A Kinetic and Conformational Study on the Interaction of Tetrahydropteridines with Tyrosine Hydroxylase†

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A Kinetic and Conformational Study on the Interaction of Tetrahydropteridines with Tyrosine Hydroxylase†

Bjørg Almås,‡ Karen Toska,‡ Knut Teigen,‡ Viola Groehn,§ Wolfgang Pfleiderer,§ Aurora Martínez,‡ Torgeir Flatmark,‡ and Jan Haavik*,‡

Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway, and Fakultät für Chemie, Universität Konstanz, Konstanz, 78434 Germany

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ABSTRACT: Tetrahydropterins are obligatory cofactors for tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis. A series of synthetic analogues of 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) with different substituents in positions C2, N3, C4, N5, C6, C7, and N8 on the ring were used as active site probes for recombinant human TH. The enzyme tolerates rather bulky substituents at C6, as seen by the catalytic efficiency (V_{max}/K_m) and the coupling efficiency (mol of L-DOPA produced/mol of tetrahydropterin oxidized) of the cofactors. Substitutions at C2, C4, N5, and N8 abolish the cofactor activity of the pterin analogues. Molecular docking of BH_4 into the crystal structure of the catalytic domain of ligand-free rat TH results in complexes in which the pteridine ring π-stacks with Phe300 and the N3 and the amino group at C2 hydrogen bonds with Glu332. The pteridine ring also establishes interactions with Leu294 and Gln310. The distance between C4a in the pteridines and the active site iron was 4.2 ± 0.5 Å for the ensemble of docked conformers. Docking of BH_4 analogues into TH also shows that the most bulky substituents at C6 can be well-accommodated within the large hydrophobic pocket surrounded by Ala297, Ser368, Tyr371, and Trp372, without altering the positioning of the ring. The pterin ring of 7-BH_4 shows proper stacking with Phe300, but the distance between the C4a and the active site iron is 0.6 Å longer than for bound BH_4, a finding that may be related to the high degree of uncoupling observed for 7-BH_4.

Tetrahydropterins, such as 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4, Figure 1),[1] are obligatory cofactors for tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis, and have a similar function for the structurally and functionally related enzymes phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (*1*). Inborn errors of BH_4 metabolism result in reduced activity of all three hydroxylases and are associated with a rare form of hyperphenylalaninemia, as well as low levels of biogenic amines in the cerebrospinal fluid and progressive neurological symptoms (*2*). Although the exact function of BH_4 in the hydroxylation reaction is not known, it probably acts together with the active site iron to activate dioxygen prior to hydroxylation of the amino acid substrate (*3*). Steady-state kinetic studies of the TH reaction are consistent with an ordered binding of BH_4 as the first substrate, followed by dioxygen and L-tyrosine (*4*). TH is a highly regulated, homotetrameric enzyme, both on a short-term (seconds to minutes) and a long-term (minutes to hours) scale (*5*, *6*). Short-term regulatory mechanisms include activation by phosphorylation of serine residues in the N-terminal regulatory domain and feedback inhibition by catecholamines which form a tight inhibitory complex with the active site iron (*7*, *8*). We have also recently demonstrated by kinetic measurements and surface plasmon resonance analyses that both the recombinant isoform 1 of human TH (hTH1) and TH isolated from adrenal medulla are regulated by a negative cooperativity of BH_4 binding (Hill coefficient in the range of $0.4 < h < 0.6$) (*9*).

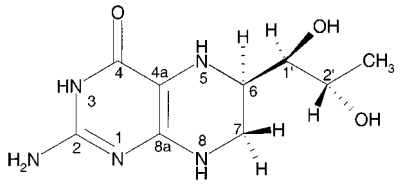

FIGURE 1: Structure of natural tetrahydrobiopterin [6(*R*)-BH_4]: 6(*R*)-(6-L-*erythro*-5,6,7,8-1′,2′-dihydroxypropyl)-5,6,7,8-tetrahydropterin.

In the crystal structure of the binary complex of the catalytic domain of rat TH and L-*erythro*-7,8-dihydrobiopterin (BH_2), an oxidized inactive cofactor analogue competitive to BH_4, the pterin binds on one side of a large hydrophobic cleft at the active site, forming an aromatic π-stacking interaction with Phe300, with a distance from the iron to the pterin C4a atom of 5.6 Å (*10*). A shorter value (3.0−4.0 Å) was estimated for the distance between the metal in Co(II)-reconstituted hTH1 and the C4a in bound 6-methyltetrahydropterin (6-MPH_4) based on NMR spectroscopy and distance geometry calculations (*11*). A recent NMR and

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* To whom correspondence should be addressed. Telephone: 47 55 58 64 32. Fax: 47 55 58 64 00. E-mail: jan.haavik@pki.uib.no.

‡ University of Bergen.

§ Universität Konstanz.

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molecular docking study on the structure of BH_2 bound to recombinant human PAH has also shown that the ring of BH_2 π-stacks with Phe254 (corresponding to Phe300 in TH) (*12*). The ring also establishes specific contacts with His264 and Leu249, and, in this structure, the N3 and the amine at C2 hydrogen bond with the carboxylic group of Glu286 of PAH (Glu332 in TH). The site for hydroxylation in the pterin, C4a, is located 4.3 ± 0.3 Å from the iron. A similar location and orientation of BH_2 is found in the recently published crystal structure of PAH complexed with the pterin analogue, although in this case the pterin does not interact directly with Glu286 but through two water molecules, and the distance between C4a in the pterin and the active site iron in PAH is 1.8 Å longer than in the NMR structure (*13*). In contrast, in the crystal structure of TH complexed with BH_2, the N3 and the amine at C2 of BH_2 do not hydrogen bond with TH residues, and the pterin seems to be rotated about 180° with respect to its conformation when bound to PAH.

Synthetic analogues of BH_4 have previously been used in structure−activity studies of the aromatic amino acid hydroxylases (*14*−*17*). Substitution of the dihydroxypropyl group in the C6-position of BH_4 seems to have minimal effects on the catalytic activity of the hydroxylases, although the kinetic parameters (*15*, *16*) may change. Generally, the introduction of hydrophobic substituents in the C6-position seems to increase the affinity for the enzymes, as determined by enzyme kinetic and fluorescence quenching studies (*15*, *16*, *18*, *19*). Substitutions at other positions on the pteridine ring invariably lead to reduced cofactor activity, which may be accompanied by a reduced coupling efficiency of substrate hydroxylation (less amino acid substrate is hydroxylated than tetrahydropterin oxidized) (*20*, *21*). This uncoupling is particularly pronounced for 7-substituted tetrahydropterins (*20*, *21*) and has been interpreted to reflect a suboptimal positioning of the cofactor at the active site.

In this study, a series of synthetic BH_4 analogues, with different substituents at positions C2, N3, C4, N5, C6, C7, and N8 on the pteridine ring, have been used to establish the structure−activity relationships for pterin cofactors of recombinant hTH1. Several 6-substituted pteridines were studied to evaluate them as BH_4 analogues in substitution therapy. Kinetic constants (K_m, V_{max}) and the coupling efficiency (mol of L-DOPA produced/mol of tetrahydropterin oxidized) of the cofactor analogues were measured to determine the structural features that result in a correct positioning for a productive cofactor function of tetrahydropterins at the active site. Finally, a molecular docking study of selected analogues into the crystal structure of the catalytic domain of TH (*22*) has been performed to further analyze the structure−activity relationships.

EXPERIMENTAL PROCEDURES

Materials. 6(*R*)-BH_4, 6(*S*)-BH_4, tetrahydroneopterin (NPH_4), tetrahydropterin (PH_4), 6-MPH_4, 6,7-dimethyl-tetrahydropterin (6,7-$DMPH_4$), 7-tetrahydrobiopterin (7-BH_4), 4-amino-tetrahydrobiopterin (4-amino-BH_4), and 5-methyl-tetrahydrobiopterin (5-methyl-BH_4) were purchased from Dr. B. Schirck's, Laboratories, Jona, Switzerland. Compounds 1−9 (Table 1) were synthesized as described by Bigham et al. (*15*) and Traub et al. (*23*, *24*), compounds 10−14 (Table 1 and Figure 2) as described by Groehn (*25*), Bömmel el al. (*26*), Fröhlich et al. (*27*, *28*), and 3-MPH_4 as described in ref *11*. Because of the low solubility of compounds 10−14, stock solutions were prepared in DMSO. All other tetrahydropterins used were water-soluble. NADH was obtained from the Sigma Chemical Co. (St. Louis, USA) and L-[3,5-^{3}H]tyrosine from Amersham International (Amersham, UK). All other chemicals were of the highest quality available.

Recombinant human TH, isoform 1 (hTH1), and a (6× histidine) tagged, truncated form lacking the 150 N-terminal amino acids (Δ150-hTH) were expressed in *Escherichia coli* and purified as previously described (*29*, *30*). Recombinant human PAH was expressed in the pMAL system of *E. coli* and purified as described (*31*). Dihydropteridine reductase (DHPR) was purchased from the Sigma Chemical Co. (St. Louis, USA); 4α-carbinolamine dehydratase was a generous gift from Dr. Sandro Ghisla, University of Konstanz, Germany.

Enzyme Assays. The TH activity was assayed at 25 °C as described (*32*), using an incubation mixture containing 10 mM NaHepes, pH 7.0, 25 μM L-[3,5-^{3}H]tyrosine, 0.5 mg/mL catalase, and 100 μM Fe(II)SO_4. The enzyme was preincubated for 3 min in this mixture before the reaction was started by addition of BH_4 or the selected analogue and 5 mM dithiothreitol (DTT) as the tetrahydropterin regenerating agent. The reaction was stopped after 3 min by the addition of a slurry of activated charcoal in 1 M HCl. After centrifugation, an aliquot of the supernatant was counted in a scintillation counter. At these conditions, the specific activity of different preparations of recombinant hTH1 was found to be 1017 ± 293 nmol min^{-1} (mg of protein)$^{-1}$ when using 500 μM 6(*R*)-BH_4 in the enzyme assay. The degree of coupling of the hydroxylation reaction was expressed as the ratio of L-DOPA formation to the oxidation of tetrahydropterin. For this assay, the reaction mixture contained 100 mM NaHepes, pH 7.0, 10 μM Fe(II)SO_4, 100 μM L-tyrosine, 150 μM NADH, and an excess of DHPR. After preincubation of the mixture for 5 min, the reaction was started with the addition of 100 μM of the selected BH_4 analogue. Oxidation of the tetrahydropterin was determined by monitoring the

[1] Abbreviations: 6-$AzMPH_4$, 6-azidomethyl-5,6,7,8-tetrahydropterin; BH_4, 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; 4-amino-BH_4, 2,4-diamino-6-dihydroxypropyl-5,6,7,8-tetrahydropteridine; 7-BH_4, 7(*R*, *S*)-tetrahydrobiopterin; DHPR, dihydropteridine reductase; 6,7-$DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DMSO, dimethylsulphoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; 2-MPH_4, 2-methyl-4-oxo-5,6,7,8-tetrahydropteridine; 3-MPH_4, 3-methyl-5,6,7,8-tetrahydropterin; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; 8-M-6,7-$DMPH_4$, 8-methyl-6,7-dimethyl-5,6,7,8-tetrahydropterin; NPH_4, 6(*R*,*S*)-5,6,7,8-tetrahydroneopterin; PAH, phenylalanine hydroxylase; PH_4, 5,6,7,8-tetrahydropterin; hTH1, human tyrosine hydroxylase isoform 1; Δ150-hTH, a 6(× histidine) tagged truncated form of hTH1 lacking the 150 N-terminal amino acids; compound 1, 6-(*R*,*S*)-6-hydroxymethyl-5,6,7,8-tetrahydropterin; compound 2, 6-methoxymethyl-5,6,7,8-tetrahydropterin; compound 3, 6-ethoxymethyl-5,6,7,8-tetrahydropterin; compound 4, 6-methoxyethoxymethyl-5,6,7,8-tetrahydropterin; compound 5, 6-ethoxycarbonyloxymethyl-5,6,7,8-tetrahydropterin; compound 6, 6-isopropoxy-5,6,7,8-tetrahydropterin; compound 7, 6-phenoxymethyl-5,6,7,8-tetrahydropterin; compound 8, 6-hydroxyacetyloxymethyl-5,6,7,8-tetrahydropterin; compound 9, (2-amino-3,4,5,6,7,8-hexahydro-4-oxo-6-pteridinyl)-methyl-L-alanine-ester; compound 10, 6-[4-(3-trifluoromethyl-3H-diazirine-3-yl-benzoyloxymethyl]-5,6,7,8-tetrahydropterin; compound 11, 6-azidobenzoyloxymethyl-5,6,7,8-tetrahydropterin; compound 12, 5(*N*-phenylthiocarbamoyl)-5,6,7,8-tetrahydropterin; compound 13, 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydrooxazolo[1,2-f]pteridine; compound 14, 6-[2-(4-benzoylphenyl)propionyloxymethyl]-5,6,7,8-tetrahydropterin; rmsd, root-mean-square deviation.

Table 1: Kinetic Constants for Tetrahydropterins in the Reaction Catalyzed by hTH1[a]

tetrahydropterin	sp	substituent	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	V_{max}/K_m (μM^{-1} min^{-1})	*n*	coupling efficiency
6(*R*)-BH_4	C6	-CHOHCHOHCH$_3$	27.4 ± 6.3	1146 ± 330	2.61	8	1.0 ± 0.07
6(*S*)-BH_4	C6	-CHOHCHOHCH$_3$	218 ± 26	492 ± 7	0.14	4	0.8
NPH_4	C6	-CHOHCHOHCH$_2$OH	93.2 ± 12.4	343 ± 53	0.23	4	nd
PH_4	C6	-H	133 ± 26	247 ± 21	0.11	4	0.64 ± 0.07
6-MPH_4	C6	-CH$_3$	35.9 ± 4.7	720 ± 79	1.25	5	nd
compound 1	C6	-CH$_2$OH	67.1 ± 4.8	1305 ± 273	1.21	4	0.92 ± 0.14
compound 2	C6	-CH$_2$OCH$_3$	8.3 ± 5.0	764 ± 106	5.74	6	0.93 ± 0.08
compound 3	C6	-CH$_2$OCH$_2$CH$_3$	15.1 ± 2.4	1050 ± 169	4.34	5	0.98 ± 0.01
compound 4	C6	-CH$_2$OCH$_2$CH$_2$OCH$_3$	81.2 ± 13.4	773 ± 108	0.59	4	0.92 ± 0.10
compound 5	C6	-CH$_2$OCOOCH$_2$CH$_3$	67.4 ± 20.4	754 ± 98	0.70	4	0.96 ± 0.14
compound 6	C6	-CH$_2$OCH$_2$CH(CH$_3$)$_2$	66.3 ± 8.2	502 ± 331	0.47	4	nd
compound 7	C6	-CH$_2$OC$_6$H$_5$	57.2 ± 3.5	543 ± 74	0.59	5	0.72 ± 0.09
compound 8	C6	-CH$_2$OCOOCH$_2$OH	47.8 ± 1.9	486 ± 227	0.63	4	nd
compound 9	C6	-CH$_2$OCOCHNH$_2$CH$_3$	80.8 ± 12.7	1265 ± 316	0.97	4	nd
compound 10	C6	-CH$_2$OCOC$_6$H$_5$CN$_2$CF$_3$	21	406	1.20	2	0.9
compound 11	C6	-CH$_2$OCOC$_6$H$_5$N$_3$	40 ± 3	521 ± 50	0.81	4	0.9
6-AzMPH_4	C6	-CH$_2$N$_3$	7.3	458	3.91	2	0.9
7-BH_4	C7	-CHOHCHOHCH$_3$	64.9 ± 14	62 ± 3	0.06	4	0.3
6,7-$DMPH_4$	C6, C7	6-CH$_3$, 7-CH$_3$	29.9 ± 4.1	283 ± 46	0.58	4	0.98 ± 0.08
3-MPH_4	C3	-CH$_3$	687 ± 50	30	0.0025	1	nd

[a] sp, position of the substituents. Except for BH_4, PH_4, 3-MPH_4, and 6,7-$DMPH_4$, all tetrahydropterins were used as mixtures of 6*R*/*S* isomers. *n*, number of experiments to calculate the kinetic constants (K_m and V_{max}). The values represent the mean with the standard errors calculated from ≥ 3 experiments or the mean from two experiments, as indicated by *n*. nd, not determined.

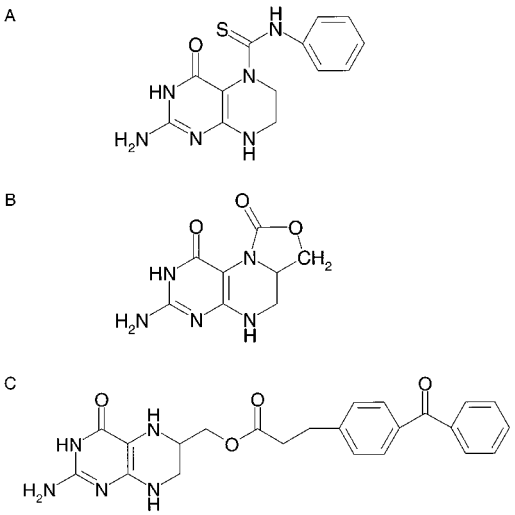

FIGURE 2: Structure of compound 12 (A), compound 13 (B), and compound 14 (C).

decrease in NADH concentration at 340 nm ($\epsilon = 6.22$ M^{-1} cm^{-1}) following regeneration of the tetrahydropterin by DHPR (*21*, *33*). The reaction was allowed to proceed for 10 min, but the coupling efficiency is reported in this work for 1-min assays. The amount of L-DOPA formed (1-min assay) was measured by HPLC with fluorometric detection (*34*). An excess of dehydratase was added to prevent an accumulation of the intermediate 4a-OH-tetrahydropterin, which may cause errors in the estimated coupling efficiency (*35*). The kinetic constants were calculated by nonlinear regression analysis using the software EnzFitter for Windows 2.0.3 from Biosoft (1999).

Molecular Docking. The DOCK 4.0.1 suite of programs (University of California, San Francisco) (*36*) was used to fit the conformers of the bound BH_4 and analogues into the crystal structure of the catalytic domain of TH (residues 164−498), except for residues 178−199 which have not been observed in the electron density map (*22*). The solvent-accessible surface of this domain was calculated by the DMS program under MidasPlus (UCSF) (*37*), and a grid was constructed with a distance of 20 Å around the Fe(III) atom, in which iron was included as a sphere of the correct radius and without including solvent-accessible crystallographic water. Although it is difficult to define the binding parameters and partial charges for metal-coordinating water, the two coordinating water molecules to the Fe(III) in TH seem to have an increased tendency to be removed by ligand binding since they have temperature factors (B-value) of 47 and 59 Å^2 (*38*). The grid is used by DOCK to evaluate the steric boundary of the protein and electrostatic and van der Waals interactions between the protein and the ligand atoms during the docking procedure, using the energy scoring function. The potential atom types and the partial charges of the ligands were assigned for the AMBER force field using InsightII (MSI Inc., CA). A grid space of 0.2 Å, a dielectric factor of 4, and a sampling size of 7000 structures were used. DOCK does not allow for flexibility of the protein during the docking procedure, but optimization of the bound ligand structure was accomplished during docking by allowing flexibility of the ligands. When indicated, docking was performed by the anchor search method (*39*), which is based on the partition of the ligand into rigid segments containing the largest set of adjacent atoms separated by nonrotable bonds, whose position is optimized during each step. The programs InsightII and WebLab Viewer (MSI) were used to prepare the figures of the docked conformers.

RESULTS

Kinetic Parameters for BH_4 Analogues in the hTH1-Catalyzed Reaction. TH is a stereospecific enzyme both for the substrate (L-amino acid) and the natural tetrahydrobiopterin cofactor (6*S*,1′*R*,2′*S*)-6-(1′,2′-dihydroxypropyl)-5,6,7,8-tetrahydropterin (BH_4). For BH_4, the natural 6*R* form has both a higher affinity and specific activity than the 6*S* form (Table 1). While for BH_4 the presence of three chiral centers allows the isolation of the pure 6*R* and 6*S* diastereoisomers by cation exchange chromatography (*40*), the separation of

Table 2: K_i Values and Type of Inhibition for Inhibitory or Weakly binding BH_4 Analogues in the Reaction Catalyzed by hTH1[a]

tetrahydropterin	sp	substituent	K_i (μM)	inhibition
2-MPH_4	C2	-CH_3		no inhibition
4-amino-BH_4	C4	-NH_2	16	competitive vs 6(*R*)-BH_4
5-methyl-BH_4	N5	-CH_3	63	competitive vs 6(*R*)-BH_4
8-M-6,7-$DMPH_4$	N8	-CH_3	617	competitive vs 6(*R*)-BH_4
compound 12	N5	-$CSNHC_6H_5$	2830	competitive vs 6(*R*)-BH_4
compound 13	N5, C6	(Figure 2)	138	competitive vs 6(*R*)-BH_4
compound 14	C6	-$CH_2OCOCH_2CH_2C_6H_5COC_6H_5$	1	competitive vs 6(*R*)-BH_4

[a] sp, position of the substituents. Compounds 13 and 14 were used as mixtures of *R*/*S* isomers. The values represent the mean from two separate experiments.

the 6*R*/*S* enantiomers or diastereoisomers of the other analogues used in this study proved unsuccessful, due to their similar chemical properties, lack of stability, and limited availability. Thus, some of the 6-substituted tetrahydropteridines studied here were in fact mixtures of 6*R* and 6*S* isomers. Moreover, we have estimated the affinity of these compounds based on their K_m values, since we are primarily interested in evaluating their eligibility as BH_4 analogues. We have previously shown that the K_m values for tetrahydropteridine binding to hTH1 and bovine PAH generally seem to be at least 3-fold higher than their corresponding K_d values, as estimated by surface plasmon resonance (*9*) and fluorescence quenching studies (*19*), but the actual values are dependent on the experimental conditions.

Except for compound 14, which does not show any cofactor activity, and the trihydroxypropyl-substituted NPH_4, which is present in mammalian tissue and shows a significantly reduced activity as compared to 6(*R*)-BH_4, most of the other 6-substituted tetrahydropterins showed K_m and V_{max} values between the values for the *S* and *R* forms of BH_4. This finding shows that the catalytic efficiency of the tetrahydropterins is not significantly affected by the length or the chemical nature of the substituent at the 6-position, which for some of the compounds (6−11) is rather bulky (Table 1). However, there seems to be a tendency for longer and more bulky side chains to result in slightly increased K_m and lowered V_{max} values. Moreover, there are some compounds showing a significantly higher affinity for hTH1 than 6(*R*)-BH_4, i.e., compounds 2 and 3 and the photolabile cofactor analogue 6-$AzMH_4$ (*41*) ($K_m = 7.3$ μM), and with these cofactors higher V_{max}/K_m ratios (3.9−5.7 μM^{-1} min^{-1}) than with the natural cofactor ($V_{max}/K_m = 2.6$ μM^{-1} min^{-1}) were obtained.

6,7-$DMPH_4$ was found to have a K_m value comparable to that of 6(*R*)-BH_4, and the K_m value for 7-BH_4 was in the range obtained for most of the 6-substituted tetrahydropterins (Table 1). However, the activity of hTH1 was significantly reduced with these 7-substituted cofactors, especially with 7-BH_4 (Table 1). Moreover, a methyl substitution at N3 dramatically reduced both the specific activity and the affinity of the enzyme, and substituents in positions C2, C4, N5, and N8 on the pteridine ring abolished the activity of hTH1 (Table 2).

The Coupling Efficiency of Tetrahydropterins in the hTH1-Catalyzed Reaction. With the exception of the 6-phenoxymethyl-derivative (compound 7), all the C6-substituted tetrahydropterins were found to give a high coupling efficiency (>90%) in the hTH1-catalyzed reaction (Table 1). It has been previously reported that the disubstituted 6,7-$DMPH_4$, but not the unsubstituted tetrahydropterin (PH_4), gave a partially uncoupled reaction with bovine TH (*20*). However, with hTH1 we have measured a partially uncoupled (0.64) reaction with PH_4, while 6,7-$DMPH_4$ showed a high coupling efficiency (Table 1). Using 3-MPH_4 as the cofactor, no significant NADH oxidation was observed. Since L-DOPA formation is measured with this compound (Table 1), it seems that its quinonoid species is not a substrate for DHPR, which is an enzyme with a reported broad substrate specificity (*21*).

Inhibitory or Weakly Binding BH_4 Analogues. 2-methyl-4-oxo-5,6,7,8-tetrahydropteridine (2-MPH_4), 4-amino-BH_4, 5-methyl-BH_4, 8-methyl-6,7-dimethyl-5,6,7,8-tetrahydropterin (8-M-6,7-$DMPH_4$) and compounds 12−14 (Figure 2), were all found to be inactive as cofactors in the hTH1 catalyzed reaction (Table 2), as well as in the reaction catalyzed by human PAH (data not shown). For 2-MPH_4, no inhibition of hTH1 activity was observed with 6(*R*)-BH_4 as the cofactor, indicating that this compound does not bind to the enzyme active site, while 4-amino-BH_4 and 5-methyl-BH_4 were found to be effective inhibitors of hTH1, competitive versus 6(*R*)-BH_4 (Table 2). Compound 14 is a very effective competitive inhibitor of hTH1 versus 6(*R*)-BH_4, with a K_i value of about 1 μM, while compounds 12 and 13, having bulky substituents in the N5-position on the pteridine ring, were found to be relatively weak inhibitors of hTH1 (Table 2).

Kinetic Parameters for Several 6-Substituted Tetrahydropterins in the Reaction Catalyzed by Δ150-hTH. To date only the crystal structure of a truncated form of rat TH, lacking the regulatory domain (and including the catalytic and tetramerization domains) has been solved (*22*). Thus, to analyze the structure−activity relationships of the tetrahydropterin cofactors, it was important to compare the properties of the full-length and the N-terminal truncated form of the enzyme. When a range of cofactors with substitutions at C6 were studied, we did not find any significant differences for the kinetic constants calculated with the full-length hTH1 (Table 1) and the truncated form Δ150-hTH (Table 3). Moreover, compound 14 was also found to be an inhibitor of the truncated form of TH competitive versus 6(*R*)-BH_4, and the K_i value obtained was similar as for hTH1 (Tables 2 and 3).

Molecular Docking of Tetrahydropterin Cofactors into the Crystal Structure of TH. 6(*R*)-BH_4 and its tetrahydropterin analogues used in this work were docked into the crystal structure (PDB code 1TOH) of the catalytic domain of recombinant rat TH (*22*) using the program DOCK 4.0 (*36*). Previous NMR studies of the conformation of diverse pterin cofactors and cofactor analogues to Co(II)-substituted hTH1 have shown that the pterins bind close to the metal at the

Table 3: Kinetic Constants for 6-Substituted Tetrahydropterins in the Reaction Catalyzed by Δ150-hTH[a]

tetrahydropterin	sp	substituent	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	V_{max}/K_m (μM^{-1} min^{-1})	*n*
6(*R*)-BH_4	C6	-$CHOHCHOHCH_3$	18 ± 2	730 ± 42	2.53	3
6-MPH_4	C6	-CH_3	55	492	0.55	2
compound 7	C6	-$CH_2OC_6H_5$	57 ± 16	310 ± 77	0.56	3
compound 10	C6	-$CH_2OCOC_6H_5N_3$	32	217	0.42	2
compound 11	C6	-$CH_2OCOC_6H_5CN_2CF_3$	59 ± 3	410 ± 54	0.43	3
compound 14	C6	-$CH_2OCOCH_2CH_2C_6H_5COC_6H_5$	K_i = 1.7 μM			2

[a] sp, position of the substituents. Except for BH_4, all tetrahydropterins were used as mixtures of 6*R*/*S* isomers. *n*, number of experiments to determine the kinetic constants (K_m and V_{max}). The values represent the mean with the standard errors calculated from three experiments or the mean from two experiments, as indicated by *n*.

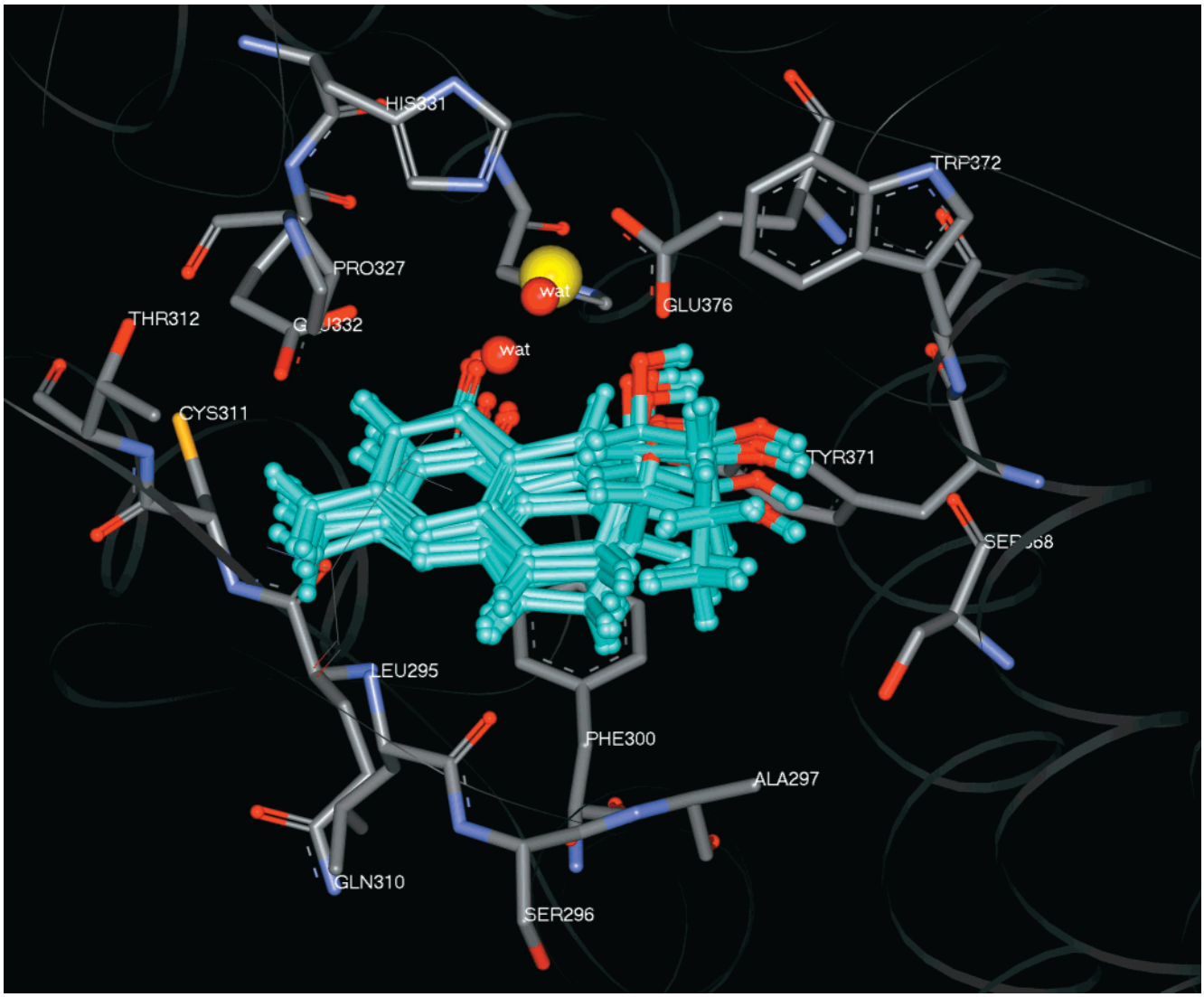

FIGURE 3: Molecular docking of 6(*R*)-BH_4 into the crystal structure of ligand-free rat TH. (A) The active site residues at the cofactor binding site are shown as sticks (nitrogen atoms in blue, oxygen atoms in red, and carbon atoms in black), and the iron is shown in yellow. For clarity, Leu294 is shown as wires. 6(*R*)-BH_4 is shown as light blue sticks (oxygens in red). Docking by DOCK 4.0 was performed in a vacuum, using the solvent-accessible area of the receptor (TH), and the water molecules shown in red are those found coordinating to the iron in the ligand-free crystal structure of rat TH (PDB code 1TOH) which are replaced after docking.

active site (distances around 6 Å for observable protons, i.e., H7 and protons at the C6 substituents) (*11*). Thus, a grid region of 20 Å around the active site was defined for the docking procedure. 6(*R*)-BH_4 (1′*R*,2′*S*) was then docked without restraints for its location in the enzyme and allowing flexibility of the ligand and optimization of the final bound structure. The 30 top-scoring docked conformers of 6(*R*)-BH_4 were found at the same binding site of the enzyme, with an rmsd value for all the atoms of 4 Å. The docked conformers were grouped into three families showing the same orientation at the active site (Figure 3) but providing slightly different atom−iron distances, i.e., the average distance between the 4-oxo and the iron, which is in the ferric form in the crystal structure, was 2.5, 2.4, and 2.3 Å in each of the families, with an average distance between the C4a and the iron of 4.2 ± 0.5 Å for all the conformers. Surprisingly, the resulting docked BH_4 structure is oriented differently from that in the reported crystal structure of TH

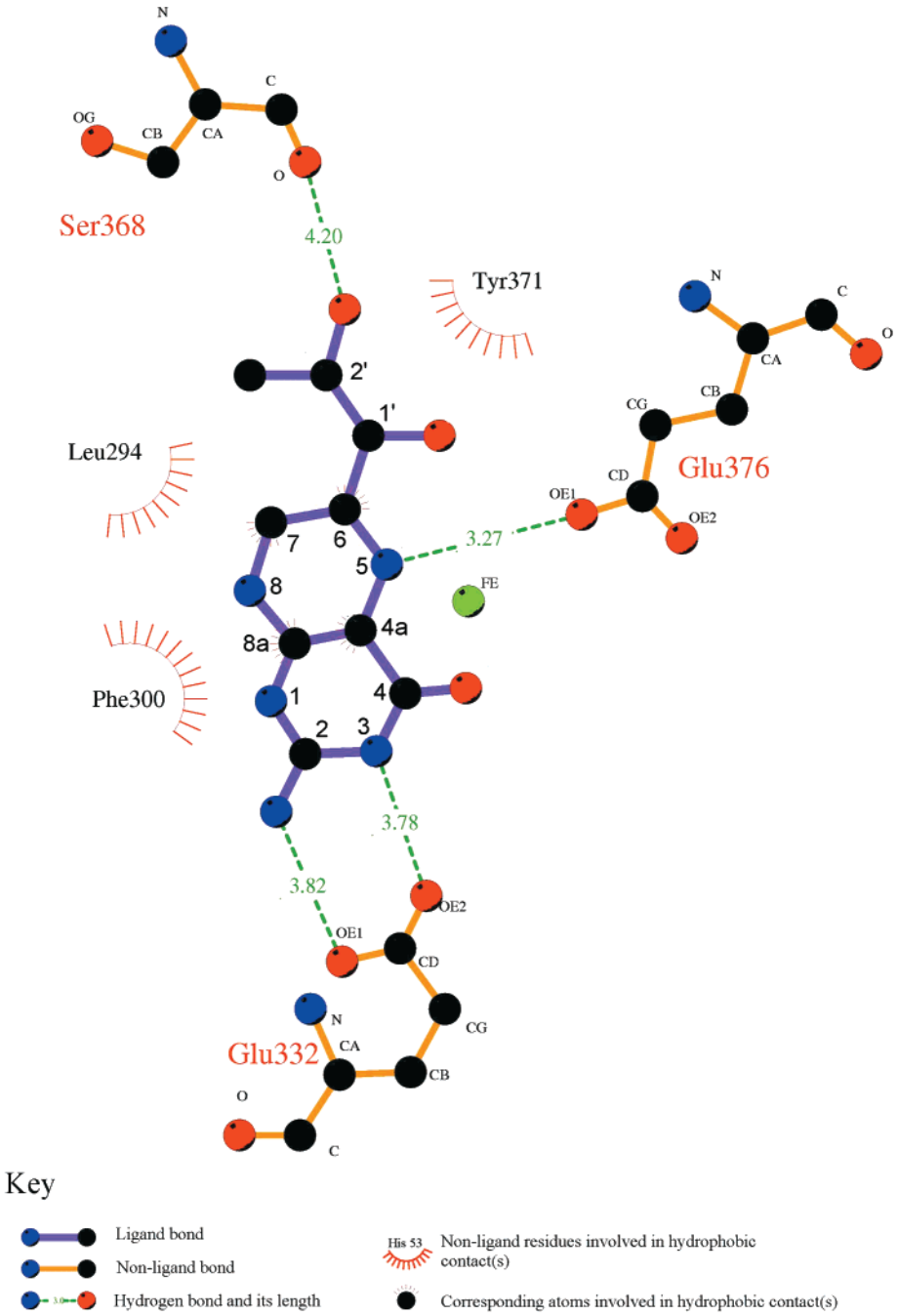

FIGURE 4: Two-dimensional view of the pterin binding site in TH, prepared with LIGPLOT (*63*). The ligand and residues making hydrogen bonds with the ligand are shown in ball-and-stick representation. Ligand or residue bonds are shown in purple and orange, respectively, and the iron atom is shown in green. Nitrogen atoms are blue, oxygen atoms are red, and carbon atoms are black.

with bound BH_2 (*10*) but similarly to the recently reported structure of the complex between PAH and BH_2 based on distances estimated either by NMR spectroscopy (*12*) or by X-ray crystallography (*13*). Thus, in the docked TH structure, the pteridine ring is rotated 180° relative to the published X-ray structure, with rotation axis along the C4a−C8a bond, and interacts differently with residues in the protein. As seen in Figure 3, in the docked structure the pterin ring π-stacks with Phe300, as in the crystal structure (*10*), with an average interatomic distance between the rings of 3.6 Å, the N3 and the amino group at C2 are at hydrogen-bonding distance from Glu332, and there are additional interactions of the ring with Leu294 and Gln310 (Figures 3 and 4). The dihydroxypropyl side chain at C6, which adopts a *cis*-conformation (*11*), binds at a large hydrophobic pocket surrounded by Ala297, Ser368, and Tyr371. The hydroxyl group at C2′ in 6(*R*)-BH_4 is at hydrogen-bonding distance from the carbonyl group of Ser368 (Figure 4). Moreover, when the top 100 scoring structures were evaluated, it was found that in five of them the pterin ring is flipped 180° with respect to the most abundant conformer, with the axis for the rotation perpendicular to the direction of the C4a−C8a bond, along the pteridine ring plane.

The BH_4 analogues with cofactor activity (Table 1) were docked into the crystal structure of the truncated TH by two different procedures, i.e., (i) similar to the docking of 6(*R*)-BH_4 (above) without any restraints and (ii) using the anchor search method (*39*), in which the position of the pteridine

ring was fixed (anchored) to the binding site for the ring in the top-score docked structure of bound 6(*R*)-BH_4, the side chains were grown stepwise and, finally, the position of the whole bound ligand was optimized (see Experimental Procedures). Both procedures gave similar results for most of the tetrahydropterin analogues, except for compound 11 (see below).

The most remarkable differences between the pterin ring position of the docked conformers of the analogues, with respect to that obtained for 6(*R*)-BH_4, was found for 6(*S*)-BH_4, 7-BH_4, and PH_4, while the pterin rings of the tetrahydropterins with various C6-substituents presented the same conformation and distances to the iron as in 6(*R*)-BH_4, when docked as 6*R* stereoisomers (Figure 5). As seen from Figure 5, panel A, in which the high-score docked structures for both diastereoisomers 6(*R*)-BH_4 and 6(*S*)-BH_4 are shown, the dihydroxypropyl substitution at the 6*S* position seems to impede a proper stacking of the pterin ring with Phe300, and the pterin ring of this analogue is displaced upward with respect to that of docked 6(*R*)-BH_4, resulting in a slightly shorter distance (about 0.5 Å) from the C4a atom to the iron. This fact may explain the relatively low affinity of 6(*S*)-BH_4, as well as its lower coupling efficiency. The pterin ring in the docked structures of 7(*R*)-BH_4 showed a proper stacking with Phe300, although it also adopted a slightly different orientation than 6(*R*)-BH_4 (Figure 5, panel B) resulting in a longer distance between the C4a and the metal than for the natural cofactor (i.e., 4.8 versus 4.2 Å), which may be related to the high degree of uncoupling found for this analogue. PH_4 also gives a partially uncoupled hydroxylation reaction (Table 1), and docking of this tetrahydropterin resulted in some structures that were inverted 180° with respect to the orientation of the ring found for the other cofactors, although the 4-oxo still pointed toward the iron (Figure 5, panel C). No significant differences were found after docking in the positioning of the ring for the disubstituted 6,7-$DMPH_4$ when compared to the natural cofactor (data not shown).

The docked conformers of the cofactor analogue 6-$AzMPH_4$, which shows the highest affinity for hTH1 among the tetrahydropterins studied (Table 1), present a similar orientation of the pterin ring toward the iron as in 6(*R*)-BH_4 and proper stacking with Phe300 (Figure 5, panel D). The polar azido group seems to orient upward toward the negative charged Glu376, which coordinates the active site iron (ref *22* and Figure 3). Moreover, after docking by either procedure, compounds 1−10 presented the same orientation of the pterin ring as found for 6(*R*)-BH_4, and the substituents at the 6-position occupy the same binding site as the dihydroxypropyl side chain of the natural cofactor (shown in Figure 5, panel E, for compound 3). However, the very bulky substituent at the 6-position of compound 11 is found to bind at the same hydrophobic pocket as the other 6-substituted compounds when the docking procedure is performed without restrictions, while it orients upward, toward the iron binding site, when the anchor search method is used (Figure 5, panel F).

DISCUSSION

Structure−Activity Relationships for BH_4 Analogues as Cofactors for hTH1. Most of the structure−activity studies on synthetic BH_4 analogues with TH have been performed using either crude enzyme or partially purified enzyme preparations solubilized by limited proteolysis (*5*, *15*, *17*). Although such studies have provided some information on the interaction of cofactor analogues with the hydroxylase, the interpretation of the results is complicated by the heterogeneity of the preparations used. Thus, later studies have demonstrated that the bovine as well as the rat enzyme are isolated in a partially phosphorylated state and contain tightly associated catecholamines that bind by bidentate coordination to the enzyme active site (*7*, *42*, *43*). Furthermore, it has also been shown that limited proteolysis generates a "core-enzyme" that resembles phosphorylated TH in its kinetic properties, i.e., showing a higher K_i for catecholamines and an increased affinity for the tetrahydropterin cofactor, as compared to the catecholamine containing full-length enzyme (*5*, *44*). The four human isoforms, hTH1−4, have all been expressed in *E. coli* and are isolated as nonphosphorylated enzymes with no catecholamines bound. The use of recombinant enzyme thus represents a more well-defined system for structure−activity studies of tetrahydropterin cofactor analogues in the reaction catalyzed by TH. Furthermore, a number of new cofactor analogues has been synthesized and tested in terms of catalytic and coupling efficiency.

The recent elucidation of the crystal structure of an N-terminal truncated form of TH (*22*) and its binary complex with BH_2 (*10*), provides a framework to rationally interpret cofactor structure−activity relationships. Considering the reported crystal structure of the enzyme−pterin complex, some of the findings derived from the present activity studies with the cofactor analogues were, however, surprising. The fact that the 2-MPH_4 analogue does not bind to the enzyme, although the amino group at C2 does not seem to be involved in important interactions with protein residues in the crystal structure, was unexpected. Moreover, from this structure it is also deduced that substituents at the 7-position (even a methyl group) would come in steric conflict with Leu295 (*10*), but the 6,7-$DMPH_4$ binds in fact with high affinity to hTH1. On the other hand, the structures for the bound conformers of the BH_4 analogues that we have obtained by molecular docking into the crystal structure of the ligand-free enzyme, using the energy score function in DOCK, fit with the cofactor properties of the tetrahydropterins studied and are in agreement with the metal−proton distances estimated by NMR for 6-MPH_4, 3-MPH_4, and BH_2 bound to Co(II) reconstituted hTH1 (*11*). Moreover, the interactions encountered between the NH(3) and the amino group at C-2 in the pyrimidine ring of the pterins and Glu332, in addition to the π-stacking interaction with Phe300, are in agreement with similar carboxylate-mediated binding modes in other pyrimidine and pterin binding enzymes (*45*−*47*) and with the results from site-directed mutagenesis of TH (*48*). Also, the specific interaction of the hydroxyl at C2′ and Ser368, a residue that is not conserved among the aromatic amino acid hydroxylases, may be related to the specific regulatory properties elicited by the natural cofactor in TH (*1*, *5*). The encountered binding mode of the tetrahydropterins also explain the higher affinity and higher catalytic efficiency of the enzyme with the 6(*R*)-diastereoisomer of BH_4 since a large substitution at 6(*S*) comes in steric conflict with the normal stacking interaction with Phe300. Thus, our results

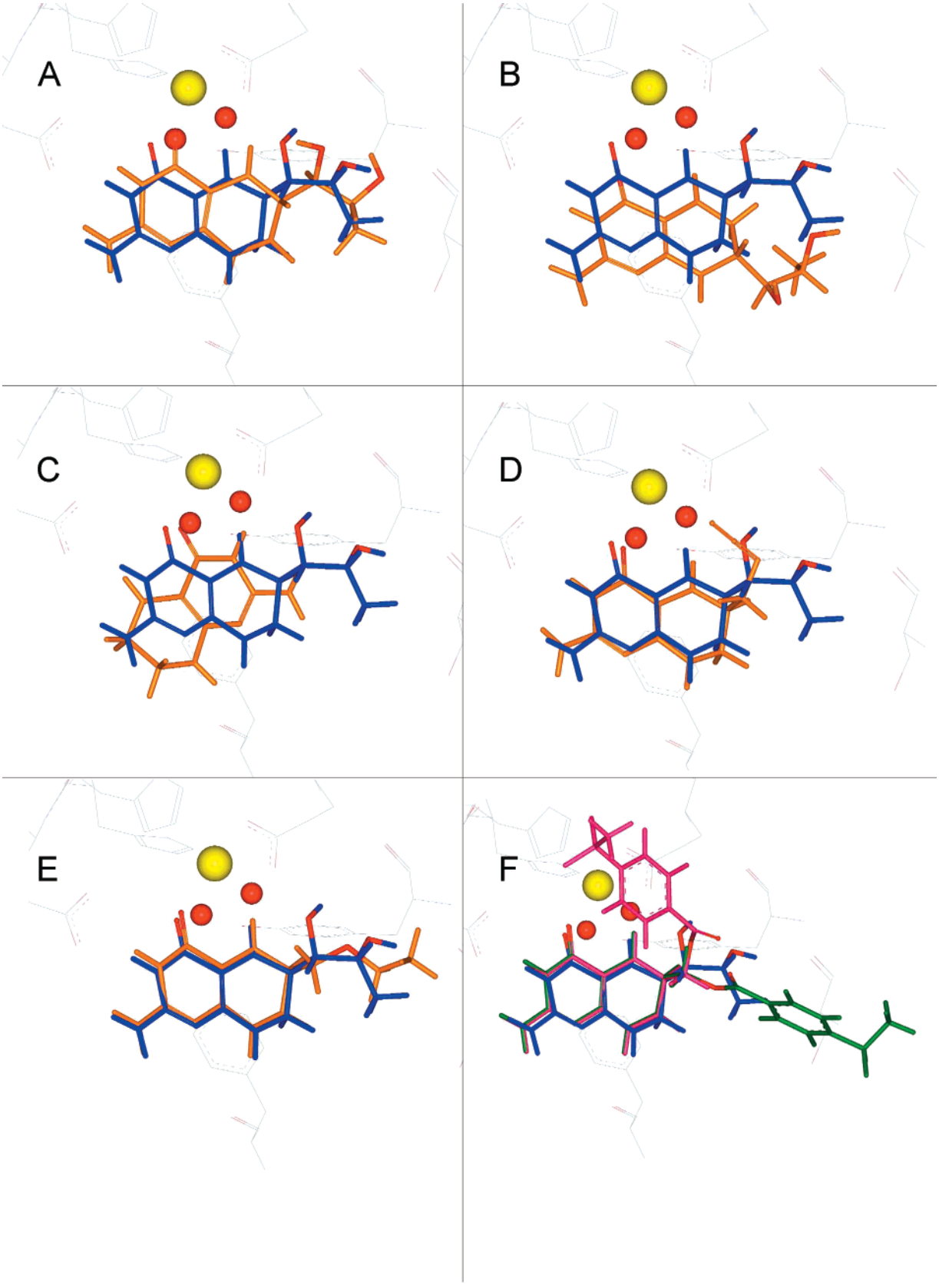

FIGURE 5: High-score docked conformers of 6(*S*)-BH_4 (A), 7(*R*)-BH_4 (B), PH_4 (C), 6-AzMPH_4 (D), compound 3 (E), and compound 11 (F). The top-scoring structures obtained for each tetrahydropterin analogue by DOCK 4.0 are shown, as compared with that obtained for 6(*R*)-BH_4 (light blue sticks, oxygen in red). Water molecules are shown in red (see legend to Figure 3). For compound 11, the structure obtained by docking without restrictions (green sticks) and by the anchor search method (pink sticks) are shown.

are in agreement with a binding of BH_4 to TH in a similar orientation as that of BH_2 bound to human PAH, as recently obtained by NMR spectroscopy and molecular docking (*12*) and by X-ray crystallography (*13*), which is 180° inverted from the orientation of BH_2 in the crystal structure of its complex with TH (*10*), with the rotation axis along the C4a−

C8a bond. The crystal structure of the BH_2−PAH complex has been refined to 2.0-Å resolution (*13*), which is significantly better than the resolution of the BH_2−TH complex (2.3 Å) performed by the same group (*10*). The authors have in fact discussed the possibility that BH_2 might bind to TH in alternate modes in the crystal and in solution (*13*). It is interesting to note that alternate modes of binding have been found for pterins bound to dihydrofolate reductase. Thus, methotrexate binds to this enzyme with its pteridine ring flipped 180° as compared to bound folate (*49*). Moreover, two tautomeric forms of folate bind to dihydrofolate reductase in a very similar way to methotrexate, and the relative populations of the productive and the unproductive forms are pH-dependent (*50*).

The protein structures and the chemical reactions catalyzed by the mammalian pterin-dependent hydroxylases are sufficiently similar to assume that all three enzymes have essentially the same catalytic mechanism (*1*). Although the details are not yet clear, the experimental evidence so far points to a common tri-bi sequential mechanism for both TH and PAH, and is consistent with formation of a 4a-peroxytetrahydropterin as the rate-limiting step (*3*, *4*, *12*). Some differences in the positioning of BH_2 are encountered between the structures of the BH_2−PAH complex resolved by either NMR/molecular docking (*12*) or X-ray crystallography (*13*). Nevertheless, the structural studies on the two enzymes have demonstrated that the cofactor is in an ideal orientation and conformation for the dioxygen to bind in a bridging position between the iron and the pterin at the C4a position (which is located at 4.2−6.1 Å from the metal), forming a putative iron−4a-peroxy-tetrahydropteridine intermediate. This transient intermediate would be either the hydroxylating species itself (*51*), or the precursor of a ferryl oxo intermediate, that would be the immediate hydroxylating species (*52*). The results obtained in this work by molecular docking are compatible with the formation of an iron−4a-peroxy-tetrahydropteridine intermediate.

As found in this work by molecular docking of the BH_4 analogues, TH is able to accommodate a large variety of substituents in the C6(*R*)-position in the large hydrophobic pocket lined by Ala297, Ser368, Tyr371, and Trp372. Among the analogues with substituents at the 6-position studied, only the photolabile and high affinity binding compound 14 carrying a benzophenone group at the C6-position (*18*) is completely inactive as a cofactor for hTH1. Independent of the docking procedure (i.e., without restraints or using the anchor search method) homogeneously "bound" conformations were obtained for all tetrahydropterins, except for compound 11. In general, compounds with relatively small, hydrophobic substituents in the C6-position, i.e., compounds 2 and 3, and also 6-AzMPH_4, seem to bind to the enzyme with high affinity (lowest K_m values). However, tetrahydropterins with large, bulky side chains, like compounds 10 and 11 were also found to function well as cofactors (*18*), in agreement with previous studies (*15*, *16*) showing that the nature of the substituent in the C6-position on the pteridine ring can be varied extensively with little effect on the cofactor activity in the TH catalyzed reaction.

Coupling Efficiency of TH. The coupling efficiency in the TH reaction is a sensitive probe for a correct positioning of the substrate and cofactor at the active site and an important property when considering the potential usefulness of the pterins in substitution therapy. Partial uncoupling of the TH reaction has been observed with several amino acid substrate analogues (*53*) as well as with cofactor analogues (*20*, *21*). Even compounds with very large and bulky side chains at C6, such as compounds 10 and 11, resulted in highly coupled hydroxylation reactions and only compound 7 gave some degree of uncoupling.

The 7-substituted isomer of BH_4 (7-BH_4), found in the urine of patients with a mild form of hyperphenylalaninemia caused by a deficiency of 4α-carbinolamine dehydratase (*54*), was found to cause a pronounced uncoupling of the hTH1 reaction, in accordance with previous reports (*20*, *21*). Thus, uncoupling seems to be a general property of the 7-substituted tetrahydrobiopterin in the TH as well as the PAH-catalyzed reactions (*20*, *21*, *33*). A structural explanation of the uncoupling effect is offered by docking of 7-BH_4 into the crystal structure of TH, which results in conformers with different orientation of the pterin ring and longer C4a-Fe(III) distances than for BH_4. The surrounding solvent molecules may thus become acceptors of the activated oxygen in the 7(*R*)-supported reactions, resulting in production of reactive oxygen species (ROS), by analogy with the uncoupled hydroxylation reaction catalyzed by the cytochrome P-450 monooxygenases (*55*). In the present study, we have also found a partial uncoupling of both the TH- and PAH-catalyzed reaction with PH_4, which is in contrast to a previous report in which PH_4 was found to give a completely coupled reaction with bovine TH (*20*). In the same study it was also reported that the disubstituted 6,7-$DMPH_4$ gave an uncoupled reaction with TH, while we here have found a fully coupled reaction of hTH1 and of PAH with this cofactor. The reason for the discrepancies between these studies is currently not clear, but may be related to the fact that Shiman et al. (*20*) used bovine adrenal TH in their studies, which is isolated with tightly bound catecholamine inhibitors (see above) and it is phosphorylated. Thus, our kinetic and docking experiments with PH_4 support the conclusion that the presence of a substituent in the C6-position on the pteridine ring represents an important determinant for a correct functional positioning of tetrahydropterins at the active site of TH. A similar conclusion has previously been reached for PAH (*56*). Uncoupling of rat TH has also been induced by mutating the active site residue Ser395 (*52*). However, it is not clear whether this mutation is also accompanied by an altered binding of the substrate.

Nonfunctional Tetrahydropteridines. The compound 2-MPH_4 showed neither cofactor activity nor inhibition of hTH1 activity with BH_4 as the cofactor. Recent NMR studies have shown no change in the relaxation rates of 2-MPH_4 protons in the presence of Co(II)-reconstituted hTH1 (unpublished results). These findings indicate that the analogue does not bind to hTH1, as expected, given the important hydrogen-bonding interactions that the amino group at C2 seem to establish with the enzyme (Figure 4). Substitutions with an oxygen (*57*), hydrogen (*57*), or dimethylamine (*58*) at the C2-position have previously been found to completely eliminate PAH activity (*57*, *58*), while a methylamine substituent is tolerated (*58*), further supporting a common orientation of the pterins in TH and PAH.

Substitution of the 4-oxo group by an amino group was found to abolish the cofactor activity of BH_4 with both hTH1 and recombinant human PAH. Although earlier studies had

shown that the 2,4-diamino-6,7-dimethyltetrahydropterin showed almost the same activity as BH_4 with rat PAH (*57*), our results are in agreement with more recent studies showing that rat PAH is inhibited by 4-amino-BH_4 with an IC_{50} value of 967 μM (*59*). This is probably because the preparation of 2,4-diamino-6,7-dimethyltetrahydropterin used in the former study subsequently was found to be highly contaminated with the 2-amino-4-keto compound (J. E. Ayling, personal communication). Moreover, no activity was observed with any of the 5-substituted compounds, i.e., 5-MBH_4, and compounds 12 and 13, which were all found to be relatively weak competitive inhibitors versus BH_4. As previously shown for rat or bovine TH, a methyl substitution at the N8 position of the pterin ring completely abolished the activity (*60*). However, based on our docking results, the binding of analogues with substitutions at N8 should not be impeded when the N atom is preserved since this atom is exposed to the solvent (Figure 3). Thus, 8-M-$DMPH_4$ is a competitive inhibitor with respect to BH_4.

Implications for the Use of Synthetic Tetrahydropterins in Vivo. BH_4 or its structural analogues have been used in the diagnosis, as well as the treatment of a variety of medical disorders, characterized by local or generalized BH_4 deficiency. However, high doses of BH_4 are required to raise the level in the central nervous system, probably due to a rather poor entry of BH_4 across the blood-brain barrier. Synthetic analogues of BH_4 have been tested as potential therapeutic agents, and one aim has been to identify a tetrahydropterin with improved cofactor properties for the hydroxylases, which can penetrate the blood-brain barrier at a higher efficiency than BH_4 (*15*). 6-MPH_4 has to some extent been used in substitution therapy, although with limited success, and there has also been a report on increased levels of circulating liver enzymes after treatment with this analogue, suggesting a potential adverse effect on hepatocytes (*61*). PH_4 has in the present work been shown to cause an uncoupled reaction with hTH1, with the potential generation of toxic side products. Thus, the coupling efficiency may represent an important parameter when evaluating tetrahydropterins as potential drugs in the treatment of BH_4 deficiency, both to determine the efficiency of the hydroxylation reaction and to evaluate a possible toxicity of the candidate tetrahydropterin. Among the tetrahydropterins analyzed in the present study, one analogue, i.e., compound 3, is of particular interest in this context. It has a favorable K_m value and catalytic efficiency, it supports a fully coupled hydroxylation reaction, and it is efficiently regenerated by dihydropteridine reductase (*15*). It has also a higher lipophilicity than BH_4 and may thus represent a good candidate for BH_4-substitution therapy.

A further limitation of pteridine substitution therapy for defects in BH_4 metabolism has also recently been discovered. Thus, Furukawa et al. (*62*) have reported that the levels of TH protein, as well as the levels of total biopterin in dopaminergic neurons, are reduced in patients with DOPA-responsive dystonia as a result of mutation in the GTP cyclohydrolase I gene. The mechanism of this reduction was not resolved, but it was attributed to a reduced stability or expression of TH. This provides an additional explanation for the limited success of previous attempts to correct the monoamine metabolism in patients with BH_4 deficiency and it also implies that further studies are required to understand the regulatory properties of tetrahydropterins in addition to their catalytic potential.

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