COMPUTATIONAL STUDIES ON TETRAHYDROBIOPTERIN AND TETRAHYDRONEOPTERIN

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This thesis is dedicated to my family.
ABSTRACT OF THE THESIS

Computational Studies on Tetrahydrobiopterin and Tetrahydroneopterin

by

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Master of Science in Biology with a Concentration in Molecular Biology
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Tetrahydrobiopterin is an important coenzyme involved with all three aromatic amino acid hydroxylases, glycerol ether monoxygenase, and nitric oxide synthase. Its full name is (6R, 1’R, 2’S)-6-(1’,2’-dihydroxypropyl)-5, 6, 7, 8- tetrahydrobiopterin. Neopterin is a related pterin, formed as the result of a deficiency in one of the tetrahydrobiopterin synthesis enzymes, PTPS. Neopterin serves as a marker of the cellular immune system activation. If cellular enzymes are capable of reducing neopterin, one product would be tetrahydroneopterin which, because of its similar structure, might interfere with the normal functioning of tetrahydrobiopterin. The structures of both the tetrahydrobiopterin and tetrahydroneopterin are shown below:

In order to develop new potentially-useful pharmaceuticals and to simply understand the mechanisms of tetrahydrobiopterin (THB) cellular utilization, it is useful to investigate the conformations of these molecules and their interactions with cellular enzymes by computational means. Our research focused on obtaining 3-dimensional conformations of both tetrahydrobiopterin (THB) and tetrahydroneopterin (THN), using the quantum
mechanical computational program Gaussian 09, to find the lowest energy state conformations of the molecules studied. We began by performing an overall scan of possible configurations of THB and THN molecules, obtaining the lowest energy conformations for each molecule. Next, we ran the DFT feature of the Gaussian 09 program for further study of the conformations showing the lowest energy conformations. Our work is the first to consider effects of water on THB and THN conformations; we used two approaches. In the first, we added one or two water molecules to THB molecules to observe their effects on hydrogen bonding and structure. In the second, we simulated the effects of solvating our THB and THN molecules using the computer program COSMOtherm.

Using the program Discovery Studio 2.5, we tested the ability of THB and THN to fit into the active sites defined by x-ray crystallographic studies of the enzyme structures (docking the THB and THN onto the enzyme). We studied their binding to each of the three aromatic amino acid hydroxylases, The H-bonding patterns were very comparable to the original H-bonding patterns of the THB in the aromatic amino acid hydroxylase structures. Nitric Oxide Synthase (NOS) interacts with THB in a different manner than that with the three aromatic amino acid hydroxylases. In the reaction catalyzed by NOS, THB gets converted into a radical form, either ·BH₃ or ·BH₄⁺. We studied both ·BH₃ and ·BH₄⁺ using Gaussian 09 and obtained their lowest energy forms. We obtained partial charge and spin density distributions that showed the electron of the ·BH₄⁺ radical was much more delocalized than that of ·BH₃, and therefore more stable than ·BH₃. Docking of the neutral THB to NOS yielded much better results than docking either ·BH₃ or ·BH₄⁺ onto the enzyme active site.

We conclude that THB and THN exist in a equilibrium of conformations, varying between the keto and enol tautomers and the axial and equatorial side chain shifts. In both the neutral and the radical form of THB, the keto-axial is the lowest energy form and thus is the predominant conformation. The THB is in its neutral form before binding fully to the active site in the NOS. This is supported from the multiple docking studies showing better overlapping of THB than that of either ·BH₃ or ·BH₄⁺.

In addition to the above studies, we examined aspects of the recycling pathway, specifically the destruction of THB through the non-enzymatic reaction of the quinoid form of 7,8 dihydrobiopterin (qDHB) formed in the normal recycling reaction to give 7,8 dihydrobiopterin (DHB). We further studied the reaction of DHB to give pterin and 2-hydroxypropanal. Theoretical intermediates were proposed for both reactions. ΔGs were calculated for DHB and intermediates in both the solvated and the unsolvated forms. The results indicated that the two destructive reactions are thermodynamically favored.
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CHAPTER 1

INTRODUCTION

Tetrahydrobiopterin (THB), (6R, 1’R, 2’S)-6-(1’,2’-dihydroxypropyl)-5, 6, 7, 8-tetrahydrobiopterin, is a naturally occurring compound that, so far, has been identified in bacteria and mammals (1). It is an important coenzyme for phenylalanine hydroxylase (L-phenylalanine + O₂ to L-tyrosine + H₂O) and tyrosine hydroxylase (L-tyrosine + O₂ to 3,4-dihydroxy-L-phenylalanine + H₂O), both involved in the pathways leading to L-3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine, and for tryptophan hydroxylase (L-tryptophan + O₂ to 5-hydroxy-L-tryptophan + H₂O), which is on the pathway for serotonin and melatonin formation. THB is also a coenzyme for reactions catalyzed by glycerol ether monoxygenase and nitric oxide synthase.

Normally, all the THB synthesis pathways proceed without difficulty, and the products created by the pathways (serotonin, epinephrine) remain at required levels. Occasionally, one of the enzymes in THB synthesis, namely 6-pyruvoyltetrahydropterin synthase (PTPS) (see Figure 1) becomes deficient; hence, tetrahydrobiopterin synthesis is blocked or slowed as a consequence of inflammatory reactions. As a result, the intermediate dihydronopterin triphosphate becomes converted into the side product, neopterin. That pathway is shown in Figure 1.

Reports suggest that neopterin levels in serum are correlated, and can predict patient mortality (2). Increased neopterin concentrations were discovered in patients with viral infections that human monocytes and macrophages produce neopterin when stimulated by interferon-γ. Therefore, measurement of neopterin concentrations in body fluids provides information about activation of T helper cell-derived cellular immune activation. Neopterin concentrations in humans reflect the degree of T helper 1 type immune activation (2).

In order to investigate both tetrahydrobiopterin (THB) and tetrahydroneopterin (THN) further, we utilized Gaussian 09 to run computational simulations, obtaining their optimized three dimensional structures in the lowest energy states, both alone and hydrated by the computer program COSMOtherm.. This work allows us to do further studies involving
computer docking of coenzymes to known conformations of the enzymes’ active sites to obtain information that might predict structures of potential pharmaceuticals. We used both THB and THN optimized structures in experiments, in which they are docked into the active site of the aromatic amino acid hydroxylase enzymes and nitric acid synthase (NOS). We have employed the Accelrys Discovery Studio program to perform the docking experiments.

Furthermore, it is important to consider the mechanism of tetrahydrobiopterin utilization, including its recycling in cells. We have studied conformations and energies of path members and intermediates of the tetrahydrobiopterin system by use of computational methods.

Our research focuses on how the THB acts, in terms of its 3-D conformations in both the bound (with enzyme) and unbound forms in the body.

**REVIEW OF THE LITERATURE**

The discovery of tetrahydrobiopterin (THB) resulted from two independent lines of research in 1955-1956. One research group was investigating an obscure protozoan, *Crithidia Jasiculata*, and found out that it required exceptionally high concentrations of folic acid for
survival (3). They deduced from this that the protozoan actually required another type of pteridine, which they named “biopterin”, which could be formed from folic acid. The second group found a pteridine through structural studies of the eye color pigments in *Drosophila melanogaster* (4). Yet, during this time, all scientists could establish was that biopterin was a pigment of some kind, and they could only hint at any possible functions that this mystery molecule might have.

In 1957 Dr. Seymour Kaufman, a researcher at the NIH, during investigations of the phenylalanine to tyrosine conversion pathway, found that NADPH (TPNH in the older notation), a known coenzyme, and another unknown coenzyme had to be involved in the reaction. Through chemical and enzymatic analysis, Kaufman concluded that the unknown coenzyme was an unconjugated pteridine (5). It took until 1963 before Kaufman’s structural studies on the unknown coenzyme isolated from rat liver, proved it to be tetrahydrobiopterin (6), THB (or BH₄, full name (6R)-2-Amino-6-[(1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1H)-one).

In 1974 Kaufman and Fisher (7) discovered that tetrahydrobiopterin was an essential coenzyme not only for phenylalanine hydroxylase, but also the other aromatic amino acid hydroxylase enzymes, tyrosine and tryptophan hydroxylases, as well. All three are involved in the production of critical neurotransmitters, of either serotonin and dopamine (and epinephrine) (7). Kaufman also played a part in the discovery that THB was a coenzyme in the oxidative cleavage of glyceryl ethers (8). In 1995, THB was found to be an essential coenzyme in the Nitric Oxide Synthase pathway (9).

Presently, it is well established that THB is synthesized from GTP (guanosine triphosphate) in a three-step pathway. The three enzymes are: GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS), and sepiapterin reductase (SR). Another pteridine, tetrahydroneopterin is a side product of the THB synthetic pathway is formed, as already discussed, because of a lack of control of tetrahydrobiopterin synthesis (caused by deficient levels of PTPS). When there is a deficient quantity of PTPS, dihydroneopterin triphosphate becomes converted into dihydroneopterin (THN).

Dihydroneopterin is released by macrophages and is an immunologic marker for the activation of the cell-mediated immune system (10). Measurement of neopterin (the
dihydroneopterin oxidation product) concentrations in body fluids such as serum or urine are elevated in infections, cardiovascular disease, rheumatoid arthritis, and certain malignant tumor diseases. Levels of neopterin well above control values are predictive of patient mortality (2).

Interferon γ is an immunologic cytokine that correlates directly with the production of neopterin. Interferon γ is produced by T-lymphocytes in response to foreign particles / invaders. Then the interferon γ stimulates monocytes and macrophages to start producing neopterin. GTP cyclohydrolase I converts guanosine triphosphate (GTP) into the intermediate dihydroneopterin triphosphate. In normal circumstances, the enzyme that converts dihydronopterin into 6-pyruvoyl tetrahydropterin, 6-pyruvoyltetrahydropterin synthase (PTPS), is abundant and GTP ultimately becomes tetrahydrobiopterin in the synthesis pathway. Yet, when there is a dearth of PTPS enzyme available, the cell coincidentally converts the dihydronopterin triphosphate into “neopterin and 7,8-dihydronopterin, after dephosphorylation and oxidation at the expense of biopterin derivatives” (11:2). Indeed, human monocytes / macrophages only have a small constitutive activity of the biopterin-forming enzyme pyruvoyl-tetrahydropterin synthase (PTPS), so that almost exclusively neopterin and 7,8-dihydronopterin become synthesized and released (11).

**EARLIER COMPUTER STUDIES OF TETRAHYDROBIOPTERIN AND TETRAHYDRODOPHTERIN**

Currently, with the modern understanding of the importance of 3-dimensional structure in the components of cells, it became clear that the 3-dimensional structure of tetrahydrobiopterin and other pterins were needed in order to examine how these pterins interacted with enzymes and other cell components. Earlier efforts to predict 3-D structures of THB and THN involved less powerful computers and molecular dynamics / semi-empirical programs than those available today. Their use led to structures with conflicting results. While using molecular dynamics simulation software, Estelberger, Mlekusch, and Reibnegger (12) stated that although he did find that weak intramolecular hydrogen bonds stabilized one of the conformers of THB, he concluded that both semi-empirical and molecular dynamic methods had severe limitations in accuracy as compared to that of
Density Functional Theory. These program inaccuracies have led to major discrepancies in reported results; for example, different authors perceptions of the existence or nonexistence of hydrogen bonding in THB. Even Estelberger, Mlekusch, and Reibnegger (12), as well as Katoh, Sueoka, and Kurihara (13) and Ziegler et al. (14), was unclear whether or not he truly found any hydrogen bonds in his THB molecules. Katoh, Sueoka, and Kurihara (13) used the molecular dynamics method to determine the full configuration of THB in neutral format. Katoh, Sueoka, and Kurihara stated that “it is difficult to find any certain hydrogen bonds in these forms” (13:30), by which they meant forms A and B of THB that they modeled with molecular dynamics (see Figure 2). Ziegler et al. (14) pointed out that they also failed to find significant hydrogen bonding in the lowest energy conformations of THB that they ran using a molecular dynamics program.

Katoh, Sueoka, and Kurihara (13) used the Molecular-Orbital Method within the MINDO/3 framework and optimized two different conformations (forms A and B) as the lowest energy conformer. As stated in his paper, "the ring structure of tetrahydrobiopterin in half-chair conformation and the R configuration of equatorial substitution of the alkyl side chain at position C6 on the ring were set up in accordance with the results of previous physical analysis of 6R-BH4 structure” (13:28). Both the side chains on both forms A and B were of the equatorial conformation, with a different dihedral of the O1’-C1’ bond around the
C1'-C6' bond. Furthermore, in both forms the hydroxyl groups around the C1'-C2' bonds were in *trans* positions. Katoh, Sueoka, and Kurihara (13) stated that forms A and B, shown in Figure 2, were compared with the theoretical structures of tetrahydropterins involved in the biosynthesis of 6R-BH$_4$. The hydroxy keto intermediates, 6-lactoyl tetrahydropterin and 6-hydroxyacetonyl tetrahydropterin, have been determined by the same framework. Figure 3 (13) shows both forms A and B of THB.

![Form A and Form B](image)


Katoh, Sueoka, and Kurihara (13) also calculated the net charges of both forms A and B. They determined that N1, C2, C4, O4, C4a, and C8a, the atoms of the pyrimidine ring, as well as C1', C2', O1' and O2', the atoms of the side chain, were "predominantly reactive than those of the other atoms of the molecule in both forms" (13:30). Yet, in spite of these findings, they stated that "it is difficult to find any certain hydrogen bonds in these forms" (13:30) by which they meant forms A and B of THB that they modeled with molecular dynamics. On the other hand, "the result of net charge calculations for forms A and B suggest
the possibility of hydrogen bonding between 1'-OH, 2'-OH, or N1' and some atom contained in the immediate environment such as the BH\textsubscript{4}-fitting domain of enzymes” (13:30).

Estelberger, Mlekusch, and Reibnegger (12) researched both tetrahydrobiopterin and tetrahydroneopterin conformational structures. In an earlier study that was done, the authors concluded that significant conformational differences exist between both compounds; the side chain at carbon atom C6, according to their results, was predicted to be in axial orientation in tetrahydrobiopterin but in equatorial orientation in tetrahydroneopterin (14). One of the original papers used both semi-empirical and molecular mechanics calculations to arrive at their conclusion (14). Yet, as Estelberger, Mlekusch, and Reibnegger (12) pointed out, the authors had many holes in their studies. He pointed out “First, as others have indicated, the possibility of intramolecular hydrogen bonds in the molecules under consideration cannot be ruled out with certainty, and secondly, all the studies cited above have investigated the structural features of the molecules in vacuo at zero temperature, i.e. only the energetic ground state, neglecting the possibility of internal rotations and vibrations which are to be expected at realistic temperatures of, say, 310 K and in an aqueous environment” (12:37).

Estelberger, Mlekusch, and Reibnegger (12) attempted to fill in the gaps from the previous study by applying molecular dynamics simulations on both tetrahydrobiopterin and tetrahydroneopterin, running at a time scale of 20ps. They established that the stereochemistry of the N5 atom in both molecules could be either pseudoaxial or pseudoequatorial, as well as the side chain being either axial or equatorial. Estelberger, Mlekusch, and Reibnegger generated 40 lowest energy conformations from their molecular mechanics program and discovered that “the most stable structures showed the E'A orientations; on average, they were more stable than E'E by about 2.5 kcal/mol, and more stable than A'A and A'E orientations by about 3.5 kcal/mol” (12:39). The amount of side chain axial conformations far exceeded the amount of side chain equatorial conformations for both molecules. It is interesting to note that Estelberger, Mlekusch, and Reibnegger (12), in spite of his criticism of the first paper (14), did not study the hydrated molecules.
NMR Determinations of Tetrahydrobiopterin Structure

Out of eight total THB configurational isomers, only one isomer is the active isomer, the 6R-L-erythro form. There have been studies of the 6R-BH$_4$ molecule under acidic conditions using NMR and CD techniques.

Bracher et al. (15) verified by Martinez et al. (16), applied nuclear magnetic resonance spectroscopy (NMR) to the study of the conformation of tetrahydrobiopterin in solution at neutral pH. By use of the nuclear Oberhauser effect (nOe), they found that the cross peaks between the methyl protons and H2’, H1’ and H7 (R form) indicate a defined conformation of THB in solution with the dihydroxypropyl side chain at C6 in an axial position curling towards the pyrazine ring.

Crystallographic Studies of Tetrahydrobiopterin Structure in Aromatic Amino Acid Hydrolases and Nitric Oxide Synthase

The X-ray crystal structures of all of the aromatic hydroxylases have been reported, and have been instrumental in determining the relative locations of the active sites for tetrahydrobiopterin and its interactions with the amino acid residues in the active site. Tetrahydrobiopterin was found bound with phenylalanine hydroxylase, whereas the 7,8 dihydrobiopterin form was found bound in both tryptophan hydroxylase and tyrosine hydroxylase. Most of the studies thus far on THB X-ray crystallography have been performed on the phenylalanine hydroxylase enzyme.

Discovery of Tyrosine Hydroxyase (TyrOH) Crystal Structure

The X-ray crystal structure of tyrosine hydroxylase complexed with tetrahydrobiopterin was discovered by Kenneth E. Goodwill, Christelle Sabatier, and Raymond C. Stevens (17). Tyrosine hydroxylase is an enzyme of the catecholamine biosynthesis pathway that hydroxylates tyrosine using molecular oxygen and tetrahydrobiopterin to produce L-dihydroxyphenylalanine (L-DOPA), and 4α-hydroxybiopterin. Since the tyrosine hydroxylase contains iron, but no heme groups,
Goodwill, Sabatier, and Stevens (17) found that the amino acid residues near the iron in the enzyme are well conserved; Goodwill, Sabatier, and Stevens (17) found that Phe300, Phe309, and Pro327 are completely conserved. Goodwill, Sabatier, and Stevens (17) stated that already known sequences of all three hydroxylases assisted greatly in determining the location of the THB binding site. Those comparisons of the sequences have shown that “TyrOH most likely diverged first from the common ancestral enzyme, with PheOH and TrpOH diverging more recently” (17:13443). Yet, the authors state that the residues in the active site seem more similar to PheOH than to TyrOH. Figures 4 (17) and 5 (17) depict how the THB is positioned in the TyroH active site.

As shown in Figures 4 and 5, the 7,8 dihydrobiopterin is extremely close to the iron (II) [grey sphere in Figure 4]) in the tyrosine hydroxylase. The main residues are Tyr371, Glu376, and Leu 295, with the keto group of DHB H-bonded to both Glu 376 and Tyr 371. The lower nitrogen of the pyrazine ring and the 1’ OH group of the side chain of DHB are H-bonded to Leu294 and Leu295. The two red spheres represent water molecules.

Goodwill, Sabatier, and Stevens (17) claim that during the binding of DHB to the active site, the DHB is seen to displace a water at 3.1 Å from the Fe. Furthermore, they point out that the binding of DHB could possibly displace one of the coordinated waters of PheOH as well (17). The resulting complex has a distance from the iron to the DHB C-4a carbon of 5.6 Å. Goodwill, Sabatier, and Stevens (17) points out that molecular oxygen could most likely interact at the same time with both the ferrous iron and the C-4a position. In discovering the TyrOH X-ray crystal structure, Goodwill, Sabatier, and Stevens stated that the TyOH structure “presents the first report of close interactions between pterin and iron in an enzyme active site” (17:13444).

**Discovery of Tryptophan Hydroxyase (TrpOH) Crystal Structure**

Tryptophan hydroxylase catalyzes the rate-limiting reaction on the pathway leading to serotonin (18). Serotonin is an important neurotransmitter in the CNS, and serves to regulate many processes, including sleep and appetite. Studies have found that lack of serotonin can lead to mood disorders, such as depression. Lately, SSRIs (selective serotonin reuptake
Figure 4. The catalytic site of tyrosine hydroxylase. Figure b is a close-up of figure a. For explanation, see the text. In figure a, the residues are His331, His336, and Glu376, as well as the hydroxylated Phe300. “The secondary structure portions containing the iron coordinating residues are shown in blue, His336 helix is shown in green. The helix which contains Phe300 and the adjacent loop region which participates in pterin binding is also shown in green.” Source: Goodwill, K.E., Sabatier, C., and Stevens, R.C. (1998) Crystal structure of tyrosine hydroxylase with bound cofactor analogue and iron at 2.3 Å resolution: Self-hydroxylation of phe300 and the pterin-binding site. *Biochemistry* 37, 13439-13445.

Figure 5. A better representation of the catalytic active site, taken from pdb (PDB ID: 2TOH) in ligand explorer. Source: Goodwill, K.E., Sabatier, C., and Stevens, R.C. (1998) Crystal structure of tyrosine hydroxylase with bound cofactor analogue and iron at 2.3 Å resolution: Self-hydroxylation of phe300 and the pterin-binding site. *Biochemistry* 37, 13439-13445.
inhibitors) have been used in treatment to upregulate TrOH expression. These studies give rise to the connection between antidepressant effects and TrpOH activity (18). Interestingly, THB has been tested as an antidepressant with inconclusive results, since THB cannot pass through the blood-brain barrier and is unstable.

Lin Wang et al. (18) were the first to obtain the X-ray crystallographic structure of tryptophan hydroxylase and thus the first to elaborate on the catalytic domain of the enzyme, shown in Figure 6 (18). From their results, they determined that the human TrpOH active site consists of an approximately 9 Å deep and 10 Å wide cavity. There is a ~12 Å long and ~7 Å wide channel where tryptophan most likely binds (18).

As predicted in previous studies (19), it was found that human TrpOH contains a catalytic Fe(III) atom about 13 Å from the keto group of bound THB, and intersection of the channel and the opening to the active site. As shown in Figure 6, "the iron III is coordinated to His272, His277 and one carboxyl oxygen atom of Glu3171. Three water molecules have been observed coordinated to the iron III: wat1, wat2, and wat3. Wat1 is axial to His272 and has a distance of 2.2 Å to the iron, Wat2 is axial to His277 and has a distance of 2.3 Å to the iron, and Wat3 is axial to Glu317 and has a distance of 2.2 Å to the iron" (18:12572).

**Discovery of Phenylalanine Hydroxyase (PheOH) Crystal Structure**

A deficiency in human L-phenylalanine hydroxylase activity is linked to the disease phenylketonuria (PKU). In most cases, the lack of phenylalanine hydroxylase activity is a result of lowered tetrahydrobiopterin levels due to mutations of enzymes involved in its synthesis (20).

THB donates electrons in the hydroxylation reaction and inhibits the activation of the enzyme by L-Phe. It was found that dihydrobiopterin (DHB) at higher concentrations than THB also hinders the L-Phe activation of the enzyme. Previous studies have found that the dihydroxypropyl side-chain of both THB and DHB is vital for this inhibitory effect (21).

In 2001 Ole Andreas Andersen, Torgeir Flatmark, and Edward Hough (22) successfully obtained a X-ray crystallographic picture of tetrahydrobiopterin bound to the catalytically active Fe(II) form of human phenylalanine hydroxylase. The final model has
Figure 6. The catalytic active site of human TrpOH with dihydrobiopterin (BH₂) bound with iron. Note that the dihydrobiopterin molecule is colored in purple, with oxygens as red and nitrogens as blue. Also, on the trpOH, the α-helices of the catalytic domain are in red, the β-strands are in blue, and the coiled regions are in yellow. Iron is colored green, along with the hydrogen bonds colored in red. The majority of the active site binding of the BH2 lies on the segment from Gly234 to Pro238. The three hydrogen bonds to that amino acid segment originate from the NH₂ group, and the two nitrogen atoms of the BH₂. Source: Wang, L., Erlandsen, H., Haavik, J., Knappskog, P.M., and Stevens, R.C. (2002) Three-dimensional structure of human tryptophan hydroxylase and its implications for the biosynthesis of the neurotransmitters serotonin and melatonin. Biochemistry 41, 12569-12574.

307 amino acids, 278 water molecules and Fe(II) in the catalytically active site. The overall fold is very similar to the corresponding ligand-free form of the enzyme (22). THB binds in the second coordination sphere of the iron, shown in Figure 7 (22).
Figure 7. Showing the schematic diagram of THB-phenylalanine hydroxylase interactions. The THB molecule is in purple, atoms in black, nitrogen atoms in blue, oxygen atoms in red, water molecules in green, iron in yellow. Source: Andersen, O.A., Flatmark, T., and Hough, E. (2001) High resolution crystal structures of the catalytic domain of human phenylalanine hydroxylase in its catalytically active Fe(II) form and binary complex with tetrahydrobiopterin. J. Mol. Biol. 314, 279-291.

Andersen, Flatmark, and Hough (22) found that the iron coordinating Wat1 (water number 1) is hydrogen bonded to O4 of THB, the non-coordinating Glu330 Oε and Tyr325 Oη in a tetrahedral fashion (for identification, refer to Figure 7). Wat2 (water number 2) forms a hydrogen bond to N5 of THB, while Wat3 forms hydrogen bonds with Glu286 Oε2 and O4 of THB (see Figure 7).

All the atoms of the cofactor including the dihydroxypropyl side chain have distinct electron densities. The pterin pyrazine is not planar as it would be if it had been oxidized; thus THB is present in the active site (22). Superposition of the structure on the human
PheOH-Fe(III) BH$_2$ complex shows that the reduced cofactor is displaced about 0.5 Å in the direction away from Ser251, and that the pterin ring is rotated about 10 Å (along the C4a-C8a bond) with the pyrimidine ring rotated towards Phe254 (22). The dihydroxypropyl side chain is predominantly equatorial.

**Comparisons Between the Three Hydroxylases**

All three of the aromatic amino acid hydroxylases utilize non-heme iron and molecular oxygen to hydroxylate their amino acid substrates using a tetrahydrobiopterin coenzyme. The enzymes from eukaryotic sources share a three domain structure (22). There is an N-terminal regulatory domain of 100-170 residues, which has a pairwise homology of about 25%. This is followed by a 270 residue catalytic domain exhibiting 80% sequence homology and a 65% pairwise sequence identity. The C-terminal 40 residues in the family form a tetramerization domain with a 60% pairwise homology. Deletion experiments with the eukaryotic enzymes have demonstrated that the highly conserved catalytic domain can promote the reaction independently of the other domains (22).

The biggest difference between the catalytic active sites of the three hydroxylases lie with the two loops (residues 263-269 and residues 363-372). "Phe313 and Ile366, can be superimposed with the positions of the corresponding residues in human PheOH (Trp326 and Val379, respectively) and rTyrOH (Trp372 and Asp425, respectively)" (18:12572).

Tyr235 is conserved in all known TrpOHs, whereas in both PheOH and TyrOH, it is a smaller hydrophobic leucine; the leucine side chains in human PheOH and rat TyrOH are located on the opposite side of the pterin as compared to those of Phe241.

Two residues are noticeably different in TrpOH compared to the other two family members. Trp372 is preserved in all TyrOH and PheOH sequences, but is a much smaller tyrosine in TrpOH. Leu294 is also conserved in all TyrOH and PheOH sequences, but is a larger tyrosine residue in TrpOH (17). Those differences in TrpOH may allow that enzyme to hydroxylate tryptophan.

Second, in the region of residues 123-129 of human TrpOH, Tyr125 δ-stacks onto Tyr235, forming an angle of 90° with respect to the aromatic rings (3.8 Å) (17). The corresponding residue is a tyrosine in most TrpOHs and PheOHs (and is a phenylalanine in most TyrOHs), but in the human PheOH structure, this tyrosine is positioned toward the
surface, instead of into the active site. These differences in the amino acid hydroxylase cofactor binding sites are consistent with the distinct cofactor structural preferences displayed by the three enzymes, even though they share a common reaction mechanism.

**Discovery of Nitric Oxide Synthase (NOS) Crystal Structure**

Since Seymour Kaufman was the ‘founding father of tetrahydrobiopterin,’ it would only seem fitting that he was one of the first scientists that linked THB to one of the essential coenzymes for nitric oxide synthase (NOS). In 1992, White and Marletta (23) were actually the first to discover that THB played a role as the coenzyme for NOS.

NOS is an enzyme that catalyzes the NADPH-dependent conversion of L-arginine to L-citrulline and nitric oxide. By 1993, the year of a major review article by Kaufman (24), it had already been discovered that NOS-mediated roles, included an endothelium-derived relaxing factor, a neurotransmitter, platelet aggregation inhibitor, and a generator of a cell-mediated immune response which slowed the growth of some tumor and bacterial cells. In 1993, scientists knew only two distinct NOS forms, the constitutive form and the cytokine-inducible form (24). Currently, NOS is known to have three distinct forms: the inducible, Ca\(^{2+}\)-independent form (iNOS), the neuronal form (nNOS) and the endothelial form (eNOS). NOS is unique in that the enzymes requires five cofactors: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH, THB, and calmodulin (23).

Each of the Nitric Oxide Synthases have a much more complicated mechanism of oxidation than the aromatic amino acid hydroxylases. It involves two sequential, mechanistically distinct, heme-based oxidations in the five-electron oxidation of L-Arg to L-citrulline (L-Cit) and NO. L-Arginine is first hydroxylated to N-hydroxy-L-arginine (NOH-L-Arg) and a proposed oxo-iron porphyrin radical intermediate [P-Fe(IV)=O]. NOH-L-Arg is then converted to L-Citruline and NO by means of one-electron oxidation (25).

Below I have listed all three of the NOS isoforms in more detail and how they were discovered.

**iNOS (INDUCIBLE FORM)**

It took until 1998 before the first X-ray crystallographic structure of iNOS was obtained by Brian R. Crane *et al.* (26). The enzyme proved to be a dimer with a ~30 A deep,
funnel-shaped active-center channel. A distal heme pocket present in the monomeric subunit structure form by refolding and recruiting components of the dimeric interface: α7a, the pterin, the NH2-terminal pterin-binding segment, and the NH2-terminal hook.

The structure supports a role for THB in maintaining the structure of iNOS but not for a directed role for THB in the actual hydroxylation of the L-arginine. This is very different from the aromatic amino acid hydroxylases.

**ENOS (Endothelial Form)**

All three NOS isoforms require THB (H4B) as a coenzyme and only the reduced pterin can sustain catalysis. Despite extensive biochemical studies, the role of pterin function in NOS remains somewhat of a mystery. Under conditions of reduced H4B availability, there is strong evidence for superoxide generation by eNOS leading to potential pathophysiology (27). In 1998, C. S. Raman *et al.* (27) obtained x-ray crystallographic structures of epithelial NOS in both its free form (w/o THB) and bound form at 1.95Å° and 1.9Å°, respectively.

A comparison of the eNOS heme groups of both pterin-free and pterin-bound shows that pterin binding is not required for dimer formation, and even fails to produce conformational changes anywhere on the enzyme including the THB binding site (27). Moreover, pterin-binding does not affect L-arginine binding and the creation of the active site/channel (27).

The overall fold of the eNOS heme domain dimer is similar to that of mouse iNOS (27). A novel feature of the eNOS structure is the presence of a Zn2+ ion tetrahedrally coordinated to pairs of symmetry-related Cys residues. A peptide of a cysteine separated by four amino acids from another cysteine is found in eNOS or iNOS from 20 different animal species (27).

The position of the Zn2+ with respect to the two hemes and two THBs of the dimer is shown in Figure 8 (27). Disruption of the metal center may cause distortion of this region of the polypeptide chain resulting in diminished affinity for THB. These structural findings may help explain why a large body of mutational data shows dramatic loss in protein stability, catalytic activity, and THB binding upon removal of the zinc ligands in NOS.

It is possible to soak THB into pterin-free eNOS heme domain crystals without disturbing the crystal lattice. Both the substrate channel and the active site are unaffected by
Figure 8. (A) Stereo view of the 2Fo-2Fc 1.9 Å\(^\circ\) omit electron density map around the zinc metal center, (B) “The ZnS4 metal center and its relationship to THB. Ser-104 is part of the loop containing the cysteine ligands and H-bonds to the C6 side chain of pterin. The stereospecific recognition of THB by NOS is dictated by the substitution at the C6 position.” Source: Raman, C.S., Li, H., Martasek, P., Kral, V., Masters, B.S., and Poulos, T.L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell* 95, 939-950.
the lack of pterin at the THB-binding site. L-Arginine can also bind at the THB site in pterin-free eNOS with a structure identical to that seen in the THB-bound form (28). There also is biochemical evidence for substrate analog (Nω-nitro- L-arginine) recognition by eNOS even in the absence of THB (27). Figure 9 shows THB interacting with all the residues in the eNOS active site.

**Figure 9.** (A) Cross talk between THB and L-Arg mediated by the heme propionate The guanidinium and amino groups of L-Arginine are held in place by H-bonding with the Glu-363. In these representations, the amino group and the THB hydrogen-bond with a heme while the pteridine ring is sandwiched between Phe-462 in one monomer and Trp-449 in another, respectively, and (B) L-Arginine binds at the THB binding site when the eNOS inhibitor SEITU is bound at the active site. Two water molecules bridge between the inhibitor and heme propionate. The ethyl group of the inhibitor forms nonbonded contacts with Val-338 and Phe-355.

The specific recognition of L-Arginine at the THB site (Figure 9A) in eNOS suggests the ability of this site to stabilize a positively charged pterin or pterin radical (27). In order for NOS to utilize a pterin radical, extensive protonation of the bound THB is necessary. Cycling between the pterin radical and THB may be achieved via electron transfer from the reductase domain while the pterin remains bound to NOS.

**nNOS (Neuronal Form)**

Crystal structural studies of neuronal nitric oxide synthase (nNOS) done before 2001 were restricted to the PDZ domain (28). The first x-ray crystal structure of the entire neuronal
nitric oxide synthase was obtained by Jian Zhang et al. in 2001 (29). Zhang et al. (29) examined the crystal structure of the FAD/ NADPH domain of rat nNOS. It was not until 2005 for the tetrahydrobiopterin binding site was successfully determined in nNOS.

Hans Matter et al. (30) obtained a 2.0 Å x-ray crystal structure of the rat NOS-I oxygenase dimer with bound THB and at 2.5 Å with bound THB and L-arginine substrate. These studies provided a model for the dimeric oxygenase domain of the human NOS-I isoform. Matter et al. (30) applied two strategies to identify and validate selective inhibitors targeting NOS-I using ligand and protein structure-based approaches. First, the structure activity relationship of a focused set of 41 pteridine counterparts were tested on three recombinant human NOS isozymes. “Systematic variations at positions 4, 5, 6, and 7 of these analogues revealed substitutions with up to 58-fold selectivity for NOS-I compared to eNOS and iNOS” (30:4784). Importantly, this selectivity was especially evident with bulky, hydrophobic substituents at 5 or 6 and alkylation of the 4-amino group with hydrophobic groups. Specific 4-amino modifications and some changes at position 6 led to the most selective inhibitors nNOS, of which alkylated 4-amino-tetrahydropteridines were especially potent and selective.

As depicted in Figure 10 (30), the cofactor THB is deeply buried in the cavity and not accessible to bulk solvent, it is oriented proximal and perpendicular to the heme (30). The main protein-ligand interaction as in other pterin-protein complexes, occurs between the planar THB ring and the Trp678 indole, stacked at 3.6 Å distance. In general, the hydrogen bond pattern corresponds to H4B bound to NOS-II or –III. The 5,6,7,8-tetrahydropteridine interacts with heme carboxylate (O4 via solvent, N3 directly); the structurally conserved water is present in related X-ray structures. The C4-carbonyl oxygen is hydrogen-bonded to Arg596 guanidine from the substrate binding helix. Figure 11 shows the THB interacting with different amino acid residues in each of the three NOS isoforms.

THB as a Radical Bound in NOS

Figure 12 shows that each NOS monomer contains a non-covalently bound THB near the dimer interface and in close proximity to the heme. This configuration seems to make
Figure 10. Stereoview of $F_{obsd} - F_{calc}$ difference electron density map contoured at 3σ showing the THB binding site in the oxygenase domain of rat nNOS. Hydrogen bonds are indicated by dotted lines and water molecules by blue spheres; atoms are colored by elements: carbon, gray; oxygen, red; nitrogen, blue. Source: Matter, H., Kumar, H.S., Fedorov, R., Frey, A., Kotsonis, P., Hartmann, E., Fröhlich, L.G., Reif, A., Pfleiderer, W., Scheurer, P., Ghosh, D.K., Schlichting, I., and Schmidt, H.H. (2005) Structural analysis of isoform-specific inhibitors targeting the tetrahydrobiopterin binding site of human nitric oxide synthases. *J. Med. Chem.* 48, 4783-4792.
Figure 11. Schematic of the interaction of THB in the rat NOS-I binding site and the amino acid differences among all three NOS isoforms. The rat NOS-I residue numbering is given in black with NOS-III numbering (grey) in comparison.
Figure 12. Tetrahydrobiopterin in its binding pocket in NOS, with substrate arginine bound (PDB 1nod) and two structural waters W1 and W2.

possible a one electron transfer from THB to the heme, enabling oxygen binding to the iron of the heme, the first step to the L-arginine oxidation. Currently it is not known how the THB radical becomes reduced back to THB (31).

As illustrated in Figure 12, the THB is anchored in NOS by hydrogen bonds from surrounding amino acids at the active site. The protons at N2 and N8 are H-bonded to backbone carbonyl oxygens of Trp457 and Ile456. The cofactor is sandwiched by the aromatic indole ring of Trp457 on one side and by Phe470 from the other. “Trp457 not only helps bind the cofactor through a π-stacking interaction but also affects the reduction of the ferric site13 by regulating the rate of electron transfer from the cofactor to the heme active site” (31:11813).

The N3-H of the THB is hydrogen-bonded to a carboxylate oxygen of one of the heme groups. The side chain of Arg375 approaches the pterin from the Phe470 side. There are two structural water molecules coordinating to O4 and N5-H of the pterin, completing an extended hydrogen-bonding network from the cofactor to the active site.
THB bound in NOS undergoes a one electron chemistry which appears to be unique. Stoll et al. (31) suggest that the NOS controls the protonation state of THB and through this regulates proton and electron transfers at the heme center and at the coenzyme active site. Stoll et al. (31) have successfully deduced the protonation state of the tetrahydrobiopterin radical in NOS, from electron paramagnetic resonance spectroscopy combined with DFT calculations. Their experimental magnetic parameters and their comparison to quantum-chemical predictions show that the radical is a cation, \( \cdot \text{BH}_4^+ \) protonated at N3 and N5. The proton on N5 was directly observed in the 1H ENDOR spectrum. In contrast, the chemically relevant proton at N3 cannot be resolved in the EPR and ENDOR spectra, as its hyperfine coupling is small and one of many similar sizes. However, the N3 protonation state was determined by its effect on the spin density distribution in the radical, and hyperfine couplings of ring nitrogens and protons.
CHAPTER 2

METHODS

Two related families of compounds, are studied in this research. The heavy atoms of the first set of compounds, the biopterin group, consist of 9 carbons, 5 nitrogens and 4 oxygens. The second family, by-products of biopterin family biosynthesis but of undetermined physiological effect is the neopterin group, consists of 9 carbons, 5 nitrogens and 5 oxygens. Each compound relative to this investigation was studied by computational chemistry, specifically using density functional theory found on Gaussian 03 (32) and later Gaussian 09 (33), installed on PC computers equipped with Pentium 4, and later with Lenovo desktops equipped with Intel core i5 processors.

The main focus of this work was on the compounds 5,6,7,8-tetrahydrobiopterin (THB) and 5,6,7,8-tetrahydroneopterin (THN). Appropriate structures were entered into the scanning program of the Gaussian program in order to determine the energies and other thermodynamic data of the different conformations. Selected conformations of lower energy were then studied by means of the Density Functional Theory program of Gaussian 09. These data represent the conformations in vacuo. The effects of hydration on selected conformations of THB and THN were studied in the hydrated state by two different methods. The first method was by use of the COSMOtherm program (34). The second method was to use Density Functional Theory settings with either THB or THN and one or two water molecules, respectively.

Four different conformations of THB and THN having the lowest energies were chosen as representatives, for studies of binding to enzymes for which THB was a coenzyme. Appropriate enzyme crystal structures were obtained from the protein data bank. Coenzymes associated with the crystal structures were removed and our computer modeled THB and THN, or other related compounds of the two families, were docked into the protein structure by means of Discovery Studio 2.5 of Accelrys (35). We also selected a low energy group for docking experiments, for both THB and THN. Then we docked the four lowest energy conformations (keto-axial, keto-equatorial, enol-axial, enol-equatorial) to phenylalanine.
hydroxylase and NOS. Figure 13 (36) shows the flow chart of COSMO-RS, the theory upon which the COSMOtherm program is based on.

**THE COSMOTHERM PROGRAM**

“To directly calculate a molecule in solution is very complicated due to the large number of solvent molecules required for a realistic representation” (37:1). Several different solvent models have been developed to study the interactions between solute and solvent. The most popular solvent models have been Continuum Solvation Models (CSM) and the Self-Consistent Reaction Field Method (SCRF), which is usually combined with CSM. CSMs describe a molecule in solution through a quantum chemical calculation of the solute.

molecule with an approximate representation of the surrounding solvent as a continuum (37). CSMs generally extend basic quantum mechanics methods to describe solutes dissolved in aqueous solution.

SCRF simulates the solvent as a polarizable range with a given dielectric constant, \( \varepsilon \). The solute is located in a cavity inside the continuum medium. The overall solvent free energy is \( \Delta G({\text{solvation}}) = \Delta G({\text{cavity}}) + \Delta G({\text{dispersion}}) + \Delta G({\text{polarization}}) \), where \( \Delta G({\text{cavity}}) \) = cavity energy, \( \Delta G({\text{dispersion}}) \) = dispersion energy, and \( \Delta G({\text{polarization}}) \) = electrostatic energy (38).

The COSMO-RS theory exceeds simple CSMs in that it integrates concepts from quantum chemistry, dielectric continuum models, electrostatic surface interactions and statistical thermodynamics. It is a fast and accurate method for extending the COSMO method to predict solute-solvent interaction energies. COSMOtherm is the software that uses the COSMO-RS method in its computation (34). We used this software to simulate the solvation of \( \text{H}_2\text{O} \) around both our THB and THN molecules. Figure 14 (36) shows an illustration of a molecular cavity in COSMO-RS.

**Density Functional Theory (DFT)**

Density Functional Theory (DFT) is a quantum mechanical modeling method used to describe the ground state properties of inorganic metals, as well as organic molecules (39). In recent years, one of the most widely used techniques in computational chemistry has been density functional theory (DFT). In comparison to Hartree-Fock, DFT is not much more demanding in computational effort and time, with much more accurate results.

DFT focuses on the electron density $\rho$, rather than the wavefunction $\psi$. The term ‘functional’ in the term DFT is derived from a mathematical function, since the “energy of the molecule is a function of the electron density, written $E[\rho]$, and the electron density is itself a function of position, $\rho (r)$” (40:395). Thus, the position is a function of a function of the energy; functionals are a function of a function.

From the equation $\rho(r) = \sum |\Psi_m (r)|^2$, the occupied orbitals are used to construct the electron density and are calculated from the Kohn - Sham equations. The Kohn-Sham equations are like the Hartree-Fock equations except for a term $V_{xc}$, called the exchange-correlation potential (40).

The Kohn-Sham equations are solved for the electron density first. Next, the Kohn-Sham equations are solved to obtain an initial set of orbitals. This is a set of orbitals is used to obtain a better approximation to the electron density and the process is repeated until the density and the exchange-correlation energy are constant to within some tolerance. It is important to note that the results of molecular orbital calculations are only approximate, with deviations from experimental values increasing with the size of the molecule. Therefore, one goal of computational chemistry is to gain insight into trends in properties of molecules, without necessarily striving for ultimate accuracy.

The DFT method we used, B3LYP, is based partly on the Hartree-Fock method.

The Hartree Fock equation is $F_1 \Psi_m (1) = \varepsilon_m \Psi_m (1)$. For each molecular orbital $\Psi_m$, The Fock operator $F_1$ has terms that express mathematically:

1. The kinetic energy of the electron in $\Psi_m$.
2. The potential energy of interaction between the electron in $\Psi_m$ and the nuclei in the molecule.
3. Repulsive interactions between the electron in $\Psi_m$ and other electrons in the molecule.
4. The effects of spin correlation between electrons in the molecule. (40:395)
Basis Sets

Basis sets are mainly used to describe molecular orbitals in quantum mechanics calculations. Generally, minimal basis set calculations are not very reliable because they are so small, but very large basis sets are only feasible for small molecules. The basis-set size must be limited for medium sized molecules, which provides one source of error in the calculations. Orbitals called Slater orbitals are often used as basis sets; yet, if Slater orbitals are used in polyatomic molecules, the calculations can become very time-consuming on a computer.

Presently, Gaussian functions are used instead of Slater orbitals as the main basis functions. The difference between a Gaussian function and a Slater function lie in its equation: Gaussian functions contain the factor $e^{-\zeta r^2}$ instead of the $e^{-\zeta r}$ factor in Slater functions. Overall, the computer calculations for Gaussian functions are much quicker than that for Slater functions. The term STO stand for Slater Type Orbital, and they are actually Gaussian orbitals. Some Gaussian basis sets of increasing size include "STO-3G, 3-21G, 3-21G*, 6-31G*, and 6-31G**, where the numbers and symbols are related to the number of basis functions on each atom" (41:714). STO-3G is essentially an obsolete basis set (41).

The next level of sophistication is the double-zeta basis sets. Here, the set of functions is doubled; thus, there are two functions for each orbital. The basis sets that we chose for our experiments were cc-pVDZ and cc-pVTZ, DZ for double zeta and TZ for triple zeta. For the 1st and 2nd row atoms, the cc-pVDZ (correlation consistent-polarized valence double zeta) basis set adds 1s, 1p, and 1d function. The cc-pVTZ set adds another s, p, d, and an f function, etc. Correlation Consistent Basis Sets were first discovered by Dunning in 1989 (42).

In the last 15 years, the hybrid functional B3LYP has been the most popular functional that was used. The B in B3LYP is a term devised from Becke, and LYP indicates a term devised from Lee, Yang, and Parr (43). LYP indicates three parameters that together optimize the performance (43).

In the late 1980s, Becke discovered the gradient-corrected functional; use of the gradient-corrected functional gives the generalized gradient approximation (GGA) (41). In 1993, Becke proposed a further improvement in $E_{\text{xc}}^\text{GGA}$, that included a contribution from $E_{\text{xc}}^\text{HF}$. That combination gave rise to hybrid functions, which give the best performance (41).
**GAUSSIAN PROGRAM**

Gaussian 03 (32) and Gaussian 09 (33), the latter an improved version of the former, is a program which is able to calculate structures and thermodynamic data for molecules of interest using quantum mechanical theory. Classical physics, including mechanics, optics, thermodynamics, electricity and magnetism, are not able to account for very small objects or objects moving at very high velocities. Quantum mechanics is based on the discovery by de Broglie that all particles move as waves (41). The de Broglie discovery as used by Schrodinger to develop a wave mechanical model of the atom which is the basis of the quantum mechanical calculations used in Gaussian 03, Gaussian 09 and similar programs.

The Gaussian program can run both single point calculations and whole geometry optimizations. A single point calculation is performed only at a single fixed molecular geometry. In a geometry-optimization, however, the program will try to locate an overall minimum in the electronic energy. A geometry optimization calculation is composed of many single point calculations, with each single point energy calculation followed by an energy-gradient calculation. The geometry-optimization continues until the minimum energy has been found from an energy surface (41). "In a vibrational – frequency calculation, the program calculates the molecular vibration frequencies; a vibrational frequency calculation must be preceded by a geometry optimization, since vibrational frequencies calculated for a geometry that is not at an energy minimum are meaningless” (41:725). A transition-state optimization aims to discover both the geometry and electronic energies of the transition state in a chemical reaction.

The geometry optimization process locates the energy minimum that lies closest to the starting geometry. The global minimum has the lowest energy of all the conformers, whereas the local minimum is the minimum energy for all the geometries in the same region.

In our conformation calculations on Gaussian, we used mainly the basis set cc-pVDZ, and some of the cc-pVTZ. We ran DFT with the hybrid functional B3LYP. All our calculations were performed without any solvent present.

**BACKGROUND ON GAUSSIAN**

We used the computational chemistry software Gaussian mainly to obtain the lowest structure optimizations, frequencies, and single point energies of our THB and THN
molecules. In obtaining the lowest energy conformations and frequency calculations, Gaussian employs the quantum mechanics method to carry out the complex calculations. Quantum Mechanics (QM) integrates the disciplines in Chemistry, Physics, and Math to extend beyond the scope of Classical Physics. Instead of focusing on the tangible elements such as mass, velocity, force, etc…, QM is more theory based and emphasizes more on particles and waves. Newtonian mechanics, thermodynamics, and Maxwell's theory of electromagnetism are all examples of classical physics. Many theories in classical physics break down when applied to extremely small objects such as atoms or to objects moving near the speed of light. QM’s mathematical foundation is based on the Schrodinger equation (41).

“QM is the exact mathematical representation that describes the behavior of a particle. In principle, QM can predict any property of an individual atom or molecule exactly” (38:6). Density Functional Theory is a quantum mechanical modeling method used in physics and chemistry to investigate the electronic structure (principally the ground state) of many-body systems, in particular atoms, molecules, and the condensed phases. Using DFT, the properties of a multiple electron system can be determined by using functionals (functions of another function) which in this case is the spatially dependent electron density. Hence the name density functional theory comes from the use of functionals of the electron density. DFT is among the most popular and versatile methods available in condensed-matter physics, computational physics, and computational chemistry.

B3LYP is one of the energy functionals of the density functional methods. It is actually the most popular hybrid functional that is used by computational chemists. Besides choosing B3LYP as the energy functional for our DFT calculations, Gaussian also requires a basis set in order to run the QM calculations. A basis set can be defined as a set of functions used to create molecular orbitals, whether the orbitals are centered around atoms, bonds or lone pairs. There are generally two categories for basis sets, minimal and extended basis sets. A typical representative of the minimal basis set would be STO-3G. Yet, according to Wolfram Koch and Max C. Holthausen (44), “One should expect no more than only qualitative results from minimal sets and nowadays they are hardly used anymore” (44:100). The next level of sophistication are the double-zeta basis sets. Here, the set of functions is doubled; thus, there are two functions for each orbital. The basis sets that we chose for our experiments were cc-pVDZ and cc-pVTZ, DZ for double zeta and TZ for triple zeta.
For the 1st and 2nd row atoms, the cc-pVDZ (correlation consistent-polarized *valence* double zeta) basis set adds 1s, 1p, and 1d function. The cc-pVTZ set adds another s, p, d, and an f function, etc.
CHAPTER 3

RESULTS

In this section, we report research results obtained by using the program Gaussian 09 (33) for the study of the three dimensional conformations of tetrahydrobiopterin (THB) and related tetrahydronopterin. The most likely conformations (with lowest energy) are tested for ability to bind to the THB-dependent enzymes phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitric oxide synthase. We began by surveying all conformers of THB and THN identified by the scanning feature of Gaussian 09. The basic structures identified - keto/axial, keto/equatorial, enol/axial and enol/equatorial - are depicted in Figure 15.

Figure 15. The four basic conformations of tetrahydrobiopterin: (A) keto/axial; (B) keto/equatorial; (C) enol/axial; (D) enol/equatorial. In A and C, the solid triangle denotes a position above the plane of the ring system; in all four conformers, the hydrogens of carbon-6 are shown below the plane of the ring system by dashed triangles, meaning that the chirality of carbon-6 is R.
The dihydroxypropyl side chain shifting is shown to be very apparent in our total energy scans. In the example I give, in Figure 16, there are peaks of different sizes and shapes. I have specifically labeled three particular energy points as examples, namely A, B, C as seen in the graph. If one carefully discerns the sample pictures, A, B, C, one can see that the side chain position and conformation differs greatly. In A, both hydroxyl groups point to the right, and the methyl group points away from the viewer. In B, one hydroxyl group points away from the viewer, while the other hydroxyl group points to the right. In C, both hydroxyl groups point in upwards, with one Hydrogen pointing away and the other Hydrogen pointing towards the viewer. Structure C is has the highest energy structure most likely due to the steric repulsion of the close hydroxyl groups.

The conformations shown in Table 1 demonstrate that the major cause of the differences in energy reflect the positioning of the atoms of the 3-carbon side chain. The presence of amine groups in THB and THN requires consideration of ionized forms of these pterins through binding of hydrogen ions (H+). Both Mono- and di-positively charged forms of THB and THN are possible. Accordingly, we have determined the energies of each charged form; these are also shown in Table 1.

As shown in Table 1, we found that after solvation, the ΔGs of the aqueous as compared to the ΔGs of the gas phase shifted by 3 kcal/mol or less. The relative energies shifted higher for tetrahydrobiopterin from its gas phase to the aqueous phase, whereas the ΔGs shifted lower for tetrahydroneopterin in similar fashion. Following the solvent correction, the keto-equatorial conformation was found to be the most stable and lowest energy, having relative energies of 0 kcal/mol. The keto-enol tautomerization in both THB and THN favors the keto form. Most if not all these compounds were run on Gaussian in their ionized forms, whether it was with one or two extra hydrogens attached. Since THB and THN are naturally found in living organisms in aqueous solution, ionization of both of these molecules should be evident.

Furthermore, we experimented with diols and solvated them as well, both THB and THN forms. These diols would be formed if water reacted with the carbonyl carbon of THB and THN. After combining the THB and THN free energies with the calculated free energy
Figure 16. A scan showing different conformations for tetrahydrobiopterin in a keto-equatorial conformation. Note that the lowest energy point is around -851.493 Hartrees and the highest is around -851.473 Hartrees. Scans for the keto-axial conformations for THB and keto-axial and keto-equatorial conformations for THN were also run (see Figures 30 and 31 in the Appendix).
Table 1. Thermodynamic Properties for Unhydrated and Hydrated Forms of Tetrahydrobiopterin and Tetrahydroneopterin in Various Ionization States

<table>
<thead>
<tr>
<th>Name and Configuration</th>
<th>A</th>
<th>b</th>
<th>c</th>
<th>R</th>
<th>ΔE (kcal/mol)</th>
<th>ΔG (gas) (kcal/mol)</th>
<th>ΔG (aqueous) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetrahydrobiopterin</strong></td>
<td>NH2</td>
<td>NH</td>
<td>NH</td>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Keto-equatorial</td>
<td>NH3</td>
<td>NH2</td>
<td>NH</td>
<td>H</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Keto-axial</td>
<td>NH3</td>
<td>NH2</td>
<td>NH</td>
<td>H</td>
<td>4.5</td>
<td>3.5</td>
<td>5</td>
</tr>
<tr>
<td>Enol-equatorial</td>
<td>NH3</td>
<td>NH2</td>
<td>NH</td>
<td>H</td>
<td>0.5</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td>Enol-axial</td>
<td>NH3</td>
<td>NH2</td>
<td>NH</td>
<td>H</td>
<td>8.3</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td><strong>Tetrahydroneopterin</strong></td>
<td>NH2</td>
<td>NH</td>
<td>NH</td>
<td>OH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Keto-equatorial</td>
<td>NH3</td>
<td>NH</td>
<td>NH2</td>
<td>OH</td>
<td>0.5</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Keto-axial</td>
<td>NH3</td>
<td>NH</td>
<td>NH2</td>
<td>OH</td>
<td>8.3</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>Enol-equatorial</td>
<td>NH3</td>
<td>NH</td>
<td>NH2</td>
<td>OH</td>
<td>1.9</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Enol-axial</td>
<td>NH3</td>
<td>NH</td>
<td>NH2</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of water, the hydration of THB or THN to form the diol is found to be highly unfavorable, with ΔG’s of 31 and 33 kcal/mol, respectively.

After eventually discovering that ionized forms of THB did not dock as well in active sites obtained from x-ray crystallographic studies of THB-dependent enzymes, the lowest energy forms of the non-ionized form of THB (which did bind well in docking studies) were chosen for more detailed examination.

We chose four lower energy configurations of tetrahydrobiopterin (THB) and tetrahydroneopterin (THN) for detailed studies of their conformations and thermodynamic
properties. Figure 17 shows four structures for THB and four additional structures for THN. As seen, THB and THN may exist in either the keto or enol form with the hydroxylated side chain either axial or equatorial to the tetrahydropyrazine ring; all possible combinations are shown. All of the tetrahydrobiopterin and tetrahydroneopterin conformations are in their neutral, unsolvated forms. In the Gaussian program, calculated energy values are given in units of Hartrees, which is why I have listed the energies in this unit. However, examining Hartrees by itself is useless and irrelevant to the scientist. In order to find the lowest energy, one must denote the lowest Hartree energy as zero, so it is relative to all the other energy values to be significant.

Note that the below conformations of THB and THN have very small deviations in their respective energies expressed in Hartrees. THB structures have energies around -851.490 Hartrees and THN have energies around -926.705 Hartrees. A Hartree (or the Hartree energy) is the atomic unit of energy equal to 4.3597439422x10^{-18} J. (45). Taking the lowest energy value (-851.493 Hartrees) for THB shows that the keto axial and the enol equatorial conformations are the most stable forms. For THN, the same analysis shows that the keto-axial is the most stable form, followed by the enol-equatorial conformation. This information is important because molecular shape will determine the ability for these tetrahydropterins to bind to enzyme active sites.

Since these unbound pterins in the cell are found in an aqueous environment, it would not be representative of the real organismal atmosphere if we only ran our THB and THN molecules in a vacuum. We approached the solvation problem in two distinct ways: first, we added one or two water molecules directly around our THB and THN molecules and ran the optimizations on the Gaussian program; second, we used a COSMOtherm program to approximate solvation of our THB and THN molecules as a uniform water shell. The program applied to THB and one or two water molecules yielded the results shown in Figure 18.

As for the hydration studies with COSMOtherm, we estimated the energies of hydrated keto-enol tautomers of THB and THN with side chains equatorial or axial to the pyrazine ring and with variable positions of their alcohol groups. From these results, we chose the lowest energy conformers for further study; the results are shown in Table 1. We
Figure 17. Tetrahydrobiopterin and Tetrahydroneopterin in all four conformations and at the lowest energy states.
Tetrahydrobiopterin (R, R, S)

Keto-axial

(a) Tetrahydrobiopterin as the axial form of the keto tautomer

Energy value: -851.493 Hartrees or 
-2.235595 x 10^6 kJ/mol

Keto-equatorial

(b) Tetrahydrobiopterin as the form equatorial form of the keto

Energy value: -851.486 Hartrees or 
-2.23558 x 10^6 kJ/mol

(c) Tetrahydrobiopterin as the axial form of the enol tautomer

Energy value: -851.4839 Hartrees 
-2.235571 x 10^6 kJ/mol

(d) Tetrahydrobiopterin as the equatorial form of the enol tautomer

Energy value: -851.493 Hartrees or 
-2.235595 x 10^6 kJ/mol
Tetrahydroneopterin (S,R,S)

Keto-axial

![Keto-axial structure]

Energy value: -926.7103 Hartrees
-2.433078 x 10^6 kJ/mol

(f) Tetrahydroneopterin as the equatorial form of the keto tautomer

Energy Value: -926.703 Hartrees
-2.433059 x 10^6 kJ/mol

Keto-equatorial

![Keto-equatorial structure]

Enol-axial

![Enol-axial structure]

Energy value: -926.7022 Hartrees
or -2.433057 x 10^6 kJ/mol

(g) Tetrahydroneopterin as the axial form of the enol tautomer

(h) Tetrahydroneopterin as the equatorial form of the enol tautomer

Energy Value: -926.705 Hartrees
or -2.433064 x 10^6 kJ/mol
Figure 18. Keto-equatorial and enol-axial THB with one or two waters added. Our results suggest that several conformations of both THB and THN exist in an equilibrium both in the unhydrated and in the hydrated systems. The water molecules were found to hydrogen bond with side chain alcohol groups and with the enol hydrogen.
have already established from above that the keto-equatorial was the most stable configuration, using both the ionized forms of tetrahydrobiopterin and tetrahydronopterin.

After solvation, the relative $\Delta G$ values of all forms of THB increased dramatically. Relative $\Delta G$ values of the THN conformers were also affected by hydration, with lower values for two forms and an increase in $\Delta G$ for the enol-axial form.

Studies on docking Gaussian-derived tetrahydrobiopterin and tetrahydronopterin to active sites of enzymes with conformations determined by x-ray crystallography.

The next steps of our investigation entail the docking of our Gaussian THB and THN molecules into the active sites (made devoid of THB or THN as described in Methods) of the subject hydroxylases (phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase) and nitric oxide synthases.

We have utilized a molecular dynamics program, Accelrys Discovery Studio (35), to simulate the docking of our Gaussian THB and THN molecules to active sites of selected x-ray crystallographically determined enzyme three-dimensional structures (as described in the Methods section). First we consider the aromatic amino acid hydroxylases.

If our THB and THN Gaussian molecules do successfully dock into the conformation of the enzyme active site, we can most likely conclude that those conformations are the stable and prevalent forms that bind to the hydroxylase active site. If they do not successfully dock, then we can conclude there may be other factors besides the conformations of the THB and THN molecules that enable them to bind to the active site. We first studied phenylalanine hydroxylase docking to THB and THN structures, after which we follow this with discussions of studies of tyrosine hydroxylase and tryptophan hydroxylase docking.

The four lowest energy conformations of THB were docked into the crystal structure of phenylalanine hydroxylase from the protein database (PDB number ). Figures 19 and 20 depict the results of the docking to the enzyme of our Gaussian 09-derived keto equatorial form of THB, the THB conformation most similar to the form actually appearing in the crystallographic structure. It is noteworthy that all four conformers of THB used in these studies yielded THB in the same side-chain conformation when bound to the active site. The keto and enol forms did not change.
The dihydroxypropyl side chain lies a bit differently for both pictures, reflecting results from two separate runs. However, the hydrogen binding patterns overall are very similar. Note the dotted green lines which represent hydrogen bonding from the substrate to the amino acid residues in the phenylalanine hydroxylase active site. After comparing to the original X-ray crystal structure of the phenylalanine hydroxylase bound with THB, the hydrogen binding patterns are seen to be very similar.
Similar docking experiments using tyrosine hydroxylase and tryptophan hydroxylase structures yielded very similar results. Interestingly, the THB with protonated amine group bound to the active sites in similar fashion. We found no published X-ray crystallographic structures for glyceryl ether monooxygenase and, consequently, were unable to study its binding of THB.

We now shift our attention to the nitric oxide synthases. We have noted, in the Introduction, that tetrahydrobiopterin binds to nitric oxide synthase (NOS) in a unique manner. As stated previously, Stoll et al. (31) have found that once tetrahydrobiopterin interacts with the NOS, in the process of the catalyzed reaction, it forms radicals. The possible radical forms are either ·BH₃ or ·BH₄⁺. We thought it interesting to study the binding behavior of both of these radical forms to NOS isoforms. First, we optimized our ·BH₃ and ·BH₄⁺ using DFT on the Gaussian 9 program. The results are shown below. The energies (ΔE) are given relative to the keto-axial structure that calculated to have the lowest energy, which is defined as 0. Results are shown in Figure 21 and Table 2.

Figure 21. ·BH₃ and ·BH₄⁺ keto-axial and keto-equatorial in lowest energy conformations.
Table 2. Shows the Predominant Conformations and the Equilibrium Constants

<table>
<thead>
<tr>
<th>BH₃ (radical)</th>
<th>Hartrees</th>
<th>Joules</th>
<th>Change</th>
<th>ΔG (J)</th>
<th>Keq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enol-axial</td>
<td>-850.85552</td>
<td>-3.70952E-15</td>
<td>Enol (axial-&gt;equatorial)</td>
<td>-1.03326E-20</td>
<td>0.081230566</td>
</tr>
<tr>
<td>Enol-equatorial</td>
<td>-850.85315</td>
<td>-3.70951E-15</td>
<td>Keto (axial-&gt;equatorial)</td>
<td>-5.34069E-21</td>
<td>2.31517E-06</td>
</tr>
<tr>
<td>Keto-axial</td>
<td>-850.86485</td>
<td>-3.70956E-15</td>
<td>Axial (enol-&gt;keto)</td>
<td>4.06765E-20</td>
<td>19593.72425</td>
</tr>
<tr>
<td>Keto-equatorial</td>
<td>-850.8526</td>
<td>-3.7095E-15</td>
<td>Equatorial (enol-&gt;keto)</td>
<td>-2.39786E-21</td>
<td>0.558446166</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enol-axial-&gt;keto-equatorial</td>
<td>-1.27305E-20</td>
<td>0.045362898</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BH₄⁺ (radical)</th>
<th>Hartrees</th>
<th>Joules</th>
<th>Change</th>
<th>ΔG (J)</th>
<th>Keq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enol-axial</td>
<td>-851.27244</td>
<td>-3.71134E-15</td>
<td>Enol (axial-&gt;equatorial)</td>
<td>2.91231E-20</td>
<td>1183.114989</td>
</tr>
<tr>
<td>Enol-equatorial</td>
<td>-851.27912</td>
<td>-3.71136E-15</td>
<td>Keto (axial-&gt;equatorial)</td>
<td>-4.88292E-21</td>
<td>0.305324706</td>
</tr>
<tr>
<td>Keto-axial</td>
<td>-851.28352</td>
<td>-3.71138E-15</td>
<td>Axial (enol-&gt;keto)</td>
<td>4.83060E-20</td>
<td>125076.7099</td>
</tr>
<tr>
<td>Keto-equatorial</td>
<td>-851.2824</td>
<td>-3.71138E-15</td>
<td>Equatorial (enol-&gt;keto)</td>
<td>1.43E-20</td>
<td>32.27835843</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enol-axial-&gt;keto-equatorial</td>
<td>4.34231E-20</td>
<td>38189.00968</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enol-equatorial-&gt; keto-a</td>
<td>1.91829E-20</td>
<td>105.7181348</td>
</tr>
</tbody>
</table>

In Table 2, note I have highlighted in blue the significant values, namely examining the keto-axial conformation equilibrium constants. As seen from Table 2, when keto-axial is on the reactant side, the $K_{eq}$ is a very low number. In contrast, when keto-axial is on the product side of the equation, the $K_{eq}$ is a very high number. Thus, keto-axial is the predominant form for both the $\cdot$BH₃ and $\cdot$BH₄⁺ radicals.

As seen in Table 2, the conformation of the THB dihydropropane group is predominantly axial when the radicals are the keto tautomers. Conversely, the conformation of the dihydropropane group is equatorial when the radicals are the enol tautomer. The ΔΔE values indicate that the keto tautomer is favored over the enol tautomer. Hydration studies were not performed on the radicals since the radicals are likely to be stabilized on the surface of the enzyme.

It was observed that the relative ΔΔE differences from the conformation of the lowest energy state (keto-axial) was much greater for the $\cdot$BH₃ than the $\cdot$BH₄⁺. Besides examining the changes of the relative energies, we also studied the spin densities and partial charges. Figure 22 shows the numbering system of the THB.

Referring to Figure 22, I have constructed Table 3 that shows the spin densities for all the atoms, starting from N1 to C8α. Note the position of the dihydroxypropyl side chain did
Figure 22. Numbering of the different atoms on both the aromatic ring and the pyrazine ring of THB.

Table 3. Table Showing the Spin Densities on Atoms Ranging from N1 to C8α

<table>
<thead>
<tr>
<th>Name and Configuration</th>
<th>N1</th>
<th>C2</th>
<th>N3</th>
<th>C4</th>
<th>C4α</th>
<th>N5</th>
<th>C6</th>
<th>C7</th>
<th>N8</th>
<th>C8α</th>
<th>Amino group</th>
<th>aromatic ring O</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keto-equatorial</td>
<td>-0.041797</td>
<td>0.10216</td>
<td>-0.0223</td>
<td>0.035</td>
<td>-0.048058</td>
<td>0.53246</td>
<td>0.023596</td>
<td>0.00897</td>
<td>0.10239</td>
<td>0.20651</td>
<td>0.069991</td>
<td>0.032487</td>
<td>1.00141</td>
</tr>
<tr>
<td>Keto-axial</td>
<td>-0.035459</td>
<td>0.09305</td>
<td>-0.0225</td>
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<td>-0.016863</td>
<td>0.517054</td>
<td>-0.028735</td>
<td>0.0031</td>
<td>0.10065</td>
<td>0.1895</td>
<td>0.061893</td>
<td>0.033028</td>
<td>0.92641</td>
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<tr>
<td>Enol-equatorial</td>
<td>-0.042965</td>
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<td>-0.0302</td>
<td>0.1412</td>
<td>-0.048302</td>
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<td>0.11097</td>
<td>-0.0414</td>
<td>0.13769</td>
<td>-0.069242</td>
<td>0.574936</td>
<td>-0.033841</td>
<td>0.00719</td>
<td>0.04122</td>
<td>0.158</td>
<td>0.03245</td>
<td>0.071171</td>
<td>0.94833</td>
</tr>
<tr>
<td>BH4</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Keto-equatorial</td>
<td>0.015362</td>
<td>0.03108</td>
<td>-0.0173</td>
<td>-0.0069</td>
<td>0.28329</td>
<td>0.351239</td>
<td>-0.013357</td>
<td>0.00034</td>
<td>0.10834</td>
<td>0.04926</td>
<td>0.113506</td>
<td>0.027808</td>
<td>0.94262</td>
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<td>Keto-axial</td>
<td>0.013974</td>
<td>0.0327</td>
<td>-0.0177</td>
<td>-0.0036</td>
<td>0.262989</td>
<td>0.365193</td>
<td>-0.017368</td>
<td>-0.0011</td>
<td>0.10624</td>
<td>0.05457</td>
<td>0.114288</td>
<td>0.028635</td>
<td>0.93885</td>
</tr>
<tr>
<td>Enol-equatorial</td>
<td>-0.003838</td>
<td>0.04259</td>
<td>0.04266</td>
<td>0.05189</td>
<td>0.223188</td>
<td>0.376728</td>
<td>-0.017011</td>
<td>0.00436</td>
<td>0.08003</td>
<td>0.04421</td>
<td>0.049232</td>
<td>0.130148</td>
<td>1.02419</td>
</tr>
<tr>
<td>Enol-axial</td>
<td>-0.004114</td>
<td>0.03802</td>
<td>0.05009</td>
<td>0.04433</td>
<td>0.24853</td>
<td>0.358404</td>
<td>-0.017243</td>
<td>-0.0005</td>
<td>0.08819</td>
<td>0.03518</td>
<td>0.049218</td>
<td>0.133965</td>
<td>1.0241</td>
</tr>
</tbody>
</table>
not influence the partial charge distribution. For example, a .57 spin density would represent a 57% chance of finding the radical on N5. Table 3 shows all the important spin density distributions in both \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \) radicals. Note that there is significant spin densities on the amino group and on the aromatic ring oxygen atom as well for \( \cdot \text{BH}_4^+ \).

Although the suggested mechanisms of nitric oxide synthase activity have electron transfers between the enzyme heme group, THB and NADP+ on the surface of the enzyme, we thought it would be interesting to dock our THB radicals to NOS structures. The \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \) radicals were docked into the X-ray crystallography model of various Nitric Oxide Synthases in a similar procedure to that which was done with the three aromatic amino acid hydroxylases. The first of these, shown in Figure 23, employs the keto-axial for the radicals since, as shown in Table 2, they are the lowest energy radical forms. The type of NOS that we used for this experiment was 1nod (PDB code) which was the inducible NOS form.

Again the dotted green lines represent hydrogen bonding from the substrate to the NOS active site. After comparing to the original X-ray crystal structure of the NOS bound with THB, the hydrogen binding patterns are very similar. These experiments using Discovery Studio were repeated ten times and the form of the docked THB or THB radical was excised and superimposed on results from previous runs. The results are shown in Figure 24. Again the type of NOS that was utilized was the inducible NOS form (PDB code 1nod).

It can be clearly seen from Figure 24 that the THB overlapped much better than did either \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \).
Figure 23. Keto-axial docked to NOS for ·BH$_3$, ·BH$_4^+$, and THB.
Figure 24. ·BH$_3$, ·BH$_4^+$, and THB docked to NOS each 10 times.
CHAPTER 4

CONCLUSION / DISCUSSION

Both tetrahydrobiopterin and tetrahydroneopterin exist in an equilibrium of conformations, varying between the keto and enol tautomers and the axial and equatorial side chain shifts. These molecules are never static, but are always fluid and shifting from one conformation to another and back. Free energies do show that some conformations should be more abundant than others at equilibrium. As deduced from our results, it is clear that the structure of a molecule is extremely crucial in determining the chemical characteristics of a particular molecule. For instance, the chemical characteristics of both tetrahydrobiopterin and tetrahydroneopterin are quite different, due to their distinct conformations.

Tetrahydrobiopterin has the R,R,S stereochemistry, whereas tetrahydroneopterin has the S, R,S stereochemistry. The stereochemistry of both molecules denotes the positions of the hydroxyl groups on carbon 1 and carbon 2 of the hydroxypropyl side chain. Since the first ring of both pterins are rigid and aromatic, the second ring has a bit of flexibility with carbon 6 shifting either upwards or downwards, compared to the plane of the molecule. Most of the shifting occurs in the highly flexible side chain. The dihydroxypropyl side chain shifting is very apparent in our total energy scans.

Next, we ran the ionized versions of THB and THN, with either one or two positive charges. Since these pterins are naturally found in the living cell, their real environment is an aqueous environment. It is highly probable that these pterins will be protonated by either one or two hydrogens in the aqueous solution. The program that was used to simulate the aqueous solution was COSMOtherm (34), which adds a shell of uniform water molecules around the molecule of interest. Thus, each of these pterins were run under Gaussian first to optimize their structures, then run on COSMOtherm to obtain the free energies of the solvated species. After analyzing the data, it was apparent that the free energies had increased for tetrahydrobiopterin, from $\Delta G$ of the vacuum to $\Delta G$ of solvent. In contrast, the free energies for tetrahydroneopterin had decreased, from $\Delta G$ of the vacuum to $\Delta G$ of solvent. Overall, the $\Delta G$s of the aqueous as compared to the $\Delta G$s of the gas phase shifted by 3 kcal/mol or less.
For both tetrahydrobiopterin and tetrahydroneopterin, the lowest energy structure was the keto-equatorial conformation. Diols were solvated as well, but were discarded because their ΔGs were deemed highly unfavorable at around 31 and 33 kcal/mol.

Most if not all our research was performed using the non-ionized version of the pterins, since the ionized versions docked poorly to our enzymes. We eventually chose the four lowest energy configurations of tetrahydrobiopterin (THB) and for tetrahydroneopterin (THN) for detailed studies of their conformations and thermodynamic properties, and their lowest energies.

In addition to running experiments with the pterins in a uniform water solvent shell with COSMOtherm, we also ran the pterins with one or two water molecules to investigate any presence of hydrogen bonding. We chose structures had the best potential for hydrogen bonding, which were the keto-equatorial and enol-equatorial conformations. When one water molecule was added to the keto-equatorial forms, it hydrogen bonded to the carboxyl group and the nitrogen and hydroxyl groups. When one water molecule was added to the enol-equatorial forms, it hydrogen bonded to the hydroxyl groups on the side chain. However, when two water molecules were added, the water molecules both hydrogen bonded to themselves and to the hydroxyl groups, forming a cage-like structure. The water molecules were originally set up far apart from each other to eliminate any random-binding results.

As discussed previously, THB binds distinctly to all three different hydroxylases, since the all the active sites have dissimilar residues. After binding the Gaussian-derived pterins and observing that the binding locations and hydrogen bonding patterns are very similar to that found in their x-ray crystallographic structures, we concluded that the binding has successfully mimicked the actual binding.

The pterins, specifically tetrahydrobiopterin, bind to Nitric Oxide Synthase (NOS) in a unique manner. As stated previously, Stoll et al. (31) have found that once tetrahydrobiopterin interacts with the NOS it undergoes reactions to become a radical form. The radical form can be either ·BH₃ or ·BH₄⁺ but is most likely the latter (31). We wanted to test the binding of the radicals to the NOS active site, so we performed docking experiments with our radicals. First, we optimized our ·BH₃ and ·BH₄⁺ under Gaussian. I chose specifically Keto-axial and Keto-equatorial conformations to the radicals in our experiment.
Once we obtained the optimized radical structures, we compared the relative energies of the radicals and determined that overall, the \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \) keto-axial conformation were the lowest energy forms.

We also observed the spin densities to determine on which atoms the radical would most likely be. From Table 2, it can be clearly seen that radicals are more likely to be found on N5 of \( \cdot \text{BH}_3 \), than that of \( \cdot \text{BH}_4^+ \), having 20% higher probability. For \( \cdot \text{BH}_3 \), it appears that the radical lies in the vicinity of N5 for about 50%-55% of the time, and shifts to the C8α atom for about 15-20%. As for the rest, the radical shifts from C2 to C7 to N8 for about the same time, 10%. As for \( \cdot \text{BH}_4^+ \), the radicals lie around N5 for about 35% of the time, shifting to the C4α atom for around 25%. The radical lies around N8 for the rest of the 10% of the time. Another interesting observation is that both the \( \cdot \text{BH}_3 \) keto tautomers have very similar spin densities, as well as the \( \cdot \text{BH}_3 \) enol tautomers. That observation applied to the \( \cdot \text{BH}_4^+ \) keto and enol tautomers as well. Apparently, the tautomerization plays an important role in determining the location of the radical, more than the position of the side chain. After data analysis, we conclude that there is more delocalization of the radicals on the \( \cdot \text{BH}_4^+ \) than the \( \cdot \text{BH}_3 \). Radicals lie on N5 on \( \cdot \text{BH}_3 \) for about half the time, in contrast for radicals having a 30-35% probability on the same atom.

Since keto-axial was found to be the lowest energy form for both \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \), we used the keto-axial conformation of both forms to dock to the X-ray crystallographic NOS enzyme. After docking our keto-axial radicals to the active site of the NOS enzyme, we compared our docking results with that of the hydrogen-bonding patterns in the original X-ray crystallographic NOS. After the comparison, we concluded that the hydrogen binding pattern were very similar and thus the docking was a success.

More importantly, we docked our \( \cdot \text{BH}_3 \) and \( \cdot \text{BH}_4^+ \) radicals ten times and isolated the docked radical, observing the quality of the 10 overlapping molecules; the better the overlapping, the more accurate the docking was. Thus, \( \cdot \text{BH}_3 \) docked the poorest, having 4 out of 10 molecules with decent overlapping. \( \cdot \text{BH}_4^+ \) had better overlapping, with 6 out of 10 being good overlaps. I even ran the neutral THB molecule, docking it 10 times in similar fashion. Neutral tetrahydrobiopterin actually had the best overlapping result, with nearly 8 or
9 out of 10 for good overlaps. We believe the reason is that the THB is in its neutral form before binding fully to the active site in the NOS, shifting it from neutral to the radical forms. Thus, right when the THB binds to the NOS, it is still in the neutral conformation. Again, these results are supported from the multiple docking studies showing better overlapping of THB than that of either ·BH$_3$ or ·BH$_4^+$. In conclusion, in both the neutral and the radical form of THB, the keto-axial is the lowest energy and thus is the predominant conformation.

Overall, these docking experiments are important because they may lead to the development of competitive inhibitors with therapeutic effects for certain pathological conditions. There are many implications for these computational studies, for instance, for a drug company desiring to create a drug that inhibits the binding of the original ligand. As we have seen, there are certain hydrogen bonding patterns specific for THB bound to phenylalanine hydroxylase or to nitric oxide synthase.

If one designs a drug that mimics the hydrogen bonding patterns of THB, and then synthesizes certain parts that makes the binding even stronger than the THB, then that drug will probably be a successfully inhibitor. Upon close examination, effective substitutions could probably be made of the oxygen group on the C4, the amino group, and the two hydroxyl groups on the side chain. One possible substitution could be another hydroxyl group, for example, to replace the amino NH$_2$ group. Other substitutions could be a strong electronegative group, such as fluorine, to take the place of the side chain hydroxyl groups. The main problem would be that the substituents cannot take up more space than the THB in the active site, otherwise the new molecule may not bind properly. However, if those any of the substitutions on the new molecule induce strong hydrogen bonding and the bonds are even stronger than that of THB to the enzyme, than the new molecule can possibly be a new inhibitor (R. Metzger, personal communication).
CHAPTER 5

STUDIES OF THE THB RECYCLING PATHWAY

Since dihydrobiopterin is formed during the oxygenation of various substrates in the hydroxylation pathway, it must be regenerated to THB. This involves a dehydration reaction in which 4-hydroxytetrahydrobiopterin is transformed to quinoid 7,8 dihydrobiopterin, which can undergo non-enzymatic isomerization to 7,8-dihydrobiopterin, as shown in Figure 25 (R. Metzger, personal communication).

We intend to study details of the two forms of dihydrobiopterin in greater detail. We will run the two forms of dihydrobiopterin (quinoid dihydrobiopterin and 7,8 dihydrobiopterin) under Gaussian DFT to determine their lowest energy conformations. Their 3-dimensional conformations will enable us to better understand the mechanism of the pathway and this form was used for some of the x-ray crystallographic studies of pterins bound to the active sites of hydroxylase enzymes. Figure 25 shows the difference in structure of the dihydrobiopterins and the tetrahydrobiopterin.

Figure 25 shows the full cycle of the tetrahydrobiopterin recycling pathway, starting from tetrahydrobiopterin. This reacts with oxygen to form the intermediate with the oxygen adduct on the carbon 4 of the intermediate. Next, the intermediate serves as a coenzyme for L-tryptophan to form 5-hydroxy-L-tryptophan. In that process, the intermediate is thereby converted into 4α-hydroxytetrahydrobiopterin. The 4α-hydroxytetrahydrobiopterin then interacts with pterin-4α-carbinolamine dehydratase to form the quinoid form of 7,8 dihydrobiopterin, shown in Figure 25. In order for the original tetrahydrobiopterin to be properly reformed again, NADPH dehydrobiopteridine reductase converts the quinoid 7,8 dihydrobiopterin back into tetrahydrobiopterin. Figure 26 show some examples of conformations for qDHB run under Gaussian. Figure 27 shows a proposed mechanism with our postulated intermediates through which the quinoid form of 7,8 dihydrobiopterin double bond changes from a C-4:N-5 to the N-5:C-6 of 7,8-dihydrobiopterin.
SEROTONIN SYNTHESIS FROM L-TRYPTOPHAN REQUIRES TETRAHYDROBIOPTERIN

L-tryptophan \[\rightarrow\] 5-hydroxy-L-tryptophan

\[\text{NADP}^+ \rightarrow \text{NADPH} \]

NADP(H) dehydropteridine reductase

7,8-dihydrobiopterin (quinoid form)

(or quinoid dihydrobiopterin)

\[\text{H}_2\text{O} \rightarrow \text{H}_2\text{O} \]

pterin-4α-carbinolamine dehydratase

\[\text{H}_2\text{O} \rightarrow \text{H}_2\text{O} \]

tetrahydrobiopterin

\[\text{NADPH} \rightarrow \text{NADP}^+ \]

dihydrofolate reductase

non-enzymatic rearrangement

Figure 25. Full cycle of tetrahydrobiopterin recycling pathway. Source: R. Metzger, personal communication.
Figure 26. Showing both keto-axial and keto-equatorial conformations for quinoid 7,8 dihydrobiopterin.
Figure 27. Proposed mechanism of quinoid 7,8 dihydrobiopterin conversion into 7,8 dihydrobiopterin. This will be the first step of the dihydrobiopterin breakdown pathway.

Note the shifting of the hydrogen on the C6 of the pyrazine ring of quinoid 7,8 dihydrobiopterin eventually transferring to the carboxyl group of the aromatic ring. Also, the double bond is shifted from the left side of the top-most nitrogen atom on the pyrazine ring to the right side.
The 7,8-dihydrobiopterin is unstable. It can be reconverted to tetrahydrobiopterin by the NADPH dependent dihydrofolate reductase, but if it is not returned to THB, it will decompose non-enzymatically as shown in Figure 28. Several possible forms of the liberated side chain group are shown in Figure 29.

Figure 28. The proposed mechanism by which 7,8 dihydrobiopterin breaks down and forms the intermediates. These intermediates will break down further into pterin and a three carbon diol or hydroxyaldehyde derivative. Several possible forms of the liberated side chain group are shown in Figure 29. This is the second and final step of the dihydrobiopterin breakdown pathway.
Figure 29. Finally, here are the proposed individual pterin molecule and its corresponding side chain cleaved off.

We have tabulated all the $\Delta E$ and $\Delta G$ of these compounds, and have combined all the data in Tables 4 and 5.

So far, we have proposed theoretical intermediates for both the pathway from quinoid THB to 7,8 DHB and the pathway from 7,8 DHB to pterin and cleaved side chain. Yet the question is: which intermediates that we have proposed are the most feasible and most likely to occur? To answer the question, we first used Gaussian 09 to calculate the thermodynamic functions $\Delta E$, $\Delta G$ and $\Delta H$. In the Appendix, we show all our molecule structures including proposed intermediates in the unhydrated phase, run using Gaussian 09.

Since Murata and Landge (46) performed all his simulation experiments using the quinoid 7,8-DHB to 7,8-DHB, in the gaseous (i.e. unhydrated) phase, we have compared our own unhydrated $\Delta E$ values with his. Our values for $\Delta E$, namely 76 kJ/mole, are considerably...
Table 4. Table Showing the qDHB, 7,8 DHB, Pterin, Side Chains, and All Their Intermediates in Solvated Form

<table>
<thead>
<tr>
<th>filename</th>
<th>E_gas kcal/mol</th>
<th>G_mix kcal/mol</th>
<th>G_mix kcal/mol</th>
<th>ΔG (kcal/mol)</th>
<th>K</th>
</tr>
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<tbody>
<tr>
<td>quinoid form 7,8-dihydrobiopterin</td>
<td>0  -533741.8</td>
<td>-533770.5</td>
<td>-28.70</td>
<td></td>
<td></td>
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<tr>
<td>DHB_keto_eq_1+.log</td>
<td>not converged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHB_keto_eq_.log</td>
<td>not converged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHB_keto_eq_freq1.log</td>
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<td>-3.62</td>
<td>461</td>
</tr>
<tr>
<td>DHB_keto_eq_freq2.log</td>
<td>0  -533750.2</td>
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<td>-3.87</td>
<td>705.48</td>
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<tr>
<td>DHB_keto_eq_freq3.log</td>
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<td>-534115.8</td>
<td>-23.21</td>
<td>-5.49</td>
<td>1.10E+04</td>
</tr>
<tr>
<td>7,8-dihydrobiopterin</td>
<td>0  -533765.1</td>
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<td>0.02</td>
<td>1.0329</td>
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<td>-83.56</td>
<td>58.35</td>
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<td></td>
</tr>
<tr>
<td>Pterin2_keto.log</td>
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<td>-364913.4</td>
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<td>-14.66</td>
<td>11.97</td>
<td>1.55E-09</td>
</tr>
</tbody>
</table>

starting compound

isomerization

isomerization intermediate 1

isomerization intermediate 2

isomerization intermediate 3

final compound first sequence

scission intermediate D

scission intermediate E

scission intermediate F

scission intermediate G

scission intermediate: 5,6-dihydropterin

scission final product: pterin

scission 1,2-propanediol

scission 2-(S)-hydroxypropional

scission hydrated 2-(S)-hydroxypropanal
Table 5. Table Showing the Energy Differences Between the qDHB and 7,8 DHB, and the Proposed Pterin and Side Chain, All in Unsolvated Form. See Appendix for Tables 6-8 with All the Intermediates

<table>
<thead>
<tr>
<th>Name of molecules in energy difference</th>
<th>ΔE</th>
<th>ΔG</th>
<th>ΔH</th>
<th>ΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keto-eq quinoid DHB -&gt; 7,8 DHB</td>
<td>0.03</td>
<td>-0.03</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-76.40</td>
<td>-73.53</td>
<td>-73.53</td>
<td>0.09 kJ/mol</td>
</tr>
<tr>
<td></td>
<td>-18.25</td>
<td>-17.56</td>
<td>-17.56</td>
<td>0.02 kcal/mol</td>
</tr>
<tr>
<td>7,8 DHB -&gt; pterin + hydrolyzed aldehyde</td>
<td>76.41</td>
<td>76.41</td>
<td>75.82</td>
<td>Hartrees</td>
</tr>
<tr>
<td></td>
<td>2.01E+05</td>
<td>2.01E+05</td>
<td>1.99E+05</td>
<td>kJ/mol</td>
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<tr>
<td></td>
<td>4.79E+04</td>
<td>4.79E+04</td>
<td>4.76E+04</td>
<td>kcal/mol</td>
</tr>
<tr>
<td>7,8 DHB -&gt; pterin + aldehyde</td>
<td>-0.03</td>
<td>-0.01</td>
<td>0.01</td>
<td>Hartrees</td>
</tr>
<tr>
<td></td>
<td>-90.84</td>
<td>-21.00</td>
<td>15.75</td>
<td>kJ/mol</td>
</tr>
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<td></td>
<td>-21.71</td>
<td>-5.02</td>
<td>3.77</td>
<td>kcal/mol</td>
</tr>
<tr>
<td>7,8 DHB -&gt; pterin + alcohol</td>
<td>0.57</td>
<td>1.18</td>
<td>0.56</td>
<td>Hartrees</td>
</tr>
<tr>
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<td>355.42</td>
<td>743.60</td>
<td>350.78</td>
<td>kcal/mol</td>
</tr>
</tbody>
</table>

higher than the 30 kJ/mole reported by Murata and Landge (46). In examining Figure 27, showing quinoid DHB becoming converted non-enzymatically into 7,8 DHB, a big energy change of 76 kJ/mol is quite possible. However, the interpretation of both sets of data is the same, namely, 7,8-DHB is considerably more stable than the quinoid DHB.

Thus begs the question, whose experiment and values were the more accurate ones? We believe that our results were the more accurate, due to our use of a far better computational quantum chemistry program (Gaussian 09). Murata and Landge (46) failed to completely elucidate the kinds of computational programs, or even their names, that they employed. They did state that they used *ab initio* methods in their calculations, but *ab initio* procedures are less accurate than full quantum mechanical methods, such as DFT.

We also calculated the entropy changes between the starting products and the intermediates shown in Figure 27 and discovered that they are negligible. Since the main change between the quinoid dihydrobiopterin and the 7,8 dihydrobiopterin is just a switch of the double bond in the pyrazine ring, the low ΔS value supports the very small entropy.
change (shown in Table 5). Because the reactions of importance occur in aqueous solution, we have used the program COSMOtherm to simulate solvation of all the molecules, including quinoid DHB, 7,8 DHB, proposed reaction intermediates, pterins and their corresponding side chains.

We have calculated the $\Delta G$ of the differences between the intermediates and both the quinoid DHB and the 7,8-DHB. $\Delta G$ results still demonstrate the greater stability of the 7,8-DHB compared to quinoid DHB. The intermediates in their solvated states yielded $\Delta G$ values that were higher than that of the starting reactant, viz. the quinoid dihydrobiopterin. From these data, we have calculated estimated rate constants for both reactions studied using the equation $k = e^{-\Delta G/RT}$, where the constant $R$ is 0.00198 kcal/mole and $T$ is 298°C.

The $\Delta G$s for the decomposition of 7,8-DHB were calculated from the difference between 7,8 DHB and the products pterin and 2-hydroxypropanal. The results were converted from units of Hartrees to those of both kJ/mol and kcal/mol. After obtaining the results, it was clear that both the $\Delta G$s for the hydrolyzed aldehyde and the alcohol with pterin were unrealistic (range in the thousands). We conclude that the most feasible side chain product was most likely the aldehyde, for which $\Delta G$s values were more reasonable (lower than 21kJ/mol).
ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Robert Metzger, for his endless guidance and patience, along with my thesis committee members Dr. Tom Huxford and Dr. Andrew Cooksy. I would also like to thank Dr. James Otto for the use of the Accelrys Discovery Studio program. Lastly, I would like to thank the Department of Computer Science for providing the Intel core i5 processors in GMSC 425 to run the Gaussian program.
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34. COSMOlogic. (2007) *COSMOTHERM 1.05*, COSMOClogic GmbH & Co. KG, Leverkusen Germany


APPENDIX

EXTRA GRAPHS AND TABLES
OTHER TYPE OF SCANS FOR THB AND THN

Figure 30. THB keto axial scan.

Figure 31. THN keto-equatorial scan.
Table 6. Table of $K_{eq}$ for BH3 and BH4

<table>
<thead>
<tr>
<th>BH3 (radical)</th>
<th>Hartrees</th>
<th>Joules</th>
<th>Change</th>
<th>$\Delta G$ (J)</th>
<th>$R$ (J/mol K)</th>
<th>$T$ (K)</th>
<th>$RT$ (J/mol)</th>
<th>$-(\Delta G/RT)$</th>
<th>Avogadro’s no.</th>
<th>$-(\Delta G/RT)$ per mol</th>
<th>$K_{eq}$</th>
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<tbody>
<tr>
<td>Enol-axial</td>
<td>-850.85552</td>
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<td>Enol (axial-&gt;equatorial)</td>
<td>-1.0326E-20</td>
<td>8.31446</td>
<td>298.15</td>
<td>2478.956249</td>
<td>-4.16813E-24</td>
<td>6.02E+23</td>
<td>-2.510463677</td>
<td>0.081230566</td>
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<td>-3.70951E-15</td>
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<td>298.15</td>
<td>2478.956249</td>
<td>-4.16813E-24</td>
<td>6.02E+23</td>
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<td>0.081230566</td>
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<table>
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<th>BH4+ (radical)</th>
<th>Hartrees</th>
<th>Joules</th>
<th>Change</th>
<th>$\Delta G$ (J)</th>
<th>$R$ (J/mol K)</th>
<th>$T$ (K)</th>
<th>$RT$ (J/mol)</th>
<th>$-(\Delta G/RT)$</th>
<th>Avogadro’s no.</th>
<th>$-(\Delta G/RT)$ per mol</th>
<th>$K_{eq}$</th>
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<tbody>
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<td>Enol-equatorial</td>
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Table 7. Table of Solvated qDHB, 7,8 DHB, Intermediates, Pterin and Their Side Chains

<table>
<thead>
<tr>
<th>filename</th>
<th>charge</th>
<th>multiplicity</th>
<th>E_gas kcal/mol</th>
<th>G_mix kcal/mol</th>
<th>ΔG_mix kcal/mol</th>
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</thead>
<tbody>
<tr>
<td>quinoid form 7,8-dihydrobiopterin</td>
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<td>1</td>
<td>-533741.772</td>
<td>-533770.476</td>
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<td>not converged</td>
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<td>7,8-dihydrobiopterin</td>
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<td>Pterin1_keto.log</td>
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<td>Pterin2_keto.log</td>
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<td>alcohol.log</td>
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<td>aldehyde.log</td>
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Table 8. Table of Unsolvated qDHB, 7,8 DHB, Intermediates, Pterin and Their Side Chains.

<table>
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<tr>
<th>Type of molecule</th>
<th>∆E (Hartrees)</th>
<th>∆G</th>
<th>∆H</th>
<th>∆E (Hartrees)</th>
<th>∆G</th>
<th>∆H</th>
<th>∆E (Hartrees)</th>
<th>∆G</th>
<th>∆H</th>
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<tbody>
<tr>
<td>keto-eq qDHB</td>
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<td>-850.001</td>
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<td>keto-eq intermed 1</td>
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<td>7,8 DHB keto form</td>
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<td>Pterin 1</td>
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THERMODYNAMIC PROPERTIES FOR REACTION OF QUINOID-DHB AND 7,8-DHB (7,8-DIHYDROBIOPTERIN)