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Mechanistic Aspects Regarding the Elimination of H_2O_2 from C(4a)-Hydroperoxyflavin. The Role of a Proton Shuttle Required for H_2O_2 Elimination

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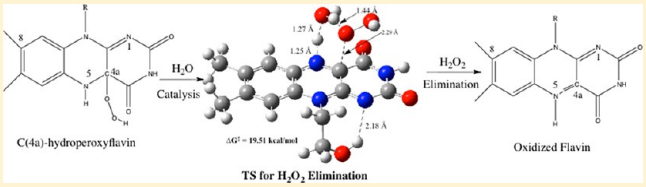
Supporting Information

ABSTRACT: DFT calculations presented for C(4a)-hydroperoxyflavin (C(4a)-FLHOOH) at the B3LYP/6-311+G(d,p) level suggest a new mechanism for the elimination of H_2O_2. The calculated activation barrier for a concerted four-centered elimination ($\Delta E^{\ddagger}$ = 32.86 kcal/mol) strongly suggests that in the absence of interactions with the local environment a spontaneous elimination is not feasible. A proton shuttle from the N5 hydrogen to the proximal oxygen of the OOH moiety involving three water molecules has an activation barrier that is reduced to 17.11 kcal/mol. Calculations that utilize CH_3OH to model the role of a local Thr or Ser residue shows that an alcohol functionality hydrogen bonded to the N5 H-atom can catalyze the elimination of H_2O_2 with a free energy of activation of 21.5 kcal/mol. Interaction of amines and amide residues (CH_3NH_2 and $CH_3(C{=}O)NH_2$) with the N5 locus of C(4a)-hydroperoxyflavin markedly reduce the activation barrier for H_2O_2 elimination relative to the concerted pathway. Proton transfer from a COOH group ($\Delta G^{\ddagger}$ = 8.36 kcal/mol) or the NH_2 group of a positively charged Arg model ($\Delta G^{\ddagger}$ = 9.99 kcal/mol) to the proximal oxygen of the OOH moiety of C(4a)-FLHOOH in the TS for H_2O_2 elimination strongly enhances elimination of H_2O_2.



INTRODUCTION

The distinguishing feature of flavoprotein monooxygenases is their ability to form a stable C(4a)-hydroperoxyflavin (C(4a)-FLHOOH), which can be considered as an activated form of oxygen capable of incorporating a single oxygen atom into an organic substance.[1−4] In certain enzymes, such as the aromatic hydroxylases of the *p*-hydroxybenzoate hydroxylase family, formation of this intermediate is usually detected only after binding of the aromatic substrate to be hydroxylated. Conversely, Baeyer−Villiger monooxygenases, flavin-containing monooxygenases, and *N*-hydroxylating monooxygenases are unique in that they require the presence of a bound $NADP^+$ in their oxidative half-reaction to stabilize the C(4a)-FLHOOH for sufficient periods to allow the arrival of an incoming substrate. While monooxygenases can form and stabilize the C(4a)-hydroperoxyflavin adduct, most flavoenzyme oxidases directly produce hydrogen peroxide typically without detectable intermediates. Remarkably, pyranose 2-oxidase represents a unique case of well-characterized formation and stabilization of C(4a)-hydroperoxyflavin in an oxidase.[5,6]

Scheme 1 summarizes the general steps leading to formation of C(4a)-FLHOOH. The two-electron reduced flavin and oxygen undergo an initial one-electron transfer to produce a radical pair between the neutral flavin semiquinone and superoxide radical that can collapse to the C-(4a)-$FLHOO^-$ peroxy anion. This compound must be protonated to generate what is thought to be the principal oxygenating reagent, C(4a)-hydroperoxyflavin. Kinetics studies of the N5-deuteriated enzyme and solvent isotope effects demonstrated that bond breakage of N5−H controls the overall process of H_2O_2 elimination and thus the stability of the C(4a)-FLHOOH.[7] Collectively, these and other recently reported experiments start to provide a more unified picture of the tuned reaction of oxygen in the context of the diversified flavoenzyme active sites.[1−10] The fundamental concept is that the formation and decay of C(4a)-hydroperoxyflavin primarily depends on the H-bonding environment and accessibility around the flavin C(4a)-N5 locus, which is the site directly involved in the reaction.[1b] This is the notion that has motivated our theoretical investigation of the effect on C(4a)-hydroperoxyflavin stability that is influenced by different local functional groups, positioned in direct contact with the C(4a)-N5 atoms. We demonstrate that the concerted elimination of H_2O_2 from C(4a)-FLHOOH by simply transferring the N5−H proton to the departing HOO^- fragment has an excessive activation barrier ($\Delta G^{\ddagger}$ = 29.47 kcal/mol) and consequently requires catalysis by protic groups that can function as proton shuttles. While at some point it must have been suggested that the hydrogen bonding interaction of local residues could actually catalyze the elimination of H_2O_2, conventional wisdom suggests that in the absence of such H-bonding interactions elimination

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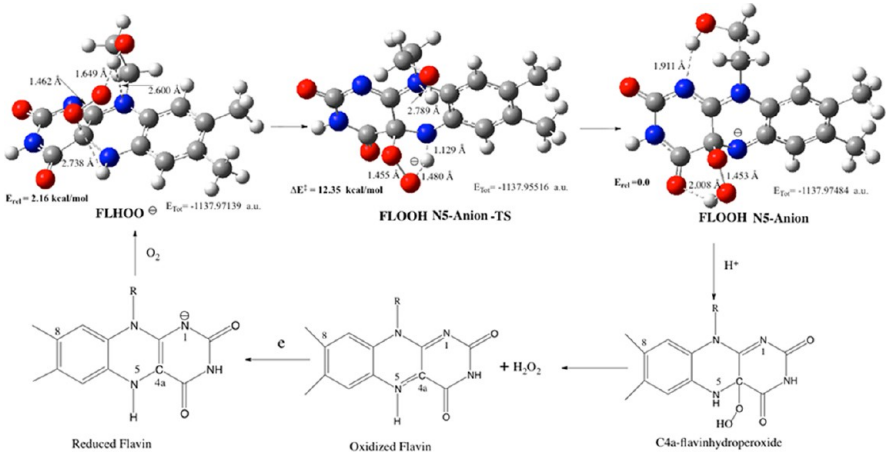
Scheme 1. Reaction of Reduced Flavin with Oxygen Produces the Oxygen Anion C(4a)-FLHOO^{-a}



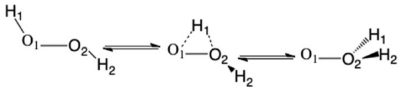
[a]The potential exists for the flavin N5−H moiety to transfer its proton to the distal oxygen producing the C(4a)-FLHOOH N5-anion, which is 2.16 kcal/mol lower in energy and can form with an activation energy of only 12.35 kcal/mol. However, HOO^- cannot be an effective leaving group from this N5-anion and N5 protonation is required to generate the chemical entity C(4a)-hydroperoxyflavin that is able to function in substrate oxygenation.

of H_2O_2 is very rapid. The current studies represent a marked departure from existing ideas and show that such H-bonding interactions can both stabilize C(4a)-FLHOOH and catalyze the elimination of H_2O_2. These novel ideas now provide a framework for the experimentalist to more closely examine related enzymatic systems.

RESULTS AND DISCUSSION

(a). Concerted H_2O_2 Elimination. Although several points of view concerning the stability and lifetime of C(4a)-FLHOOH (Figure 1) currently exist, one thought is that some form of H-bonding interaction is essential for both formation and stabilization of C(4a)-FLHOOH or else H_2O_2 elimination is very rapid.[1a] We examined first the activation barrier for a concerted elimination in the absence of any stabilization due to local environment interactions (Figure 2).

A concerted elimination pathway would require that the proton affinities of the participating fragments should be comparable in magnitude with that of the more basic HOO^- residue being somewhat greater. Our DFT calculations predict a PA for the N5 anion of C(4a)-FLHOOH of 339.7 kcal/mol whereas that of the departing HOO^- is 380.7 kcal/mol so this should not constitute a problem. However, a simple transfer of the N5−H to the proximal oxygen in a concerted fashion would require considerable bond distortion because of the distance that separates the two atoms (r_{O-H} = 2.847 Å) in GS-1 (Figure 1). Although the distal oxygen of the OOH moiety in GS-1 is closer (r_{O-H} = 2.666 Å), this would result in a structure resembling water oxide, a high-energy isomer of H_2O_2. We have shown previously that a 1,2-hydrogen shift to an adjacent lone pair of electrons is a symmetry forbidden process that has an unusually high activation barrier. For example, the 1,2 hydrogen shift in H_2O_2 ($\Delta E^{\ddagger}$ = 53.4 kcal/mol)[12a] producing the water oxide isomer has a barrier for reversion to H_2O_2 of only 3.2 kcal/mol (eq 1).[12b]



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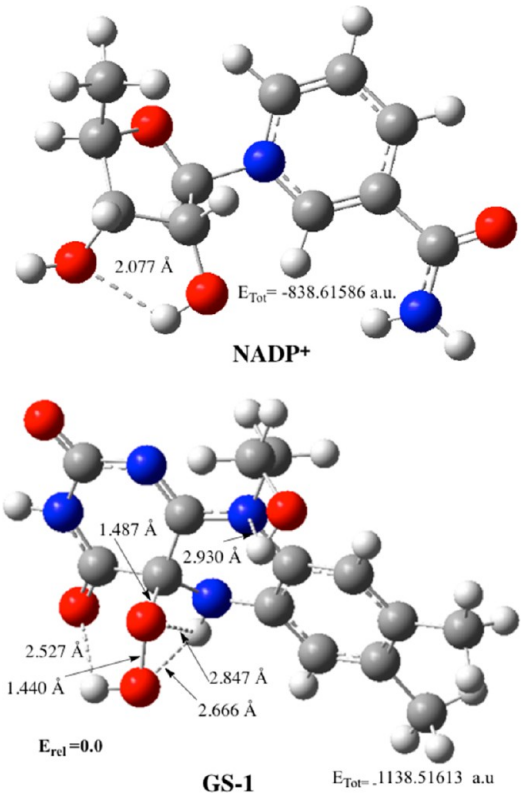

Figure 1. Ground state flavin hydroperoxide model (GS-1) and $NADP^+$ fully optimized at the B3YLP/6-311+G(d,p) level of theory. As we have done in the past[11] a β-hydroxyethyl group was used to model the ribityl side chain of native FAD.

Consistent with this reasoning, we found that the classical activation barrier for concerted elimination involving transfer of the N5−H to the proximal oxygen (TS-2) is quite high (32.86 kcal/mol; Figure 2). Since the N−H bond is only slightly elongated (r_{N-H} = 1.060 Å) and the O−H bond in the developing H_2O_2 is hardly formed, the barrier height is due largely to the extended C−O bond of 2.479 Å. When the N5−H is transferred to the distal oxygen producing a water oxide-

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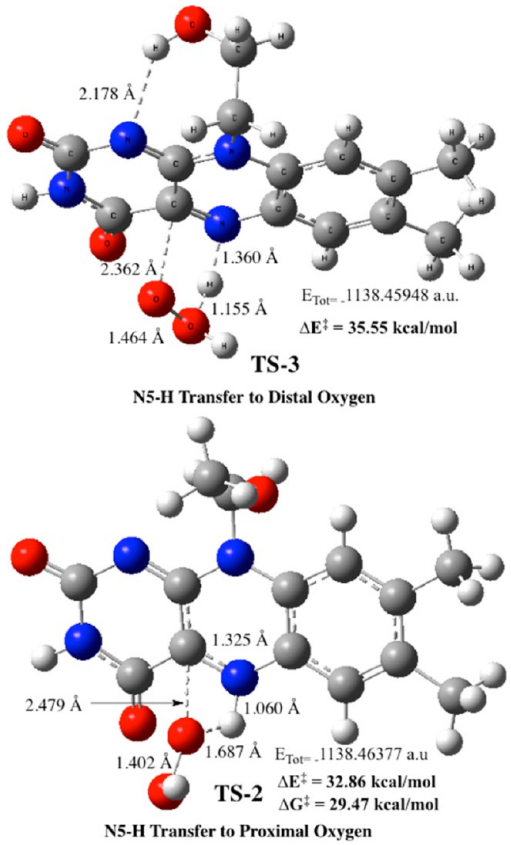

Figure 2. Transition structures for concerted transfer of the N5−H to the distal (TS-3) and proximal (TS-2) oxygens of C(4a)-FLHOOH with the elimination of H_2O_2.

like structure (TS-3), the N−H bond is more elongated (r_{N-H} = 1.360 Å) but the barrier is even greater ($\Delta E^{‡}$ = 35.55 kcal/mol). Elimination barriers of this magnitude clearly indicate that a rapid spontaneous concerted elimination of H_2O_2 is very unlikely and that C(4a)-FLHOOH should have a lifetime measured in weeks not seconds. These data suggest that we need to revise our thoughts[7] suggesting that H_2O_2 elimination in the absence of some stabilizing influence would be very rapid.

(b). Water Catalyzed H_2O_2 Elimination. Next, we examined the effect of water on the stability of gas phase FLHOOH. In GS-4a (Figure 3), the H_2O molecule is H-bonded to the N5−H (r_{O-H} = 1.961 Å) and to the adjacent C=O (1.950 Å). The activation barrier for the elimination of H_2O_2 is now markedly reduced ($\Delta\Delta E^{‡}$ = 10.68 kcal/mol) from those noted above for concerted eliminations in the absence of catalysis.

The N5−H of TS-5a is evenly transferred to the water oxygen (r_{N-H} = 1.245 Å; r_{O-H} = 1.266 Å) as the water proton shifts to the departing OOH fragment to produce H_2O_2. We also see that the C−O bond to the departing OOH fragment is quite long (r_{C-O} = 2.294 Å). The very large single imaginary frequency (ν_i 1189.8i cm^{-1}) suggests the major contribution to the reaction coordinate is light atom motion.[14] With a free energy of activation of 19.51 kcal/mol the estimated half-life of C(4a)-FLHOOH is about 22 s. This effectively demonstrates the catalytic role that even a single water molecule can play in H_2O_2 elimination from C(4a)-hydroperoxyflavin.

If a second molecule of water is included the activation barrier for H_2O_2 elimination actually increases slightly ($\Delta\Delta E^{‡}$ = 0.89 kcal/mol) although this could simply be due to the fact that we do not have the optimal juxtaposition of the two waters (the optimized structures are given in Supporting Information, Figure S1). However, when a third water molecule is included the activation barrier for H_2O_2 elimination is reduced ($\Delta E^{‡}$ = 17.11 kcal/mol) and a calculated free energy of activation of 16.24 kcal/mol suggests a half-life of only ~0.1 s. In this case, elimination of H_2O_2 involves N5−H abstraction (1.336 Å) followed by extensive C−O bond elongation in the TS (2.444 Å) and a proton relay involving all three water molecules (Figure 3). These data are consistent with experiment[13] where under pulse radiolysis conditions in aqueous solution the breakdown of C(4a)-FLHOOH into FL_{Ox} and H_2O_2 is very rapid (2.5 ms). While we have not exhaustively searched for the global minimum in this model calculation and those below, we wish to emphasize that our primary goal in this study was to demonstrate that these H-bonding interactions do result in catalysis and the absolute magnitude of the individual barriers for H_2O_2 elimination was of secondary importance. For that same reason we did not include additional water molecules in the model calculations below even though this would obviously lower the activation barrier for elimination.

(c). Alcohol Catalyzed H_2O_2 Elimination. We next examined the effect of a hydroxyl functionality on the elimination barrier using methanol as a model. In GS-6 (Figure 4) the OH group of CH_3OH is H-bonded to the N5−H and the distal oxygen of the hydroperoxide group. The activation barrier for H_2O_2 elimination ($\Delta E^{‡}$ = 24.42 kcal/mol) is greater than that shown above for one-water catalysis but involves the same type of proton shuttle from the N5−H to the alcohol oxygen that transfers its H atom to the leaving HOO^- fragment. If the OH proton is transferred to the distal oxygen instead of the proximal oxygen, as expected, the barrier increases ($\Delta\Delta E^{‡}$ = 3.60 kcal/mol; TS-8) due to the unfavorable formation of an H atom bridged between the two oxygens of the peroxide in a water oxide-like structure (see above). The predicted 12 min ($\Delta G^{‡}$ = 21.50 kcal/mol for TS-7) half-life of the FLHOOH for the alcohol-catalyzed reaction is much longer than that in a fully solvated environment (0.1 s; Figure 3). These observations are consistent with the general conclusion reached by Chaiyen and co-workers,[7b,c] who have shown that the stabilization of the C(4a)-hydroperoxyflavin intermediate in pyranose 2-oxidase critically depends on a Thr interacting with N5. Replacement of this amino acid with Ser, Ala, or Gly effectively abolished C(4a)-hydroperoxyflavin formation and/or stabilization, in agreement with the idea that deprotection of the N5−C4a locus and increased solvent accessibility facilitates H_2O_2 elimination.

(d). Altering the Basicity and Acidity of the Interacting Group. Having established the catalytic effect on the rate of C(4a)-FLHOOH decay of protic groups interacting with N5−H, we evaluated the dependency of this effect on enhanced propensities to donate and accept protons using as model groups a protonated carboxylate and a deprotonated amine, respectively. The GS complex (GS-9) for C(4a)-FLHOOH and methylamine shows the N5−H interacting with the amine nitrogen with a fairly strong H-bond (r_{N-H} = 1.985 Å; Figure 5). The increased basicity of the nitrogen renders the N5−H almost completely transferred to the NH_2 group (r_{N-H} = 1.548 Å) in TS-10. The TS comes earlier along the reaction coordinate with a C−O bond length of only 1.747 Å resulting in a lower activation energy ($\Delta G^{‡}$ = 15.18 kcal/mol). This represents a half-life of only 1.5 × 10^{-2}

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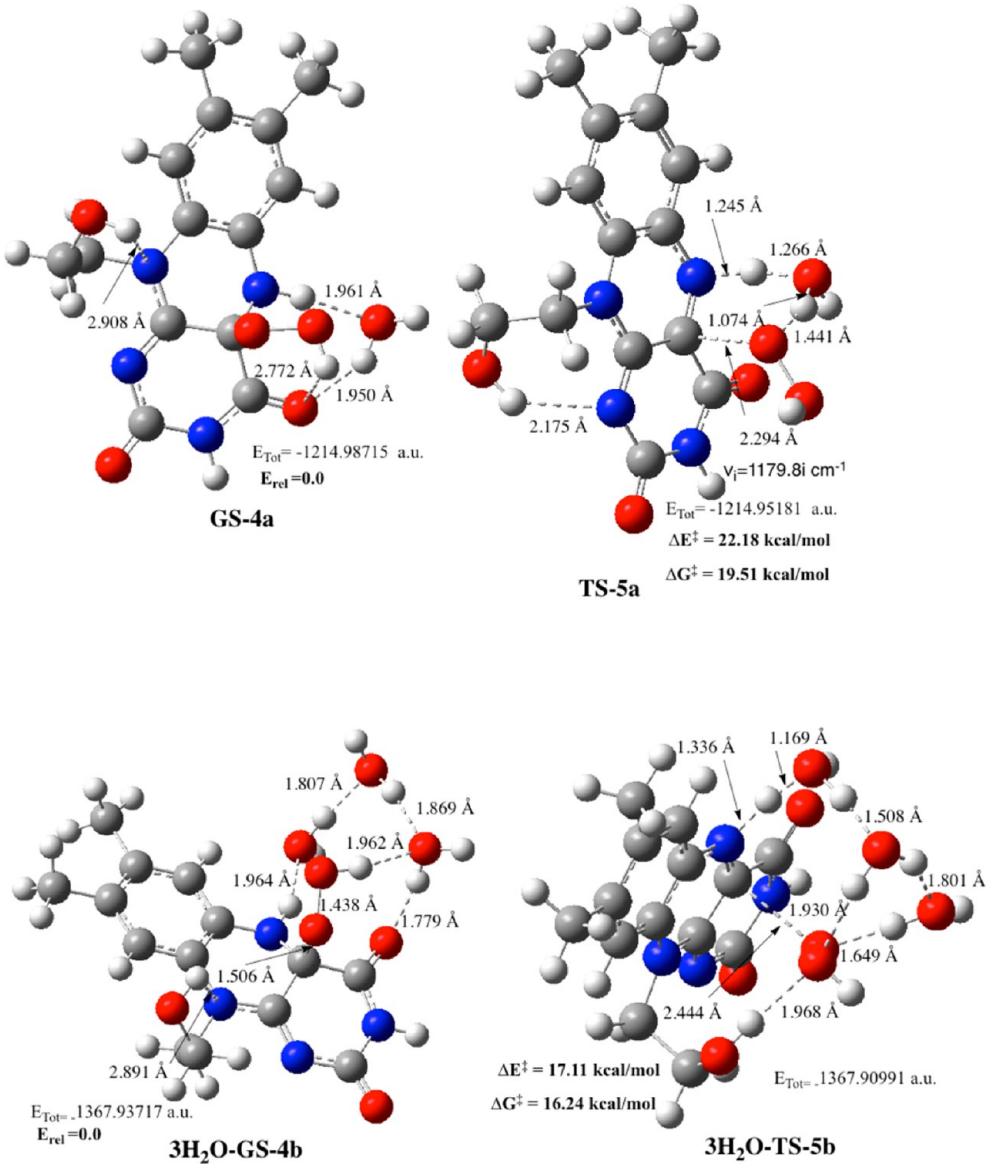

Figure 3. (a) Ground state (GS-4a, upper left) and transition structure (TS-5a, upper right) for the elimination of H_2O_2 involving a proton shuttle from one water molecule. (b) Ground state ($3H_2O$-GS-4b) and transition structure ($3H_2O$-TS-5b) for the elimination of H_2O_2 involving a proton shuttle involving three waters.

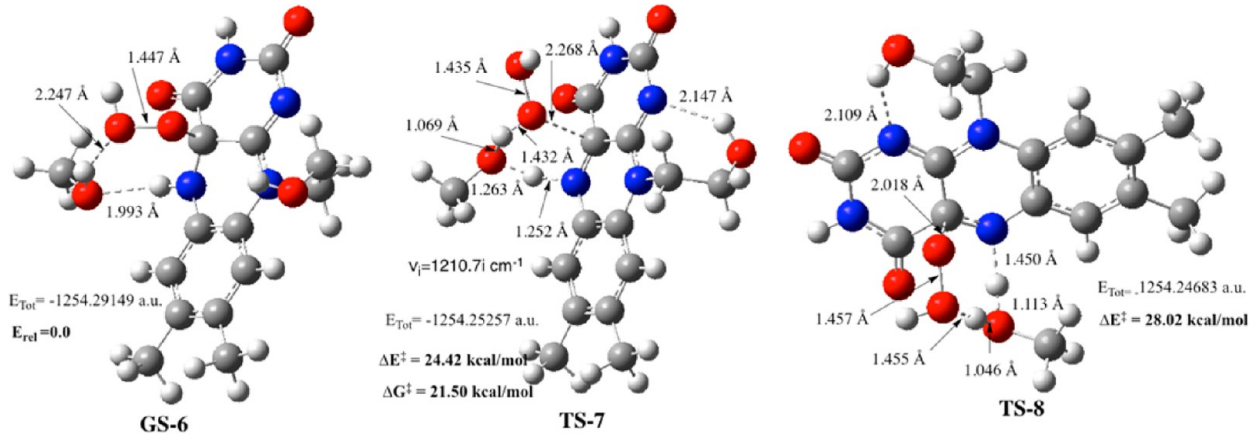

Figure 4. Ground state (GS-6) and transition structure for the N5−H transfer to the proximal (TS-7) and distal (TS-8) oxygens involving a proton shuttle from methanol to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

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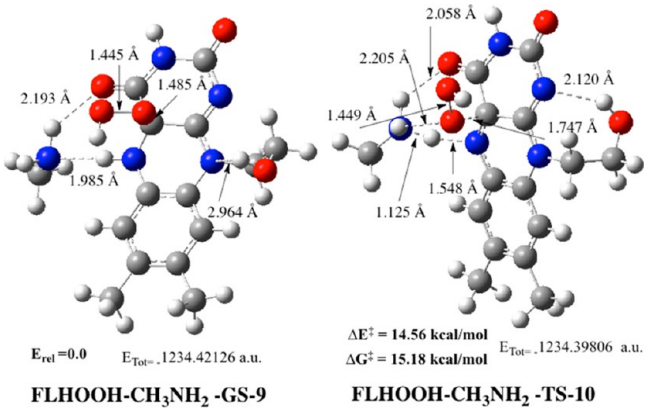

Figure 5. Ground state (GS-9) and transition structure for the N5−H transfer to the proximal (TS-10) oxygen involving a proton shuttle from methylamine to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

seconds indicating that an NH_2 functional group in close proximity and interacting with the N5−H/OOH locus should result in rapid elimination of H_2O_2.

We next addressed the effect of proton transfer directly to the OOH leaving group as the initiating interaction. The possibility exists for a carboxylic acid group to interact at the active site, and we chose acetic acid to play this role. In GS-11, the OH group of HOAc (Figure 6) is directly interacting with

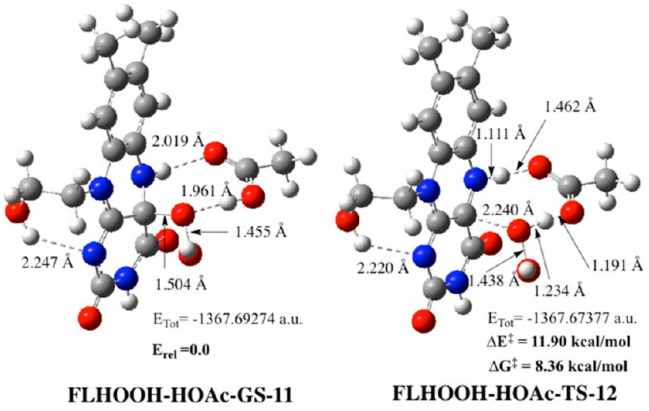

Figure 6. Ground state (GS-11) and transition structure for the N5−H transfer to the proximal (TS-12) oxygen involving a proton shuttle from acetic acid to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

the proximal oxygen of the peroxy group (r_{O-H} =1.961 Å), and its carbonyl oxygen is now acting as an H-bond acceptor to the N5−H donor. In TS-12 the COOH proton is about one-half transferred (r_{O-H} = 1.234 Å) while the N5−H bond remains nearly intact (r_{N-H} = 1.111 Å; TS-10). This leads to very low-energy TS ($\Delta G^{\ddagger}$ = 8.36 kcal/mol; Table 1) that implies that C(4a)-FLHOOH has only a fleeting existence under these conditions.

(e). Arg Catalyzed H_2O_2 Elimination. Arginine is one of the more pervasive local residues involved in enzymatic catalysis so we elected to include a guanidine group as an Arg model that could act in the same acceptor−donor fashion as noted above for HOAc. Although Arg is generally involved in its protonated state, from a simple mechanistic perspective, we also wanted to examine the effect of its charge on the barrier to

Table 1. Activation Barriers, Free Energies of Activation, and TS Single Imaginary Frequencies for H_2O_2 Elimination from C(4a)-FLHOOH

catalyst	$\Delta E^{\ddagger}$ kcal/mol	$\Delta G^{\ddagger}$ kcal/mol	frequency
none (proximal)	32.86 (TS-2)	29.47	ν_i = 368.8i cm^{-1}
none (distal)	35.55 (TS-3)		ν_i = 745.3i cm^{-1}
H_2O	22.18 (TS-5a)	19.51	ν_i = 1179.8i cm^{-1}
$2H_2O$	23.07		ν_i = 1021.0i cm^{-1}
$3H_2O$	17.11 (TS-5b)	16.24	
CH_3OH (proximal)	24.42 (TS-7)	21.5	ν_i = 1210.7i cm^{-1}
CH_3OH (distal)	28.02 (TS-8)		ν_i = 324.7i cm^{-1}
CH_3NH_2	14.56 (TS-10)	15.18	ν_i = 180.4i cm^{-1}
CH_3COOH	11.90 (TS-12)	8.36	ν_i = 881.7 cm^{-1}
neutral Arg	13.58 (TS-14)	12.15	
Arg	9.99 (TS-16)	7.59	ν_i = 339.2i cm^{-1}
$CH_3(C{=}O)NH_2$	30.85 (NH_2) (TS-18)		ν_i = 1235.3 cm^{-1}
$CH_3(C{=}O)NH_2$	19.25 (C=O) (TS-20)		ν_i = 344.6 cm^{-1}
$NADP^+$ model (NH_2)	25.63 (TS-26)		ν_i = 47.96i cm^{-1}
$NADP^+$ model (C=O, NH_2)	14.56 (TS-28)	10.1	ν_i = 949.4i cm^{-1}

H_2O_2 elimination. To determine the role of basicity on H_2O_2 elimination, as noted above for an amine residue, we include the ground state complex of C(4a)-FLHOOH with guanidine. The basic imine nitrogen in our neutral Arg model is strongly H-bonded (r_{N-H}= 1.686 Å) to the peroxide hydrogen in GS-13 (Figure 7). The transition structure for H_2O_2 elimination is initiated by N5−H bond breaking as this H atom is largely transferred to the imine nitrogen (N−H−N distances of 1.481 and 1.145 Å). The C−O bond is essentially intact (r_{C-O} = 1.535 Å) as a proton is transferred from the adjacent NH_2 group in a concerted fashion. The barrier for TS-14 is surprisingly low ($\Delta G^{\ddagger}$ = 13.58 kcal/mol) for this acceptor−

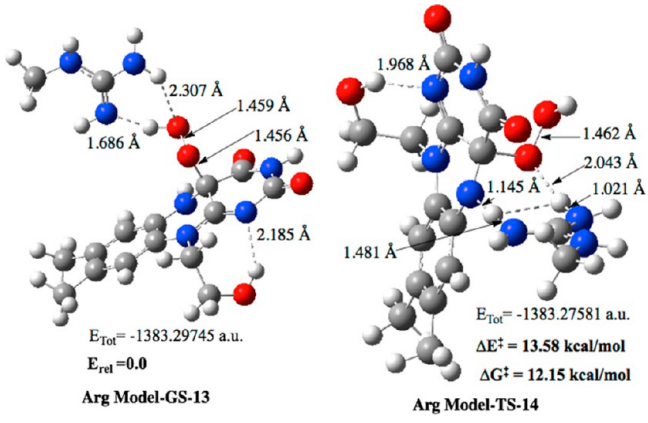

Figure 7. Ground state (GS-13) and transition structure for the N5−H transfer to the proximal (TS-14) oxygen involving a proton shuttle from the neutral Arg model to the leaving HOO^- anion to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

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donor TS that is somewhat reflective of an E2-elimination reaction where considerable negative charge is building on N5 in the TS.

Positively charged groups are known to enhance the reaction with oxygen and are often found in oxygen-reacting flavoenzymes.[1c] Consequently, we examined the effect a guanidinium group (an Arg-mimic) directly interacting with flavin N5−C(4a). The ground state complex has both oxygen atoms of the C(4a)-FLHOOH hydrogen bonded to the two NH_2 groups of the guanidinium model (GS-15; Figure 8). The

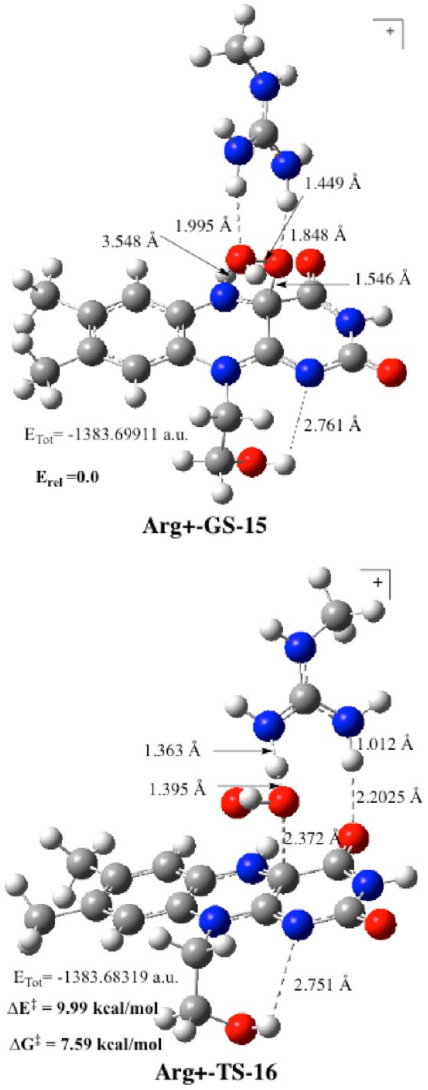

Figure 8. Ground state (GS-15) and transition structure for the N5−H transfer to the proximal oxygen (TS-16) involving a proton shuttle from the positively charged Arg to the leaving HOO^- anion to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

elimination process is peculiar in that the N5−H is not involved initially but instead a proton is directly transferred to the proximal oxygen producing H_2O_2, as noted above for TS-12 involving HOAc. While the pK_a of Arg is 12.5, the incipient HOO^- anion is still capable of abstracting a proton from the positively charged guanidinium moiety. Consistently, the calculated proton affinity of the neutral Arg model at the G3 level is 241.5 kcal/mol whereas that of the fully developed HOO^- anion is 375.6 kcal/mol. Some bonding of the protonated OOH leaving group to the flavin carbon 4a remains in the TS although the C−O bond is extensively elongated (r_{C-O} = 2.372 Å). Despite this, TS-16 has the lowest observed activation barrier noted thus far as evidenced by Table 1 and provides a very realistic example of how an Arg residue could initiate H_2O_2 elimination.

(f). Amide Group Catalysis. We finally included the amide functional group as part of our survey on interactions that may facilitate H_2O_2 elimination from C(4a)-FLHOOH. Two primary possibilities exist for catalyzing the loss of H_2O_2. In the first instance, we allowed the NH_2 group of acetamide to engage the N5−H hydrogen and the adjacent C4−C═O of the flavin. The corresponding TS-18 (Figure 9) exhibits some characteristics of the CH_3NH_2 TS-10 but has a prohibitive barrier of 30.85 kcal/mol.

The second mode of elimination is quite interesting in that the amide carbonyl oxygen can act as the base to abstract the N5−H hydrogen in concert with its NH_2 group providing the proton to the proximal oxygen to afford H_2O_2. The classical barrier ($\Delta E^{\ddagger}$ = 19.25 kcal/mol) is greater than that for the other proton shuttles noted above (Table 1) but is much reduced relative to the amide NH_2 interaction.

A large class of monooxygenases that include N-hydroxylating enzymes, flavin-containing monooxygenases, and Baeyer−Villiger monooxygenases use $NADP^+$ as the essential cofactor for C(4a)-hydroperoxyflavin stabilization. Therefore, we extended our analysis to the amide functionality bonded to a positively charged pyridinium ring (Figures 10−11). The formal charge on the ring nitrogen atom is β to the amide functional group in the same manner as that in $NADP^+$. In GS-25, the NH_2 of the amide group is H-bonded to both distal OOH oxygen (2.606 Å) and the C4 C═O (1.972 Å). Since the amide carbonyl oxygen is pointed away from the flavin, the N5−H hydrogen is not involved in the elimination process and consequently the activation barrier is prohibitively high ($\Delta E^{\ddagger}$ = 25.63 kcal/mol).

As noted above for acetamide, the carbamide carbonyl oxygen proves to be more effective than the NH_2 group in catalyzing H_2O_2 elimination. This is also evident in the present case but with a slight modification. In GS-27 the C═O moiety is H-bonded to the N5−H hydrogen in a position to effect proton transfer to the proximal hydroperoxy oxygen in TS-28 (Figure 11). As expected, the positive charge resulted in increased acidity of the NH_2 group reducing the barrier for H_2O_2 elimination to 14.56 kcal/mol. However, in TS-28 the N5−H hydrogen is only slightly elongated (r_{N-H} = 1.058 A) and the C−O bond is essentially broken (2.308 Å) closely resembling the case of the above Arg TS (TS-16) that had an even lower activation barrier ($\Delta E^{\ddagger}$ = 9.99 kcal/mol). Indeed, it is the transfer of an amine NH_2 proton (r_{N-H} = 1.272 Å) that induces elimination, and the role of the N5−H comes into play late along the reaction coordinate. With a free energy of activation of only 10.1 kcal/mol the lifetime of C(4a)-FLHOOH interacting with the pyridinium carbamide NH_2 would be measured in milliseconds rather than minutes.

(g). Complexation of FLHOOH with $NADP^+$. The different H_2O_2 elimination pathways that were identified above have to be analyzed in light of the fact that the presence of $NADP^+$ can have a stabilizing influence on C(4a)-FLHOOH, preventing H_2O_2 elimination in many enzymatic processes. A number of X-ray studies suggest that in these enzymes substrate hydroxylation takes place in the region enveloped by the nicotinamide-ribose moiety of the cofactor and the C4a-N5

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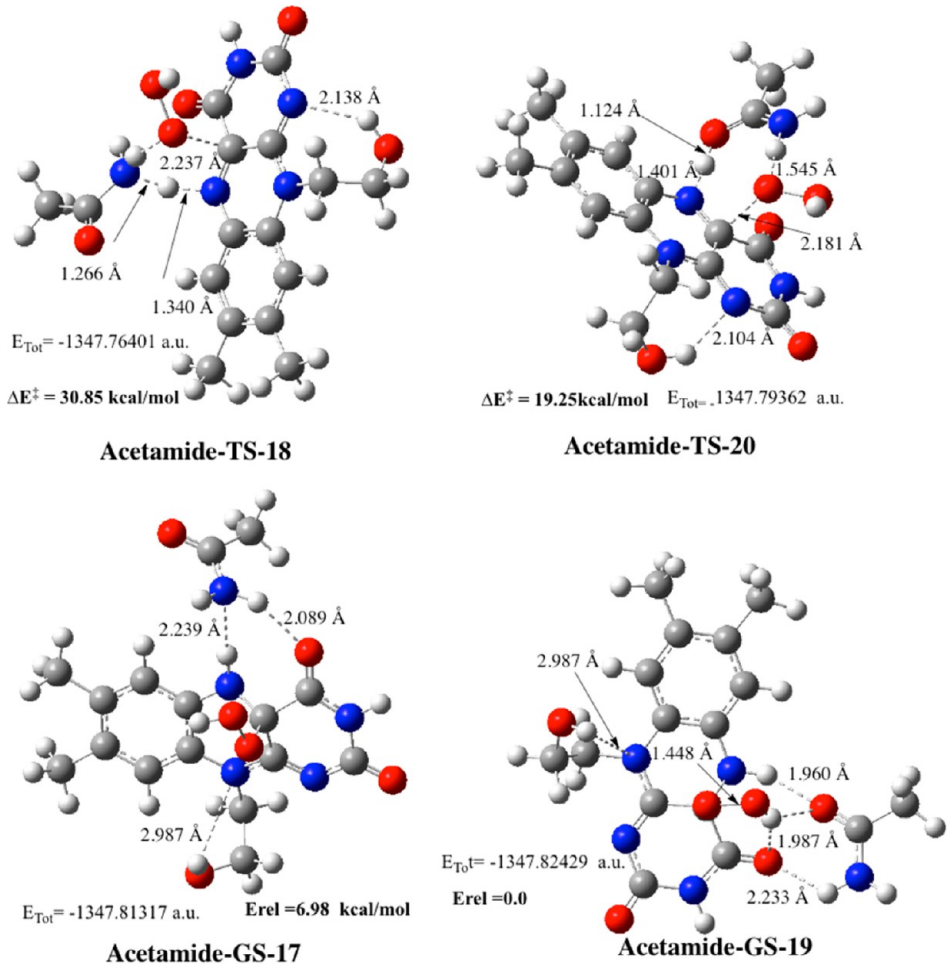

Figure 9. Ground states (GS-17 and 19) for the C(4a)-FLHOOH complex with acetamide and transition structures for the N5−H transfer (TS-18 and TS-20) in concert with a proton shuttle from the NH_2 group to the leaving HOO^- anion to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

locus of the flavin. Here, we used as a reference model the 2.3 Å resolution crystal structure of reduced ornithine hydroxylase SidA bound to $NADP^+$.[15] The nicotinamide ring is juxtaposed to the dimethylbenzyl ring of the flavin prosthetic group, with the $NADP^+$ carbamide C═O oxygen apparently H-bonded to the protonated N5−H of the reduced flavin. Such an "inward" C═O orientation and N5-carbamide interaction are expected to lead to a low-energy TS and no C(4a)-FLHOOH stabilization (TS-20 and TS-28 in Figures 9 and 11) in contrast to the opposite orientation that is unable to trigger a similar catalytic effect on C(4a)-FLHOOH decay (TS-18 and TS-26 in Figures 9 and 10). However, a point of consideration must be that the crystallographic structure refers to the active-site configuration that precedes the reaction with oxygen and formation of the C(4a)-FLHOOH adduct. Geometry optimization (without any constraints) of the $NADP^+$ complex with modeled C(4a)-FLHOOH produced a structure with the NH_2 carbamide group H-bonded to the pyrimidine ring oxygen of flavin (2.476 Å; Figure 12) which corresponds to the C(4a)-FLHOOH-stabilizing orientation of GS-17 and GS-25 (Figures 9 and 10). Even more important, the nicotinamide-ribose moiety of $NADP^+$ encapsulates the oxygen atoms of the C(4a)-FLHOOH, effectively shielding the HOO^- leaving group. This configuration can be expected to hamper proton shuttling, which, as seen above, is at the heart of all catalytic pathways, independently from the nature of the group that interacts with the N5−C(4a) locus.

■ CONCLUSIONS

These combined data require a reevaluation of some of the more generally accepted ideas concerning the manner in which C(4a)-hydroperoxyflavin is both produced and stabilized at the active site of a number of enzymatic systems. Once formed, C(4a)-FLHOOH would appear to be quite stable and therefore exhibit a rather extensive lifetime. A concerted elimination of H_2O_2 in the absence of a protic source has an activation barrier for H_2O_2 elimination far in excess of that which would allow a facile or spontaneous elimination. Elimination of H_2O_2 with a reasonable rate of reaction requires some form of a proton shuttle in order to effect a proton transfer to the HOO^- fragment to produce a neutral H_2O_2 product. While all of the functional groups that we have examined can effectively stabilize the hydroperoxide, they also can catalyze the elimination of H_2O_2, and consequently the order in which such groups interact with the OOH moiety can first potentially assist in its formation and then in a different mode of interaction also catalyze H_2O_2 elimination. We have observed two fundamental modes of H_2O_2 elimination from C(4a)-FLHOOH. The major pathway involves a proton shuttle with extensive N5−H elongation in concert with a proton shift to

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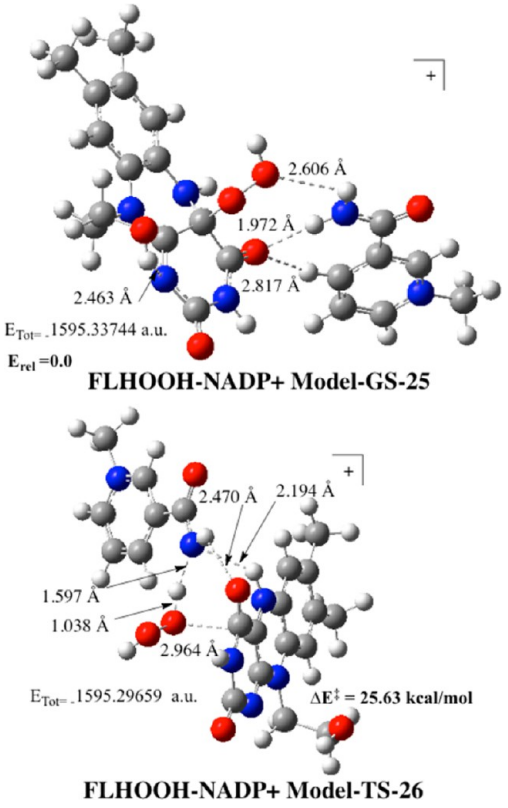

Figure 10. Ground state (GS-25) for the FLHOOH complex with the *N*-methyl pyridinium amide and transition structure for the NH_2 transfer to the HOO group (TS-26) in concert with C−O bond elongation to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

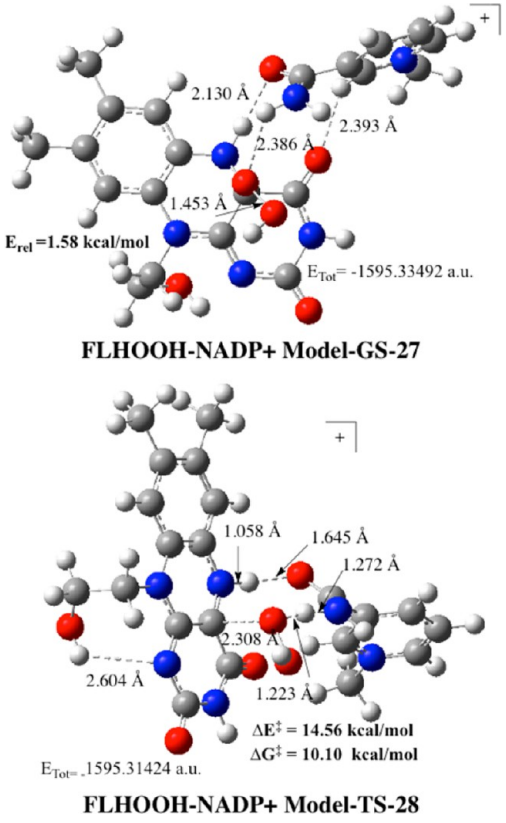

Figure 11. Ground state (GS-27) for the FLHOOH complex with the *N*-methyl pyridinium amide and transition structure for the NH_2 transfer to the HOO group (TS-28) in concert with N5−H hydrogen transfer to the amide carbonyl oxygen and C−O bond elongation to effect the elimination of H_2O_2 from C(4a)-FLHOOH.

the departing HOO^- leaving group to produce H_2O_2 and oxidized flavin (Scheme 1). These data are consistent with the mechanism for H_2O_2 elimination from C(4a)-FLHOOH suggested by Sucharitakul and co-workers[7a] involving a proton transfer from the flavin N5−H but must also involve a proton shuttle from some form of catalyst to protonate the departing OOH anion. The second pathway occurs with a protonated carboxylic acid or positively charged protic functionalities exemplified by Arg. The initiating interaction is first a proton transfer to the proximal peroxy-oxygen with extensive C−O bond elongation and very little N5−H involvement until late along the reaction coordinate.

The above mechanistic protocol is predicated on the assumption that the C(4a)-FLHOOH is fully formed and that the N5/OOH locus is accessible to local residues at the active site. The presence of a substrate and its complexation with the OOH moiety of C(4a)-FLHOOH would tend to block some of the essential proton shuttle requirements for H_2O_2 elimination. These concepts are in agreement with our recently disclosed alteration in how we perceive oxygen atom transfer from C(4a)-FLHOOH to proceed. We no longer invoke an S_N2-like attack of the nucleophilic substrate on the O−O bond of the hydroperoxide.[8,11] We have suggested a new mechanism[11a] for flavoprotein monooxygenase oxidation involving a concerted homolytic O−O bond cleavage in concert with hydroxyl radical transfer from the C(4a)-hydroperoxyflavin rather than an S_N2 displacement by the substrate on the OOH group. We have suggested that such novel rearrangements can be operating in systems as simple as

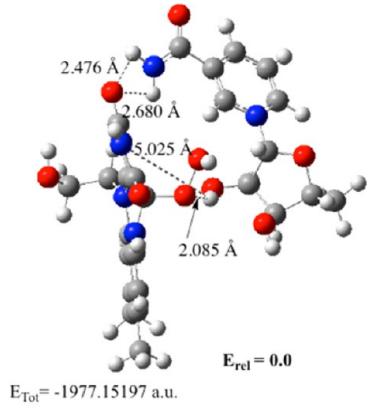

Figure 12. Fully optimized complex between reduced flavin and $NADP^+$ without geometry constraints at the B3LYP/6-311+G(d,p) level. Generated starting from the coordinates of PDB entry 4b65.[16]

Fenton oxidation[16a] or as complex as cytochrome P450 hydroxylation.[16b]

■ COMPUTATIONAL DETAILS

Quantum chemistry calculations were carried out using the Gaussian 09 program[17] system utilizing gradient geometry optimization.[18] In each case the ground states (**GS**) transition structures (**TS**) were fully optimized without geometry constraints. Calculations were performed using the B3LYP hybrid density functional[19] with a 6-311+G(d,p)

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basis set for all atoms. In prior papers[16] we have attempted to use the more recently developed DFT functionals MO6−2X and MPW1K[20] but have experienced erratic behavior when the O−O bond is being broken. The MPW1K activation barriers were consistently higher than the B3LYP, QCISD(T), or CCSD(T) barrier as discussed previously.[16] Each full structure optimization was followed by a complete frequency analysis. The activation barrier for the addition step was calculated relative to the energy of the fully optimized ground state (GS) complex.

■ ASSOCIATED CONTENT

Supporting Information

Total energies and Cartesian coordinates for all structures are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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