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Model Systems for Flavoenzyme Activity: Relationships between Cofactor Structure, Binding and Redox Properties

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Abstract: A series of flavins were synthesized bearing electron-withdrawing and -donating substituents. The electrochemical properties of these flavins in a nonpolar solvent were determined. The recognition of these flavins by a diamidopyridine (DAP) receptor and the effect this receptor has on flavin redox potential was also quantified. It was found that the DAP−flavin binding affinity and the reduction potentials ($E_{1/2}$) for both the DAP-bound and unbound flavins correlated well with functions derived from linear free energy relationships (LFERs). These results provide insight and predictive capability for the interplay of electronics and redox state-specific interactions for both abiotic and enzymatic systems.

Introduction

Flavoenzymes are a ubiquitous class of proteins that catalyze a variety of redox transformations including the oxidation of amines to imines, thiols to disulfides and the hydroxylation of aromatic species.[1−4] Flavoproteins also mediate between single-electron redox processes involving iron−heme and iron−sulfur clusters and the obligate two-electron redox processes of NADH. Flavoenzymes consist of two essential components, the apoprotein and a flavin-based redox cofactor. The latter is usually present as either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), both derived from riboflavin (vitamin B_2).

The apoprotein in flavoenzymes serves both to form a binding pocket for the cofactor and to regulate cofactor redox properties. The distinctive differences between flavin microenvironment tune the redox properties of the cofactor to meet the function of the given flavoenzyme. X-ray crystallography has provided a great deal of information about the identities and relative positions of the components of the flavoprotein microenvironment, but does not yield direct insight into the mechanism or redox/recognition properties of the enzymes.[5]

Biochemical researchers have employed artificial flavins featuring electron-donating and -withdrawing substituents as structural probes to explore the environment of flavin-binding sites and as mechanistic probes that have led into new insights into flavoenzyme function. In the former category artificial flavins have been widely used for their spectral properties, where the sensitivity of the flavin chromophore to changes in its local dielectric has yielded valuable information about active-site polarity.[6] Moreover, synthetic flavins that exist in two or more tautomeric forms have confirmed the presence or absence of particular patterns of hydrogen bonds, permanent charges, and strong dipoles through perturbation of the natural equilibria between these states.[7] In other work Massey and co-workers have studied free energy changes associated with flavodoxins reconstituted with a variety of artificial flavins. This work examined the role microenvironmental effects of the binding pocket have upon the redox couple of the cofactor, and the related effects on cofactor protonation state and stability within the active site.[8]

In experiments designed to reveal mechanistic information, it has been shown that flavin substitution can completely eliminate reactivity.[9] In other cases more subtle substitutions can be made to change the redox potential of the cofactor, where

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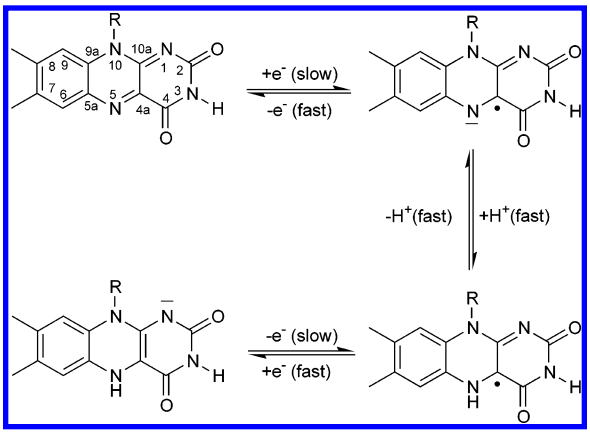
Scheme 1. Flavin Numbering and Common Oxidation/Protonation States Observed for the Flavin Cofactor



in one notable example, 7,8-dichloro-FAD was employed to show that product release is the rate-determining step for D-amino acid oxidase.[10] Recently Massey showed that not only could the reactivity of a flavoprotein be altered kinetically through cofactor replacement, it could also be altered mechanistically. In these studies, the NADPH-dependent reductase Old Yellow Enzyme (OYE), functioned as an oxygen-dependent desaturase when reconstructed with 8-cyano-FMN.[11] Flavoproteins are, however, incredibly complex pieces of biochemical machinery, and as such it is often difficult to determine precise cause and effect relationships using enzymatic systems. One way to address this problem is through the use of appropriate model systems, where interactions between receptor and substrate can be added and tuned systematically to address a given problem.

In 1998, Rizzo and co-workers studied differences in the reduction potentials within a series of flavins substituted at the 7 and/or 8 positions.[12] They showed that a linear free energy relationship (LFER) provided good correlation between the flavin reduction potential and the electronic nature of the substituents. In a later reappraisal of this work by Edmonson and Ghisla, a refined model of flavin reduction was presented that explicitly took into account electronic differences between the 7 and 8 positions.[13] Rizzo's studies were carried out in aqueous environments which differ from the flavin microenvironment provided by apoproteins in both the overall dielectric and the absence of the controlled interactions. Additionally, reduction of flavin in water initiates a series of processes that lead inevitably to production of the flavohydroquinone anion (Scheme 1).[14] Therefore, this series of experiments is valuable in the context of the many two-electron processes implicated in flavin biochemistry; however, the rich single-electron chemistry of the flavoenzymes remains relatively unexplored by a systematic LFER study. Additionally, these studies in aqueous media do not allow the effect of specific interactions on flavin redox properties to be determined.

Previously, we have shown that diaminopyridine derivatives (e.g., DAP) may be used as a simple, yet informative, mimic of apoproteins.[15] These receptors recognize the flavin through three-point hydrogen bonding to the imide moiety of the cofactor in a manner reminiscent of most natural flavoproteins such as lipoamide dehydrogenase (Figure 1).[16] Furthermore nonpolar aprotic solvents (e.g., methylene chloride and chloroform) better

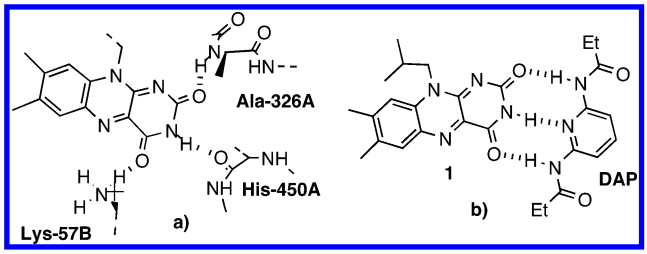


Figure 1. (a) Lipoamide dehydrogenase active site.[15] (b) DAP−flavin complex.

simulate the overall dielectric of flavin binding compared with aqueous environments. As in the natural analogues, flavin binding by DAP causes a stabilization of the radical anion form of the cofactor, manifested by a substantial cathodic shift in the reduction potential. Here we report experiments using artificial flavins bearing systematic functional group variations at the 7 and 8 positions, allowing the simplicity of the DAP−flavin model system to act as a benchmark for biochemical experiments using intact flavoproteins. In these studies, we demonstrate that the reduction potentials of the flavins free in solution, the binding of DAP with flavin in the oxidized state, and the reduction potentials of the resulting complexes can be standardized using LFERs. To our knowledge this is the first time the effects of subtle electronic modifications on redox-modulated molecular recognition has been explored in a systematic manner.

Results and Discussion

(1) DAP−Flavin Binding. The association constants for all 10 flavins were determined with DAP using ^{1}H NMR titration (Table 1).[17] In these studies, the downfield shift of the imide (N(3)) proton resonance was fitted to a 1:1 binding isotherm using iterative nonlinear curve-fitting methods, giving values accurate to within 5%.

Analysis of the data shows that in general the measured binding constant increases with increasing electron-donating capacity of the substituents at the 7 and 8 positions of the flavin. This can be rationalized in a qualitative fashion from a hydrogen-bond donor−acceptor viewpoint.[18] The nature of the interaction between DAP and flavin is a three-point hydrogen-bonded network.[19] Of the three hydrogen bonds, flavin provides two sites that act as hydrogen-bond acceptors, the O(2) and O(4) carbonyl oxygens, but only one site that can operate as a hydrogen-bond donor, the N(3) imide proton. Electron-donating groups will have the effect of increasing the electron density throughout the flavin system. As a direct consequence, this effect will increase the electron density at the imide proton, making this contact a poor hydrogen-bond donor. However the greater electron density at the two carbonyl oxygen atoms will make

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(17) K_a and $E_{1/2}$ values were determined in noncompetitive solvents with similar dielectric constants ($CDCl_3$ and CH_2Cl_2) due to their suitability for NMR and electrochemical work, respectively. Previously, we have shown that neither the interchange between these solvents nor the presence of the electrolyte necessary for conducting the electrochemical experiments has any measurable effect on the results obtained. See: Deans, R.; Niemz, A.; Breinlinger, E. C.; Rotello, V. M. *J. Am. Chem. Soc.* **1997**, *119*, 10863−10864.

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Table 1. Summary of Electrochemical and Binding Data for Substituted Flavins

flavin number	C7 (meta)	C8 (para)	C7 σ_m[a]	C8 σ_p[a]	flavin$_{ox}$ + DAP K_a (M^{-1})[b]	flavin $E_{1/2}$ (mV)[c]	flavin + DAP $E_{1/2}$ (mV)[d]	flavin$_{red}$+DAP K_a (M^{-1})[e]
1a	Br	Br	0.37	0.26	365	−1039	−944	14 800
1b	Cl	Cl	0.37	0.24	362	−1048	−949	17 100
1c	Me	Cl	−0.06	0.24	404	−1163	−1071	14 500
1d	H	H	0.00	0.00	432	−1206	−1109	18 900
1e	Me	H	−0.06	0.00	480	−1235	−1146	15 400
1f	Cl	MeO	0.37	−0.28	456	−1231	−1153	9 500
1g	Cl	NMe_2	0.37	−0.83	792	−1258	−1181	15 900
1h	Me	Me	−0.06	−0.14	517	−1286	−1194	18 600
1i	MeO	MeO	0.10	−0.28	606	−1358	−1278	13 700
1j	Me	NMe_2	−0.06	−0.83	960	−1378	−1304	17 100

[a] Values obtained from Hansch, Leo and co-workers.[21] [b] Values ±5%, $CDCl_3$, 23 °C. [c] [Flavin] = 5 mM; $E_{1/2}$ ± 3 mV vs ferrocene, CH_2Cl_2, 23 °C, 0.1 M TBAP used as electrolyte. [d] [Flavin] = 5 mM, [DAP] = 50 mM; $E_{1/2}$ ± 3 mV vs ferrocene, CH_2Cl_2, 23 °C, 0.1 M TBAP used as electrolyte. [e] Obtained from the relationship $K_a(\text{red})/K_a(\text{ox}) = \exp[(nF/RT)(E_{1/2}(\text{bound}) - E_{1/2}(\text{unbound}))]$.

them able to form stronger hydrogen bonds with the amide protons of the receptor. In other words, electron-donating groups diminish the strength of one hydrogen bond while simultaneously promoting the other two, with the net result being that substitution of this type enhances DAP−flavin complexation.[20]

(2) Electrochemical Studies. We next compared the redox behavior of the flavins both alone and in the presence of DAP. The experiments were carried out using cyclic voltammetry and square-wave voltammetry methods, using the ferrocene−ferrocenium couple as an internal standard.[17] The half-wave reduction potentials ($E_{1/2}$) corresponding to the conversion of the oxidized substrate to the corresponding radical anion for both the receptor-bound and free flavins are recorded in Table 1; a 10-fold excess of receptor was used to ensure complete binding. As expected, substitution at the 7 and 8 positions of the flavin results in pronounced differences in the values of $E_{1/2}$, with electron-donating and -withdrawing substituents making the reduction potentials more and less negative, respectively.

Another dramatic difference in the redox behavior of the DAP-bound flavins compared to the free flavins lie in the shapes of the cyclic voltammograms (Figure 2). For the free "natural" flavin (7,8-dimethyl flavin **1h**) one reduction wave and two distinct reoxidation waves were observed. In an earlier study,[22] we showed that this behavior arose from an electrochemical−chemical−electrochemical (e−c−e) process where the radical anion formed at the electrode surface rapidly deprotonates oxidized flavin in the bulk medium. The protonated flavin radical produced in this process undergoes a further one-electron reduction at the electrode surface to form the relatively stable fully reduced flavin anion. In the present study, we observed that the tendency to undergo this process depends strongly upon the reduction potential of each flavin: those with strongly electron-donating substituents (Figure 2a) have the most negative reduction potentials and also show the most pronounced e−c−e behavior. In contrast, the low reduction potentials caused by electron-withdrawing substituents give rise to fully electrochemically reversible formation of the radical anion. The reason for this change in behavior lies in changes in basicity of the N(5) position of the flavin radical anion, with electron-withdrawing groups having the effect of removing much of the charge from this nitrogen and thus lowering the pK_a at this position to below that of the imide of the oxidized form.

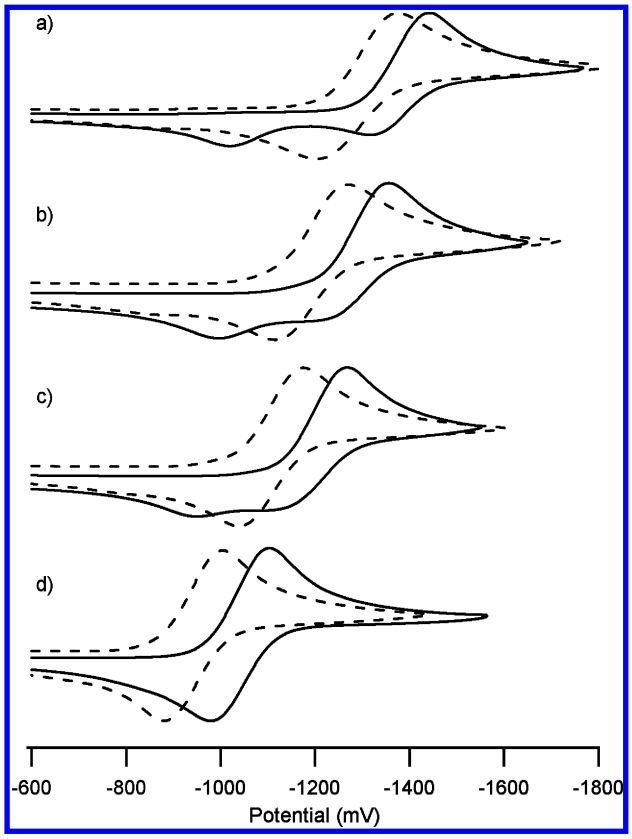

Figure 2. Cyclic voltammetry responses of selected 7,8-substituted flavins vs ferrocene, CH_2Cl_2, 23 °C, 0.1 M TBAP used as electrolyte. The solid lines indicate each flavin free in solution (5 mM), the dashed lines are the same flavins in the presence of DAP (50 mM). (a) **1j**, (b) **1h**, (c) **1d**, (d) **1b**.

In contrast, the redox behavior for all of the flavins in the presence of DAP are much simpler, displaying only a single electrochemical wave in their voltammograms, indicative of a fully electrochemically reversible conversion to and from the radical anion. There are two reasons for this difference in electrochemical behavior: (i) binding to DAP effectively blocks the imide proton of the oxidized flavin, preventing proton transfer between the flavin radical anion and the fully oxidized flavin and (ii) binding to flavin in the radical anionic form dramatically increases the extent of delocalization of the anion into the pyrimidine moiety, significantly lowering the reactivity of the N(5) position.

(3) Linear Free Energy Analysis and Biochemical Implications. As mentioned previously, Rizzo and co-workers linked

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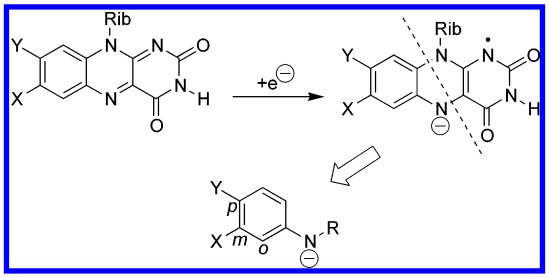

Figure 3. Simplification of the flavin radical anion into a pseudo-phenyl system.

the reduction potential of a series of flavins to a LFER.[12] In this study, the aromatic ring of the flavin was treated as a phenyl system, with the N(5) position bearing the bulk of the negative charge upon reduction to the radical anion form. In comparison to this position, the C(7) and C(8) positions of a flavin are analogous to the meta and para positions respectively of a simpler phenyl system (Figure 3).

This simplification of the flavin system allowed Rizzo to use the corresponding Hammett σ constants to correlate his data through the relationship:

$$E_{1/2}(\mathrm{X,Y}) = \rho\{\sigma_{\mathrm{m}}(\mathrm{X}) + \sigma_{\mathrm{p}}(\mathrm{Y})\} + E_{1/2}(\mathrm{H,H}) \qquad (1)$$

While this treatment gave good qualitative agreement between the experimental data and values predicted from this relationship, Edmonson and Ghisla showed that better correlation could be achieved for the prediction of flavin reduction potentials in an aqueous environment through a simple separation of the Hammett parameters, such that each position had an independent sensitivity factor (ρ):[13]

$$E_{1/2}(\mathrm{X,Y}) = \rho_{\mathrm{m}}\sigma_{\mathrm{m}}(\mathrm{X}) + \rho_{\mathrm{p}}\sigma_{\mathrm{p}}(\mathrm{Y}) + E_{1/2}(\mathrm{H,H}) \qquad (2)$$

We applied a variation of the latter method to analyze the data from our experiments, with replacement of electrochemical half-wave potentials with ΔG values, allowing us to analyze both the electrochemical and ^{1}H NMR derived data sets using one general equation:

$$\Delta G(\mathrm{X,Y}) = \rho_{\mathrm{m}}\sigma_{\mathrm{m}}(\mathrm{X}) + \rho_{\mathrm{p}}\sigma_{\mathrm{p}}(\mathrm{Y}) + \Delta G(\mathrm{H,H}) \qquad (3)$$

Using the generalized LFER of eq 3, the system-specific ρ values were iteratively varied to give an optimized relationship between the experimental and Hammett-derived values for the reduction of the free flavins in an aprotic solvent (Table 2). Overall the best fit for both the bound and unbound reduction potentials was found for $\rho_{\mathrm{m}} = -6.9$ kcal/mol/σ and $\rho_{\mathrm{p}} = -4.6$ kcal/mol/σ, suggesting that flavin reduction is more sensitive to electronic changes at the C(7) position than C(8). The only data that did not correlate well using these values were those where MeO was at C(8), where presumably the bulky nature of this substituent interferes with resonance donation. If these points are excluded from the analysis, $r^2 > 0.98$ is found for both the bound and unbound flavin reductions (Figure 4). These results contrast strongly with those reported by Edmonson and Ghisla for the reduction of flavins in an aqueous environment, where changes at the C(8) position were shown to be the dominant factor in controlling flavin electrochemistry ($\rho_{\mathrm{m}} = -3.2$ kcal/mol/σ, $\rho_{\mathrm{p}} = -4.7$ kcal/mol/σ). We believe this change in behavior arises from a combination of the change in dielectric

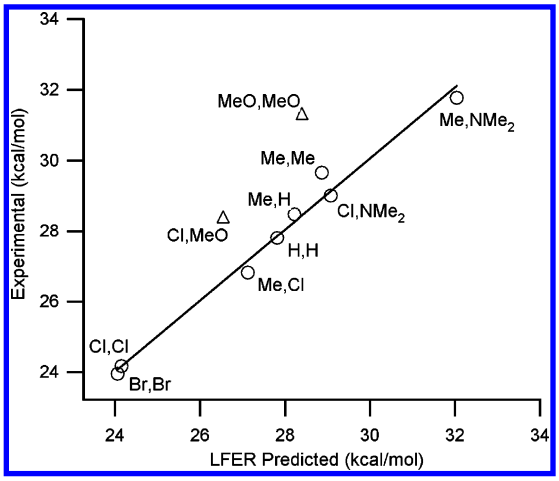

Figure 4. Correlation between experimental and linear free energy derived values for the reduction potentials of flavins in methylene chloride free in solution. Slope = 1.01 (except for methoxy-substituted flavins), $r^2 = 0.9831$.

Table 2. Summary of Experimental and LFER Predicted Free Energy Changes for Reduction of Flavins **1a**−**h**

flavin number	C7 $\sigma_{\mathrm{m}}\rho_{\mathrm{m}}$[a]	C8 $\sigma_{\mathrm{p}}\rho_{\mathrm{p}}$[b]	ΔG_{calc}[c] (kcal/mol)	ΔG_{exp}[d] (kcal/mol)
1a	−2.55	−1.20	24.1	24.0
1b	−2.55	−1.10	24.2	24.2
1c	0.41	−1.10	27.1	26.8
1d	0.00	0.00	27.8	27.8
1e	0.41	0.00	28.2	28.5
1f	−2.55	1.29	26.6	28.4
1g	−2.55	3.82	29.1	29.0
1h	0.41	0.64	28.9	29.7
1i	−0.69	1.29	28.4	31.3
1j	0.41	3.82	32.0	31.8

[a] Energetic contribution from the C7 substituent, values obtained using $\rho_{\mathrm{m}} = -6.9$ kcal/mol/σ. [b] Energetic contribution from the C8 substituent, values obtained using $\rho_{\mathrm{p}} = -4.6$ kcal/mol/σ. [c] Total LFER predicted energy change for reduction, obtained using eq 3. [d] Experimental free energy change for reduction, obtained from values listed in Table 1 using the relationship $\Delta G = -nFE_{1/2}$.

of the medium and the difference in hydrogen bonding between the aqueous and nonaqueous environments.

The results obtained from ^{1}H NMR titrations for the DAP−flavin complex were analyzed using eq 3 after unit conversion. It was found that the experimental data were fitted best when the LFER was substituted with the values $\rho_{\mathrm{m}} = 0.15$ kcal/mol/σ and $\rho_{\mathrm{p}} = 0.45$ kcal/mol/σ; thus, for binding to DAP the C(8) position dominates. A graph showing the correlation between the values obtained from these optimized ρ values and those determined experimentally is presented in Figure 5 (see also Table 3). Again an excellent correlation of $r^2 > 0.98$ is observed with the range of free energies produced, showing that effective communication does take place between the imide portion of the flavin and the distant C(7) and C(8) positions.

The final set of experimental data corresponding to flavin reduction in the presence of the receptor was also fitted to the standardized LFER (eq 3). Optimized values for the two sensitivity parameters were found to be identical to those obtained for the unbound flavins with $\rho_{\mathrm{m}} = -6.9$ kcal/mol/σ and $\rho_{\mathrm{p}} = -4.6$ kcal/mol/σ (Figure 6, see also Table 4), with the only difference being that the values are offset by 3 kcal/mol. This suggests that the two events occurring within the electrochemical cell, namely binding between flavin and receptor and reduction of the cofactor to the radical anion, are indepen-

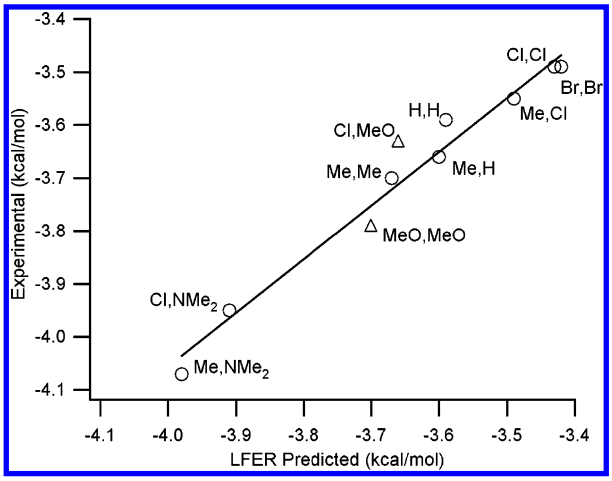

Figure 5. Plots showing correlation between experimental and linear free energy derived values for the change in free energy upon binding between flavins and DAP in $CDCl_3$. Slope = 1.02, $r^2 = 0.9841$.

Table 3. Experimental and LFER-predicted Free Energy of DAP−Flavin Association in $CDCl_3$

flavin number	C7 $\sigma_m\rho_m$[a]	C8 $\sigma_p\rho_p$[b]	ΔG_{calc}[c] (kcal/mol)	ΔG_{exp}[d] (kcal/mol)
1a	0.06	0.12	−3.42	−3.49
1b	0.06	0.11	−3.43	−3.49
1c	−0.01	0.11	−3.49	−3.55
1d	0.00	0.00	−3.59	−3.59
1e	−0.01	0.00	−3.60	−3.66
1f	0.06	−0.13	−3.66	−3.63
1g	0.06	−0.37	−3.91	−3.95
1h	−0.01	−0.06	−3.67	−3.70
1i	0.02	−0.13	−3.70	−3.79
1j	−0.01	−0.37	−3.98	−4.07

[a] Energetic contribution from the C7 substituent, values obtained using $\rho_m = 0.15$ kcal/mol/σ. [b] Energetic contribution from the C8 substituent, values obtained using $\rho_p = 0.45$ kcal/mol/σ. [c] Total LFER predicted energy change upon binding, obtained using eq 3. [d] Experimental free energy change upon binding, obtained from values listed in Table 1 using the relationship $\Delta G = -RT \ln K_a$.

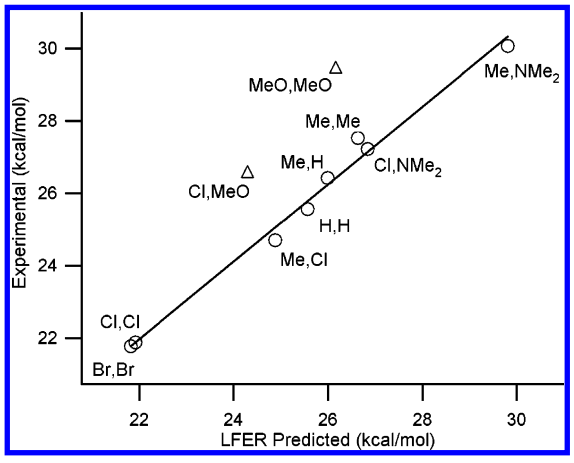

Figure 6. Plot showing correlation between experimental and linear free energy derived values for the reduction potentials of flavins in methylene chloride in the presence of DAP. Slope = 1.07 (methoxy-substituted flavins not included), $r^2 = 0.9884$.

dent. This is plausible due to the nature of the hydrogen-bonding system at the molecular orbital level. Interaction with the receptor causes a significant polarization of the HOMO away from N(5) to maximize the hydrogen-bond ability of the O(2) and O(4) positions. Moreover, this causes a redistribution of the spin density of the SOMO containing the unpaired electron

Table 4. Experimental and LFER Predicted Free Energy Changes for the Flavin Reduction in the Presence of DAP

flavin number	C7 $\sigma_m\rho_m$[a]	C8 $\sigma_p\rho_p$[b]	ΔG_{calc}[c] (kcal/mol)	ΔG_{exp}[d] (kcal/mol)
1a	−2.55	−1.20	21.8	21.8
1b	−2.55	−1.10	21.9	21.9
1c	0.41	−1.10	24.9	24.7
1d	0.00	0.00	25.6	25.6
1e	0.41	0.00	26.0	26.4
1f	−2.55	1.29	24.3	26.6
1g	−2.55	3.82	26.8	27.2
1h	0.41	0.64	26.6	27.5
1i	−0.69	1.29	26.2	29.5
1j	0.41	3.82	29.8	30.1

[a] Energetic contribution from the C7 substituent, values obtained using $\rho_m = -6.9$ kcal/mol/σ. [b] Energetic contribution from the C8 substituent, values obtained using $\rho_p = -4.6$ kcal/mol/σ. [c] Total LFER predicted energy change for reduction, obtained using eq 3. [d] Experimental free energy change for reduction, obtained from values listed in Table 1 using the relationship $\Delta G = -nFE_{1/2}$.

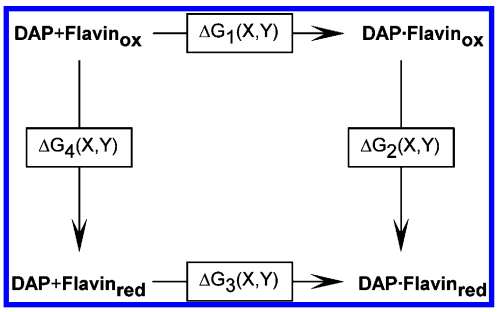

Figure 7. Thermodynamic cycle showing the relationship between the flavin−DAP binding and reduction processes.

(gained upon reduction) back into areas of the molecule where the HOMO has vacated, maintaining wave function orthogonality.[23] This redistribution in turn will cause significant disruption in the extent of communication between the phenyl and pyrimidine subunits of the flavin.

One way to test this hypothesis of independence and noncommunication between the phenyl and pyrimidine subunits of the flavin is by examination of the binding constants between receptor and cofactor in the reduced form. If the hypothesis is correct, this lack of communication would diminish the correlation between experimental values and those based upon the standard LFER. Although we were not able to quantify the interaction between the flavin radical anion and DAP through direct experimental observation, we were able to obtain values for this process through use of a relationship that makes direct use of the three experimental parameters:

$$K_a(\text{red})/K_a(\text{ox}) = \exp[(nF/RT)(E^{1/2}(\text{bound}) - E^{1/2}(\text{unbound}))] \quad (4)$$

Using a thermodynamic cycle (Figure 7) it is possible to derive a LFER that corresponds to the free energy change associated with binding DAP to the flavin radical anion (ΔG_3(X,Y)) from the optimized parameters for (i) the binding to flavin in the oxidized form (ΔG_1(X,Y)), (ii) the reduction processes corresponding to the DAP-bound flavin (ΔG_2(X,Y)), and (iii) the free flavin (ΔG_4(X,Y)). Inspection of this cycle tells us that if the level of electronic communication between

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(23) Niemz, A.; Rotello, V. M. *J. Am. Chem. Soc.* **1997**, *119*, 6833−6836.

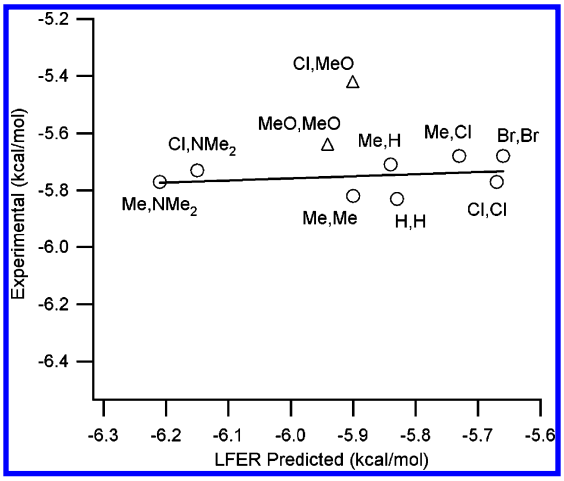

Figure 8. Plot showing the lack of correlation between experimental and linear free energy-derived values for the DAP binding of flavins in the reduced form.

Table 5. Summary of Experimental and LFER Predicted Free Energy Changes for the Free Energy Change upon Association of Flavins in the Radical Anionic Form and DAP

flavin number	C7 $\sigma_m\rho_m$[a]	C8 $\sigma_p\rho_p$[b]	ΔG_{calc}[c] (kcal/mol)	ΔG_{exp}[d] (kcal/mol)
1a	0.06	0.12	−5.66	−5.68
1b	0.06	0.11	−5.67	−5.77
1c	−0.01	0.11	−5.73	−5.68
1d	0.00	0.00	−5.83	−5.83
1e	−0.01	0.00	−5.84	−5.71
1f	0.06	−0.13	−5.90	−5.42
1g	0.06	−0.37	−6.15	−5.73
1h	−0.01	−0.06	−5.90	−5.82
1i	0.02	−0.13	−5.94	−5.64
1j	−0.01	−0.37	−6.21	−5.77

[a] Energetic contribution from the C7 substituent, values obtained using $\rho_m = 0.15$ kcal/mol/σ. [b] Energetic contribution from the C8 substituent, values obtained using $\rho_p = 0.45$ kcal/mol/σ. [c] Total LFER predicted energy change upon binding, obtained using eq 3. [d] Experimental free energy change upon binding, obtained from values listed in Table 1. using the relationship $\Delta G = -RT \ln K_a$.

the phenyl and pyrimidine moieties of the flavin remains constant, we then obtain eq 5. Substituting the optimized parameters into this relationship gives eq 6, which simplifies to give a LFER with the same ρ_m and ρ_p values as were optimized for the binding to the oxidized flavins (eq 7).

$$\Delta G_1(X,Y) + \Delta G_2(X,Y) = \Delta G_3(X,Y) + \Delta G_4(X,Y) \quad (5)$$

$$(0.15\sigma_m(X) + 0.45\sigma_p(Y) + \Delta G_1(H,H)) + (\Delta G_2(H,H) - 6.9\sigma_m(X) - 4.6\sigma_p(Y)) = \Delta G_3(X,Y) + (\Delta G_4(H,H) - 6.9\sigma_m(X) - 4.6\sigma_p(Y)) \quad (6)$$

$$\Delta G_3(X,Y) = 0.15\sigma_m(X) + 0.45\sigma_p(Y) + \Delta G_1(H,H) + \Delta G_2(H,H) - \Delta G_4(H,H) \quad (7)$$

Experimental values for the free energy change associated with DAP−flavin$_{red}$ binding obtained using eq 4 are compared to LFER-derived predicted values from eq 7 in Table 5 and also graphically in Figure 8. As anticipated, there is little similarity between the two data sets, with the experimental values scattered over a very small energy range compared to values predicted from eq 7. This shows that upon reduction of the flavin to the radical anion, communication between the phenyl and pyrimidine subsystems is effectively eliminated.

The experiments described herein show that in the absence of any additional specific interactions, in media consistent with the dielectric of the flavin binding site of most flavoproteins, structural variations at the C(7) and C(8) positions of the flavin structure lead to a series of well defined and predictable changes in the properties of the cofactor. First, binding between the imide functionality of the cofactor and a model of the residues that would bind to these positions in a native protein, is affected strongly by the changes examined, meaning that a significant degree of electronic delocalization exists through the full extent of the neutral molecule. Moreover this suggests that binding to the corresponding residues in the active site would interplay strongly with microenvironmental effects that would perturb this delocalization in the form of secondary specific interactions.

Second, the single-electron reduction of both the free and bound flavins to the corresponding radical anions also relates strongly (but differently) to the identity of the substituents at the C(7) and C(8) positions. Third, while the sensitivity factor for substituents at C(8) remains essentially constant for the reduction in both aprotic ($\rho_p = -4.6$ kcal/mol/σ) and aqueous environments ($\rho_p = -4.7$ kcal/mol/σ),[13] the contribution from the C(7) position is much more variable. The sensitivity factor for the latter position in a low dielectric medium (e.g., CH_2Cl_2, $\rho_m = -6.9$ kcal/mol/σ), which mimics the binding pocket of a natural flavoenzyme, is twice that of measurements recorded in water ($\rho_m = -3.2$ kcal/mol/σ).[13] As the C(7) position is out of direct conjugation with N(5), the role differing substituents (attached at C(7)) have on modulating the flavin's properties has been largely overlooked. However, the variability in sensitivity on moving from pure association with the binding site to electrochemical activity suggests that more importance should be given to this site than at present when designing experiments to modify and tune flavoprotein reactivity.

Conclusions

Ten flavins bearing a variety of substitutions at the C(7) and C(8) positions were synthesized. It was found that these substitutions had marked effects on (i) the redox properties of the flavin, (ii) the affinity for a synthetic receptor designed to mimic the flavins enzymatic partner, and (iii) the redox properties of the bound species. Subsequently, it was found that the trends in all three physical properties affected could be analyzed through use of linear free energy relationships. These relationships showed that the redox behavior of the flavins had greater sensitivity to substituents at the C(7) position than C(8). For binding to the oxidized flavin the opposite effect was observed with C(8) substituents modulating the affinity for the receptor to a much greater extent than C(7) substituents. Further analysis of the data showed that in the reduced form the communication between the C(7) and C(8) positions of the flavin and the imide moiety was effectively eliminated.

To our knowledge the work presented here provides the most controlled examination of substitution effects on the electrochemical properties of a series of flavins yet reported. In addition this paper is the first to explicitly examine a redox modulated molecular recognition process in detail using linear free energy relationships and, is thus a key step on the path towards the rational design of electrochemically controllable switches and

sensors. It is hoped that this work will likewise prove valuableto those workers employing flavins and their analogues as mechanistic probes and as therapeutic agents.

Currently we are continuing our investigations in this area through extensive use of computational methods to achieve a greater understanding of the root causes of the observed behavior. Further experimental work will focus on the effects these substituents have upon the affinity for more complex guests that better mimic the flavoprotein active sites through employment of specific $\pi-\pi$ and donor atom$-\pi$ interactions with the flavin system. The results of these investigations will be disclosed in due course.

Experimental Section

Materials and General Methods. Solutions utilized in electrochemical experiments were prepared using reagent grade CH_2Cl_2 dried via distillation over CaH_2. Tetrabutylammonium perchlorate (TBAP, obtained from SACHEM, electrometric grade) was dissolved in $CHCl_3$, washed with distilled water, recrystallized twice from ethyl acetate, and dried for several days under high vacuum. Other chemicals were reagent grade, obtained from Aldrich, and used without further purification.

NMR Titrations (General).[24] These experiments were performed in a noncompetitive solvent under constant concentration conditions of the individual flavin at 298 K. Flavin stock solution ($CDCl_3$ solvent; 2 mL, 5 mM) was used to prepare 20 mM solutions of the receptor molecule (DAP = 8.85 mg). Then 600 μL of the stock solution was transferred to an NMR tube and the spectrum of this solution recorded in a Bruker AC200 200 MHz NMR spectrometer. Aliquots of the receptor solution were added, with spectra recorded for each addition, and the incremental downfield change in chemical shift of the imide proton noted.

The resulting data were analyzed to reveal the binding constant K_a by application of nonlinear least-squares curve fitting.[25] The data for each curve gave excellent agreement with the 1:1 binding isotherm, using the relationship

$$\partial_{obs} = \partial_H + \frac{(\partial_{HG} - \partial_H)\left(\left([H_t] + [G_t] + \frac{1}{K_a}\right) - \left(\left([H_t] + [G_t] + \frac{1}{K_a}\right)^2 - 4[H_t] + [G_t]\right)^{1/2}\right)}{2[H_t]}$$

where $[G_t]$ and $[H_t]$, correspond respectively to the total concentrations of guest and host; δ_{obs}, the observed chemical shift, δ_H represents the chemical shift of the imide proton of the host in the absence of guest, and δ_{HG} the chemical shift of the followed proton in the fully bound complex, estimated from extrapolation from the limiting experimental value.[26]

Electrochemistry. All electrochemical experiments were carried out on a Cypress System potentiostat. A 1-mm platinum button and a gold-plated electrode were utilized as working and counter electrodes, respectively. A silver wire pseudoreference electrode was used, and all potentials are referenced versus the ferrocene/ferrocenium couple. The sweep rate was 200 mV/s, and the studies were run on an argon-purged temperature-controlled cell. Solutions of the flavin being studied alone and of the comparable complex with DAP were prepared, maintaining a constant concentration of the flavin. Maximal shifts were observed at ~5 equiv of DAP; 10 equiv was used to ensure complete binding. The solutions were degassed by bubbling argon through them for at least 10 min, at which time cyclic and square wave voltammograms were recorded (for representative voltammograms, see Supporting Information).

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Supporting Information Available: Synthesis of flavins and cyclic voltammetry for flavins and flavin−DAP complexes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) For further information see: Conners, K. *Binding Constants*; Wiley and Sons: New York, 1987.