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An N1–Hydrogen Bonding Model for Flavin Coenzyme

Fengli Guo, Bryan H. Chang and Carmelo J. Rizzo*

Department of Chemistry and Center for Molecular Toxicology, Vanderbilt University, VU Station B 351822, Nashville, TN 37235-1822, USA

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Abstract—A model flavin possessing a specific hydrogen bond to the N1-position has been synthesized. The redox potential has been measured in aqueous buffer and found to be shifted +21 mV as compared to a similar flavin lacking this hydrogen bond. The reaction of the N1–hydrogen-bonding model with sulfite and 1-benzyl-dihydronicotinamide were examined and compared with the non-hydrogen-bonded flavin. The N1-hydrogen bond did not accelerate the rate of sulfite ion or hydride addition to N5, however the N5–sulfite complex was stabilized by nearly 4-fold over a non-hydrogen-bonding model. The model flavins were also studied computationally.

The mechanism by which the protein environment influences the redox and catalytic properties of flavin coenzyme are poorly understood.[1] Specific interactions between the protein and the flavin such as hydrogen bonding are believed to be important in defining the reactivity of a particular flavoenzyme. Massey and Hemmerich suggested one criterion by which flavoenzymes could be classified pertained to the presence of a specific hydrogen bond to the N1- or N5-position. Hydrogen bonding to N1 activates the flavin cofactor to addition reactions directly to N5 while a hydrogen bond to N5 activates the 4a-position.[2]

A number of flavin models have been developed to study how specific hydrogen bonds to the isoalloxazine ring system influences its chemistry. These include elegant host–guest systems to examine the influence of hydrogen bonding to the pyrimidine portion of the isoalloxazine ring system as well as models designed to have a specific hydrogen bond to N5.[3,4] We have prepared flavin model **1** to probe the influence of a specific hydrogen bond to the N1-position (Fig. 1). Flavin **2** served as a reference compound, since it will have similar electronic[5] and conformational effects[6] as **1**, however is not capable of forming an intramolecular hydrogen bond.

Flavins **3a**–**c** and **4** were previously developed as N1–hydrogen bonding models (Fig. 1). Müller examined **3a** using vapor phase UV photoelectron spectroscopy and showed that the π- and σ-orbital framework of **3a** are significantly altered by N1–hydrogen bonding.[7] Shinkai subsequently examined related models **3b** and **c** in solution and found that the appended hydrogen-bonding group had no effect on their chemistry.[8] Model **4**, also studied by Shinkai, was only mildly effective as an N1–hydrogen bonding model.[8] Molecular modeling and X-ray crystallographic analysis showed that the N10-aryl group is orthogonal to the flavin ring system, and is therefore not in the optimal geometry for hydrogen bonding to N1 in the oxidized state.

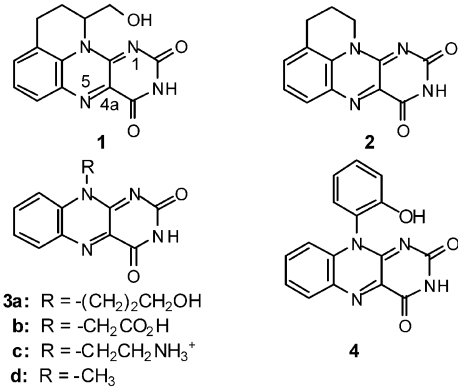

Figure 1. N1–Hydrogen bonding models of flavin coenzyme.

*Corresponding author. Fax: +1-615-343-1234; e-mail: c.j.rizzo@vanderbilt.edu

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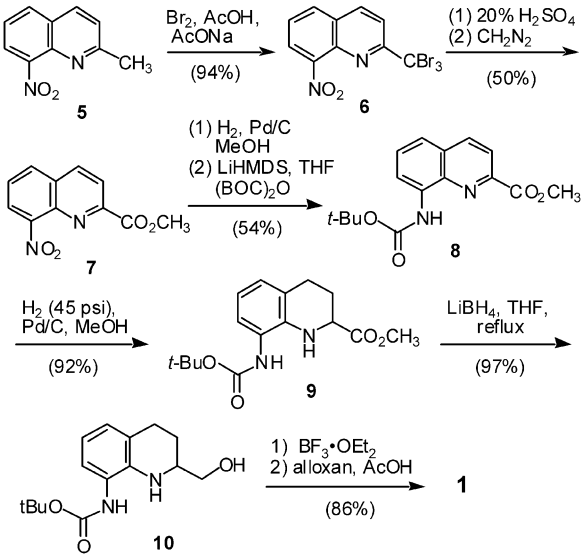


Scheme 1. Synthesis of N1–hydrogen-bonding flavin model.

The synthesis of **1** is outlined in Scheme 1 and begins with the bromination of 8-nitroquinaldine (**5**) to give **6**. Hydrolysis and esterification provided methyl ester **7** in 50% overall yield.[9] The nitro group was then reduced and protected as the BOC-derivative (**8**). At this stage, the quinoline ring was reduced under 45 psi hydrogen pressure over Pd. When the reduction of the nitro group and quinoline was attempted in a single step, low yields of the desired product were obtained, possibly due to poisoning of the catalyst by the 8-amino-tetrahydroquinoline product. Reduction of **9** with lithium borohydride in THF at reflux gave alcohol **10** in high yield. Optimal conditions for the removal of the BOC-group involved treatment of **10** with boron trifluoride etherate in CH_2Cl_2.[10] The product from the deprotection was condensed without purification with alloxan in glacial acetic acid to give **1** in 86% overall yield.[11]

The redox potentials of flavins **1** and **2** (Table 1) were measured by cyclic voltammetry in 100 mM, pH 7.4 HEPES buffer using a standard three-electrode cell with a glassy carbon working electrode. The redox potential of **1** was found to be shifted +21 mV (0.5 kcal/mol) from that of **2**. We attribute this shift to specific hydrogen bonding to the N1-position. The redox potential of **1** under these conditions, was nearly that of the unsubstituted flavin **3d**.[6] Our data should be contrasted to that of Frerman and co-workers who examined the redox chemistry of a bacterial electron-transfer flavoprotein (ETF) from *Paracoccus denitrificans*.[12] This particular ETF possesses a rare intramolecular hydrogen bond from the C4′ hydroxyl group of the ribityl chain to the flavin N1-position (Fig. 2).[13] The ETF was reconstituted with an unnatural 4′-deoxy-FAD, which presumably eliminated the critical hydrogen-bonding interaction. As a result of this modification, the oxidized-semiquinone couple was destabilized by 116 mV (2.7 kcal/mol), over 5-fold greater than we observed for the two electron reduction of model **1**. Zhou and Swenson examined the role of electrostatic interactions on the redox potential of a bacterial flavodoxin from *Desulfovibrio vulgaris*.[14] Mutant proteins in which aspartate and glutamate residues near the FMN binding site were systematically neutralized resulted in less negative midpoint potentials for the semiquinone/hydroquinone couple; the oxidize/semiquinone couple was not significantly influenced by the changes. The calculated electrostatic surface potential of the ETF from *P. denitrificans* was found to be highly negative.[13] Eliminating the N1–hydrogen bond would greater expose the one-electron reduced semiquinone anion to the electrostatic field. Thus, electrostatic may play a significant role in the destabilization of the semiquinone in the reconstituted ETF lacking the N1–hydrogen bond. It was also reported that the long wavelength absorbance of the ETF reconstituted with the unnatural cofactor was red shifted 7 nm versus that of the native protein (443 vs 436 nm). However, we observed the opposite behavior for model **1** where the presence of the hydrogen bond caused a 4 nm red shift relative to **2**.

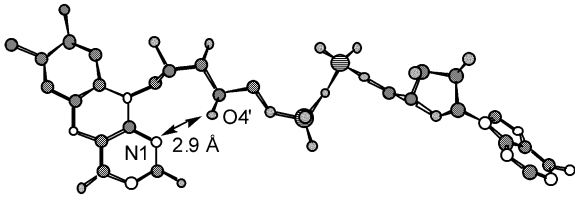


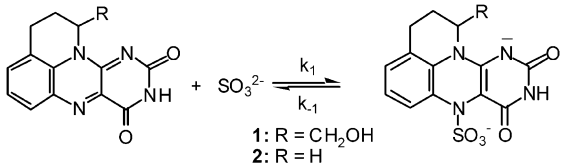
Figure 2. Crystal structure of the FAD cofactor of an electron-transfer flavoprotein (ETF) at 2.6 Å resolution.

We have examined two reactions of flavins **1** and **2**, the addition of sulfite and the oxidation of the NAD(P)H analogue 1-benzyl-dihydronicotinamide (Tables 2 and 3). These reactions involve a direct nucleophilic addition to N5 and should be accelerated by hydrogen bonding to the N1-position. We observed that the formation of the sulfite–flavin complex was approximately four times more favorable for N1–hydrogen bonding model **1** than for **2** which lacks this hydrogen bonding interaction.[15]

Table 1. Midpoint potential (mV) and UV data of flavin models in 100 mM, pH 7.4 HEPES buffer at room temperature. $E^{\circ\prime}$ versus Ag/AgCl (−197 mV vs NHE), scan rate = 20 mV/s

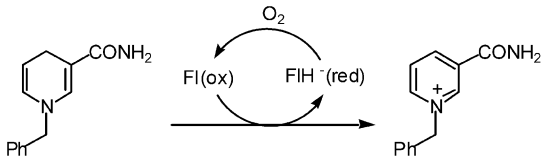
Flavin	E_p^a	E_p^c	$E^{\circ\prime}$	λ (ε)
1	−446	−376	−411	440 (8400), 372 (9100), 265 (30,000)
2	−450	−414	−432	436 (6300), 370 (7000), 265 (23,300)
3d	−428	−386	−407	429 (6500), 346 (4800), 261 (21,300)

Table 2. Reaction of flavins 1 and 2 with sulfite in 100 mM, pH 7.4 phosphate buffer at 30 °C



Flavin	K_d (M)	k_1 (min^{-1} M^{-1})	k_{-1} (min^{-1})
1	0.371 ± 0.019	0.493 ± 0.012	0.183 ± 0.007
2	1.47 ± 0.173	0.443 ± 0.343	0.651 ± 0.504

Table 3. Oxidation of 1-benzyldihydronicotinamide by flavins **1** and **2** in 100 mM, pH 7.4 phosphate buffer



Flavin	k_1 (min^{-1} M^{-1})	
	30 °C	15 °C
1	1494±47	646±32
2	1541±58	612±34
3d	2576±109	928±34

Interestingly, the rate of the sulfite addition reaction was not accelerated by the presence of the N1– hydrogen bond.

The oxidation of 1-benzyl-dihydronicotinamide was examined under aerobic conditions.[16] The produced flavin hydroquinone is quickly reoxidized under these conditions thus satisfying a first order equation; dihydronicotinamides are not oxidized under these conditions. Again, no significant increase in rate was observed for the oxidation of 1-benzyl-dihydronicotinamide by **1** as compared to **2** at 30 or 15 °C. We also examined flavin model **3d**, which has a similar redox potential as **1**. This ensured that flavins with relatively small differences in redox potential would give measurable differences in reaction rates.

Hydrogen bonding effects should be enhanced in organic solvents. Shinkai reported the reaction of N1–hydrogen bonding model **4** with 1-benzyl-dihydronicotinamide in acetonitrile, although at a considerably slower rate than in aqueous buffer. Reactions with non-hydrogen bonding substrates were not observed. Unfortunately, we were unable to observe a satisfactory decay curve for the reaction of **1** or **2** with 1-benzyl-dihydronicotinamide in acetonitrile.

We have examined the conformation of **1** using a variety of computational methods. Molecular mechanics and AM1 calculations were used to obtain starting geometries for a hydrogen-bonding (**1a**) and non-hydrogen-bonding conformation (**1b**). These conformations were then optimized using the B3LYP/6-31G* hybrid density functional method as implemented in Gaussian 98W;[17] the optimized conformations are shown in Figure 3. This analysis predicts the hydrogen-bonding conformation to be favored by greater than 5 kcal/mol. These calculations did not include solvent. The calculated N1–H and N1–O distances are 1.98 and 2.78 Å, respectively, and the N1–H–O angle is 136.6°. The N1–H and N1–O distances are well within hydrogen-bonding range and compare favorably with the N1–O4′ distances found in *P. denitrificans* ETF crystal structure (2.89 Å). The N1–H–O angle of **1**, however, is significantly smaller than optimal. In addition, the proton involved in the critical hydrogen bonding interaction is displaced 0.58 Å from the best plane comprised of the six-ring atoms of the pyrimidine ring.

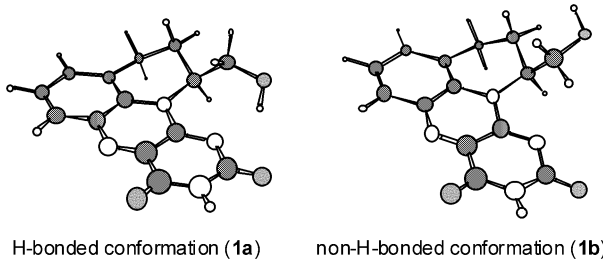


Figure 3. Hydrogen-bonded and non-hydrogen-bonded conformations of flavin **1**.

A recent computational study of some 27 oxidized flavin models showed the two-electron reduction potential is linearly correlated with the energy of their LUMO (R = 0.96);[18] the experimentally determined reduction potentials of the flavins varied over a 300 mV range while the computed LUMO energies spanned about 1 eV. The computed FMO energies for our flavins are listed in Table 4.[19] Although we can not determine the redox potential of the non-hydrogen-bonding conformation **1b**, we would expect it to be similar to that of **2**. We find that the calculated LUMO energies of the non-hydrogen-bonding conformation (**1b**) and **2** are in reasonable agreement. The experimentally determined reduction potential of **1** is similar to that of flavin model **3d** (−411 vs −407 mV vs Ag/AgCl). The calculated LUMO energies of the hydrogen-bonding conformation **1a** is very close to that of **3d** in agreement with similar $E^{\circ\prime}$ values. The reduction potential of **1** would be dependent on the equilibrium of conformations **1a** and **1b**. We have not been able to determine this value at this time, however the computational results lead us to believe that the hydrogen-bonding conformation **1a** is the predominant form.

The geometry of reference flavin **2** with a molecule of water hydrogen bonded to the N1-position was also computed (Fig. 4). The geometry of the hydrogen-bonded state is not as favorable as that in **1a** due to steric

Table 4. B3LYP/6-31G* calculated FMO energies of flavin models

Flavin	HOMO (eV)	LUMO (eV)
1a	−6.606	−3.095
1b	−6.410	−2.967
2	−6.382	−2.890
2·H_2O	−6.506	−3.004
3d	−6.508	−3.012

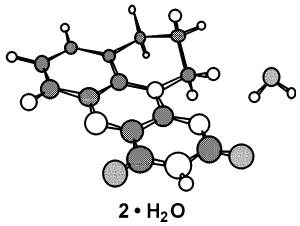


Figure 4. B3LYP/6-31G* optimized geometry of model **2** hydrogen bonded to a water at N1.

interactions with the rigidly held N10-methylene group. The calculated N1–H and N1–O distances are 2.16 and 3.03 Å, respectively, and the N1–H–O angle is 147.9°. The calculated LUMO energy of **2·H$_2$O** is intermediate to that of **2** and **1a**. Since solvent may be hydrogen bonded to **2**, the effect of hydrogen bonding in **1** may be underestimated when compared to **2**.

In summary, we have synthesized N1–hydrogen-bonding model **1** and compared its redox chemistry with related model **2** which lacks the N1–hydrogen-bonding interaction. A shift in reduction potential and a significant stabilization of the N5–sulfite complex indicates the presence of the desired hydrogen bond. Contrary to previous predictions, no measurable rate enhancement for the addition of nucleophile to N5 was observed. Deprotonated dihydroflavins are often drawn as the N1-anion, however this charge is actually delocalized into both pyrimidine carbonyls. The B3LYP/6-31+G* optimized geometry of dihydro-lumiflavin anion predicts nearly equal distribution of the negative charge on N1 and both carbonyl oxygens.[20] Flavoproteins often show hydrogen bonds to these carbonyl groups. A bifurcated hydrogen bond to N1 and the C2–carbonyl is common. Hydrogen bonding to the C2 and C4 carbonyl groups may play a significant role in activation of N5 toward nucleophilic addition.

Acknowledgements

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References and Notes

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19. In addition to **1**, **2** and **3d**, the optimized geometries of nine other oxidized flavin analogues were calculated (B3LYP/6-31G*). The calculated LUMO energies of these eleven flavins was found to be linearly correlated with the published two-electron reduction potentials ($R = 0.977$) as previously observed. Details of our study will be reported elsewhere.
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