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Model Systems for Flavoenzyme Activity. Control of Flavin Recognition via Specific Electrostatic Interactions

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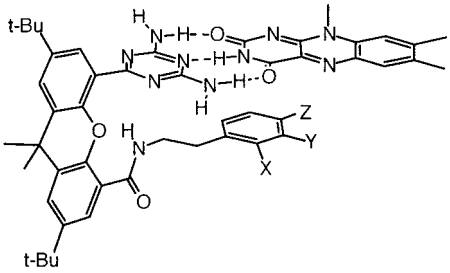
ABSTRACT



A model system has been used to study the interactions of dipole-containing aromatic systems with oxidized and reduced flavin. Ab initio computational and experimental studies show that dipole orientation within the host is a critical determinant for recognition and redox behavior of the flavin guest.

The interaction of aromatic rings with aromatic[1] and nonaromatic moieties[2] is an important motif in molecular recognition.[3] These complex interactions are composed of dipoles, quadrupoles, and higher order influences[4] that can be either attractive or repulsive in nature, differing dramatically in both their distance dependence and their directionality.[5] The numerous variables involved results in a complicated additivity of forces, making direct interpretation and prediction of these interactions difficult.

Side chain−flavin and substrate−flavin interactions are a common motif in flavoenzyme structures, with aromatic stacking[6] and aromatic−dipole interactions both prominent features. One example of the importance of specific flavin−protein aromatic stacking is found in the *Desulfovibrio vulgaris* flavodoxin (Figure 1a), where Swenson et al. have shown that the Y98F active site mutation results in a substantial change in the recognition and redox activity of the adjacent FMN cofactor.[7] In this system, as well as other flavoenzymes, both dipolar and quadrupolar interactions are possible between the electron-poor flavin and adjacent

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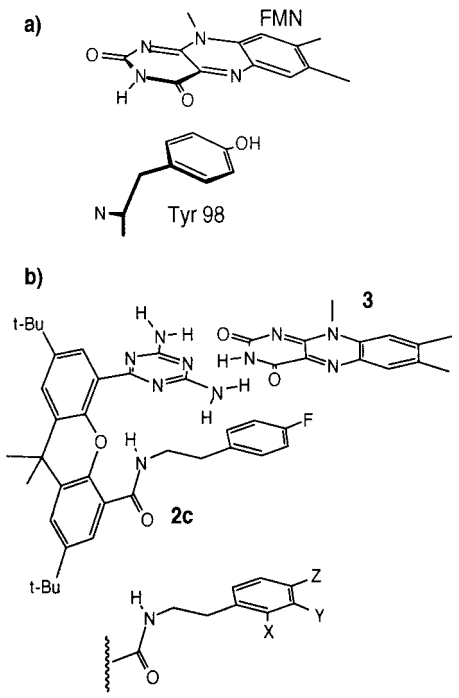

	x	y	z
1	H	H	H
2a	F	H	H
2b	H	F	H
2c	H	H	F

Figure 1. (a) Tyrosine takes part in the aromatic−dipole interaction in the FMN binding site of the *D. vulgaris* flavodoxin.[7] (b) The analogous host−guest complexes of receptors **1** and **2** with flavin $\mathbf{3}_{ox}$.

aromatic residues.[8] The orientation and nature of these intermolecular contacts determines their resultant effect on the microenvironment of the active site, providing specific recognition based on oxidation state and concomitant regulation of the redox behavior of the cofactor.

The selectivity of the apoprotein for a specific cofactor oxidation state provides the enzyme with the ability to control cofactor redox potentials. This selectivity can be partially deciphered via mutagenesis studies of intact biological systems.[9] These results, however, are difficult to attribute to a single interaction due to multiple secondary effects that arise when residues are altered.[10] Model systems that are able to focus on a single interaction thus serve as complements to biological studies.[11]

To address the issue of the roles of specific electrostatic factors on flavin−aromatic interactions, we have developed a model system that directly controls the orientation of polar aromatics in relation to the redox active flavin nucleus (Figure 1b). This model utilizes hydrogen bonding to align the flavin nucleus over aromatic groups featuring oriented dipolar functionality, without significantly changing the overall electron density of the aromatic stacking moiety. This allows the direct quantification of dipole/quadrupole−flavin energetics in reference to unsubstituted receptor **1**. Comparison of the binding of oxidized and reduced species then affords insight into the stabilizing and destabilizing effects of dipolar interactions on flavin redox properties.

Preliminary insight into the general role of dipole orientation on flavin recognition was obtained through computational analysis of the fluoro-substituted compounds **2a**−**c**. Following initial PM3 optimization of the entire $\mathbf{1}\cdot\mathbf{3}_{ox}$ complex, the imide proton of **3** and the amide proton of the receptor were constrained to the PM3-predicted distance and the resulting fragment geometry optimized at the HF 3-21G* level. The structures of the receptor−flavin complexes $\mathbf{2a}-\mathbf{c}\cdot\mathbf{3}_{ox}$ were then optimized in a similar fashion, with multiple starting points used to determine the minimum energy binding modes. To investigate the trend in the reduced species, the corresponding radical anions were likewise optimized. After ab initio geometry optimization, DFT B3LYP 6-31G* single-point calculations were used for each of the systems to obtain energies of complexation and electrostatic potential surfaces[12] (Figure 2).

In the oxidized series we see a predicted trend of increasing stability as the fluoro substituent moves from *ortho* to *para*. The structure and calculated energy difference of the receptor $\mathbf{2a}\cdot\mathbf{3}_{ox}$ fragment indicates that a dipole−dipole repulsion between the fluoro substituent and the carbonyl oxygen (O(4)) forces the aromatic moiety out of plane with the flavin nucleus. The interaction of the *meta*-substituted receptor **2b** with flavin is energetically more favorable than that of receptor **2a**, due in part to the interaction of the fluorine atom with the weakly positive N(10) position of the flavin ring system.

The strongest favorable computationally predicted interaction is seen for the *para*-substituted receptor **2c**. In this receptor, a strong donor atom−π interaction can be seen between the fluoro substituent and the highly electropositive portion of the flavin.[13]

A similar trend is seen in the reduced series of host−guest species. A pronounced repulsion is seen in the *ortho*-substituted dyad $\mathbf{2a}\cdot\mathbf{3}_{red}$ as compared to the oxidized system, evidenced by a "swinging out" of the phenyl ring from underneath the flavin ring system. This loss of aromatic stacking is also observed for the *meta*- and *para*-substituted scaffolds, indicating similar repulsion of the phenyl ring from the flavin radical anion. Some stability is gained in the *para*-substituted dyad through two edge-to-face hydrogen bond interactions between the phenyl hydrogens and flavin carbonyl O(2), thus generating the most stable complex.

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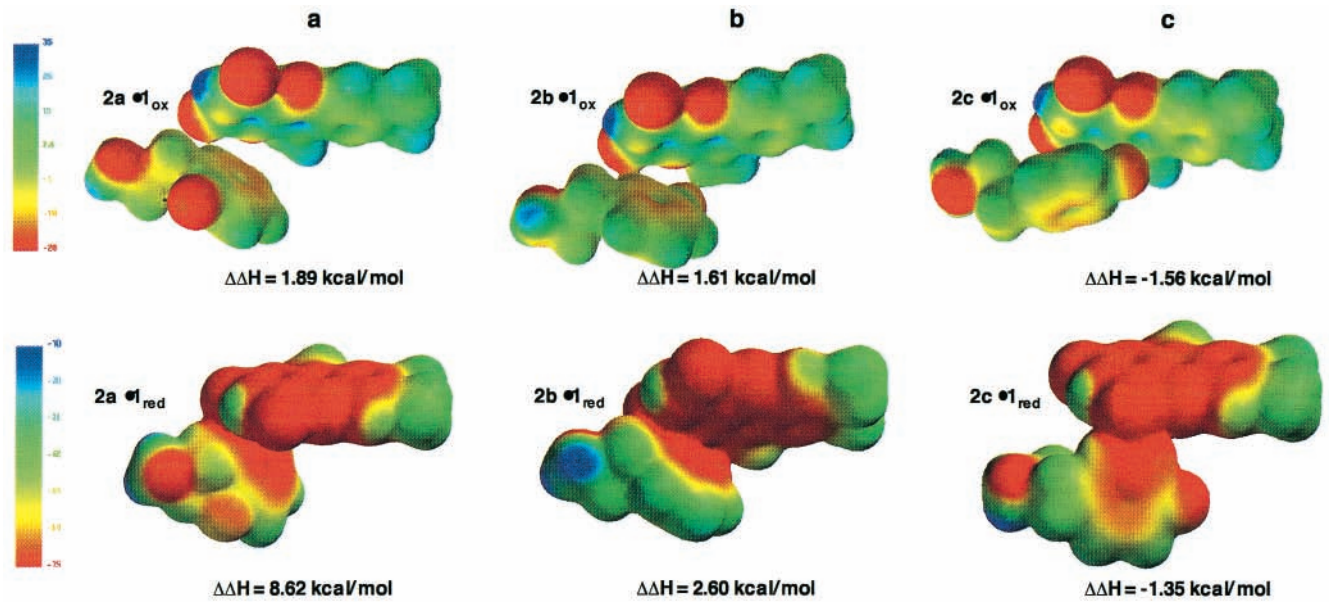

Figure 2. The position of the fluoro substituent leads to differing atom−flavin interactions: (a) *ortho*, (b) *meta*, and (c) *para*. Structures represent the lowest energy conformations. Reported energies are relative to the unsubstituted receptor **1**.

Overall, the energies derived from the calculations suggest a trend of increased recognition for *ortho*-, to *meta*-, to *para*-substituted complexes that should experimentally be observed as increased binding affinities.

To test the hypothesis of substituent position dependency and to verify ourcalculations, the receptors **1** and **2a**−**c** were synthesized and binding affinities of the dyads **1**·$\mathbf{3_{ox}}$ and **2a**−**c**·$\mathbf{3_{ox}}$ were quantified by ^{1}H NMR titrations in $CDCl_3$. Electrochemical studies on the redox active flavin were then performed to measure the change in reduction potential for the flavin. These changes were used to calculate free energies of binding as well as the association constants of the reduced cofactors **1**·$\mathbf{3_{red}}$ and **2a**−**c**·$\mathbf{3_{red}}$ (Table 1).

Table 1. Binding Constants for Complexes of Oxidized and Reduced Flavin **3** with Receptors **1** and **2**

receptor	ring substit	$K_{a(ox)}$ (M^{-1})[a]	$\Delta G_{(ox)}$ (kcal/mol)	$\Delta E_{1/2}$ (mV)[b]	$\Delta G_{(red)}$ (kcal/mol)	$K_{a(red)}$ (M^{-1})
1	H	920	-4.04	21	-4.45	1839
2a	*o*-F	690	-3.87	12	-4.07	966
2b	*m*-F	1270	-4.23	23	-4.69	2761
2c	*p*-F	1920	-4.48	18	-4.80	3330

[a] $CDCl_3$, 23 °C, all titrations are ±5%, based on standard error of the curve fits. [b] Concentrations used: **1**, **2** = 0.0025 M, **3** = 0.0005 M in 0.1 M TBAP solution in CH_2Cl_2 at 23 °C. $\Delta E_{1/2}$ is for the bound vs unbound flavin **3** referenced to ferrocene ($E_{1/2(unbound)}$ = −1293 mV).[14]

In agreement with the ab initio predictions, there is a dependence on the geometry of the fluoro substituent on the binding affinities of the complexes (Figure 3). An increase in binding is observed as the substituent moves from *ortho* (**2a**) to *meta* (**2b**) to *para* (**2c**) for both fluoro and related moieties.[15] In the oxidized family of receptors, we see an increase in free energy of 0.61 kcal/mol from *ortho* to *para*. This is of a lower magnitude than the predicted values (3.45 kcal/mol), presumably due to entropic effects arising from the flexibility of the ethyl chain and solvation effects not accounted for in the calculations. Upon reduction of the flavin cofactor, the geometry dependence trend is maintained. Similar differences of calculated vs observed energy changes as compared to the oxidized series are apparent (9.97 to 0.73 kcal/mol, respectively). An increase in binding for each of the reduced scaffolds, highlighted by the positive $\Delta E_{1/2}$

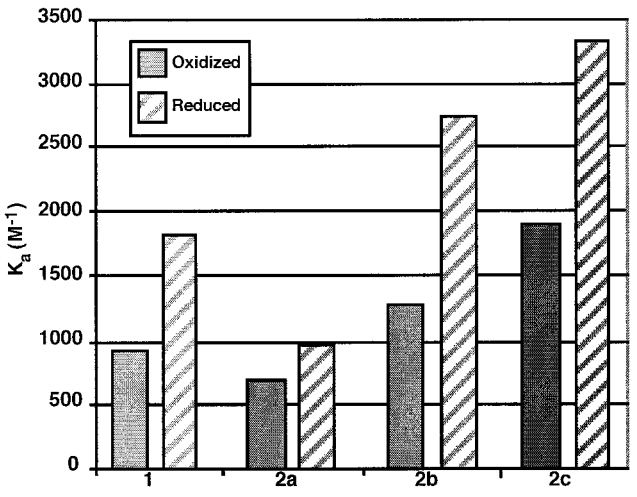

Figure 3. Binding constants for the oxidized and reduced species.

values, reflects the additional strength of the hydrogen bonds formed in the reduced complex as compared to the oxidized. These bonds are strong enough to maintain the bound state and compensate for the loss of aromatic stacking and π-donor interactions with the reduced cofactor, in agreement with previous model systems.[16]

The reduction in binding for the oxidized *ortho*-substituted host system when compared with that of the unsubstituted phenethylamine complex **1·3**$_{ox}$ clearly supports the theory that the unfavorable interaction caused by the proximity of the fluoro substituent of **2a** to the carbonyl group of flavin **3**$_{ox}$ is of a higher magnitude than any favorable aromatic stacking (present in **1·3**$_{ox}$). In the case of the *meta*-substituted system (**2b·3**$_{ox}$), a modest increase in binding affinity is seen for the electron-deficient fluoro receptor as compared to the unsubstituted system **1·3**$_{ox}$. This slight increase in binding, although still within the general trend of the fluoro series, is unexpected on the basis of the results of our calculations. This can possibly be attributed to the entropic benefits of having several low-energy conformational states in the experimental system. The *para*-substituted system, **2c·3**$_{ox}$, exhibits the expected increase in binding when compared with the unsubstituted phenethylamine dyad **1·3**$_{ox}$. This shows favorable responses due to both aromatic stacking and donor atom$-\pi$ interactions, concomitant with previous studies.[17]

In the reduced *ortho*-substituted system (**2a·3**$_{red}$), a greater difference in free energy from the unsubstituted dyad for the reduced as compared to the oxidized system is observed experimentally. This is in good agreement with the calculations, which show a large unfavorable interaction for this complex. The change in reduction potential to the negative (as compared to the **1·3** dyad) indicates an increased difficulty in reducing the flavin cofactor and also correlates with the substantial decrease in stability of the reduced complex in vacuo. The binding affinities of the *meta*- and *para*-substituted systems (**2b·3**$_{red}$ and **2c·3**$_{red}$, respectively) follow the general trend observed for the oxidized series, in which the increased hydrogen bonding typical of this triazine−flavin pair in the reduced system increases the overall binding affinity. This again supports our hypothesis that the binding strength is affected by the position of the fluoro moiety. The $\Delta E_{1/2}$ values for both the *meta* and *para* positions are similar to those of unsubstituted ring, suggesting specifically that the dipole−quadrapole interaction of the phenyl ring with the flavin in these systems is lost.

In summary, we have predicted and experimentally determined the effects of dipole orientation on aromatic stacking in both the oxidized and radical anion states of flavin. Since the electrostatic topology of the flavin surface is not uniform, not all areas are expected to interact equally with dipoles. Computational and experimental results confirm this assertation, showing (experimentally) a modulation of recognition of up to 0.61 kcal/mol in the oxidized systems and 0.73 kcal/mol in the reduced complexes, indicating that proper placement of dipoles is critical to recognition processes. By quantifying the individual interactions present in enzyme active sites, the requirement for particular residues in the local environment of the cofactor can be more readily interpreted and targeted in future experiments.

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Supporting Information Available: Synthesis, ^{1}H NMR, and elemental analyses for precursors and target molecules and NMR titration and electrochemical conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Binding constants for the reduced species were calculated by single electron-transfer processes using the following formula: $K_a(\text{red})/K_a(\text{ox}) = e^{(nF/RT)(E_{1/2}(\text{bound})-E_{1/2}(\text{unbound}))}$, where K_a(red) and K_a(ox) are the binding constants for the reduced and oxidized species, respectively.

(15) A parallel, but less dramatic, substituent geometry dependency was observed for the receptor with a methoxy substituent.

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