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Model Systems for Flavoenzyme Activity. 2-Aminopyridines as Spectroscopic Models for Flavoenzyme Active Sites

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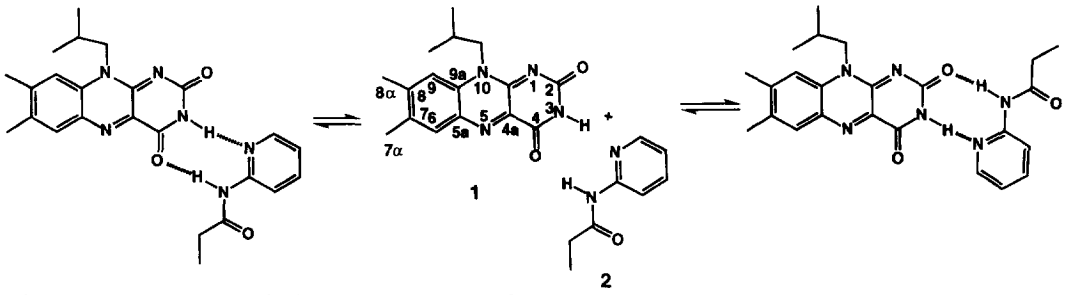
Abstract: *Acylated 2-aminopyridines provide a model for specific flavoenzyme-cofactor interactions, allowing isolation and observation of the effects of hydrogen bonding on flavin NMR. We describe here the use of one of these receptors to determine the relative hydrogen bond affinities of O(2) and O(4) of the flavin. Additionally, the receptor allows the effects of hydrogen bonding at O(2) and O(4) on the electron distribution in the flavin nucleus to be determined using ^{13}C NMR.*

Flavoenzymes are proteins that use the $FADH_2$-FAD redox cycle to catalyze a variety of biological redox transformations,[1] including the oxidation of amines to imines, the dehydrogenation of lipid esters and amides, hydroxylation of aromatic substrates, and the oxidation of thiols to disulfides. They also serve as electron transfer proteins, mediating between obligate one-electron (i.e. heme-Fe, FeS-cluster) and two-electron redox cofactors (i.e. NADH).

In flavoenzymes, enzyme-cofactor interactions such as hydrogen bonding are responsible for determining the redox-properties of enzyme-bound flavin.[2] Variations in the electron density distribution of the ring system in the fully oxidized flavin are an important determinant of enzyme mechanism.[3] Since there is an essentially linear correlation between ^{13}C shift and π-electron density,[4] an important tool for determining electron density distribution in enzyme-bound flavins is ^{13}C NMR spectroscopy of the apoenzymes reconstituted with isotopically labeled flavins.[5] Because the effect of hydrogen bonding, π-stacking and protein environment (dipolar interactions) on the π-electron density are additive, direct attribution of chemical shift changes to specific interactions is often not possible with the enzymatic systems.

In previous research, we have shown that diaminopyridines hydrogen bond selectively to the O(2), N(3) and O(4) positions of flavins in aprotic media.[6] This binding was accompanied by changes in the ^{13}C NMR of the bound flavin. Use of this system to probe the effects of binding at specific positions, however, was limited by the simultaneous binding at O(2) and O(4). We report here the use of the flavin **1**-2-aminopyridine **2**[7] complex (Scheme 1) to determine the relative hydrogen bond affinities of O(2) and O(4) of the flavin, as well as the effects of specific hydrogen bonds on ^{13}C NMR shifts, and hence electron distributions, within the flavin nucleus.



Scheme 1. Binding modes in the flavin 1-receptor 2 complex

Complexation between flavin 1 and receptor 2 was established using ^1H NMR spectroscopy. These studies were performed in a non-competitive solvent ($CDCl_3$), to allow observation of specific hydrogen bond interactions, and effectively model flavoenzyme active site hydrophobicity. Addition of aliquots of receptor 2 to flavin 1 resulted in a steady downfield movement of H(3) of the flavin, indicating formation of the hydrogen bound complex. Non-linear least-squares curve fitting[8] of the shift values provided a combined binding constant for both recognition modes of 21 M^{-1}.

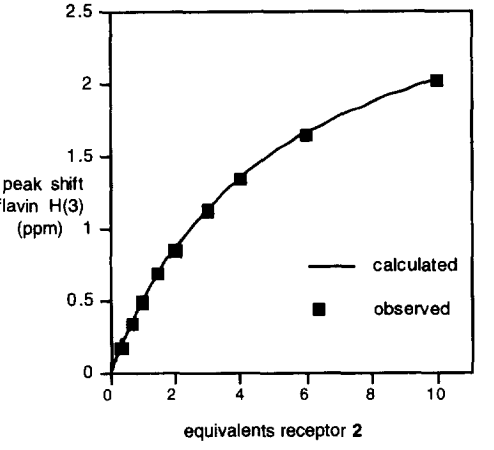


Figure 1. NMR titration of flavin 1 with receptor 2 in $CDCl_3$ at 298K, concentration of flavin 1 = 10^{-3}M. Solid line represents the calculated 1:1 binding isotherm.

Hydrogen bond interactions between flavin and apoenzyme are important determinants of flavoenzyme activity. NMR spectroscopy of ^{13}C labeled flavins bound to various flavoenzymes has been employed extensively to quantify the modulation of the cofactor by the protein.[9] Subtle effects of enzyme-cofactor hydrogen bonding interactions can be explored using this technique. In previous studies, the interpretation of the spectra obtained from flavoproteins has relied heavily on NMR data of unbound flavins in protic and aprotic media. The use of unbound flavins as benchmarks for interpreting hydrogen bonding interactions has two drawbacks. First, the change in dielectric constant of the solvents complicates the interpretation of chemical shift changes. Second, the use of a hydrogen-bonding solvent such as water does not allow specific hydrogen bond patterns to be studied individually.

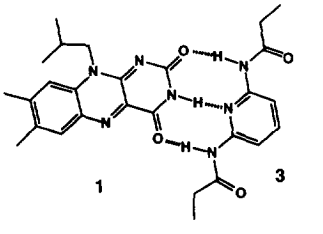


Figure 2. Flavin 1-receptor 3 complex.

To provide a system that maintains constant solvent dielectric, and allows observation of specific hydrogen bonding interactions, we have studied the ^{13}C NMR of the flavin 1-receptor 2

complex.[10] Table 1 summarizes the observed changes in peak position of the flavin **1** ^{13}C resonances upon addition of receptor **2**. For comparison, the peak positions of the flavin **1**-receptor **3**[11] (Figure 2) complex and FMN in water[12] are listed as well.

Table 1. ^{13}C NMR Shifts of Free and Host-Bound Flavins

	Chemical Shift (ppm)					
Atom	Flavin **1**[a,b]	**1**+ Host **2**[b,c]	$\delta_{1+2}-\delta_1$	**1**+ Host **3**[b,c]	$\delta_{1+3}-\delta_1$	FMN[d]
C(2)	154.99	156.96	1.97	156.31	1.32	159.8
C(4)	159.55	160.34	0.80	160.90	1.35	163.7
C(4a)	136.17	136.45	0.28	135.90	-0.24	136.2
C(5a)	134.94	134.96	0.02	135.18	0.24	136.4
C(6)	132.88	132.87	-0.01	132.93	0.05	131.8
C(7)	136.99	136.82	-0.17	137.28	0.29	140.4
C(7α)	19.48	19.45	-0.03	19.48	0.00	19.9
C(8)	147.98	147.67	-0.31	148.38	0.40	151.7
C(8α)	21.69	21.64	-0.05	21.76	0.07	22.2
C(9)	115.84	115.82	-0.02	115.91	0.07	118.3
C(10a)	150.81	150.91	0.10	150.82	0.01	152.1

a) in $CDCl_3$, [**1**]= 10^{-2}M, 296°K; b) δ values ± 0.01 ppm c) in $CDCl_3$, [**1**]=10^{-2}M, [**2**]=10^{-2}M; d) in H_2O, pH 8.[9]

There is a strong downfield movement in the resonances of the carbonyl carbons C(2) and C(4), resulting from hydrogen bond formation at these positions. These movements are between one half and one third of the values observed with FMN in water. Comparison of these shift changes with those of receptor **3** (which provides essentially equivalent binding at O(2) and O(4)) indicates an approximate binding preference of 2.5:1 for O(2) versus O(4). This model system provides the first experimental evidence for this binding preference, which is in accord with the greater calculated (AM1) electrostatic potential at O(2) (-0.519) compared with O(4) (-0.471).

The marked binding preference for O(2) versus O(4) allows the effects of hydrogen bonding at these two sites on the ^{13}C NMR of flavins to be determined individually through comparison with the flavin **1**-receptor **3** complex. Significant shielding upon formation of the flavin **1**-receptor **2** complex is observed in the positions C(7) and C(8). These upfield shifts indicate a considerable increase of flavin electron density at these positions resulting from hydrogen bonding at the relatively distant O(2) position. These shifts are opposite to those observed in the receptor **3**-flavin complex, indicating that binding at O(4) has a very strong deshielding effect at these positions.

An important feature observed in the flavin **1**-host **2** complex is the downfield shift of C(4a), which differs in direction from that of the flavin **1**-receptor **3** complex. This indicates that hydrogen bonding at O(2) is responsible for this deshielding effect, denoting a decrease in electron density at C(4a). This is mechanistically important, since this activates the position toward nucleophilic addition. Enzymes that react through this mechanism, including lipoamide dehydrogenase,[13] which reacts via addition of thiol to C(4a), likewise show a downfield shift of the resonance position of this

carbon. The data overall indicate that hydrogen bonding at O(2) favors two-electron processes, while bonding at O(4) disfavors these reactions.

We have designed a system that enables us to quantify the effects of specific hydrogen bonding on flavin properties. Since it employs both flavin and receptor soluble in a solvent of relatively low dielectric constant, it resembles more closely the enzyme-bound cofactor which is buried in the hydrophobic interior of the protein. The ^{13}C NMR data revealed the influence of hydrogen bond interactions at O(2) and O(4) on the π-electron density distribution in the flavin isoalloxazine ring system. The results differ in a number of respects to the previous benchmarks of flavins in protic media. These changes can be attributed to the non-specificity of the hydrogen bond interactions between FMN and bulk solvent and the change in dielectric constant of the medium.

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(10) Spectra were obtained on a Bruker MSL 300 spectrometer equipped with a 10mm broadband probe. All samples were dissolved in $CDCl_3$, and spectra acquired at 298°K. Concentrations of flavin **1** and receptor **2** were 10mM, respectively. WALTZ-16 composite pulse proton decoupling was employed with a recycling delay of 9 s to prevent saturation of the tertiary and carbonyl carbons. Typically, around 7000 scans per spectrum were acquired.

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