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Model Systems for Flavoenzyme Activity: Interplay of Hydrogen Bonding and Aromatic Stacking in Cofactor Redox Modulation

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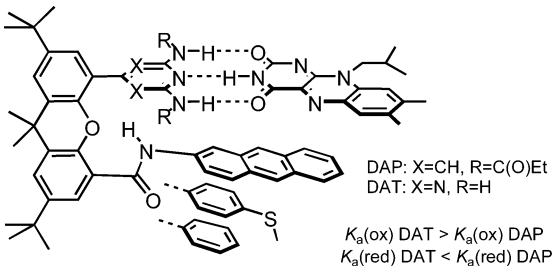
ABSTRACT



A model system has been developed to study the synergy between aromatic stacking and hydrogen bonding in the binding of a flavin derivative. The results show that the identity of both the hydrogen bonding and π-stacking units strongly determine the overall receptor affinity for flavin in both the oxidized and radical anion forms.

Flavoenzymes are a class of biological catalysts performing a diverse range of functions in living systems. These functions range from the oxidation of amines and thiols to imines and disulfides, facilitation of electron-transfer reactions with Fe-heme units and FeS-clusters, neurotransmitter regulation, and xenobiotic detoxification.[1] At the heart of a flavoenzyme is the cofactor flavin, usually present as flavin mononucleotide (FMN) or flavin adenine dinucloeotide (FAD), noncovalently bound into the active site of the protein through an array of specific interactions. It is the identity of these interactions that determines the redox properties of the cofactor and thus the overall utility of the enzyme.

Intact flavoprotein systems have been successfully used to probe qualitative cause−effect relationships between active site structure and flavoprotein functionality.[2] However, many standard biochemical techniques such as site-directed mutagenesis cause unavoidable changes to the subtle yet significant secondary interactions that exist within the system as well as affecting the primary interaction of interest. One way to examine the weight that specific noncovalent interactions have upon modulation of the physical properties of the cofactor in a quantitative manner is through the development of model systems.[3]

Within the reported active sites of the flavodoxin family a common three-point hydrogen bonding pattern is displayed between the imide functionality of the cofactor and the

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(1) For reviews of flavoprotein function, see: (a) Walsh, C. *Acc. Chem. Res.* **1980**, *13*, 148. (b) Ghisla, S.; Massey, V. *Eur. J. Biochem.* **1989**, *181*, 1. (c) Bruice, T. C. *Isr. J. Chem.* **1984**, *24*, 54. (d) Mueller, F. In *Topics in Current Chemistry*; Boschke, F. L., Ed.; Springer-Verlag: Berlin, 1983; Vol. 108, p 71. (e) Ziegler, D. M. *Trends Pharm. Sci.* **1990**, 321.

(2) Ghisla, S.; Massey, V. *Biochem. J.* **1986**, *239*, 1.

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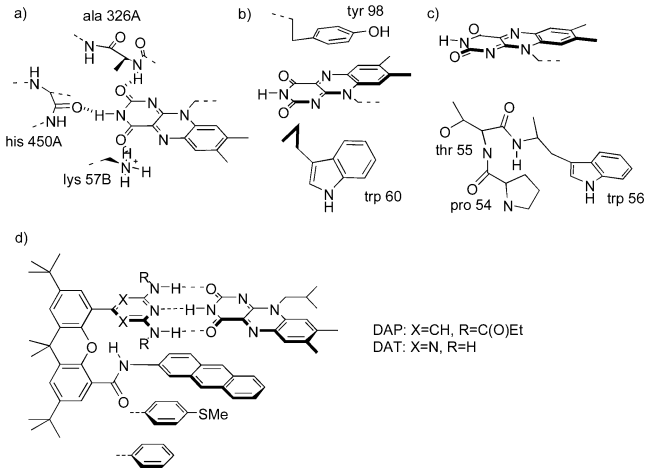

Figure 1. (a) Example of hydrogen-bonded contacts to the imide portion of flavin, taken from the structure of lipoamide dehydrogenase.[8] (b) Example of $\pi-\pi$ interactions between protein and cofactor, taken from the structure of *Desulfovibrio vulgaris*.[9] (c) Example of a donor atom$-\pi$ interaction between a flavoprotein residue and cofactor, taken from a red algae *Chrondus-Crispus*.[10] (d) Adaptable general model of flavoprotein active sites.

apoprotein. The identity of these contacts can result in significant stabilization of the radical anionic state (Figure 1a) and thus acts to tune the reactivity of the flavin. In previous work, we developed and evaluated a simple model based on the dipropamide of 2,6-diaminopyridine (DAP) to replicate the effects of the natural three-point hydrogen-bonding pattern on the reduction potential of the cofactor.[4] It was found that the binding constant between DAP and the flavin radical anion was enhanced by a factor 500 times larger than that observed for flavin in the oxidized form. In addition, the reduction potential of the complex was observed to take place at a potential some 155 mV lower than that of the flavin alone, effectively mimicking the tuning function of a natural flavoprotein.

In other work, we showed that other noncovalent contacts commonly observed in the active sites of flavodoxins such as those observed between tryptophan and tyrosine residues (Figure 2b) or donor atoms in the form of carbonyl groups (Figure 2c) or disulfides, and the electron poor aromatic surfaces of the cofactor could be replicated using a more complex model. These systems employed a 2,6-diaminotriazine (DAT) unit appended onto a xanthene scaffold to help orient and anchor the cofactor in place through a similar three-point hydrogen-bonding interaction as used previously between flavin and DAP. The receptor design is such that any interaction of interest can be placed in contact with the electron-deficient aromatic surface of the flavin, with the general result being that strongly electron-donating substituents cause dramatic increases in binding to the oxidized flavin but reduced association constants with the same flavin in the radical anionic form.[5]

(4) Niemz, A.; Imbriglio, J.; Rotello, V. M. *J. Am. Chem. Soc.* **1997**, *119*, 887.

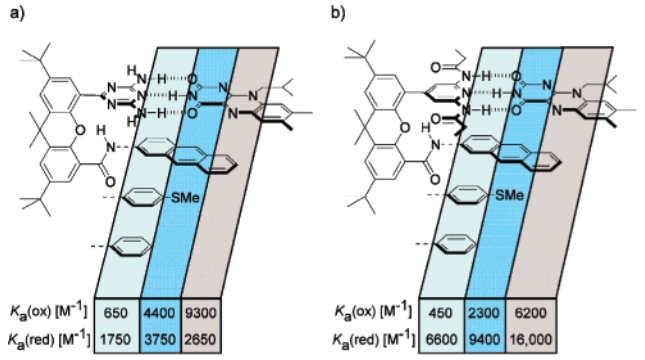

Figure 2. Binding constants observed for (a) the DAT-based receptors and (b) the DAP-based receptors with flavin in both its oxidized and radical anion states. The colored areas depict approximate zones of influence according to the maximum extent of π-overlap.

More recently, we showed that the reduction potential of 1,8-naphthalimide, an electron-deficient aromatic imide similar to flavin, was much more strongly stabilized by a simple DAP receptor than the DAT analogue.[6] As a result, while both receptors showed identical binding constants with the oxidized imide (160 M^{-1}) the increase in binding constant upon reduction of the imide was far more pronounced for the DAP system (46 000 M^{-1}, a ~300-fold increase) than DAT (900 M^{-1}, a ~6-fold increase). Extensive use of computational methods showed that these effects could be attributed to a greater polarizability within the DAP−imide complex than the DAT−imide system. In light of this work, we have developed an improved class of flavin receptor that combines the strong electronic modulating ability of a DAP-based recognition unit with the general utility afforded by the modular nature of a xanthene scaffold to give the most powerful synthetic flavoprotein model yet reported. In this paper, we compare and contrast energetic data associated with this new DAP-based xanthene system with values obtained for the older DAT-containing analogues. Three different receptors were synthesized and evaluated for each of the DAP and DAT systems, through variations of the aromatic unit suspended below the flavin π-system via: (i) an anthracyl unit to typify the strong $\pi-\pi$ interactions observed in *Desulfovibrio vulgaris*, (ii) a thiomethylphenyl unit to illustrate a general donor atom$-\pi$ effect as seen in flavodoxins such as *Chrondus-Crispus* or flavocytochrome-C sulfide dehydrogenase,[7] and (iii) a simple phenyl unit which does not extend significantly below the flavin as a control.

The association constants (K_a(ox)) between N(10)-isobutylflavin and all six receptors were quantified through NMR titration in $CDCl_3$ by following the downfield changes in

(5) (a) Breinlinger, E. C.; Rotello, V. M. *J. Am. Chem. Soc.* **1997**, *119*, 1165. (b) Breinlinger, E. C.; Keenan, C. J.; Rotello, V. M. *J. Am. Chem. Soc.* **1998**, *120*, 8606. (c) Goodman, A. J.; Breinlinger, E. C.; McIntosh, C. M.; Grimaldi, L. N.; Rotello, V. M. *Org. Lett.* **2001**, *3*, 1531.

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(7) Chen, Z.-W.; Koh, M.; van Driessche, G.; van Beeumen, J.; Bartsch, R.; Meyer, T.; Cusanovich, M.; Mathews, F. *Science* **1994**, *266*, 430.

chemical shift that occurred to the N(3) imide proton of the flavin as the amount of receptor present was increased. In addition, the half-wave reduction potentials of the free flavin ($E_{1/2(\text{unbound})}$) and the corresponding potentials for flavin in the presence of each receptor ($E_{1/2(\text{bound})}$) were determined in dichloromethane through cyclic voltammetry. Taken together, these three pieces of experimental data were used to obtain a binding constant for each receptor with flavin in the radical anionic form through the following relationship.

$$K_a(\text{red}) = K_a(\text{ox})e^{[(nF/RT)(E_{1/2}(\text{bound})-E_{1/2}(\text{unbound}))]}$$

An examination of the measured binding constants between the fully oxidized flavin and the six receptors (Figure 2) shows that as expected the addition of either a donor atom-π interaction or an enhanced $\pi-\pi$ interaction causes an increase in binding relative to the receptors containing an unsubstituted phenyl unit below the flavin. Moreover, the three receptors containing the DAT−imide recognition unit displayed binding constants around 1.5 times greater than the DAP-containing counterparts.

This effect may be explained from an examination of the ^{1}H NMR spectra of the receptors themselves. The primary difference lies in the chemical shift of the proton associated with the amide group that attaches the aromatic units to the xanthene skeleton. This resonance occurs at around 9.5 ppm for the DAT series but significantly further upfield at 8.7 ppm for the DAP-based receptors. This suggests that in the DAT series this proton is more strongly hydrogen bound to the ether oxygen atom of the xanthene giving a more preorganized structure and would hence lead to stronger guest binding from an entropic standpoint (Figure 3).

Reduction of the flavin to the corresponding radical anion causes large changes in the strength of the interaction between each host and guest. Within the DAT series of receptors, binding to the unsubstituted phenyl receptor is more than doubled compared to the value obtained for the oxidized guest; however, addition of functionality through either a donor atom or an extended π-surface both result in lower binding constants for the reduced form in comparison to the oxidized guest.

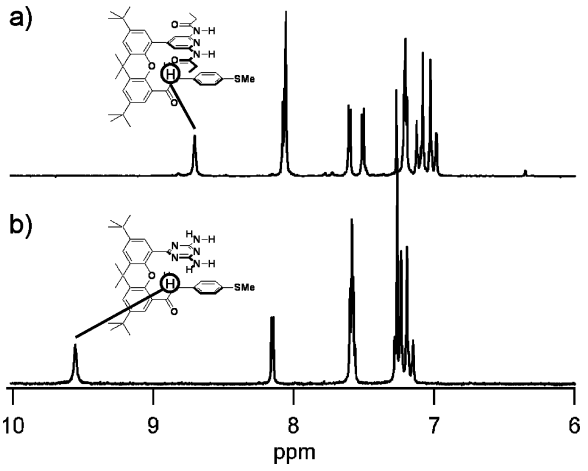

Figure 3. Comparison of the NMR spectra of the *p*-thiomethylphenyl (a) DAP- and (b) DAT-based receptors show the latter to be more preorganized due to the presence of an intramolecular hydrogen bond between the amide proton and the ether oxygen of the xanthene.

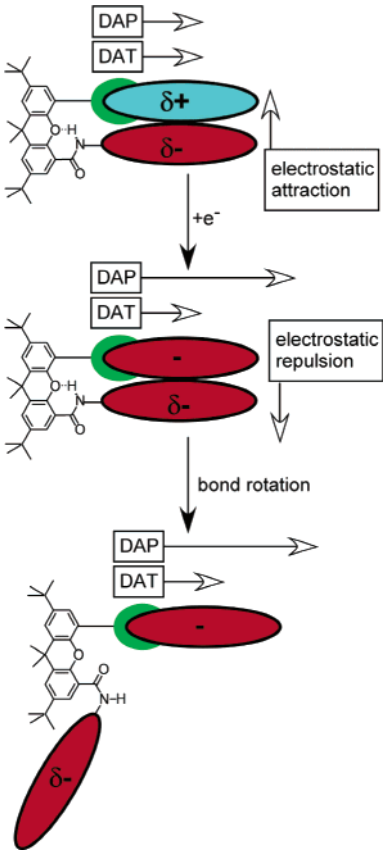

Figure 4. Cartoon showing the interplay of forces that occurs upon reduction of flavin once bound to the xanthene based receptors.

In contrast, binding flavin with the DAP-based receptors all show significant enhancement when the cofactor is in the radical anion state. This behavior may be rationalized through a dissection of the binding constant data into individual energetic contributions from the hydrogen bonding and π-stacking units.

For flavin in the neutral state, all of the intermolecular forces promote binding, with the hydrogen-bonding interactions between receptor and substrate reinforced by the complementarity between the electron poor π-surface of the cofactor and the aromatic units appended to the xanthene. Upon reduction of the cofactor, however, the flavin becomes

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(9) (a) Muller, F. In *Chemistry and Biochemistry of Flavoenzymes*; Muller, F., Ed.; CRC: Ann Arbor, MI, 1992; Vol. III, p 557. (b) Muller, F.; Vervoort, J.; Mierlo, P.; Mayhew, S.; van Berkel, W.; Bacher, A. In *Flavins and Flavoproteins (1990)*; Kurti, B., Ronchi., Zanetti, G., Ed.; De Gruyer: Berlin, 1991 p 261.
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extremely electron rich; thus, interaction with the π-stacking recognition units becomes actively disfavored. With the DAT unit in place to bind the imide moiety of the flavin guest, little enhancement in the strength of the hydrogen bonding interactions takes place, even less upon contributions from the stronger π-stacking units, leading to an overall diminution in observed binding for the *p*-thiomethylphenyl and anthracyl containing DAT receptors. However, as mentioned previously, the DAP recognition element is highly responsive to changes in the oxidation state of guests with massive increases in affinity being reported upon guest reduction. Thus, for the three receptors bearing the DAP system, the binding enhancement that occurs within the hydrogen-bonding units strongly outweigh the change from attraction to repulsion occurring simultaneously between the π-systems.

A second effect that may play a contributory role to the overall observed effect lies in an extension of the role the differences in receptor structure. The DAP systems have appended π-stacking elements that are loosely preorganized relative to the DAT systems where evidence of hydrogen bonding between the amide proton and the xanthene oxygen gives a more ordered class of receptor. The advantages this difference lends to the DAT receptors for oxidized flavin become disadvantages once the flavin is reduced. The loosely preorganized DAP receptors may alleviate much of the inter-π cloud repulsion through low energy bond rotations, for the same rotations to occur within the DAT receptors the amide−oxygen hydrogen bond must be broken giving significant enthalpic cost to the process (Figure 4).

Finally, an examination of the differences in the free energy changes associated with flavin binding in the presence of the two classes of receptor reveal a subtle synergy between the hydrogen bonding and π-stacking effects (Table 1). If the two recognition events were electronically independent then these free energy differences would be constant. However, our results show that the anthracyl receptors, which display the largest π-stacking effects, show larger differences in stabilization than the phenyl and *p*-thiomethylphenyl systems. This effect may be again attributed to the strong mutual polarization between the DAP group and the guest, which in this case shifts the electron density away from the N(5) position toward the imide functionality.

Table 1. Binding Energies and Energy Differences (kcal/mol)

		Phen[a]	Thio[b]	Anth[c]
DAT	ΔG(ox)	−3.84	−4.97	−5.41
	ΔG(red)	−4.42	−4.87	−4.67
DAP	ΔG(ox)	−3.62	−4.58	−5.17
	ΔG(red)	−5.21	−5.42	−5.73
$\Delta G(\text{ox})_{\text{DAP}} - \Delta G(\text{ox})_{\text{DAT}}$		0.22	0.38	0.24
$\Delta G(\text{red})_{\text{DAP}} - \Delta G(\text{red})_{\text{DAT}}$		−0.79	−0.54	−1.06

[a] Phenyl-substituted receptors. [b] *p*-Thiomethylphenyl-substituted receptors. [c] Anthracyl-substituted receptors.

As a result, less negative charge occupies the region overlapping the electron-rich anthracene unit, thus lessening the repulsion.

In summary, we have synthesized a flavoenzyme model bearing a diamidopyridine recognition element and several aromatic units to replicate $\pi-\pi$ and donor atom-π interactions commonly found in nature. In contrast to an earlier series of models that incorporated a passive diaminotriazine unit to bind imides, substantial increases in binding occurred upon electrochemical reduction of the guest. It was shown that a subtle interplay occurs between the hydrogen bonding network and the aromatic units which allows for maximum stabilization of the cofactor in the reduced state. Our further efforts will involve investigating these systems using high level computational methods and the application of the results of this work to the rational design of redox active molecular devices. The results of our subsequent investigations will be disclosed in due course.

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Supporting Information Available: Synthesis of DAT and DAP systems; ^{1}H NMR, IR, HRMS, as well as ^{1}H NMR titrations and electrochemical data with flavin "guests". This material is available free of charge via the Internet at http://pubs.acs.org.

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