Model Systems for Flavoenzyme Activity. Stabilization of the Flavin Radical Anion through Specific Hydrogen Bond Interactions

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Flavoenzymes such as the flavin monooxygenases (FMOs) and dehydrogenases (FDHs) are proteins that use the $FADH_2$−FAD redox cycle to catalyze a variety of oxygen-dependent transformations.[1,2] These processes include the oxidation of amines to imines (the monoamine oxidases, MAOs), the dehydrogenation of lipid esters and amides (the lipid dehydrogenases), and the hydroxylation of aromatic substrates (the hydroxylases).

Due to the structural and mechanistic complexity of the native enzymatic systems, there is considerable debate regarding the reaction pathways and mechanisms utilized by flavoproteins.[3] One way insight into the redox chemistry of these systems has been obtained is through synthetic models.[4] The relative simplicity of these models allows us to focus on individual interactions and pathways in much greater detail than is possible with their biological prototypes, increasing our understanding of large and complex flavoenzyme systems *at the molecular/atomic level*.[5]

We have designed synthetic receptors to isolate, quantify, and observe the effects of individual interactions on flavoenzyme behavior. Our initial research efforts are directed toward the creation of synthetic receptors designed to reproduce the specific hydrogen bond patterns present in flavoenzymes. These proteins modulate the redox behavior of the flavin cofactor through stabilization of reactive intermediates. In the case of the flavin radical anion, this is believed to occur through hydrogen bond stabilization of electron-rich sites on the flavin nucleus, including O(2) and O(4) (Figure 1).[3d,3f,6]

To quantify the energetic effects of hydrogen bonding on flavin redox processes, we have synthesized a family of receptors designed to model these interactions. Receptor **1** faithfully reproduces the enzyme−cofactor hydrogen bond interactions at O(2), N(3), and O(4) of a number of systems of known structure, such as lipoamide dehydrogenase (LipDH)[7]

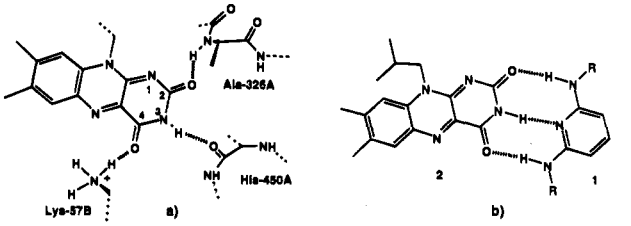

Figure 1. (a) Active site of lipoamide dehydrogenase. (b) Flavin **2**−receptor **1** complex.

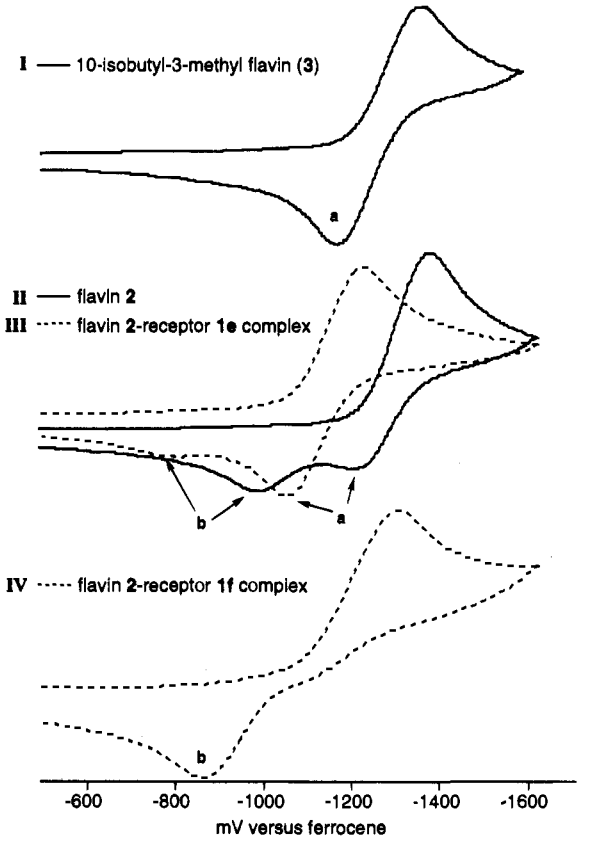

Figure 2. Cyclic voltammetry of the 3-methyl-10-isobutyl flavin **3** (trace I), the flavin **2** (trace II), and the complexes of flavin **2** with receptors **1e** (trace III) and **1f** (trace IV). CH_2Cl_2 was used as solvent, with tetrabutylammonium perchlorate carrier (0.1 M). Concentration of flavin **2**, 1×10^{-3} M, receptors **1e** and **1f**, 5×10^{-3} M; scan rate, 500 mV/s; $T = 23$ °C.

(Figure 1).[8] In LipDH, as well as other flavoproteins, the majority of these hydrogen bonds are to main chain amides. This limits the effectiveness of mutation studies for determining the role these interactions play in enzymatic processes.

To quantify the receptor−flavin hydrogen bonding interactions, we determined the association constants (K_a values) of diaminopyridine and its amide and urea derivatives **1a**−**g**[9] with the 10-isobutyl flavin **2**[10] via NMR titration in $CDCl_3$ (Table 1).[11,12]

With host−guest complexation verified, we next studied the ability of the hydrogen bond contacts to stabilize partially reduced flavins. Cyclic voltammetry (CV) studies of the 3-methyl-10-isobutyl flavin **3** in dichloromethane[14] (Figure 2, trace I) show reversible formation of the flavin radical anion.[15,16] CV studies of the 10-isobutyl flavin **2** (Figure 2, trace II) show a single, one-electron reduction peak, but two separate oxidation

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(2) Ziegler, D. M. *Trends. Pharm. Sci.* **1990**, 321.
(3) For studies on flavoenzyme mechanisms, see: (a)Bruice, T. C. *Prog Bioorg. Chem.* **1975**, *4*, 1. (b) Silverman, R. B. *J. Biol. Chem.* **1983**, *258*. (c) Kim, J. M.; Bogdan, M. A.; Mariano, P. S. *J. Am. Chem. Soc.* **1993**, *115*, 10591. (d) Hemmerich, P. *The Enzymes, Vol. XII*; Boyer, P. D., Ed.; Academic Press: New York, 1976; p 191. (e) Bach, R.; Su, M.-D. *J. Am. Chem. Soc.* **1994**, *116*, 5392. (f) Hemmerich, P. *Biochem. Soc. Trans.* **1980**, *8*, 246.
(4) For previously published model systems for flavoenzymes, see: (a) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7670. (b) Schultz, P. G. *Acc. Chem. Res.* **1989**, *22*, 287. (c) Shinkai, S.; Ishikawa, Y.; Shinkai, H.; Tsuno, T.; Makishima, H.; Ueda, K.; Manabe, O. *J. Am. Chem. Soc.* **1984**, *106*, 1801. (d) Tabushi, I.; Kodera, M. *J. Am. Chem. Soc.* **1987**, *109*, 4734. (e) Akiyama, T.; Simeno, F.; Murakami, M.;Yoneda, F. *J. Am. Chem. Soc.* **1992**, *114*, 6613. (f) Seward, E. M.; Hopkins, R. B.; Sauerer,W.; Tam, S.-W.; Diederich, F. *J. Am. Chem. Soc.* **1987**, *102*, 1783. (g) Takeda, J.; Ohta, S.; Hirobe, M. *J. Am. Chem. Soc.* **1987**, *109*, 7677.
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(8) For other examples of diaminopyridine−imide interactions, see: Hamilton, A. D.; Van Engen, D. *J. Am. Chem. Soc.* **1987**, *109*, 5035. Muehldorf, A. V.; Van Engen, D.; Warner, J. C.; Hamilton, A. D. *J. Am. Chem. Soc.* **1988**, *110*, 6561 and references therein.

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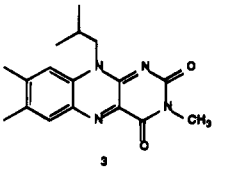
Table 1. Binding Constants and Reduction Potentials for Flavin **2**–Receptor **1** Complexes

receptor	R_1	R_2	K_a (M^{-1})[a]	$E_{1/2}$ (mV)[b,c]	$\Delta E_{1/2}$ (mV)[b,c]	$\Delta\Delta G_{red}$ (kcal/mol)
none				−1297	0	0
1a	H	H	55	−1231	66	−1.52
1b	H	$-C(O)CH(CH_3)_2$	141	−1218	79	−1.82
1c	$-C(O)CH(CH_3)_2$	$-C(O)CH(CH_3)_2$	194	−1193	104	−2.40
1d	$-C(O)CH(CH_3)_2$	$-C(O)N(H)C(CH_3)_3$	162	−1185	112	−2.58
1e	$-C(O)CH_2CH_3$	$-C(O)CH_2CH_3$	537	−1142	155	−3.58
1f	$-C(O)CCl_3$	$-C(O)CCl_3$	45	−1183[d]	114	−2.63
1g	$-C(O)CF_3$	$-C(O)CF_3$	30	−1232[d]	65	−1.50

[a] $CDCl_3$, 23 °C, H(3) peak followed. [b] In CH_2Cl_2, tetrabutylammonium perchlorate carrier (0.1 M), [**2**] = 1 × 10^{-3} M, 23 °C. [c] [**1**]= 5 × 10^{-3} M. [d] $E_{1/2}$ estimated from peak potential for the reduction couple.[13]

couples. Peak a is the reversible oxidation couple of **2**, while peak b represents an intermediate formed via tautomerization of H(3).[17] Addition of receptor **1e** provides a significant reduction in voltage for both the reduction wave potential and the $E_{1/2}$ for the reversible redox couple (Figure 2, trace III). All of the flavin **2**–receptor **1** complexes showed much less negative reduction potentials, indicating substantial stabilization (1.50–3.58 kcal/mol) of the flavin radical anion (Table 1).[18] This provides a direct analogy to flavoenzyme stabilization of the cofactor during redox reactions. To verify that this stabilization is not a simple media effect, we examined the reduction of the 3-methylflavin **3**, which cannot efficiently form hydrogen-bonded complexes to diaminopyridines **1**. Addition of receptor **1a–g** to solutions of the 3-methylflavin **3** provided no substantial change in reduction potential ($\Delta E_{1/2}$ < 20 mV), demonstrating that specific hydrogen bond contacts are required for the stabilization of the flavin **2** radical anion by receptor **1**.



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While all receptors lowered the potential for the reduction wave, their effect on the oxidation wave differed dramatically. Addition of receptors **1a–e** (i.e., receptor **1e**, Figure 2, trace III) increased the proportion of the reversible oxidation couple relative to the irreversible. This is apparently the result of stabilization of the radical anion through complexation, disfavoring proton migration. Receptors **1f** (Figure 2, trace IV) and **1g**, in contrast, actually promote proton transfer, completely eliminating the reversible redox pathway.[19] The cause of this strikingly different behavior is currently under study.

In summary, we have examined the recognition of flavins by synthetic receptors. Complexation of flavin by these receptors lowered the reduction potential of the flavin, mimicking the behavior of flavoenzyme–cofactor complexes. The use of these and other synthetic receptors to model other flavoenzyme functions is underway and will be reported in due course.

Acknowledgment. We thank Richard Walton for synthesizing the 3-methyl-10-isobutyl flavin used in these studies. We also thank Professors Michael Maroney and David Curran for helpful conversations.

Supplementary Material Available: ^1H NMR titration data and cyclic voltammetry plots for receptor **1a–g**–flavin **2** complexes (8 pages). This material is contained in many libraries on microfiche, immediately follows the article in the microfilm version of this journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(9) These receptors were synthesized from 2,6-diaminopyridine using the appropriate acid chlorides (**1b,c,e**) and anhydrides (**1f,g**). Receptor **1d** was made via reaction of 2,6-diaminopyridine with *tert*-butylisocyanate, followed by acylation with isobutyryl chloride.

(10) Synthesized according to the method of Yoneda et. al.: Yoneda, F.; Sakuma, Y.; Ichiba, M.; Shinomura, K. *J. Am. Chem. Soc.* **1976**, *98*, 830.

(11) Performed via addition of aliquots of hosts **1a–g** to a solution of flavin **2**. The plot of the chemical shifts of H(3) as a function of receptor concentration provided a titration curve. Association constants were determined through nonlinear least-squares curve fitting. All curves provided a good fit to the 1:1 binding isotherm.

(12) Evidence for the three-point binding geometry shown in Figure 1 is provided by the strong intermolecular NOESY crosspeak observed between H(3) of flavin **2** and the amide protons of receptors **1** in solutions containing both components. Reverse titrations which followed the chemical shift of the amine/amide protons of receptors **1** upon addition of flavin **2** yielded numerically identical results to those in Table 1, providing further evidence for the hydrogen bonding mode presented.

(13) Referenced to ferrocene as an internal standard: Gagné, R.; Koval, C. Lisensky, G. C. *Inorg. Chem.* **1980**, *19*, 2854. Due to the loss of reversible oxidation couple, $E_{1/2}$ values for receptors **1f,g** were estimated from the reduction wave potential.

(14) For a previous study of flavin reduction in an aprotic medium (dimethyl sulfoxide), see: Tatawandi, S.; Santhanam, K.; Bard, A. *Electroanal. Chem. Interfac. Electrochem.* **1968**, *17*, 411.

(15) Established through comparison of ΔE_p for the flavin with that of ferrocene, cf. ref 10.

(16) It has recently been suggested that the initially formed reduction product is actually the dianion: Male, R.; Samotowka, M.; Allendoerfer, R. *Electroanalysis* **1989**, *1*, 333. If this is the case, all results and conclusions remain valid, except for ΔG values, which would be doubled.

(17) Dudley, K.; Ehrenberg, A.; Hemmerich, P.; Müller, F. *Helv. Chim. Acta* **1964**, *47*, 1354.

(18) Addition of aliquots of receptors **1** to solutions of flavin **2** provided steadily increasing values of $\Delta E_{1/2}$. This demonstrates that the on–off rate for the receptor **1**–flavin radical anion complex is fast relative to the electrochemical time scale. Maximum values for $\Delta E_{1/2}$ for all receptors occurred between 2–4 equiv of added receptor. This indicates considerably stronger binding of receptors **1a–g** to the flavin radical anion than to the oxidized flavin **2**, since only a relatively small fraction of the oxidized flavin is complexed at the receptor concentrations used for the CV studies. The markedly enhanced binding observed in our electrochemical studies is most likely due to the enhanced hydrogen bonding between the anionic flavin radical and the receptor. Further study of receptor–flavin radical anion recognition is currently underway.

(19) For an example of a flavin–metal complex with similar behavior, see: Sawyer, D.; Kowai, R.; McCreery, R. *Experentia*, **1971**, Suppl. 18, 563.