded in the calculation of distances. Distances from the paramagnetic center to the protons of L-Phe in the complex [TH1-Co(II) · BH₂ · Phe] were also calculated, and the values obtained ranged from 7.0 (± 1.3) $\text{\AA}$ for the aromatic protons to 7.9 (± 1.4) $\text{\AA}$ for the H β protons, in good agreement with previous calculations (3).

Intramolecular nuclear Overhauser effects in the enzyme-bound BH₂ and L-Phe

The intramolecular proton distances of the bound BH₂ and L-Phe were measured from TRNOESY spectra of the complex [TH1-Zn(II) · BH₂ · Phe], since Zn(II) is diamagnetic and binds with high affinity to apo-TH1 ($K_d=0.3 \mu\text{M}$) at the same site as Fe(II) (3,5). Transferred NOEs from the bound to the free state were observed for both BH₂ and L-Phe as cross-peaks in the NOESY spectra, reflecting negative NOEs (Figure 2). A similar value of p_B for both ligands also in-

Figure 2. Two-dimensional nuclear Overhauser effect (NOESY) spectra of L-Phe and L-erythro-7,8-dihydrobiopterin (BH₂) in the presence of TH1. The 400-MHz ¹H-NMR spectra were taken at 273 K in samples (0.5 ml) prepared in D₂O containing 0.3 mM TH1, 5 mM BH₂, 5 mM L-Phe, 0.3 mM ZnCl₂, 0.1 M KCl and 0.1 M deuterated K-phosphate at pH* 7.4. The mixing time was 100 ms; other parameters given in the Materials and Methods section. (Upper) Region showing cross-peaks between resonances of L-Phe. (Lower) Region showing cross-peaks between the H7, H1' and H2' and the methyl resonance of BH₂.

icates that the cross-relaxation rate of the H2,6-H3,5 protons of L-Phe, separated by $2.46 \pm 0.02 \text{ \AA}$ (20), can be used as a reference to calculate the intramolecular proton distances both in BH₂ and L-Phe (Table 2).

We have also looked for intermolecular interactions between BH₂ and L-Phe in the NOESY spectra. However, the intensity of cross-peaks at the expected ppm values for these interactions was at the noise level of the protein cross-peaks, which precludes their unambiguous assignment to intermolecular interaction between the substrate and the pterin. Without distance constraints between L-Phe and BH₂ an infinite number of positions may be adopted by both ligands around the active site metal and a plausible structural model of L-Phe and BH₂ simultaneously bound to the enzyme could not be computed.

Table 2. Calculated interproton distances in the [TH1-Zn(II) · BH₂ · Phe] complex based on NOESY spectra

Proton pair	Distances (Å)
<i>L</i> -Phe:	
H3,5-H2,6	2.46 ± 0.02 ^a
H2,6-Hα	3.73 ± 0.3
H3,5-Hα	4.34 ± 0.4
H2,6-Hβ (pro-S)	3.83 ± 0.3
H2,6-Hβ (pro-R)	3.61 ± 0.3
Hα-Hβ (pro-S)	3.56 ± 0.3
Hα-Hβ (pro-R)	3.61 ± 0.3
<i>BH</i> ₂ :	
H1'-CH ₃	3.39 ± 0.3
H1'-H7 ^b	2.72 ± 0.2
H1'-H2'	2.63 ± 0.2
H7 ^b -H2'	3.08 ± 0.3
H7 ^b -H1'	2.50 ± 0.2

^aInternal standard from neutron diffraction (26).^bAverage position of the two H7 protons.

Molecular modeling of bound pterins

BH₂ was built within the Builder module (Insight II) and its covalent geometry (bond lengths, angles, planarity of the rings) was optimized using the Mopac module with the MNDO framework. Distance geometry calculations for BH₂ were performed using the three measured metal-proton distances (Table 1) and the five interproton distance constraints (Table 2) supplemented by two chirality constraints, R and S, for the C1' and C2' chiral centers (*L*-erythro-1',2'-dihydroxypropyl), respectively. Thirty sets of coordinates were computed in the embedding step of the calculation and refined (22). The refined set of structures was analyzed for the presence of chiral and distance constraints violations as well as covalent geometry violations. Three conformers with more than two violations of distance restraints were excluded, while for the rest of the conformers violations were limited to <0.2 Å and were all accepted. Two families of conformers (A and B) could be discerned (Figures 3A and 3B), the major difference between the two being the orientation of the pterin rings towards the metal at the active site. This distance was shorter in family A (C4a is at 3.6–4.1 Å, N5 at 3.3–3.8 Å and O4 at 3.3–4.1 Å from the metal) than in family B (C4a is at 8.6–9.0 Å, N5 at 7.8–8.1 Å, and O4 at 10.7–11 Å from the metal). For all the conformations it was determined that the dihedral angle (Θ) around the C1'-C2' bond for the two hydroxyl groups in the side-chain at C6 was -75° to -83°.

Assuming that the dihydroxypropyl side-chain at C6 has the same conformation in both enzyme-

Figure 3. Conformations of *L*-erythro-7,8-dihydrobiopterin (BH₂) bound at the active site of metal(Co(II) or Zn(II))-reconstituted TH1 based on the experimental distances given in Tables 1 and 2. Family A (A) and family B (B), shorter and longer distance from the pteridin ring to the metal ion, respectively (see Results), with the distances to the metal (in Å) for one conformer in each family. The asterisk shows the position of the N3.

bound BH₂ and (6*R*,1'*R*,2'*S*)-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydropterin (BH₄), we have also modeled the conformation of BH₄ bound to TH1. The ring was built according to the structure determined by Matsuura *et al.* (24) and Williams and Storm (25) and the torsion angles [O2'-C2'-C1'-O1'] and [C3'-C2'-C1'-C6] were set to the corresponding values measured for the TH1-bound BH₂. Two families of BH₄ conformers (A and B) were also obtained, with similar distances from C4a to the metal as calculated for the BH₂ conformers (see above).

We have previously obtained data on the binding of the synthetic pterin cofactor (6*R,S*)-methyltetrahydropterin (6-MPH₄) to TH1 at near anaerobic conditions in the absence of amino acid substrate (12). The distances between the metal and the H7R, H7S, H6 and methyl protons at C6 of 6-MPH₄ were calculated to range from 3.8 (±0.6) Å to 5.3 (±0.9) Å. With these distances

we modeled the conformation of TH1-bound 6-MPH₄ and compare it with that of BH₂. The structure of the pterin ring of 6-MPH₄ was built according to the proposed structure of several tetrahydropterin salts as determined by X-ray diffraction (26,27) and high resolution NMR experiments (25), in which the C7 at the pyrazine ring is nearly coplanar with the pyrimidine ring and the N8 atom essentially sp² hybridized. The forty accepted conformers were also grouped into two families, i.e. family A with C4a-metal distances ranging from 3.0 to 4.0 Å (Figure 4A) and family B in which this distance ranges from 7.0 to 7.5 Å (Figure 4B). Both families included conformers with equatorial and axial positions of the methyl group at C6, since both solutions are within the experimental error for the relative metal-proton distances. However, from previous ¹H-NMR studies it has been shown that the methyl at C6 prefers equatorial-like orientations (25), as shown in Figure 4A.

In order to investigate if the ambiguity in the distances from the pterin ring to the metal for

both BH₂ and 6-MPH₄ is a result of the lack of distance restraints from the pyrimidine ring, we synthesized 5,6,7,8-tetrahydro-3-methylpterin, with stable protons at this ring. This pterin had cofactor activity with $K_m=685\pm40$ mM and $V_{max}=15\pm1$ nmol L-DOPA min⁻¹ mg⁻¹ (3% of the activity with BH₄ as cofactor and remained reduced in D₂O even in the presence of 50 μM apo-TH1 (Figure 5). On addition of Co(II) (up to 30 μM) to this sample a decrease in 1/T₁ and 1/T₂ of the proton resonances was observed and the distances from 5,6,7,8-tetrahydro-3-methylpterin protons to the paramagnetic center were estimated (Table 3). On modeling the conformation of the enzyme-bound 5,6,7,8-tetrahydro-3-methylpterin we found that the distance between the Co(II) in TH1 and the methyl was only compatible with conformers in which C4a is separated 4.0-4.5 Å from the metal, equivalent to family A of conformers for both BH₂ and 6-MPH₄ (see Figures 3 and 4).

Figure 4. Conformations of 6-methyltetrahydropterin (6-MPH₄) bound at the active site of Co(II)-reconstituted TH1 based on the experimental distances measured in ref. 16. Family A (A) and family B (B), shorter and longer distance from the pteridin ring to the metal ion, respectively (see Results and Discussion), with equatorial position for the methyl group at C6. For one conformer in each family, the distances in Å to the metal are shown. The asterisk shows the position of the N3.

Figure 5. ¹H-NMR spectrum of 5,6,7,8-tetrahydro-3-methylpterin in the presence of apo-TH1. The 400-MHz ¹H-NMR spectrum (25 transients) was taken at 273 K in samples (0.5 ml) prepared in D₂O containing 0.05 mM TH1 and 5 mM 5,6,7,8-tetrahydro-3-methylpterin.

Table 3. Calculated distances from Co(II) to protons of 5,6,7,8-tetrahydro-3-methylpterin in the [TH1-Co(II) 5,6,7,8-tetrahydro-3-methylpterin] complex

nucleus	$\delta(\text{ppm})^a$	$1/fT_{1\rho}$ (s ⁻¹)	r (Å) ^b
H6	4.17	90.0	5.6 ± 1.0
H7 ^c	3.50	22.0	7.1 ± 1.4
CH ₃ (N3)	3.29	23.4	7.0 ± 1.3

^{a,b}See Table 1.

Discussion

In the present study the conformation of pterins (BH₂ and 6-MPH₄) when bound to the active site of metal-reconstituted TH1 has been computed based on ¹H-NMR experiments. The binding of BH₂ was studied in the presence of L-Phe as amino acid substrate and the conformation of enzyme-bound L-Phe obtained in an earlier study (3) was confirmed. Two distance geometry solutions (families A and B) satisfying the NMR data sets were obtained for the conformation of BH₂ (and by assumption also for BH₄), as well as for 6-MPH₄ bound in the absence of L-Phe. This ambiguity seems to result from the lack of distance restraints from the pyrimidine ring. Since the tetrahydropterin reduces the enzyme-bound Fe (III) to Fe(II) in TH, both in the presence and absence of amino acid substrate (28), and BH₄ is able to displace dopamine directly coordinated at the active site iron in TH1 (23), it is likely that the pterin binds at a distance from the metal corresponding to that in the family A of conformers. This is further supported by the conformation obtained for enzyme-bound 5,6,7,8-tetrahydro-3-methylpterin. The estimated distances from the metal to the pterin ring are slightly different depending on the pterin analogue used and are shorter for 6-MPH₄, which has good cofactor activity (1,2,14), and longest for 5,6,7,8-tetrahydro-3-methylpterin, which shows very low affinity and low cofactor activity (this work). These differences in the metal-pterin ring distances for the various cofactor analogues may result from the experimental error associated to the determination of absolute metal-proton distances by the paramagnetic probe-T₁ method (about 15%). In addition, this results might also indicate that the N3 at the pyrimidine ring interacts with the enzyme, as found in other pterin binding proteins as chicken liver dihydrofolate reductase in which a glutamic acid side chain forms hydrogen bonds to both N3 and exocyclic N2 of the pterin (29). In this context, the residue Glu332, located at the active site of TH (11) may be involved in the bind-

ing of the pterin. This amino acid is conserved in the homologous enzyme phenylalanine hydroxylase, in which it has been shown to be of critical importance for pterin binding (30).

In addition to the substitutions on the tetrahydropterin ring, the C6 chirality and the conformation of the side-chain at C6 seem to be critical for the affinity and the regulatory properties of the cofactor (1,2,31). The conformation around the C1'-C2' in the dihydroxypropyl-side chain at C6 in BH₄ has been found to be *trans* both by X-ray analysis (24) and theoretical calculations by the molecular-orbital method (32). Moreover, these hydroxyl groups in BH₂ bound to 4a-hydroxycarbinolamine dehydratase are *trans* and only the 1' interacts with the protein through a hydrogen bond (33). For this enzyme, however, there are small effects of substitutions at C6 of the pterin on the catalytic efficiency of the enzyme (34). The data obtained in the present NMR study are consistent with these two hydroxyl groups in TH1-bound BH₂ being almost-*cis* ($\Theta = -75^\circ$ to -83°), indicating that their conformation might be altered on binding to the enzyme. The hydroxyls in bioppterin are also bound in almost-*cis* conformation to dihydrofolate reductase (29), an enzyme showing a narrow specificity for substitutions at C6 (35).

Due to the metal coordinating ability of the pterins, both in small pteridine-containing metal complexes (36,37) and in the copper-inhibited phenylalanine hydroxylase of *Chromobacterium violaceum* (38), the possibility of a direct coordination of the pterins to the divalent metal in TH (iron in the active form) was considered. For all the conformers of BH₂ or 6-MPH₄, the estimated distance between either the 4-oxo in the pyrimidine ring or the N5 in the pyrazine ring and the metal were 3.3-4.1 Å. Although these distances indicate that a metal-pterin coordination is unlikely, the experimental error associated to the determination of absolute metal-proton distances prevents a definite conclusion.

It has been proposed that a 4a-hydroperoxy-tetrahydropterin, or a similar species also involving the iron atom at the active site, is the hydroxylating intermediate in the TH catalyzed reaction (1,10), although this species has not been directly observed with any tetrahydropterin dependent enzyme. Both the pterin ring and the aromatic ring of L-Phe ((3) and this work) seem to be placed in the second coordination sphere of the metal in TH1, supporting a key role of the iron in the hydroxylation reaction. A dioxygen

molecule bound to the iron forming a peroxo- or superoxo-iron species comes close ($<3 \text{ \AA}$) from both the C4a atom in the pterin [the 4a-hydroxy-tetrahydropterin is the first product of the reaction (8-10)] and either the C3- or the C4-position of the aromatic ring of the substrate. The site of initial hydroxylation in the substrate is determined primarily by steric factors, with a ratio for addition at C4- *versus* C3-position of 24:1 for L-Phe as the substrate (39-40). Thus, the results of our structural studies are consistent with a hydroxylating species involving the active site iron in TH, in agreement with recent kinetic data (40).

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