Theoretical Study of the Antioxidant Properties of Pyridoxine

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Molecules acting as antioxidants capable of scavenging reactive oxygen species (ROS) are of utmost importance in the living cell. The antioxidative properties of pyridoxine (vitamin B_6) have recently been discovered. In this study, we have analyzed the reactivity of pyridoxine toward the ROS $^{\bullet}OH$, $^{\bullet}OOH$, and $^{\bullet}O_2^-$ at the density functional theory level (functionals B3LYP and MPW1B95). Two reaction types have been studied as follows: addition to the aromatic ring atoms and hydrogen/proton abstraction. Our results show that $^{\bullet}OH$ is the most reactive species, while $^{\bullet}OOH$ displays low reactivity and $^{\bullet}O_2^-$ does not react at all with pyridoxine. The most exergonic reactions are those where $^{\bullet}H$ is removed from the CH_2OH groups or the ring-bound OH group and range from −33 to −39 kcal/mol. The most exergonic addition reactions occur by attacking the carbon atoms bonded to nitrogen but with an energy gain of only 6 kcal/mol.

Introduction

Radicals are necessary intermediates in a variety of normal biochemical reactions. When generated in excess or not appropriately controlled, radical species can cause severe damage to a broad range of macromolecules. A prominent feature of radicals is that they have extremely high chemical reactivity, due to the presence of unpaired electrons, which explains not only their normal biological activities but also how they inflict damage on cells. Some of the radicals that are most abundantly produced in biochemical reactions are the so-called reactive oxygen species (ROS). These species are highly reactive and are produced in a variety of biochemical processes. For instance, oxygen-derived radicals are continuously generated as part of normal aerobic life, in mitochondria as oxygen is reduced along the electron transport chain, and as necessary intermediates in a variety of enzymatic reactions. However, there are situations in which oxygen radicals are overproduced in cells. White blood cells such as neutrophiles specialize in producing oxygen radicals, which are used in host defense against invading pathogens. Cells exposed to abnormal environments such as hypoxia or hyperoxia generate high and often damaging levels of ROS. A number of drugs have oxidizing effects on cells and lead to production of oxygen radicals. Ionizing radiation is well-known to generate oxygen radicals within biological systems. Because of the overproduction or poor control of these species, they can cause damage in all macromolecules, including lipids, proteins, and nucleic acids. One of the best known toxic effects of oxygen radicals is damage to cellular membranes in the form of lipid peroxidation.[1]

Vitamin B_6, also called pyridoxine, is one of eight water-soluble B vitamins. It is the precursor of the biologically active derivatives pyridoxal-5′-phosphate and pyridoxamine-5′-phosphate, with functional roles in a number of enzymes.[2] Moreover, this group of vitamins assists in the metabolism of proteins, fats, and carbohydrates. Vitamin B complexes also play an important role in maintaining muscle tone in the gastrointestinal tract and promoting the health of the nervous system, skin (preventing dandruff, eczema, and psoriasis), hair, eyes, mouth, and liver. Vitamins B_{12} (riboflavin), B_6, and B_9 (folic acid) work closely together to control homocysteine levels in blood, which is detrimental to the heart muscle. In addition, pyridoxine is essential for normal brain development and function, in that it participates in the biosynthesis of neurotransmitters. Pyridoxine is an especially important vitamin for maintaining healthy nerve and muscle cells, and it aids in the production of nucleic acids DNA and RNA. It is necessary for proper absorption of vitamin B_{12} and for the production of red blood cells and the immune system. Pyridoxine is also required for the balance of hormonal changes in women, by assisting in mood control as well as behavior. It assists in the balancing of sodium and potassium and is furthermore linked to cancer immunity.[3]

Pyridoxine, although not classified as an antioxidant compound, has recently been shown to have highly effective antioxidant properties.[4] It was proposed that this effect could be related to possible coenzymatic activities, which cannot be observed in chemical tests, although their antioxidant mechanisms are not clear. ROS are constantly formed in the human body and must be removed by antioxidants. A lack of balance in the oxidant−antioxidant activity is involved in many free radical-mediated pathologies. Lately, pyridoxine biosynthesis-deficient mutants of fungi and yeast have been shown to be sensitive to ROS, such as singlet oxygen[5,6] and hydrogen peroxide.[7] This suggests that vitamin B_6 and its derivatives are also involved in stress tolerance in living organisms, especially in alleviating oxidative stress. In eukaryotes, stress resistance has been implied to involve pyridoxine-dependent singlet oxygen quenching,[8] whereby the pyridoxine itself would act as antioxidant.[6,8] Pyridoxine itself was in these tests found to be the most reactive of the vitamin B_6 species pyridoxine, pyridoxal, pyridoxamine, and pyridoxal-5-phosphate; twice as effective as pyridoxal 5-phosphate; and as effective as vitamin E.[9] Pyridoxine and, even more so, pyridoxal, have also proven to be efficient superoxide (or $^{\bullet}OOH$) scavengers in in vitro chemical assays and to prevent lipid peroxidation.[10]

To provide more insight into the antioxidant properties of pyridoxine, we here present a theoretical study of its capacity as a quencher of $^{\bullet}OH$, $^{\bullet}OOH$, and $^{\bullet}O_2^-$ radicals, within the density functional theory framework. Two possible reactions have been studied. On one hand, addition reactions to atoms of

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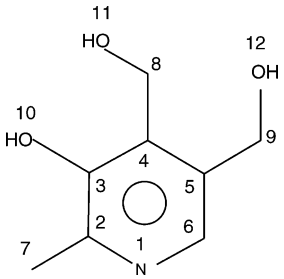

Figure 1. Pyridoxine (vitamin B_6), including atomic numbering.

TABLE 1: ΔG_{aq}^{298}, Calculated Using B3LYP, in kcal/mol, for the Addition Reactions of •OH and •OOH

	complex	TS	products
B_6(N1)−•OH	5.19 (6.06)	23.50 (22.93)	23.78 (19.86)
B_6(C6)−•OH	6.12 (7.03)	7.43 (8.24)	−6.37 (−9.60)
B_6(C6)−•OOH		27.78 (29.45)	22.48 (21.16)

[a] Energies are relative to isolated reactants. In parentheses, MPW1B95 energies are given.

TABLE 2: ΔG_{aq}^{298} of the Products, Calculated Using B3LYP, in kcal/mol, for the H Abstraction Reactions[a]

	reaction 1	reaction 2	reaction 3
•B_6(C7)	−29.95 (−29.53)	2.43 (3.72)	19.41 (21.78)
•B_6(C8)	−39.72 (−40.07)	−7.27 (−6.82)	9.72 (11.25)
•B_6(C9)	−39.22 (−40.50)	−6.77 (−7.25)	10.21 (10.81)
	reaction 4	**reaction 5**	**reaction 6**
B_6^-(C6)	108.47 (107.96)	99.71 (99.54)	52.99 (51.18)
B_6^-(O10)	58.15 (63.85)	49.40 (55.42)	2.68 (7.06)

[a] Energies are relative to isolated reactants. In parentheses, MPW1B95 energies are given.

the aromatic ring of pyridoxine have been studied, and on the other, hydrogen/proton abstraction from the side groups has been studied.

Methods

The study is carried out using the gradient-corrected hybrid functional B3LYP[11−13] within density functional theory.[14,15] Structure optimizations are carried out in the gas phase, using the 6-31+G(d,p) basis set. Harmonic vibrational frequencies are obtained by analytical differentiation of gradients, to determine whether the structures found are minima or transition states (TSs) and to extract zero-point energies and Gibbs free energy contributions. Intrinsic reaction coordinate calculations[16,17] are performed to assess that the calculated TSs connect the appropriate reactants and products. Single-point calculations using the 6-311+G(2df,p) basis set and the integral equation formalism of the polarized continuum model of Tomasi and co-workers[18,19] were performed on the optimized structures to estimate the effects of bulk solvation. To take into account the influence of enthalpy and entrophy, the Gibbs free energy contributions from the gas phase were added to give the final free energies, ΔG_{aq}^{298}. The GAUSSIAN03[20] package was used throughout the study.

To validate the results obtained with the B3LYP functional, calculations using the meta functional MPW1B95, developed by Truhlar and co-workers,[21−24] have been carried out for some of the systems, using the same approach as outlined above. The attack of ROS to pyridoxine may be classified into two groups. On one hand, we have the attack and binding to the aromatic ring, and on the other, we have the abstraction of hydrogens from the groups attached to the ring. Throughout the study, the atoms in the pyridoxine aromatic ring are labeled as shown in Figure 1. To compare the results obtained with B3LYP and MPW1B95, the following reactions have been chosen. For the case of the addition reactions of •OH, the additions to atoms 1 (highest barrier) and 6 (lowest barrier) have been chosen. For the addition reactions of •OOH, addition to atom 6 has been chosen. The obtained results are given in Table 1. The abstraction reactions are described in Hydrogen Abstraction Reactions. To compare the results of both functionals, the reactions chosen are those with highest and lowest ΔG values. The obtained results are shown in Table 2.

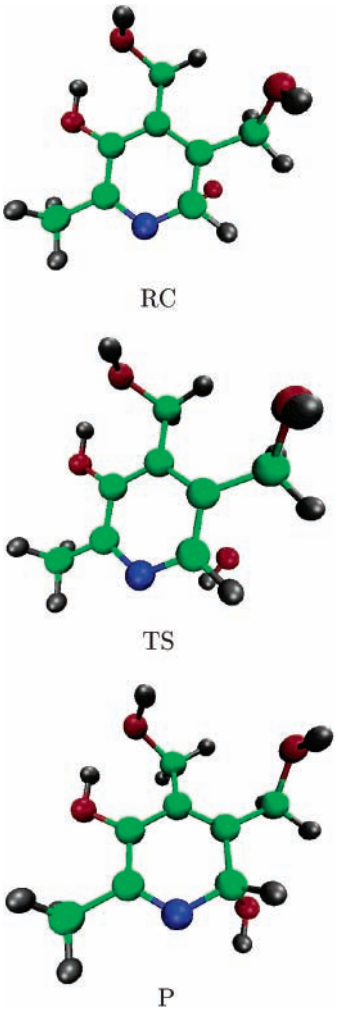

Figure 2. From top to bottom, reactant complex, TS, and product of the •OH addition to C6.

According to the results given in the tables, it is observed that both methods give similar results. Except for the proton abstraction reaction from O10, the differences between the two methods are around 1 kcal/mol, showing the reliability of the B3LYP functional to perform these calculations. All data in the remaining systems and discussion of the results are thus based on the B3LYP calculations.

Results

The attack of ROS to pyridoxine may be classified into two groups. On one hand, we have the attack and binding to the aromatic ring, and on the other, we have the abstraction of hydrogens from the groups attached to the ring. All possible paths have been tested for all three radicals, and the results are described below. In Addition of •OH, •OOH, and $•O_2^-$ to the Aromatic Ring, the attack of the radicals to the ring atoms is

TABLE 3: Distances between the O Atom of •OH or •OOH and the Pyridoxine Ring Atom (R), in Å[a]

	complex				TS					products			
	R	θ	ΔG_{aq}^{298}	SD	*R*	θ	ΔG_{aq}^{298}	freq.	SD	*R*	θ	ΔG_{aq}^{298}	SD
B_6(N1)−•OH	2.29	2.0	5.19	0.775	1.61	3.1	23.50	474.3i	0.176	1.44	2.8	23.78	0.002
B_6(C2)−•OH	2.49	4.0	6.70	0.755	2.10	7.1	9.11	253.7i		1.44	1.2	−6.63	0.034
B_6(C3)−•OH	2.46	4.0	9.25	0.755	2.04	7.0	9.36	368.6i	0.483	1.43	11.6	−1.71	0.012
B_6(C4)−•OH	2.24	3.9	9.25	0.707	2.17	4.3	10.40	74.3i	0.659	1.47	5.8	−3.95	0.030
B_6(C5)−•OH	2.60	1.4	6.12	0.755	2.07	4.4	8.83	222.6i	0.571	1.46	2.8	−2.39	0.032
B_6(C6)−•OH	2.49	1.4	6.12	0.755	2.06	5.1	7.43	285.4i	0.526	1.44	3.5	−6.37	0.022
B_6(C2)−•OOH					1.84	15.5	30.14	546.4i	0.368	1.47	10.3	24.78	0.047
B_6(C3)−•OOH					1.82	11.7	30.88	475.7i	0.336	1.46	4.9	28.66	0.044
B_6(C4)−•OOH					1.83	7.2	31.65	460.9i	0.391	1.48	5.2	26.88	0.045
B_6(C5)−•OOH					1.81	9.5	31.33	530.9i	0.377	1.48	4.6	29.00	0.052
B_6(C6)−•OOH					1.84	11.9	27.78	533.5i	0.382	1.45	3.6	22.48	0.035

[a] The maximum dihedral angle in the aromatic ring, θ, is in degrees. ΔG_{aq}^{298} is in kcal/mol. Spin density (SD)* of the O atom of •OH and •OOH. For the TSs, the imaginary frequencies in cm^{-1} are given. *SDs of isolated OH and OOH are 1.020 and 1.070, respectively.

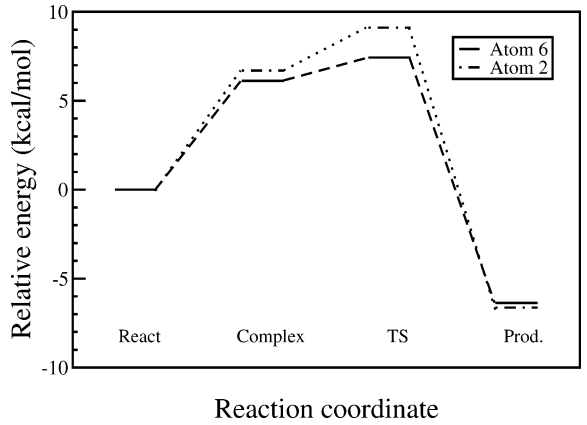

Figure 3. Reaction paths ΔG_{aq}^{298} for •OH addition to C2 and C6.

described, and in Hydrogen Abstraction Reactions, the hydrogen abstraction reactions are described.

Addition of •OH, •OOH, and $•O_2^-$ to the Aromatic Ring. Reaction paths for the attack by •OH and •OOH radicals to all six atoms in the aromatic ring of pyridoxine have been characterized. Addition of either of these two radicals to atoms in the ring breaks the aromaticity. The $•O_2^-$ radical, on the other hand, does not bind to any atoms of the ring. All attempts to calculate the adduct of superoxide to any of the atoms of the ring yielded dissociated pyridoxine and the superoxide radical anion, which may be due to the repulsion between the ring π system and the negative charge of the radical.

In Figure 2, the reactant complex, TS, and product for the addition of •OH to C6 are depicted as representative structures for the remaining addition reactions. In Table 3, the distance between the attacking radical and the ring atom under attack is given along the reaction path, together with the maximum dihedral angle of four neighboring atoms, within the aromatic ring, θ. The free energies in water, for all possible additions, ΔG_{aq}^{298}, and the spin density on the attacking oxygen atom are given as well. For the TS, we report the corresponding imaginary frequencies. The full reaction paths of the two most exergonic reactions, i.e., •OH addition to atoms 2 and 6, are depicted in Figure 3.

The reactions proceed in one step, as can be seen in Figure 3. We will in the following focus on the addition of •OH and •OOH radicals to atom 6, keeping in mind that all additions occur in a similar fashion. In the case of the addition of •OH, an initial reactant complex is formed, where the distance between the O atom of the •OH radical and the carbon C6 is 2.49 Å. This distance is shortened to 2.06 Å; in the TS, with an imaginary frequency of 285.4i cm^{-1}, and 1.44 Å in the product.

TABLE 4: ΔG_{aq}^{298} of Reactions 1−6, in kcal/mol[a]

	reaction				reaction		
	1	2	3		4	5	6
•B_6(C6)	−10.62	21.75	38.74	B_6^-(6)	108.47	99.71	52.99
•B_6(C7)	−29.95	2.43	19.41	B_6^-(7)	94.30	86.80	38.83
•B_6(C8)	−39.72	−7.27	9.72	B_6^-(8)			
•B_6(C9)	−39.22	−6.77	10.21	B_6^-(9)			
•B_6(O10)	−33.32	−0.95	16.04	B_6^-(10)	58.15	49.40	2.68
•B_6(O11)	−8.70	23.67	40.66	B_6^-(11)	62.00	53.24	6.52
•B_6(O12)	−14.55	21.75	34.81	B_6^-(12)	81.05	72.30	25.58

[a] Parentheses refer to the atom from which $•H/H^+$ is abstracted (cf. Figure 1).

Similar distances are seen for the attack on all carbons in the ring, whereas for binding to nitrogen the TS is much shorter (only 1.61 Å). The planarity of the aromatic ring along the reaction remains relatively constant. In isolated pyridoxine, the largest dihedral angle is 1.4°. In the case of the addition to C6, the complex has the same maximum dihedral angle, 1.4°, while it increases to 5.1° in the TS and decreases finally to 3.5° in the product. The largest value of the dihedral angle is for the C3 adduct, 11.6°.

From the ΔG values in Table 3, one can observe that •OH will not add to N1 but only to carbons of the aromatic rings, preferently C2 and C6, the neighboring atoms to the nitrogen. The C2 and C6 addition products are the most exergonic, whereas the lowest barrier to addition is found for the C3 adduct, only 0.1 kcal/mol in aqueous solution. The resulting product is, however, also the thermodynamically least stable one. All activation energies, in going from RC to TS, are very low, less than 3 kcal/mol, indicative of fast addition reactions. When going from reactants to products, there is a transfer of the unpaired spin from •OH to the ring atoms. In the reactants, the spin density of the isolated •OH radical is 1.020 (in the oxygen atom). In the RC and TS, the value is reduced to 0.755 and 0.526, respectively. The remaining spin density is delocalized in the aromatic ring. Finally, in the product, the unpaired spin density is delocalized throughout the aromatic ring.

The hydroperoxyl radical, on the other hand, is found to have large positive ΔG_{aq}^{298} values for all additions and activation energies approximately 30 kcal/mol above the isolated reactants. These reactions are hence not likely to occur. As compared to the addition of the hydroxyl radical, no stable addition complexes could furthermore be found; all attempts ended up in the separated reactants irrespective of initial structures.

On the basis of the current energetics and optimized structures, we conclude that only the hydroxyl radical will be capable of forming adducts to the pyridoxine moiety. These reactions are associated with low barriers and slightly exergonic

TABLE 5: Data for Reactions 1−9[a]

	reactants	TS						complex			products		
reaction 1	R_{X-H}	R_{X-H}	R_{O-H}	ΔG_{aq}^{298}	freq.	SD_O	SD_X	ΔG_{aq}^{298}	SD_O	SD_X	ΔG_{aq}^{298}	SD_O	SD_X
$^{\bullet}B_6$(C6)	1.087	1.189	1.379	7.76	399.7i	0.610	0.300	−0.97	0.001	0.831	−10.62	0.000	0.855
$^{\bullet}B_6$(C7)	1.094	1.178	1.434	8.69	476.9i	0.644	0.294	−20.24	0.000	0.724	−29.95	0.000	0.699
$^{\bullet}B_6$(C8)	1.089	1.321	1.324	0.91	1661.1i	0.135	0.256	−30.09	0.001	0.563	−39.72	0.000	0.570
$^{\bullet}B_6$(C9)	1.092							−28.30	0.001	0.603	−39.22	0.000	0.585
$^{\bullet}B_6$(O10)	0.974	1.293	1.134	0.91	1661.1i	0.135	0.116	−21.88	0.001	0.333	−33.32	0.000	0.325
$^{\bullet}B_6$(O11)	0.964	1.353	1.106	8.67	1250.1i	0.014	0.308	−0.05	0.001	0.716	−8.70	0.000	0.771
$^{\bullet}B_6$(O12)	0.963	1.144	1.293	8.67	1250.1i	0.014	0.085	−9.55	0.000	0.839	−14.55	0.000	0.836

[a] Distances between the O atom of $^{\bullet}$OH or $^{\bullet}$OOH and the abstracted pyridoxine H, R_{O-H}, and between the pyridoxine atom and H, R_{X-H}, in Å. Spin densities (SD) of the O atom of $^{\bullet}$OH or $^{\bullet}$OOH and of H-abstracted pyridoxine atom (X). For the TSs, the imaginary frequencies in cm^{-1} are given. ΔG_{aq}^{298} in kcal/mol.

free energies. The most likely sites of attack are atoms C2 and C6, on either side of the ring nitrogen.

Hydrogen Abstraction Reactions. Hydrogen/proton abstraction reactions by $^{\bullet}$OH, $^{\bullet}$OOH, and $^{\bullet}O_2^-$ may be divided in two groups. On one hand, the radicals may abstract a hydrogen atom from pyridoxine, in which case the radical nature would be transferred to the pyridoxine. These reactions are summarized below.

$$B_6 + {}^{\bullet}OH \rightarrow {}^{\bullet}B_6 + H_2O \quad (1)$$

$$B_6 + {}^{\bullet}OOH \rightarrow {}^{\bullet}B_6 + H_2O_2 \quad (2)$$

$$B_6 + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}B_6 + HOO^- \quad (3)$$

On the other hand, the ROS could in principle abstract a proton, in which case we have a charge transfer reaction. The reactions of this second group would be the following, of which the first two are the least likely to occur:

$$B_6 + {}^{\bullet}OH \rightarrow B_6^- + H_2O^{\bullet+} \quad (4)$$

$$B_6 + {}^{\bullet}OOH \rightarrow B_6^- + H_2O_2^{\bullet+} \quad (5)$$

$$B_6 + {}^{\bullet}O_2^- \rightarrow B_6^- + {}^{\bullet}OOH \quad (6)$$

The ΔG_{aq}^{298} values of these reactions are given in Table 4. As can be seen, $^{\bullet}$OH can abstract $^{\bullet}$H from all atoms. The most likely sites of abstraction are the methylene carbons C8 and C9, with ΔG_{aq}^{298} around −40 kcal/mol. $^{\bullet}$OOH can also abstract $^{\bullet}$H from C8 and C9 but with ΔG_{aq}^{298} much smaller than those of $^{\bullet}$OH, around −7 kcal/mol. $^{\bullet}$H abstractions by $^{\bullet}O_2^-$ all have positive ΔG_{aq}^{298}, as do all proton abstractions. Again, $^{\bullet}$OH is the most reactive species, and the full reaction paths for these $^{\bullet}$H abstractions are explored in more detail.

In Table 5, geometric, electronic, and energetic values for the characterized TSs, product complexes, and isolated products of $^{\bullet}$H abstraction by $^{\bullet}$OH are given. In Figure 4, the TS, complex, and product structures for the abstraction from O10 are given, as illustrative for these reaction types. The reaction paths for abstracting hydrogen from C8 and O10 have the same TS, which may be seen in the TS depicted in Figure 4. Similarly, the abstractions from atoms O11 and O12 have the same TSs. For abstraction from C9, no TS was found. In the isolated pyridoxine molecule, the X−H distances are around 1.09 Å when X = C and 0.97 Å when X = O. In the TS, the X−H distances are increased, although to very different extent in the various systems. The TSs for abstraction from C6, C7, and O12 are all early, with short X−H bonds and long H−O distances. For the C8 TS, the H atom is exactly midway between C and O, and in

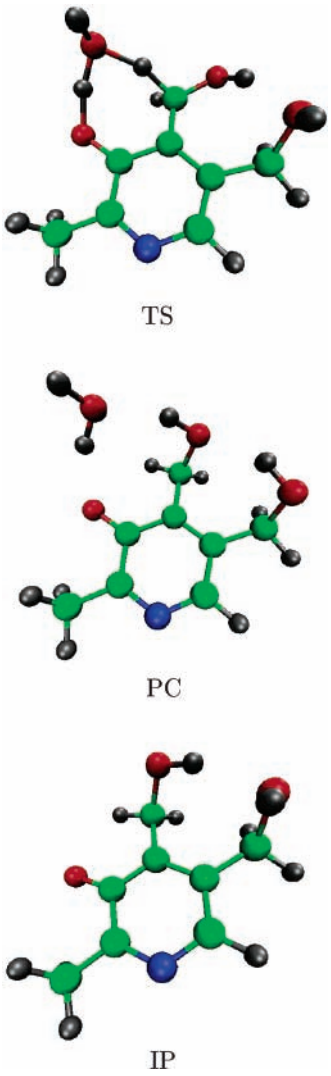

Figure 4. From top to bottom, TS, product complex, and isolated product from H abstraction at O10 by the $^{\bullet}$OH radical.

the reactions with O10 and O11, the systems are best characterized as late TSs. In the product complexes, the abstracted hydrogen forms a hydrogen bond to the remaining pyridoxine radical.

In the TSs to abstraction from C6 and C7, the least radical character is transferred to the pyridoxine moiety, only 0.3−0.4 electrons. These, along with the C8 and O10 TSs, also display the largest spin density on the X atom (approximately 0.3 electrons). For the other systems, the hydroxyl oxygen holds much less of the unpaired spin. Instead, this is delocalized throughout the pyridoxine ring system. Interesting to note is

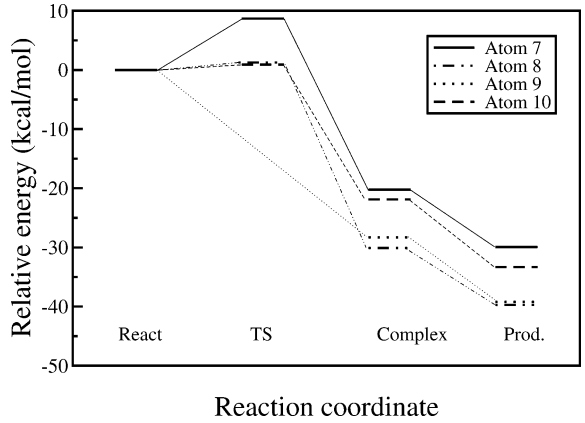

Figure 5. Reaction paths (ΔG_{aq}^{298}) for •OH-induced •H abstraction at C7, C8, C9, and O10.

that the more stable products show large delocalization of the radical character in the corresponding TSs.

The energetics of the reaction paths for abstraction from C7, C8, C9, and O10 are depicted in Figure 5. The barriers for the reactions are small (8.7 kcal/mol in the case of abstraction from C7), very small (0.91 kcal/mol for C8 and O10), or without barrier (C9), indicative of very fast reactions. The products from reactions with C8 and C9 are the most exergonic ones, with ΔG_{aq}^{298} about 40 kcal/mol. Reactions with O10 and C7 are less exergonic, with ΔG_{aq}^{298} about 33 and 30 kcal/mol respectively. ΔG_{aq}^{298} values of the remaining products are 2−3 times smaller.

Conclusions

The reactions between pyridoxine and several oxygen radicals (•OH, •OOH, and $•O_2^-$) have been investigated in this work. Two sets of reactions were explored as follows: addition reactions to the aromatic ring and H abstraction.

The first set of reactions may be carried out by •OH and •OOH radicals, while for $•O_2^-$, repulsion between the aromatic π system and the negative charge of the radical makes these additions strongly disfavored. In addition, whereas •OH additions have negative ΔG_{aq}^{298} (except addition to N), •OOH additions are all endothermic. We therefore predict that only the hydroxyl radical will be able to form these adducts. The most favored additions are those to C2 and C6, albeit the reactions are only mildly exergonic.

Also, for the second reaction type, the •H abstraction reactions, •OH is the most reactive one, while •OOH is reactive only to a very small extent and $•O_2^-$ will not react at all. The hydrogens that are easiest to abstract are those bonded to C8 and C9. Removal of those hydrogens by the hydroxyl radical is essentially barrierless, and the products have ΔG_{aq}^{298} values of approximately −40 kcal/mol, around seven times more exergonic than those of the •OOH products.

Comparing the addition and •H abstraction reactions, the latter display much more negative ΔG_{aq}^{298} values. Consequently, when •OH approaches pyridoxine, the most probable reaction will be •H abstraction from C8 and C9, although it may also abstract H from other atoms or attack the aromatic ring. •OOH is much less reactive and will only abstract •H from C8 and C9. The $•O_2^-$ radical, finally, is the least reactive species and will not react with pyridoxine at all.

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Supporting Information Available: Electronic and Gibbs free energies, optimized structures, and vibrational modes. This material is available free of charge via the Internet at http://pubs.acs.org.

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