Conformation and Interaction of Phenylalanine with the Divalent Cation at the Active Site of Human Recombinant Tyrosine Hydroxylase as Determined by Proton NMR†

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ABSTRACT: Recombinant human tyrosine hydroxylase has been purified as a metal-free apoenzyme (apo-hTH1) which tightly binds one Fe^{2+}, Co^{2+}, or Zn^{2+} per subunit with activation only by Fe^{2+} and competitive inhibition by the other cations. L-tyrosine and L-phenylalanine are alternative substrates for this enzyme, giving similar V_{max} values, although the K_M value for phenylalanine is about 8-fold greater than for tyrosine. Apo-hTH1 enhances the paramagnetic effects of Co^{2+} on $1/T_1$ and $1/T_2$ values of the protons of enzyme-bound phenylalanine both in the presence and in the absence of the oxidized form of the cofactor L-*erythro*-7,8-dihydrobiopterin (BH_2), which was used as an inactive analog of the natural cofactor (6*R*)-1-*erythro*-tetrahydrobiopterin (BH_4). No effects of hTH1–Zn^{2+} on $1/T_1$ or $1/T_2$ are found. From paramagnetic effects of hTH1–Co^{2+} on $1/T_1$ of phenylalanine protons at 250 and 600 MHz, in the presence of BH_2, a correlation time (τ_c) of 1.8 ± 0.1 ps was found. Using this τ_c value, and assuming that only one proton of the pairs H3,H5, and H2,H6 is experiencing the total paramagnetic effect (asymmetric limiting case), distances from enzyme-bound Co^{2+} to phenylalanine (±1.2 Å) of 6.1 Å (H3 or H5), 6.3 Å (H2 or H6), 7.0 Å (H4), 7.3 Å (Hα), ≥7.4 Å (Hβ-*pro-S*), and ≥7.6 Å (Hβ-*pro-R*) were calculated. The distances to H3 or H5 and to H2 or H6 are slightly increased to 6.8 and 7.0 Å, respectively, if each proton of both degenerate pairs equally experiences the paramagnetic effect of Co^{2+} (symmetric limiting case). These distances place the aromatic ring of phenylalanine in the second coordination sphere of the metal, which would permit an Fe-bound oxy or peroxy species to approach molecular contact with C3/C4, suggesting a direct role of Fe^{2+} in the hydroxylation reaction. The same correlation time and similar distances were found in the absence of BH_2 with H4 of phenylalanine slightly closer to the metal. In the ternary hTH1–Zn^{2+}·BH_2·phenylalanine complex, eight interproton distances in the enzyme-bound phenylalanine were determined by NOESY spectra at 600 MHz at 35-, 50-, and 75-ms mixing times. The conformation of enzyme-bound phenylalanine, consistent with the six Co^{2+}–proton distances and the eight interproton distances, is partially extended with torsional angles $\chi_1 = 97° \pm 3°$ and $\chi_2 = -78° \pm 2°$.

Tyrosine hydroxylase (EC 1.14.16.2) is an iron- and tetrahydrobiopterin-dependent enzyme which catalyzes the rate-limiting step in the biosynthesis of catecholamines both in brain and adrenal medulla (Kaufman & Kaufman, 1985). Human tyrosine hydroxylase exists as four different isoforms (hTH1–hTH4)[1] generated by alternative splicing events (Grima et al., 1987). The distribution of the isoforms seems to be tissue-specific, hTH1 being the predominant form in the adrenal medulla and in the *locus coeruleus* and *substantia nigra* of the human brain (Grima et al., 1987; Le Bourdellès et al., 1988; O'Malley et al., 1987). All isoenzymes have recently been expressed in *Escherichia coli* (Le Bourdellès et al., 1991; Haavik et al., 1991). The recombinant enzymes are tetramers, composed of four identical subunits of M_r approximately 55 000–59 000, and we have shown that the purified apoenzymes are rapidly activated (up to 40-fold) by the incorporation of stoichiometric amounts of Fe^{2+} (Haavik et al., 1991). All isoenzymes are competitively inhibited by other divalent metal ions, e.g., Zn^{2+}, Co^{2+}, and Ni^{2+}, which bind with affinity and stoichiometry similar to those of Fe^{2+} (Haavik et al., 1991, 1992).

Several mechanisms of hydroxylation have been proposed for tyrosine hydroxylase (Benkovic, 1980; Kaufman & Kaufman, 1985; Dix & Benkovic, 1988), but the actual catalytic mechanism is not clear. Also, little is known about the interaction of substrates and inhibitors with the enzyme. The present paper reports for the first time the conformation of an enzyme-bound substrate (phenylalanine) and its proximity to the metal on this enzyme. The conformation of enzyme-bound phenylalanine was determined in the tyrosine hydroxylase–Co^{2+}(or Zn^{2+})·L-*erythro*-7,8-dihydrobiopterin·phenylalanine (E–M^{2+}·BH_2·Phe) complex based on metal–substrate distances measured by the paramagnetic probe–T_1 method (Mildvan & Gupta, 1978; Mildvan et al., 1980) and on interproton distances measured by transferred nuclear Overhauser effects (Rosevear & Mildvan, 1989; Weber et al., 1991). Similarly, the distances from enzyme-

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[1] Abbreviations: BH_2, L-*erythro*-7,8-dihydrobiopterin; BH_4, (6*R*)-L-*erythro*-tetrahydrobiopterin; E–M^{2+}·Phe, the apoenzyme–metal·phenylalanine complex; E–M^{2+}·BH_2·Phe, the apoenzyme–metal·L-*erythro*-7,8-dihydrobiopterin·phenylalanine complex; hTH1–hTH4, human tyrosine hydroxylase isoenzymes 1–4; TSP, sodium 3-(trimethylsilyl)-propionate; TPPI, time proportional phase incrementation; NOESY, nuclear Overhauser effect spectroscopy.

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bound Co^{2+} to protons of phenylalanine in the abortive, enzyme–metal·phenylalanine complex were also determined.

While the distances from Co^{2+} to phenylalanine and, presumably, the conformations of the enzyme-bound substrate are similar in both complexes, displacement of the bound substrate by the product analog dopamine differs significantly, in accordance with the ordered kinetic scheme of tyrosine hydroxylase in which pteridine binding precedes amino acid binding (Fitzpatrick, 1991a). A preliminary report of this work has been published (Martínez et al., 1993a).

MATERIALS AND METHODS

Materials

All the reagents used, including metal salts, were of analytical grade. Before use, buffers and solutions of L-phenylalanine, L-tyrosine, and L-dopamine (from Sigma Chemical Co., St. Louis, MO) and of L-*erythro*-7,8-dihydrobiopterin (BH_2) (Dr. B. Schircks Laboratories, Jona, Switzerland) were passed through Chelex-100 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA).

Human tyrosine hydroxylase isoform 1 (hTH1) was expressed in *E. coli* and purified to homogeneity as previously described (Haavik et al., 1991; Le Bourdellès et al., 1991). The purified preparations of hTH1 used in the present study contained 0.02 ± 0.01 (mean ± SD, $n = 4$) atoms of iron/subunit, as determined by atomic absorption spectrometry. The concentration of purified hTH1 was determined by the absorbance at 280 nm ($\epsilon^{1\%} = 10.4\ cm^{-1}$) at neutral pH (Haavik et al., 1988).

Methods

Tyrosine Hydroxylase Assay. Tyrosine hydroxylase activity was measured by the procedure of Reinhard et al. (1986), using an incubation mixture containing 25 μM L-[3,5-^{3}H]-tyrosine, 500 μM (6*R*)-1-*erythro*-tetrahydrobiopterin (BH_4), 5 mM dithiothreitol, 0.5 mg/mL catalase, and 10 μM Fe^{2+} ammonium sulfate in 100 mM NaHepes buffer, pH 7.0. Under these conditions, the specific activity of the purified enzyme was found to be 400 ± 5 nmol of DOPA min^{-1} mg^{-1} when measured at 30 °C. The enzyme retained 75–80% of its activity after prolonged NMR measurements (up to 6 days at 20 °C). The activity of hTH1 with phenylalanine as substrate was measured as described (Fukami et al., 1990).

Preparation of Samples for ^{1}H-NMR Measurements. Tyrosine hydroxylase samples (≈16.4 mg) were initially prepared in H_2O containing 20 mM NaHepes, pH 7.5, and 0.15 M NaCl. The samples were then deuterated by lyophilization with 99.96% ^2H_2O low in paramagnetic impurities (Aldrich Chemical Company, Inc., Milwaukee, WI). Lyophilization did not affect activity or stability of the enzyme. The concentration of Hepes was then reduced 5-fold by concentration in Amicon Centricon 30 microconcentrators (Amicon, Danvers, MA). The final deuterated 0.5-mL samples contained 4 mM NaHepes, pH 7.5, 0.2 M NaCl, 5.0–5.2 mM phenylalanine, and 0.21 mM human tyrosine hydroxylase isoform 1 (hTH1). The pH* of the samples was determined with an Ingold electrode and represents the measured value in ^2H_2O. The pH* of the samples was adjusted to 7.5 by the addition of diluted ^2HCl or NaO^2H. When measurements were made in the presence of the oxidized cofactor BH_2, samples of 0.21 mM hTH1 in 4 mM NaHepes, pH 7.5, and 0.2 M NaCl in ^2H_2O were incubated for 5 min at 20 °C with 0.5 mM BH_2 prior to the addition of phenylalanine. The use of deuterated Tris-HCl was avoided since Tris has been shown to bind to the catalytic iron center in the homologous enzyme phenylalanine hydroxylase (Martínez et al., 1991) and to interact with Co^{2+} (Hall et al., 1962).

^{1}H Relaxation Rate Measurements of Phenylalanine. To determine the paramagnetic effects of enzyme-bound Co^{2+} on the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the proton resonances of phenylalanine bound to human tyrosine hydroxylase, the solutions prepared as described above were titrated with $CoCl_2$, and the increases in the relaxation rates were measured at 600 MHz, a frequency at which all of the proton resonances of phenylalanine were resolved. The effects of Co^{2+} concentration on the relaxation rates were also measured at 250 MHz on the aliphatic Hα proton resonance, this being the only resonance clearly resolved in the enzyme–substrate complexes at this frequency. Following the titration with $CoCl_2$, phenylalanine was displaced from the E–Co^{2+}·BH_2·Phe complex by tyrosine and from the E–Co^{2+}·Phe complex by tyrosine or by the potent active site inhibitor dopamine, permitting measurements of the outer-sphere effects on $1/T_1$ and $1/T_2$. The diamagnetic control measurements of $1/T_1$ and $1/T_2$ were made by titrating identical solutions with $ZnCl_2$.

The ^{1}H-NMR spectra were recorded on Bruker AM 250- and 600-MHz spectrometers by collecting 16K data points over a spectral width of 4000 or 10 000 Hz, respectively, with acquisition times of 1.016 s, at a probe temperature of 20 °C. Routine spectra were acquired by collecting 32 transients, with a 15-s delay to obtain fully relaxed spectra (>5 T_1). The longitudinal relaxation rates ($1/T_1$) of the proton resonances were measured by the nonselective saturation–recovery method, and the transverse relaxation rates ($1/T_2$) were determined from the widths of resonances at half-height ($\Delta\nu_{1/2}$), where $1/T_2 = \pi\Delta\nu_{1/2}$. All proton chemical shifts are reported with respect to external TSP. Other parameters, when used, are indicated in the figure captions.

The paramagnetic effects of the enzyme-bound Co^{2+} on $1/T_1$ and $1/T_2$ of the protons of phenylalanine were analyzed by plotting the increase in the relaxation rates as a function of Co^{2+} concentration. No outer-sphere effect was measured after displacing the phenylalanine. Titration of identical solutions with Zn^{2+} (added as $ZnCl_2$ from 0 to 250 μM) rather than Co^{2+} had negligible effects on the relaxation rates of the proton resonances of phenylalanine (see below). The normalized $1/T_{1P}$ values, $1/fT_{1P}$, were then used to obtain metal-to-proton distances using the general theory reviewed elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980).

The effective correlation times, τ_c, for the electron–nuclear dipolar interaction in the E–Co^{2+}·Phe and the E–Co^{2+}·BH_2·Phe complexes were determined by measuring the frequency dependence of $1/fT_{1P}$ of the aliphatic Hα proton of phenylalanine at both 250 and 600 MHz as described earlier (Mildvan & Gupta, 1978; Mildvan et al., 1980; Serpersu et al., 1989). Since the correlation time for Co^{2+} complexes is dominated by the short electron spin relaxation time of Co^{2+}, the same value of τ_c applies for all of the Co^{2+}–proton interactions.

Two-Dimensional NOE Experiments. To determine interproton distances on enzyme-bound phenylalanine in the E–Zn^{2+}·BH_2·Phe complex, transferred NOEs were measured in NOESY spectra (Jeener et al., 1979; Kumar et al., 1980) obtained at 600 MHz. The sample contained 0.21 mM tyrosine hydroxylase (hTH1), 0.5 mM BH_2, 5 mM phenylalanine, 0.21 mM $ZnCl_2$, 0.2 M NaCl, and 4 mM NaHepes at pH* 7.5 in 0.5 mL of ^2H_2O. Four sets of NOESY data were collected at mixing times of 35, 35, 50, and 75 ms. All spectra were acquired in the phase-sensitive mode using TPPI

(Marion & Wüthrich, 1983). Acquisition parameters include a 3-s relaxation delay, a 0.26-s acquisition time, a sweep width of 8064 Hz, a filter width of 30 KHz, and 16 scans per t_1 value. Raw data sets contained 4K × 1K (TPPI) time domain data points in the t_2 and t_1 dimensions, respectively. The data sets were processed on a Personal IRIS (Silicon Graphics, Inc.) using the program FELIX (Hare Research Inc.). Processing parameters include cosine bell apodization in both dimensions and zero-filling in t_1 before Fourier transformation to yield 2K × 2K (real) data matrices. Intensities of NOESY cross peaks were measured by both volume integration using FELIX and optimal slice integration using standard Bruker software. Interproton distances were calculated at each mixing time, using as a reference the distance of 2.46 ± 0.02 Å between the 2,6 and 3,5 protons of phenylalanine as measured by neutron diffraction (Al-Karaghouli & Koetzle, 1975) and extrapolation to zero mixing to minimize spin diffusion effects, as previously described (Baleja et al., 1990; Weber et al., 1991). Alternative calculations of interproton distances, based on the relative slopes of plots of cross-peak volume as a function of mixing time, yielded identical distances within experimental error.

Modeling Studies. Initially, skeletal models of enzyme-bound phenylalanine based on the metal–proton and interproton distances were manually constructed. Objective conformational searches by computer were independently carried out using the program DSPACE (Hare Research Inc.), which takes into account all measured distances and their errors together with the van der Waals radii of the atoms (Weber et al., 1991). In this approach, atomic coordinates of phenylalanine were initially randomized, followed by annealing in 4-dimensional space (to avoid local minima), minimizing deviations from all input distances. In a typical search, 25 structures for enzyme-bound phenylalanine were computed, of which 10 were judged acceptable, on the basis of their consistency with all covalent and van der Waals constraints and with the measured distances within their experimental errors.

RESULTS

Phenylalanine as a Substrate for Recombinant Human Tyrosine Hydroxylase. In the present study we have used phenylalanine in order to determine the conformation of the enzyme-bound substrate. The use of phenylalanine instead of tyrosine has several advantages: (i) Tyrosine has a low solubility at neutral pH and 20 °C (~2 mM). (ii) Tyrosine has a very high affinity for the enzyme (see below) which implies slow exchange between tyrosine in the bound and the free state in our NMR experiments. This is not the case for phenylalanine (see below). (iii) In deuterated solutions, the exchangeable hydroxyl proton in the phenol ring of tyrosine cannot easily be observed, while phenylalanine has a stable proton at the 4-position.

Phenylalanine has been shown to be a good substrate for tyrosine hydroxylase from many sources (Shiman et al., 1971; Wang et al., 1991; Fukami et al., 1990). It has also been found that phenylalanine can be utilized for the biosynthesis of catecholamines in isolated bovine adrenal chromaffin cells (Fukami et al., 1990), although the physiological significance or biological advantage of the use of this substrate is not clear. Our preparations of purified human recombinant tyrosine hydroxylase (hTH1) were able to hydroxylate phenylalanine both to tyrosine and to DOPA, with a K_M = 85 ± 5 μM and a V_{max} (for the hydroxylation to both products) = 461 ± 89 nmol min^{-1} mg^{-1} (n = 3) at pH 7.0 with BH_4 as cofactor, compared with a K_M = 11 ± 1.7 μM and a V_{max} = 486 ± 51 nmol min^{-1} mg^{-1} for tyrosine (n = 3), measured under the same conditions. We also found strong substrate inhibition at concentrations of tyrosine greater than 60 μM, while weak substrate inhibition by phenylalanine (≤25%) was found at concentrations greater than 0.8 mM. Thus, at high substrate levels (≥100 μM), the rate of phenylalanine hydroxylation by the human enzyme exceeded that of tyrosine hydroxylation, as found for tyrosine hydroxylase isolated from rat pheochromocytoma cells (PC12) (Ribeiro et al., 1991).

Binding of Divalent Cations to Apo-Tyrosine Hydroxylase. The binding of Fe^{2+} and Zn^{2+} was measured using ^{55}Fe, ^{59}Fe, and ^{65}Zn in an equilibrium binding assay as described (Haavik et al., 1991). The stoichiometry of metal ions bound to human tyrosine hydroxylase (n) was found to be 0.97 ± 0.04 mol of Fe^{2+}/mol of subunit and 1.13 ± 0.04 mol of Zn^{2+}/mol of subunit, and the dissociation constant (K_D) was 0.89 ± 0.08 μM for Fe^{2+} and 0.34 ± 0.15 μM for Zn^{2+} at pH 6.5 (Haavik et al., 1991). The stoichiometry of the binding of Co^{2+} was measured by the quenching of the intrinsic L-tryptophan fluorescence of hTH1 as described (Haavik et al., 1992) and found to be 1.03 ± 0.03 mol of Co^{2+}/mol of subunit (n = 4). The binding of Co^{2+} and Zn^{2+} was also measured in an enzymatic assay in competition with Fe^{2+} (Haavik et al., 1991), and K_i values of ~1.0 and ~0.1 μM were estimated for the binding of Co^{2+} and Zn^{2+}, respectively, at pH 7.5.

Apo-tyrosine hydroxylase is selectively activated by added Fe^{2+}, although other divalent metal ions, e.g., Co^{2+}, Zn^{2+}, Ni^{2+}, and Mn^{2+}, bind to the apoenzyme in stoichiometric amounts. Of these metal ions, Zn^{2+} and Co^{2+} have the highest affinity. Steady-state kinetic experiments show that these metal ions are competitive inhibitors of the enzyme with respect to added Fe^{2+} (data not shown), and they also compete in binding assays (Haavik et al., 1991, 1992). Since Zn^{2+} and Co^{2+} bind with similar stoichiometry to apo-tyrosine hydroxylase, but cause 12 and 31% quenching of the intrinsic tryptophan fluorescence of the enzyme, respectively, it was important to obtain additional proof that they bind to the same site. By measuring the initial reaction rate in the presence of a combination of these inhibitors and plotting the data according to Yonetani and Theorell (1964), a series of parallel lines was obtained, corresponding to an interaction constant (α) of infinity (Figure 1). This confirms that the binding of Co^{2+} and Zn^{2+} is independent and mutually exclusive, as expected if they compete for binding to the same site, i.e., the Fe^{2+} binding site.

Paramagnetic Effects of Enzyme-Bound Co^{2+} on the Relaxation Rates of Proton Resonances of Phenylalanine. (A) In the Presence of Oxidized Cofactor. Proposed kinetic mechanisms for tyrosine hydroxylase involve the ordered binding of the substrates tetrahydropterin, oxygen, and tyrosine (Dix et al., 1987; Fitzpatrick, 1991a), with the formation of a dead-end enzyme–tyrosine complex (Fitzpatrick, 1991a). The strong substrate inhibition also suggested a dead-end enzyme–metal–substrate complex. Therefore, the effects of Co^{2+} on the phenylalanine protons were first measured in the hTH1–metal·pterin cofactor·phenylalanine complex. Since some activity is observed in the enzyme as isolated, probably due to the presence of residual iron (about 0.02 atom of Fe/mol of subunit) (Haavik et al., 1991), reduced pterin cofactor could not be used in our NMR studies. Catalytic turnover was avoided by using L-*erythro*-7,8-dihydrobiopterin (BH_2), which has been shown to be a competitive inhibitor versus the tetrahydropterin cofactors and noncompetitive versus tyrosine (Nagatsu et al., 1972; Roskoski et al., 1990; Fitzpatrick,

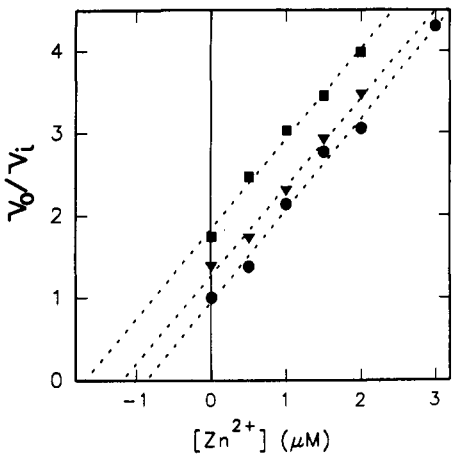

FIGURE 1: Inhibition of human tyrosine hydroxylase (hTH1; 0.16 μM subunit concentration) by Zn^{2+} and Co^{2+}, in the presence of 1 μM Fe^{2+}. The assay mixture contained either no added Co^{2+} (●) or 5 μM $CoCl_2$ (▼), or 15 μM $CoCl_2$ (■), and the concentration of $ZnSO_4$ shown. The data were plotted according to Yonetani and Theorell (1964), with the ordinate representing the enzyme activity in the absence of inhibitors (v_o), relative to the activity in the presence of Zn^{2+} or Co^{2+} (v_i). Each point represents the average of two separate experiments, using 1-min incubation and performed in duplicate, and the data were fitted using linear regression (dotted lines).

1991a). In the present study, we have also measured the inhibition of recombinant human tyrosine hydroxylase with BH_2, which shows a competitive inhibition pattern versus the natural cofactor BH_4, with a K_i = 70 μM.

The 600-MHz ^{1}H-NMR spectrum of phenylalanine (5.0 mM) in the presence of hTH1 apoenzyme (0.21 mM) and 0.5 mM BH_2 and the assignment of proton resonances of phenylalanine are shown in Figure 2. The addition of Co^{2+} results in a paramagnetic line broadening of the proton resonances, when compared with the effects of Zn^{2+} addition. Titrations with Co^{2+} measuring the $1/T_1$ of the proton resonances of phenylalanine are shown in Figure 3. Titrations with Zn^{2+}, used as the diamagnetic controls, revealed negligible effects on the $1/T_1$ or $1/T_2$ values beyond the effects of the enzyme alone. Because of the high-affinity binding of divalent cations, including Co^{2+}, to recombinant human tyrosine hydroxylase ($K_D \leq 1$ μM), which is not decreased by the presence of substrates or inhibitors (Haavik et al., 1991, 1992), it was reasonably assumed that the added metal was fully incorporated into the active site forming the E–Co^{2+}·BH_2·Phe complex. When compared with titration curves made in the absence of enzyme, it was found that the enzyme enhances the paramagnetic effects of Co^{2+} on the $1/T_1$ value of the aromatic protons of phenylalanine, with enhancement factors ranging from 1.6 to 4.5. Because of larger errors in $1/T_1$ measurements of the aliphatic Hβ protons which have very short T_1 values (≤1 s), in both the absence and the presence of the enzyme, only upper limits of $1/fT_{1P}$ values and lower limit distances to the metal could be measured for these protons.

In order to calculate the outer-sphere contribution to the relaxation rate, phenylalanine was displaced from its enzyme complex by tyrosine, used as a competitive ligand. A displacement of 44% was reached when a concentration of 1 mM tyrosine was used (Figure 3), which indicates a ratio of about 4.5 for the affinities of tyrosine and phenylalanine for the enzyme, comparable to the ratio of their K_M values of 7.7 ± 1.3 (see above). Precipitation of tyrosine occurred when it was added at higher concentrations. Taking into account the relative affinities of both substrates, their concentrations in the NMR samples, and the displacement observed at 1 mM tyrosine, a negligible outer-sphere contribution to the relaxation rates was calculated, as directly observed for the E–Co^{2+}·Phe complex (see below). The normalized paramagnetic contributions to the longitudinal ($1/fT_{1P}$) and transverse ($1/fT_{2P}$) relaxation rates of phenylalanine protons obtained for this complex are summarized in Table I.[2] On the basis of a K_M value of 85 μM for phenylalanine and a concentration of 5.0 mM used in the titrations, the enzyme (0.21 mM subunit) was calculated to be >98% saturated with phenylalanine for determining the normalized relaxation rates. Moreover, the $1/fT_{2P}$ values exceeded all of the $1/fT_{1P}$ values by several orders of magnitude, indicating that the $1/fT_{1P}$ values are not limited by the exchange rate of phenylalanine into the paramagnetic environment and do not include the contribution of τ_M, the lifetime of the complex.

Catecholamines (e.g., dopamine, noradrenaline, adrenaline) are active site inhibitors of aromatic amino acid hydroxylases, including human tyrosine hydroxylase (Haavik et al., 1991; Almås et al., 1992). Dopamine binds to iron-reconstituted hTH1 with a K_D of 2.3 μM (Almås et al., 1992) and to recombinant rat tyrosine hydroxylase with a K_D of 1 μM (Ribeiro et al., 1992). The inhibition of the aromatic amino acid hydroxylases by catecholamines is kinetically competitive with the pterin cofactor, with a K_i = 0.5 μM for hTH1 (Le Bourdellès et al., 1991), and noncompetitive with respect to the amino acid substrates (Udenfriend et al., 1965; Bublitz, 1971; Almås et al., 1992), although catecholamines displace phenylalanine bound at the active site of the enzyme phenylalanine hydroxylase in the absence of cofactor (Martínez et al., 1991). We checked the ability of dopamine to displace the phenylalanine bound in the hTH1–Co^{2+}·BH_2·Phe complex, and only a partial displacement of substrate was observed (about 22% at 4 mM dopamine, measured as the decrease in the longitudinal and transverse relaxation rates for all of the resonances) (not shown).

(*B*) *In the Absence of Oxidized Cofactor.* In order to study the conformation of phenylalanine in the proposed dead-end enzyme–substrate complex (Fitzpatrick, 1991a), the paramagnetic effects of Co^{2+} on the phenylalanine protons were also measured in the E–Co^{2+}·Phe complex. The addition of Co^{2+} to a deuterated solution containing hTH1 (0.21 mM) and phenylalanine (5.2 mM) also resulted in a paramagnetic line broadening and in an increase of the $1/T_1$ of the proton resonances of phenylalanine (Figure 4). Titrations with Zn^{2+}, used as the diamagnetic controls, had negligible effects on the $1/T_1$ or $1/T_2$ values, beyond the effects of the enzyme alone. Unlike the results with the BH_2-containing complex (see above), phenylalanine was effectively displaced from its enzyme complex by the active site inhibitor dopamine, which completely abolished the paramagnetic effects on all proton resonances of phenylalanine at concentrations of 4 mM (Figure 4), indicating no significant outer-sphere contribution to the relaxation rates. From the displacement curve obtained, a ratio of 14.5 was calculated for the relative affinities of dopamine and phenylalanine for hTH1 in the presence of Co^{2+}.[3]

The effects of Co^{2+} on $1/T_2$ values of phenylalanine protons were also measured, and the normalized paramagnetic contributions to the longitudinal ($1/fT_{1P}$) and transverse ($1/fT_{2P}$) relaxation rates obtained are summarized in Table II.[2] The $1/fT_{2P}$ values also exceeded all the $1/fT_{1P}$ values by several

[2] The linearity of the paramagnetic effects with Co^{2+} concentration (Figures 3 and 4) and the inequality of the $1/fT_{1P}$ values (Tables I and II) argue against significant contributions of spin diffusion to the relaxation rates.

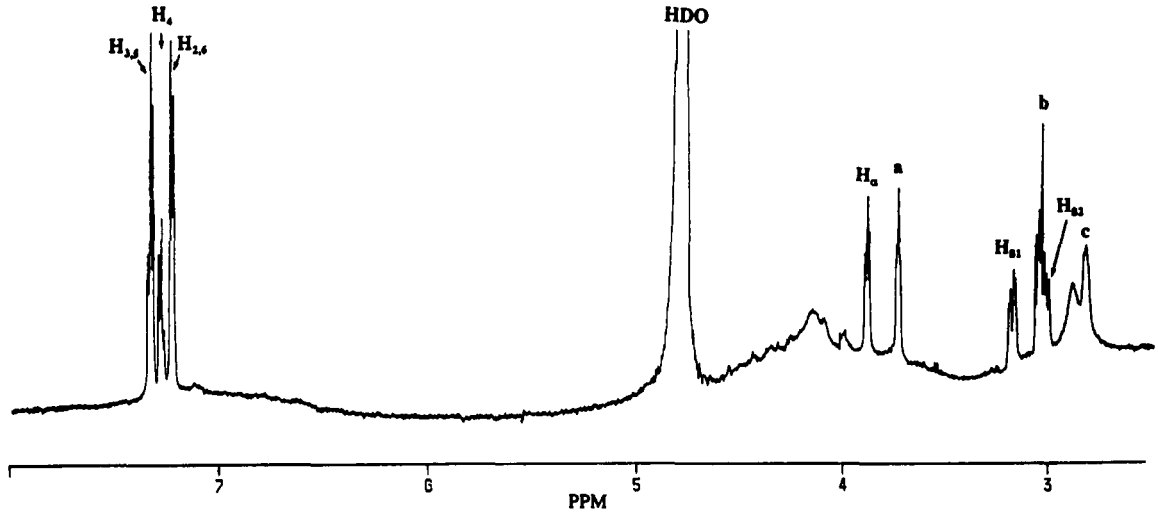

FIGURE 2: ^{1}H-NMR spectrum of phenylalanine in the presence of recombinant human tyrosine hydroxylase, isoform 1 (hTH1), and L-*erythro*-7,8-dihydrobiopterin (BH_2). The 600-MHz proton-NMR spectrum (32 transients) was taken at 293 K in samples (0.5 mL) prepared in D_2O that contained 4 mM NaHepes (pH* = 7.5), 0.2 M NaCl, 5 mM phenylalanine, 0.21 mM subunit human tyrosine hydroxylase isoform 1 (hTH1), and 0.5 mM BH_2. Peaks a and b are signals from Hepes. Signals from BH_2 are not observed at the concentration used in these experiments. The spectrum of phenylalanine in the presence of hTH1 (0.21 mM subunit) and without BH_2 is identical.

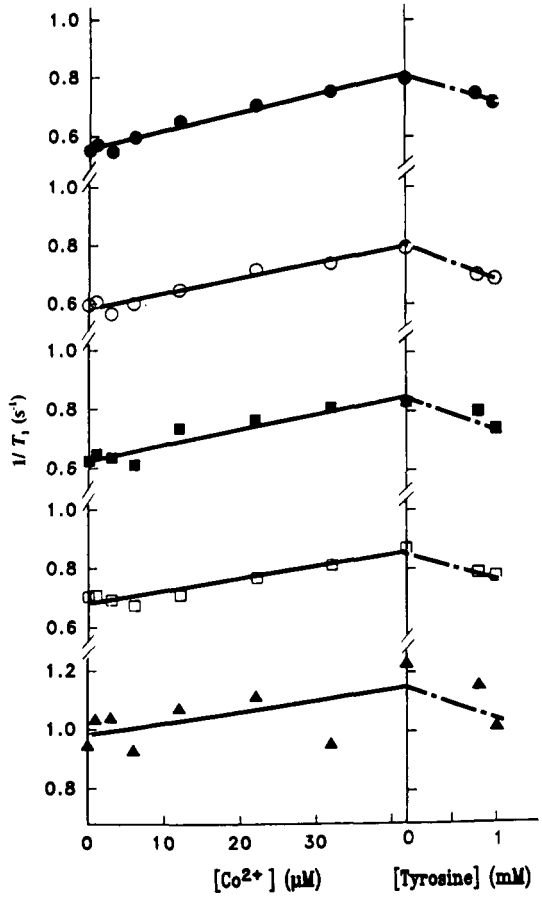

FIGURE 3: Paramagnetic effects of Co^{2+} on proton resonances of phenylalanine in the presence of human tyrosine hydroxylase, isoform 1, and BH_2 at 600 MHz and 293 K: $1/T_1$ versus $[Co^{2+}]$ for the aromatic H3,5 (●), H4 (○), and H2,6 (■) protons and the aliphatic Hα (□) and Hβ1 (▲) protons of phenylalanine, and displacement by tyrosine. The solutions contained 0.21 mM hTH1, 0.5 mM BH_2, and 5.0 mM phenylalanine in 4 mM NaHepes, pH* 7.5, and 0.2 M NaCl (prepared in ^2H_2O). The effect of displacement with tyrosine is shown.

orders of magnitude in this case, indicating $1/fT_{1P}$ to be in the fast-exchange case (Table II).

Calculation of the Correlation Time (τ_c) and of Cobalt-to-Proton Distances in the Enzyme–Co^{2+}·Phenylalanine and the Enzyme–Co^{2+}·BH_2·Phenylalanine Complexes. The correlation time in both the absence and the presence of BH_2 was calculated from the frequency dependence of $1/fT_{1P}$ of the aliphatic Hα proton at 250 and 600 MHz (Mildvan & Gupta, 1978; Mildvan et al., 1980; Serpersu et al., 1989). The $1/fT_{1P}$ values obtained at 250 MHz for this proton were 27.7 ± 3.0 s^{-1} in the E–Co^{2+}·BH_2·Phe complex and 29.7 ± 1.8 s^{-1} in the E–Co^{2+}·Phe complex. From the ratios fT_{1P}(600 MHz)/fT_{1P}(250 MHz) of 1.33 ± 0.17 and 1.38 ± 0.13 for the quaternary and ternary complexes, respectively, a similar τ_c of 1.8 ± 0.1 ps was calculated for both complexes, assuming τ_c to be independent of frequency over this range. This correlation

Table I: Distances from Co^{2+} to Protons of Phenylalanine in the Human Tyrosine Hydroxylase–Co^{2+}·BH_2·Phe Complex

nucleus	δ (ppm)[a]	$1/fT_{1P}$ (s^{-1})	$1/fT_{2P}$ (s^{-1})	r (Å)[b]
H3,5	7.39	30.3 ± 2.0	1915 ± 399	6.83 ± 1.21 (6.08 ± 1.07)[c]
H4	7.35	25.3 ± 2.3	1740 ● 205	7.03 ● 1.25
H2,6	7.31	25.5 ± 3.6	750 ± 96	7.00 ± 1.30 (6.26 ± 1.15)[c]
Hα	3.97	20.0 ± 2.6	510 ● 27	7.32 ± 1.30
Hβ1 (*pro-S*)	3.25	≤19.4	447 ± 26	≥7.40
Hβ2 (*pro-R*)	3.10	≤16		≥7.6

[a] From external TSP. [b] Distances calculated as described in the text. The τ_c value was determined as 1.8 ± 0.1 ps as described in the text. Errors in r include contributions from errors in $1/fT_{1P}$ and τ_c, which contribute ≤5%, and from the assumption that the C values used (895 ± 125) have a g value for high-spin Co^{2+} of 4 ± 2 (Mildvan et al., 1980), which contributes 15% to the total error in the absolute distances. Since the g tensor of Co^{2+} and τ_c should be the same for the effects of Co^{2+} on all protons of the substrate, the errors in the relative distances are approximately 2%. [c] Distances in parentheses: values determined if one proton experienced the total paramagnetic effect (asymmetric limiting case).

[3] Assuming that the K_M value of phenylalanine (85 μM) represents the dissociation constant for the binding of phenylalanine to hTH1 (which, in a second sphere complex, should not be strongly affected by the nature of the bound divalent cation), a dissociation constant of 6 μM is calculated for the binding of dopamine to the enzyme. This value is only slightly higher than the K_D values reported for the binding of dopamine to the iron-reconstituted enzyme (Ribeiro et al., 1992; Almás et al., 1992) and agrees with the finding that the affinity of catecholamines for the human apoenzyme decreases in the absence of iron (Almás et al., 1992).

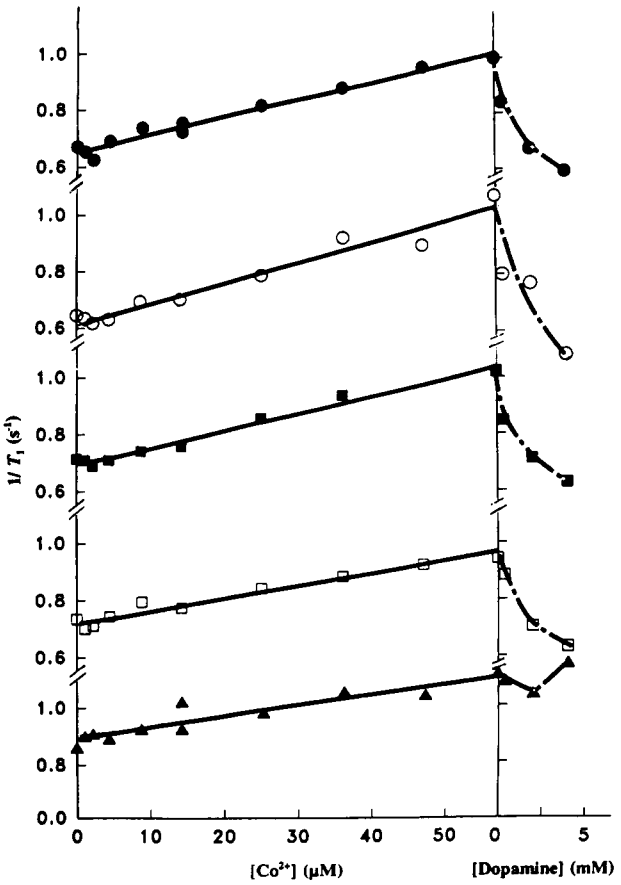

FIGURE 4: Paramagnetic effects of Co^{2+} on proton resonances of phenylalanine in the presence of human tyrosine hydroxylase, isoform 1, at 600 MHz and 293 K: $1/T_1$ versus [Co^{2+}] for the aromatic H3,5 (●), H4 (○), and H2,6 (■) protons and the aliphatic Hα (□) and Hβ1 (▲) protons of phenylalanine, and displacement by dopamine. The solutions contained 0.21 mM hTH1 and 5.2 mM phenylalanine in 4 mM NaHepes, pH* 7.5, and 0.2 M NaCl (prepared in ^2H_2O).

Table II: Distances from Co^{2+} to Protons of Phenylalanine in the Human Tyrosine Hydroxylase–Co^{2+}·Phe Complex

nucleus	δ (ppm)[a]	$1/fT_{1P}$ (s^{-1})	$1/fT_{2P}$ (s^{-1})	r (Å)[b]
H3,5	7.39	31.7 ± 1.5	1159 ± 183	6.80 ± 1.20 (6.03 ± 1.06)[c]
H4	7.35	38.4 ± 3.2	1740 ± 302	6.56 ± 1.18
H2,6	7.31	31.0 ± 1.6	925 ± 134	6.80 ± 1.21 (6.06 ± 1.06)[c]
Hα	3.97	22.3 ± 2.1	409 ± 45	7.18 ± 1.28
Hβ1 (*pro-S*)	3.25	19.5 ± 2.5	480 ± 83	7.35 ± 1.31
Hβ2 (*pro-R*)	3.10	≤17		≥7.52

[a] From external TSP. [b,c] See Table I.

time, which represents the longitudinal electron spin relaxation time (τ_c) of Co^{2+} (Mildvan et al., 1980), was used to calculate the distances from the phenylalanine protons to the Co^{2+} (Tables I and II). While uncertainties in the *relative* Co^{2+}–proton distances are small (±2%), resulting only from errors in $(1/fT_{1P})^{1/6}$, uncertainties in the *absolute* distances are much greater (±19%), resulting from errors in $(1/fT_{1P})^{1/6}$, $(\tau_c)^{1/6}$, and the effective g value of Co^{2+} of $(4 \pm 2)^{1/3}$ (Mildvan & Gupta, 1978; Serpersu et al., 1989; Weber et al., 1991).[4]

Intramolecular Nuclear Overhauser Effects on Enzyme-Bound Phenylalanine in the Presence of the Oxidized Cofactor. The hTH1–Zn^{2+}·BH_2·Phe complex was used to study in greater detail the conformation of bound phenylalanine. Transferred nuclear Overhauser effects for the protons of the

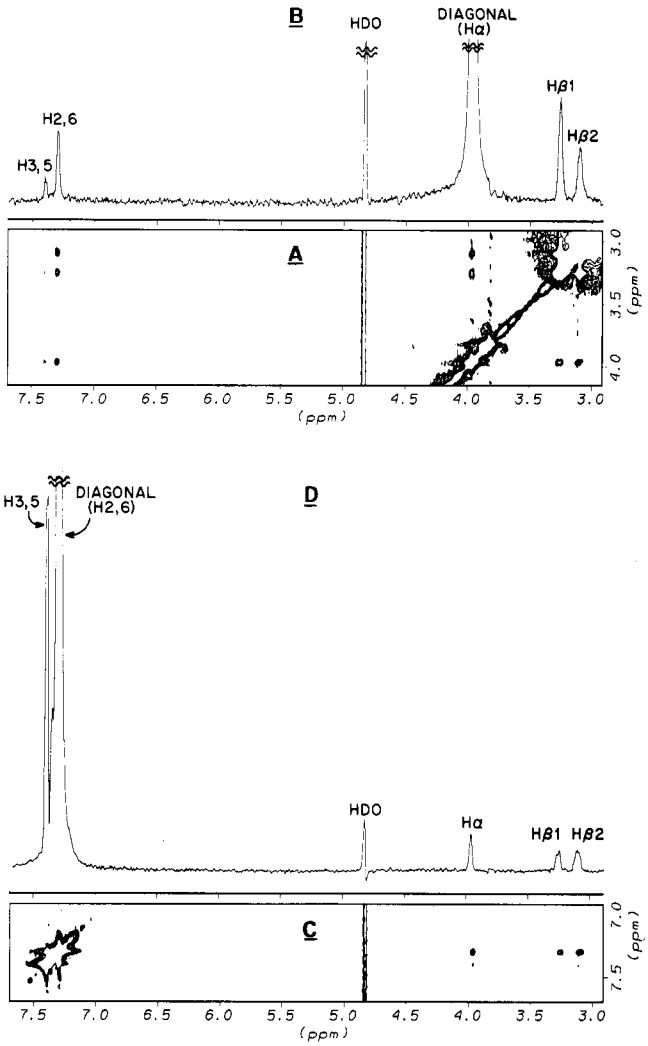

FIGURE 5: Two-dimensional nuclear Overhauser effect (NOESY) spectra at 600 MHz of phenylalanine (5 mM) in the presence of human tyrosine hydroxylase, isoform 1 (0.21 mM), Zn^{2+} (0.21 mM), and BH_2 (0.5 mM) at 600 MHz and 293 K. The mixing time was 35 ms. Other parameters are given in Methods. (A) Region showing cross peaks between aliphatic (F1) and other resonances of phenylalanine. (B) Optimum slice at 3.97 ppm in the F1 dimension showing NOEs to Hα. (C) Region showing cross peaks between aromatic (F1) and other resonances of phenylalanine. (D) Optimal slice at 7.31 ppm in the F1 dimension showing NOEs to H2,6.

bound substrate were measured at 600 MHz and 20 °C. Selected portions of a phase-sensitive NOESY spectrum obtained with a mixing time of 35 ms are shown in Figure 5A,C. Typical slices from this NOESY experiment are also displayed (Figure 5B,D). In addition to the expected cross peaks among the phenyl protons (aromatic region), cross peaks reflecting negative NOEs between the aliphatic (Hα, Hβ1, and Hβ2) and the aromatic protons (H3,5 and H2,6) and between the Hα and Hβ1,2 protons of enzyme-bound phenylalanine were clearly observed. In the absence of apoenzyme, no cross peaks reflecting negative NOEs are visible,

[4] With $1/fT_{1P}$ measurements at only two frequencies, an alternative value for τ_c, which also fits the ratio of $1/fT_{1P}$ values, is 0.38 ± 0.02 ps. This short τ_c value, while not excluded, is unprecedented for enzyme–Co^{2+} complexes for which τ_c values ranging from 0.7 to 5.1 ps have been determined by $1/fT_{1P}$ measurements at multiple frequencies (Melamud & Mildvan, 1975; Smith et al., 1980; Serpersu et al., 1988). If the lower τ_c value of 0.38 ps were correct, the distances in Tables I and II would decrease by 13%, which is within the errors given for the absolute distances and used in computing the conformations of enzyme-bound phenylalanine. Hence the structural conclusions would remain unaltered.

Table III: Interproton Distances Calculated from NOESY Data in the Human Tyrosine Hydroxylase–Zn^{2+}·BH_2·Phe Complex

proton pair	distances (Å)[a] vol integration	optimal slice integration	av
H3,5 – H2,6	2.46 ± 0.02[b]	2.46 ± 0.02[b]	2.46 ± 0.02[b]
H3,5 – Hα	4.30 ± 0.14	4.39 ± 0.34	4.35 ± 0.35
H3,5 – Hβ1 (*pro-S*)	4.96 ± 0.18	5.11 ± 0.35	5.04 ± 0.35
H3,5 – Hβ2 (*pro-R*)	4.38 ± 0.10	4.65 ± 0.25	4.52 ± 0.25
H2,6 – Hα	3.42 ± 0.12	3.43 ± 0.20	3.43 ± 0.20
H2,6 – Hβ1 (*pro-S*)	3.48 ± 0.10	3.57 ± 0.25	3.53 ± 0.28
H2,6 – Hβ2 (*pro-R*)	3.33 ± 0.08	3.48 ± 0.18	3.41 ± 0.27
Hα – Hβ1 (*pro-S*)	2.90 ± 0.09	3.05 ± 0.11	2.98 ± 0.17
Hα – Hβ2 (*pro-R*)	3.27 ± 0.14	3.38 ± 0.19	3.34 ± 0.29

[a] Distances from aliphatic to degenerate pairs of aromatic protons were treated as single proton–proton interactions. [b] Internal standard, based on neutron diffraction study of L-phenylalanine (Al-Karaghouli & Koetzle, 1975).

indicating that, in the presence of hTH1, the dipole–dipole relaxation of bound phenylalanine is transferred to the free phenylalanine by rapid chemical exchange. The intensity of the NOESY cross peaks was measured by both volume and optimal slice integration, and interproton distances were calculated by extrapolation of the distances measured at each mixing time to zero mixing time, as described previously (Baleja et al., 1990; Weber et al., 1991), using as an internal reference the distances between the 2,3 and 5,6 protons of phenylalanine of 2.46 ± 0.02 Å as measured by neutron diffraction (Al-Karaghouli & Koetzle, 1975) (Table III). The cross-peak intensities were found to increase linearly with mixing times of 35, 50, and 75 ms, and the interproton distances calculated from the slopes of such plots yielded distances indistinguishable from those given in Table III, obtained by extrapolation to zero mixing time.

Conformation of Enzyme-Bound Phenylalanine. Using the four measured and the two lower limit Co^{2+}–proton distances (Table I) together with the eight measured interproton distances (Table III), a structural model of enzyme-bound phenylalanine (23 atoms) in the presence of oxidized cofactor (BH_2) was computed.

Because the degeneracies of the chemical shifts of the 3 and 5 protons and the 2 and 6 protons of phenylalanine introduced an ambiguity in interpreting the intramolecular NOEs between aliphatic and aromatic protons, the errors in the interproton distances were enlarged during the computer searches to permit the aliphatic protons of enzyme-bound phenylalanine to interact either asymmetrically or symmetrically with the aromatic protons. Unless this was done, acceptable structures with low penalties were not obtained. Because of an analogous ambiguity in interpreting the Co^{2+}–proton distances, two limiting cases were assumed in separate conformational searches. At one extreme, the asymmetric case, it was assumed that only one proton of each degenerate proton pair (e.g., the 2,6 and 3,5 protons) experienced the entire paramagnetic effect of Co^{2+}. At the opposite extreme, the symmetric case, it was assumed that each proton of both degenerate pairs experienced equal halves of the paramagnetic effect of Co^{2+}. These limiting cases, which placed the metal either asymmetrically or symmetrically with respect to enzyme-bound phenylalanine, did not significantly influence the resulting conformation of the bound substrate. For the asymmetric case, 25 conformational searches, each starting from randomized coordinates, yielded 10 acceptable solutions (Figure 6). Similarly, for the symmetric case, 29 conformational searches yielded 10 acceptable structures (Figure 7). Within the experimental uncertainties, the conformations of enzyme-bound phenylalanine determined in both searches agreed and were partially extended with $\chi_1 = 97° \pm 3°$ and $\chi_2 = -78° \pm 2°$. In accord with the initial assumptions, the position of the bound metal with respect to enzyme-bound phenylalanine differed. In both the asymmetric and symmetric limiting cases, the aromatic ring of the substrate is in the second coordination sphere of the metal.

DISCUSSION

In the present work we have determined the conformation of phenylalanine and its proximity to the divalent cation when the amino acid is bound at the active site of recombinant human tyrosine hydroxylase isoform 1 (hTH1). Phenylalanine is shown to be a good substrate for hTH1, although its K_M for phenylalanine is greater than for tyrosine, as previously found for the enzyme purified from bovine adrenal chromaffin cells (Shiman et al., 1971; Fukami et al., 1990; Meyer et al., 1992) and for the recombinant rat enzyme (Fitzpatrick, 1991b; Wang et al., 1991). As for the bovine enzyme (Shiman et al., 1971), hTH1 has similar V_{max} values for the hydroxylation of phenylalanine and tyrosine. This finding is also consistent with the similar turnover number observed for the recombinant rat enzyme with phenylalanine, tyrosine, or 3-hydroxyphenylalanine as the substrate, 3-hydroxyphenylalanine being also an intermediate product when phenylalanine is used as a substrate (Fitzpatrick, 1991b).

An ordered sequential reaction mechanism has been proposed for rat and bovine tyrosine hydroxylase, with the order of substrate binding being tetrahydropterin, oxygen, and amino acid, both with tyrosine and phenylalanine, and it is believed that an enzyme–Fe^{2+}·BH_4·O_2·Phe complex is formed before any product is released (Kaufman & Kaufman, 1985; Fitzpatrick, 1991a,b). In our NMR studies, carried out under aerobic conditions with Co^{2+} and Zn^{2+} rather than Fe^{2+} at the active site, we observe only the interaction of the amino acid substrate with the enzyme–metal complex and the effect of the oxidized pterin cofactor on this binding. It is unlikely that dioxygen binds to the enzyme–substrate complexes under these conditions. The substrate inhibition observed at high concentrations of tyrosine and phenylalanine may be due to the binding of amino acid prior to the binding of the pterin, forming a dead-end enzyme–tyrosine complex (Fitzpatrick, 1991a). The substrate inhibition has been demonstrated to be competitive versus the reduced pterin cofactor (Fitzpatrick, 1991a). We have also found strong substrate inhibition for the recombinant human enzyme when tyrosine was used as substrate at concentrations greater than 60 μM.

As found by the paramagnetic probe–T_1 NMR method, there appear to be no significant differences in the conformation or location of phenylalanine when it binds to the Co^{2+}-reconstituted enzyme (E–Co^{2+}, pterin cofactor absent) or when it binds to the pterin complex E–Co^{2+}·BH_2, although the aromatic H4 proton of phenylalanine is slightly farther from the metal in the presence of the cofactor (Tables I and II). Although weak substrate inhibition is found when phenylalanine is used at the concentrations required in our NMR experiments (5 mM), the similarity of the conformation and location of enzyme-bound phenylalanine in the presence and absence of pterin, which reverses substrate inhibition (Fitzpatrick, 1991a), argue against significant structural alterations resulting from the high concentrations of substrate. Moreover, dopamine effectively displaces phenylalanine from its binding site in the E–Co^{2+}·Phe complex, but not from the E–Co^{2+}·BH_2·Phe complex. Catecholamines have also been found to

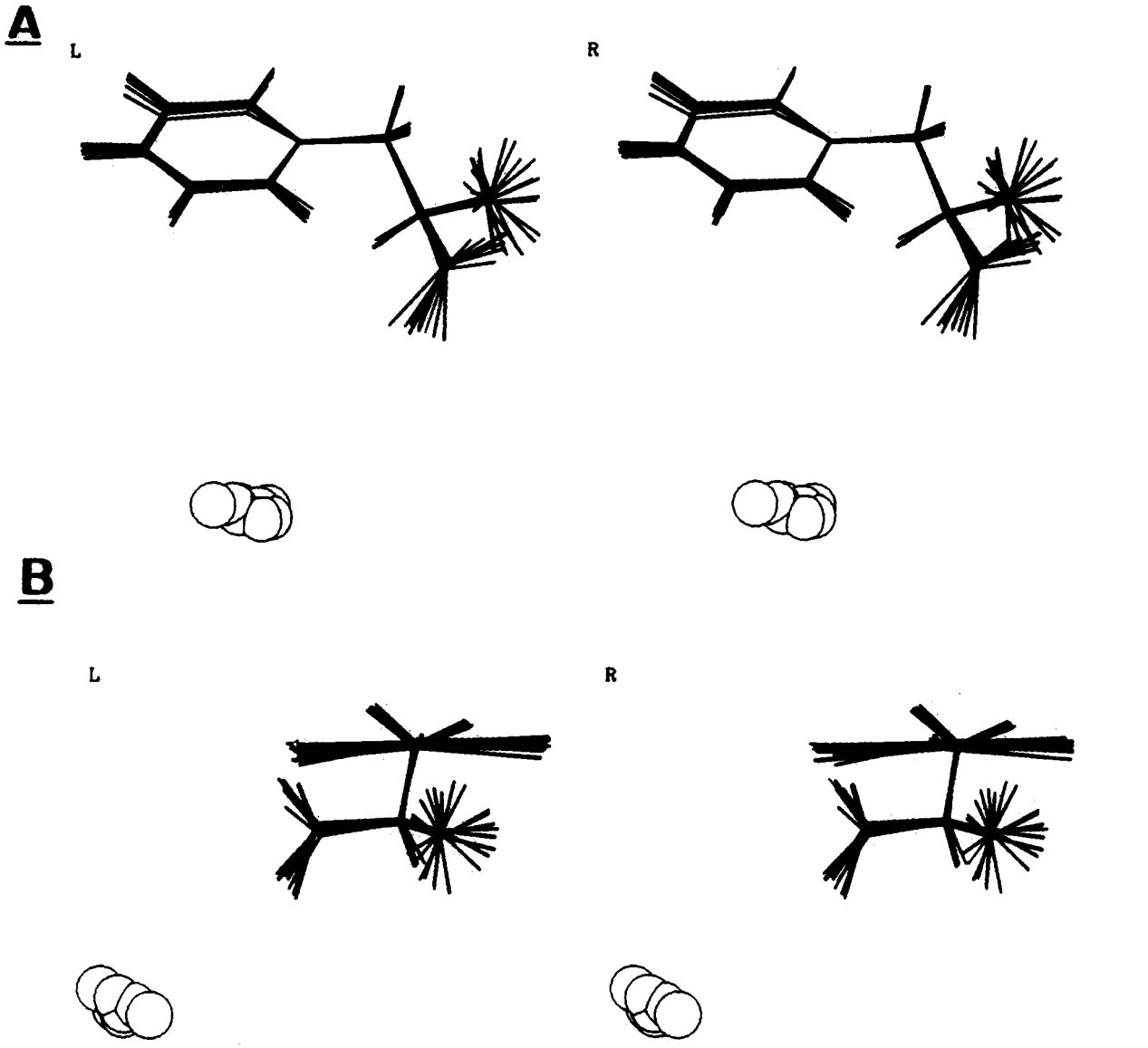

FIGURE 6: Computed conformations of phenylalanine bound at the active site of metal-reconstituted tyrosine hydroxylase in the presence of the oxidized cofactor (BH_2) based on the experimental distances of Tables I and III: asymmetric case, in which the metal is assumed to relax only one proton of each degenerate pair. The positions of the metal ions in the computed structures are shown as overlapping spheres. (A) Side view. (B) End view from the direction of H4.

displace phenylalanine bound at the active site of the structurally related enzyme phenylalanine hydroxylase in the absence of cofactor (Martínez et al., 1991). We have calculated a K_D of 6 μM for the binding of dopamine to the Co^{2+}-reconstituted enzyme in the absence of oxidized cofactor, indicating a relatively high affinity of the catecholamine for the enzyme, not uniquely dependent on iron at the active site. The inhibition of the aromatic amino acid hydroxylases by catecholamines is kinetically competitive with respect to the pterin cofactor, with a K_i of 0.5 μM for hTH1 (Le Bourdellès et al., 1991), and noncompetitive with respect to the amino acid substrates (Udenfriend et al., 1965; Bublitz, 1971; Almás et al., 1992). The kinetic competition of catecholamines (product analogs) with the tetrahydrobiopterin cofactor can be explained by a competition of both ligands for overlapping sites, possibly with catecholamines chelating the enzyme-bound iron (Andersson et al., 1988; Haavik et al., 1992).

While a precise conclusion about the binding site for catecholamines cannot be inferred from kinetic data, further information is provided by the present NMR studies. On the basis of similar distances from Co^{2+} to the protons of phenylalanine, both in the presence and absence of BH_2 (Tables I and II), the binding of the pterin does not greatly alter the location or conformation of the bound substrate. Yet, dopamine readily displaces phenylalanine from the E–Co^{2+}·Phe complex but not from the E–Co^{2+}·BH_2·Phe complex, presumably due to the additional competition between dopamine and the pterin cofactor, as found by kinetics. Thus the binding site of the product analog dopamine may overlap partially with those of both phenylalanine and the pterin cofactor in the E–Co^{2+}·BH_2·Phe complex and in the active E–Fe^{2+}·BH_4·O_2·Phe complex. Despite this overlap, the observed noncompetitive inhibition by dopamine with respect to the substrate could well result from the ordered kinetic scheme in which substrate and product bind to differing forms of the enzyme. Such overlap of sites for dopamine implies a close proximity between the binding sites of the pterin cofactor and the amino acid substrate and, possibly, a specific interaction between them, although the evidence is indirect. Such interaction would be consistent with the fact that the affinity of tyrosine hydroxylase for both tyrosine and phenylalanine is dependent on the structure of the pterin cofactor used, such that the lowest K_M values and the highest V_{max} values for the amino acids have been obtained with the natural cofactor

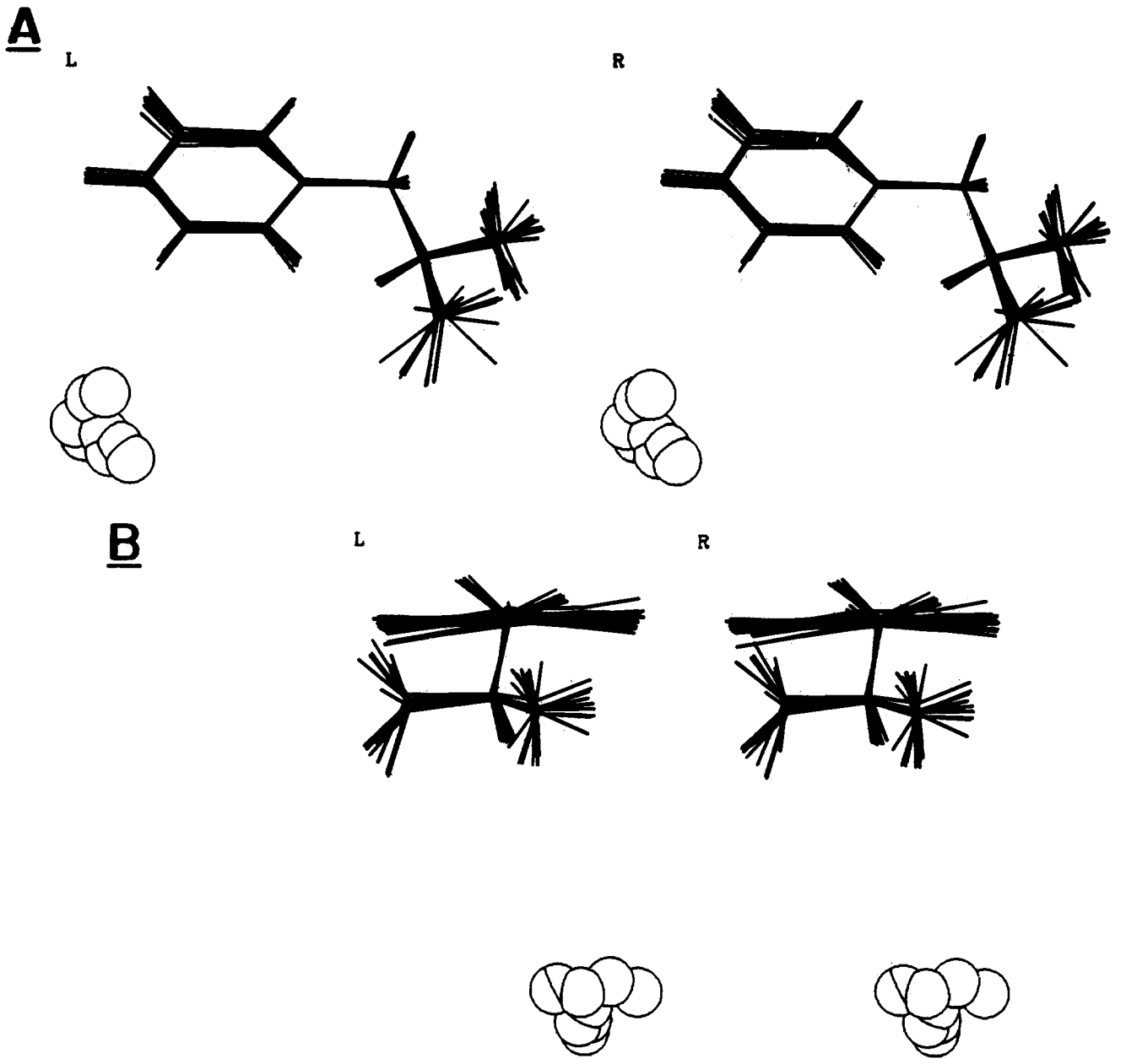

FIGURE 7: Computed conformations of phenylalanine bound at the active site of metal-reconstituted tyrosine hydroxylase in the presence of the oxidized cofactor (BH_2) based on the experimental distances of Tables I and III: symmetric case, in which the metal is assumed to relax equally both protons of each degenerate pair. The positions of the metal ions in the computed structures are shown as overlapping spheres. (A) Side view. (B) End view from the direction of H4.

tetrahydrobiopterin (Kaufman & Kaufman, 1985). Hence, the kinetic and NMR data may be accommodated by assuming that phenylalanine partially occupies the cofactor binding site in the dead-end E–Co^{2+}·Phe complex and that there is a close proximity between the bound amino acid substrate and the pterin cofactor in the E–Co^{2+}·BH_2·Phe complex and, by extension, in the catalytic E–Fe^{2+}·BH_4·O_2·Phe(or Tyr) complex.

The conformation and location of phenylalanine bound in the E–Co^{2+}·BH_2·Phe complex that we have determined from the experimental distances obtained both by the paramagnetic probe–T_1 method and by NOESY spectra have further mechanistic implications. Thus, the distances from the aromatic protons of phenylalanine to the metal in the ternary E–Co^{2+}·BH_2·Phe complex (Table I) place the aromatic ring of the substrate in the second coordination sphere of the metal (Figures 6 and 7). Since no group from the aliphatic chain of the amino acid (i.e., the amino or carboxyl group) is near enough to coordinate to the metal, the metal contributes only indirectly to the correct orientation of the amino acid. By EPR spectroscopy we have previously found changes in the redox state and the ligand field of the catalytic iron upon binding of the amino acid substrate to resting bovine tyrosine hydroxylase, both the phosphorylated and the nonphosphorylated enzyme (Haavik et al., 1988; Andersson et al., 1989), suggesting an interaction between the metal and the amino acid substrate. Changes in the ligand environment of the iron have also been observed upon binding of phenylalanine to phenylalanine hydroxylase (Wallick et al., 1984; Martínez et al., 1993b). These effects of substrates are probably indirect since phenylalanine does not seem to directly coordinate to the metal (Figures 6 and 7).

Most interestingly, the calculated distances from the metal to the aromatic ring (Tables I and II) are appropriate for an Fe(II)-bound oxy or peroxy species, acting as a hydroxylating intermediate, to approach molecular contact with C3/C4,

the positions at which phenylalanine is hydroxylated by tyrosine hydroxylase, forming both 3-hydroxyphenylalanine and tyrosine (Tong et al., 1971). Such a highly reactive oxygen-containing intermediate has been postulated to be the hydroxylating intermediate in rat liver phenylalanine hydroxylase (Davis & Kaufman, 1989). Although the precise mechanism of oxygen activation by the pteridine-dependent monooxygenases and the identity of the hydroxylating species are unknown, several structures have been proposed as hydroxylating intermediates: a 4a-hydroperoxytetrahydropterin adduct, a carbonyl oxide resulting from a ring opening of the tetrahydropterin, an activated enzyme-bound iron–oxy or peroxy adduct, and a 4a-peroxytetrahydropterin–iron species [for reviews, see Dix and Benkovic (1988) and Kaufman and Kaufman (1985)]. While none of these species has been directly detected and unequivocally proven in enzymatic systems, the isolation of the 4a-hydroxypterin product suggested that 4a-peroxytetrahydropterin may be formed in the oxygen-activation step both for tyrosine hydroxylase and phenylalanine hydroxylase (Dix et al., 1987; Haavik & Flatmark, 1987; Dix & Benkovic, 1988; Davis & Kaufman, 1989). This or another intermediate may be further activated by direct coordination to Fe^{2+}. Although our results cannot be used to unambiguously determine the identity of the hydroxylating intermediate, the proximity of the metal to the aromatic ring of phenylalanine found in this study is appropriate to support a role for the Fe^{2+} at the active site in the binding, activation, and transfer of oxygen.

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