Model Systems for Flavoenzyme Activity. The Role of N(3)−H Hydrogen Bonding in Flavin Redox Processes

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Received December 1, 1999. Revised Manuscript Received February 9, 2000

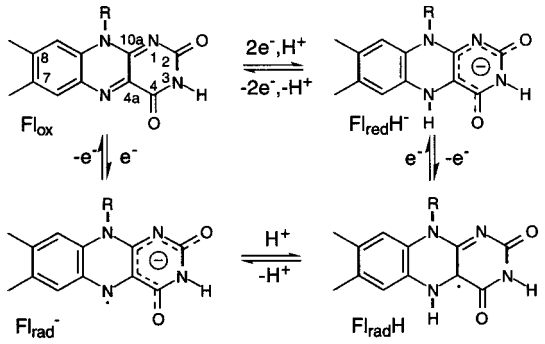
Abstract: Computational and experimental studies were performed to determine the role of N(3)H hydrogen bonding in controlling flavin redox chemistry. B3LYP calculations of a lumiflavin−DMF complex indicate that hydrogen bonding to N(3)H is redox state dependent: hydrogen bonds to this position are formed in the oxidized state, and released in the flavin radical anion system due to increased electron density in the reduced species. Electrochemical studies of flavin and *N*(3)-methyl flavin in hydrogen bonding and non-hydrogen bonding solvents confirm this prediction, demonstrating that N(3)H hydrogen bonding modulates the potential of flavin reduction to the corresponding radical anion by 80 mV (1.8 kcal/mol). Variable-temperature electrochemical studies were also performed to establish the enthalpic and entropic effects of N(3)H hydrogen bonding on flavin reduction.

Flavoenzymes are among the most structurally and functionally diverse families of redox proteins.[1] They catalyze an enormous range of biotransformations and electron-transfer processes using a single redox unit, the flavin. These redox events can have either one- or two-electron mechanisms (Scheme 1), and occur over a wide (>500 mV) potential range.[2] This corresponds to a free energy range ($\Delta\Delta G$) of *>10 kcal/mol* per electron, an extensive energetic range for a single covalent structure.

From the wide variation found in both the redox potentials and mechanisms of flavoenzyme activity, it is apparent that the redox properties of the flavin nucleus are controlled via noncovalent apoenzyme−flavin interactions. These interactions include hydrogen bonding[3] and aromatic stacking,[4] as well as other less apparent dipolar/multipolar[5] and steric effects.[6] Direct determination of the roles of these noncovalent interactions in biological systems has been complicated, however, by both the wealth of peptide backbone−flavin interactions and the intricate additivity of the multiple forces involved.

Model systems provide an important tool for exploring the role of apoenzyme−cofactor interactions in flavoenzymes.[7]

Scheme 1. Common Redox/Protonation States of Flavin Cofactors



Using synthetic model systems, researchers have recently explored the role of biomimetic multi-point noncovalent interactions[8] and conformational effects[9] on flavin redox processes. Further studies have combined synthetic and computational models to explore donor atom−π interactions[10] and hydrogen bonding in flavin and similar redox units.[11] Few model studies, however, have focused on the roles of individual enzyme−cofactor interactions on flavin redox properties.[12]

Hydrogen bonding between the apoprotein and N(3)H of the flavin cofactor is a universal aspect of flavoenzyme structure.

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10.1021/ja994204v CCC: $19.00
Published on Web 03/23/2000

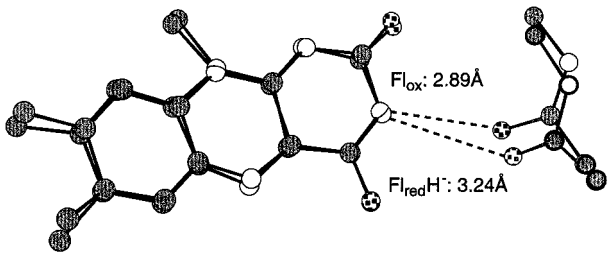

Figure 1. Flavin binding site of the *Desulfovibrio vulgaris* flavodoxin showing hydrogen bonding between N(3)H of FMN and Tyr100 of the apoprotein.[19]

This interaction has been observed in virtually all ^{15}N NMR studies of flavoenzymes by both chemical shift and ^{15}N−^{1}H coupling.[13] From X-ray crystal structures, it is apparent that the hydrogen bond acceptor for N(3)H in the vast majority of flavoenzymes is a main-chain carbonyl group (Figure 1).[14] Despite the widespread occurrence of N(3)H hydrogen bonding, the role of this interaction in mediating flavoenzyme redox processes has only recently been explored.[15] This lack of attention arises in part from an early report by Kuhn and Boulanger[16] concluding that alkylation at N(3) has no effect on the redox potential of flavins in aqueous media. This has generally been interpreted as evidence for "insulation" of this site from electronic changes concomitant with flavin redox processes.[17] Given recent studies demonstrating dramatic flavin substituent effects on flavin redox processes[18] and the large changes in carbonyl−flavin distances observed in flavoenzymes upon flavin reduction (Figure 1), we felt that this interpretation was perhaps oversimplified. We report here the use of experimental and theoretical methods to determine the role of N(3)H hydrogen bonding on the reduction of flavin to the corresponding semiquinone anion (Fl_{rad}^-).

Results and Discussion

Insight into the role of recognition at N(3)H on flavoenzyme structure and reactivity was obtained through calculations on the lumiflavin−DMF complex (Figure 2).[20] This complex provides a bridge between the carbonyl−flavin interaction commonly found in flavoproteins and our electrochemical solvent studies on the highly analogous *N*(10)-isobutyl flavins (vide infra). To provide an accurate model for hydrogen bonding to both Fl_{ox} and Fl_{rad}^-, the B3LYP self-consistent hybrid functional was used. This methodology has been shown to accurately model hydrogen bonding interactions[21] and predict molecular properties of biologically relevant radicals.[22]

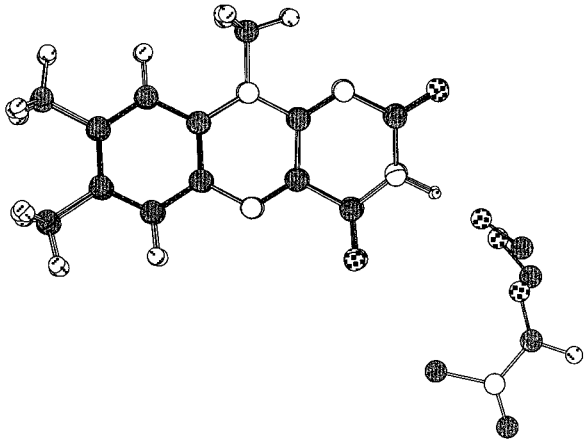

Figure 2. Lumiflavin−DMF complex at minimum, intermediate, and maximum N(3)−O distances of calculation for the Fl_{ox} state. The geometry in the Fl_{rad}^-−DMF coordinate driving was essentially identical.

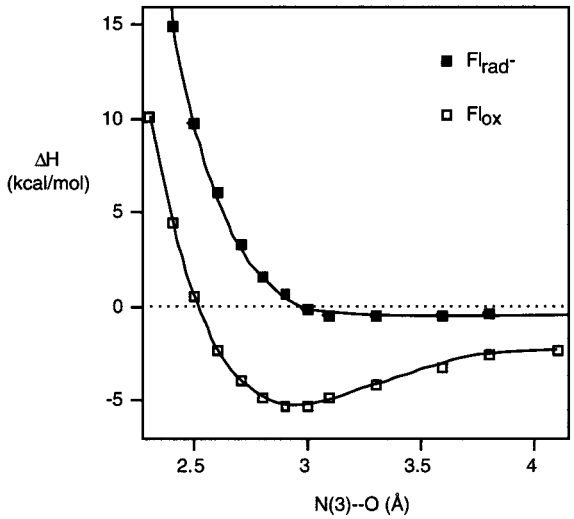

Figure 3. Plot of enthalpy versus N(3)−O distance for the lumiflavin−DMF complex, based on B3LYP/6-31+G*//HF/3-21 G* calculations.

To provide a detailed picture of the flavin−carbonyl interaction, we examined the enthalpy of interaction as a function of N(3)−O distance (Figure 3). The results for the Fl_{ox}−DMF complex were as expected for a normal hydrogen bond, with an optimal N(3)−O distance of 2.87 Å and a maximum enthalpy of binding of −5.3 kcal/mol. In contrast, there is no defined minimum for the interaction of Fl_{rad}^- with DMF, with a maximum favorable interaction of −0.5 kcal/mol found.

The difference in enthalpy of interaction between the complexes of DMF with Fl_{ox} and Fl_{rad}^- predicts that reduction would be made 4.8 kcal/mol more difficult in vacuo through hydrogen bonding at N(3). Furthermore, there is a 0.7 kcal/mol repulsive interaction predicted between DMF and Fl_{rad}^- at the optimal Fl_{ox}−DMF distance. To minimize this unfavorable interaction, the hydrogen bond distance in enzymatic systems should increase by approximately 0.3 Å to reach a very shallow

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Table 1. Reduction Potentials and Free Energies of Reduction for Flavins **1** and **2**[a]

solvent	$E_{1/2}$(**1**) (mV)[b]	$E_{1/2}$(**2**) (mV)[b]	$\Delta E_{1/2}$(mV)[c]	$\Delta\Delta G$(kcal/mol)[d]
CH_2Cl_2	−1282	−1354	−72	1.66
DMF	−1290	−1282	8	−0.18
THF	−1379	−1378	1	−0.02
Pyridine	−1344	−1344	0	−0.00

[a] Flavin concentration = 3.8×10^{-4} M, electrolyte (TBAP) concentration = 0.1 M, temperature = 296 K. [b] Relative to ferrocene as an internal standard.[26] [c] $\Delta E_{1/2}$(**1**) − $\Delta E_{1/2}$(**2**). [d] ΔG_{red}(**1**) − ΔG_{red}(**2**).

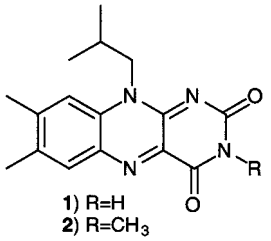

Figure 4. Flavin **1** and *N*(3)-methyl flavin **2**.

energy minimum as flavin **1** is reduced from the neutral Fl_{ox} to the anionic Fl_{rad}^-. This change in geometrical preference is consistent with that observed in flavoenzymes, where the analogous reduction of the neutral flavin (Fl_{ox}) to the fully reduced anion $Fl_{red}H^-$ results in increased N−O distances (0.35 Å movement in *D. vulgaris* flavodoxin (Figure 1)).

To explore the role of N(3)H hydrogen bonding on flavin reduction potentials, we quantified the one-electron electrochemical reduction (Fl_{ox} to Fl_{rad}^-)[23] of flavin **1** in solvents with and without hydrogen bond-accepting functionality (Table 1).[24] As N(3)H is the only hydrogen bond-donating substituent on the isoalloxazine nucleus, solvent studies are well-suited to exploring the effects of interactions at this site. To provide a control for other possible flavin−solvent interactions, parallel control studies were performed using the *N*(3)-methylated flavin **2** (Figure 4).[25]

In dichloromethane, a non-hydrogen bond accepting solvent, the reduction potential of *N*(3)-methyl flavin **2** is substantially more negative than that of flavin **1**. The shift in reduction potential observed arises from the greater electron-donating properties of the *N*(3)-methyl group of **2** relative to the proton of **1**. This electron donation stabilizes Fl_{ox} relative to Fl_{rad}^-, making flavin **2** more difficult to reduce. The 72 mV (1.7 kcal/mol) shift in redox potential is considerably larger than that observed upon alkylation at the C(7) and C(8) positions of the isoalloxazine nucleus.[27] This greater modulation is expected due to the close proximity of the methyl group of **2** to the areas of greatest change in charge density upon reduction of Fl_{ox} to Fl_{rad}^-.[7]

In contrast to the behavior observed in dichloromethane, there is little difference in reduction potential between flavins **1** and **2** in hydrogen bond-accepting solvents such as DMF and pyridine. This also holds true for THF, a solvent of similar dielectric ($\epsilon = 7.3$) to that of dichloromethane ($\epsilon = 8.9$). Since the electronic effect of alkylation at N(3) on the redox potential of flavin **2** will be comparable in each of these solvents, this indicates that hydrogen bonding to N(3)H makes the reduction of flavin **1** more difficult, as predicted from calculations (vide supra). Comparison of the difference in $E_{1/2}$ between **1** and **2** in hydrogen bond-accepting solvents with those observed in dichloromethane indicate that hydrogen bonding at N(3)H makes flavin reduction from the neutral Fl_{ox} to the anionic Fl_{rad}^- up to *80 mV* (1.8 kcal/mol) more difficult. This result mirrors recent mutagenesis studies of the *C. beijerinckii* flavodoxin, where the analogous neutral semiquinone ($Fl_{rad}H$) to fully reduced anion ($Fl_{red}H^-$) couple was explored. In these studies, disruption of hydrogen bonding at N(3) results in a less negative potential for the reduction of FMN from $Fl_{rad}H$ to $Fl_{red}H^-$ of 86 mV.[15] A quantitative comparison cannot be made to our studies, however, as indirect effects of the mutation may also cause perturbations at other flavin atoms which affect the reduction potential.[28]

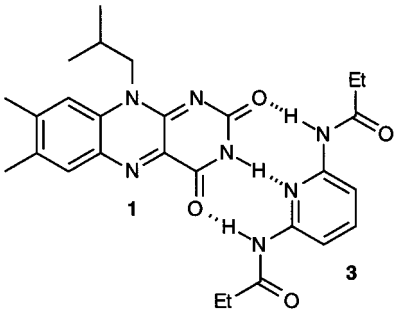

Figure 5. Flavin **1**−receptor **3** complex.

The similarity in reduction potentials of **1** and **2** in hydrogen bond-accepting solvents is consistent with results previously observed for the two-electron reduction (Fl_{ox} to $Fl_{red}H^-$) of flavin in aqueous media; this correspondence in behavior is a consequence of reduction of Fl_{ox} to Fl_{rad}^- being the rate-limiting step in the ece (electrochemical−chemical−electrochemical) reduction of Fl_{ox} to $Fl_{red}H^-$ in protic media.[21] From these studies, instead, we can conclude that the similarity of redox potentials between **1** and **2** in aqueous media results from a cancellation of effects, rather than "insulation" of the N(3) position.

The dramatic effect of hydrogen bonding at N(3)H on the reduction potential of flavin **1** demonstrates the role of this enzyme−cofactor interaction in regulating flavoenzyme properties. This effect also sheds light upon earlier host−guest model studies. In these investigations, we observed that formation of the flavin **1**−receptor **3** complex (Figure 5) made the reduction potential of **1** 155 mV less negative, corresponding to a 3.6 kcal/mol stabilization of Fl_{rad}^-.[29] This complex has three hydrogen bonds: two are to the flavin carbonyl oxygens O(2) and O(4) and one is to N(3)H. The results of these two sets of hydrogen bonds are offsetting, with hydrogen bonding to O(2) and O(4) making flavin reduction easier, and hydrogen bonding to N(3)H making reduction more difficult. From comparison of our previous results with the shift in potential observed in this study for pyridine (a base of very similar hydrogen bond accepting nature to **3**),[30] we can now estimate that the hydrogen

(23) Niemz, A.; Imbriglio, J.; Rotello, V. *J. Am. Chem. Soc.* **1997**, *119*, 887−892.

(24) Previous studies have shown that flavin **1** does not dimerize or aggregate at the concentrations used in this study: Breinlinger, E.; Rotello, V. *J. Am. Chem. Soc.* **1997**, *119*, 1165−1166.

(25) Dutra, J.; Cuello, A.; Rotello, V. *Tetrahedron Lett.* **1997**, *38*, 4003−4004. Niemz, A.; Imbriglio, J.; Rotello, V. *J. Am. Chem. Soc.* **1997**, *119*, 887−892.

(26) Since the comparison of reduction potentials between flavin **1** and **2** is calculated separately for each solvent system, any solvent effect experienced by the ferrocene is canceled out.

(27) Hasford, J.; Kemnitzer, W.; Rizzo, C. *J. Org. Chem.* **1997**, *62*, 5244−5245.

(28) The change in reduction potential cannot be fully attributed to interruption of the hydrogen bond to N(3) due to indirect effects of the mutation along the protein backbone. The authors suggest that the mutated residue may function not only to provide a hydrogen bond to flavin at N(3) but also to constrain the protein backbone into a conformation that optimizes additional hydrogen bonds to the flavin cofactor at O(4) and N(5), complicating direct assignment of energetic contributions.

(29) Niemz, A.; Rotello, V. *J. Mol. Recognit.* **1996**, *9*, 158−162.

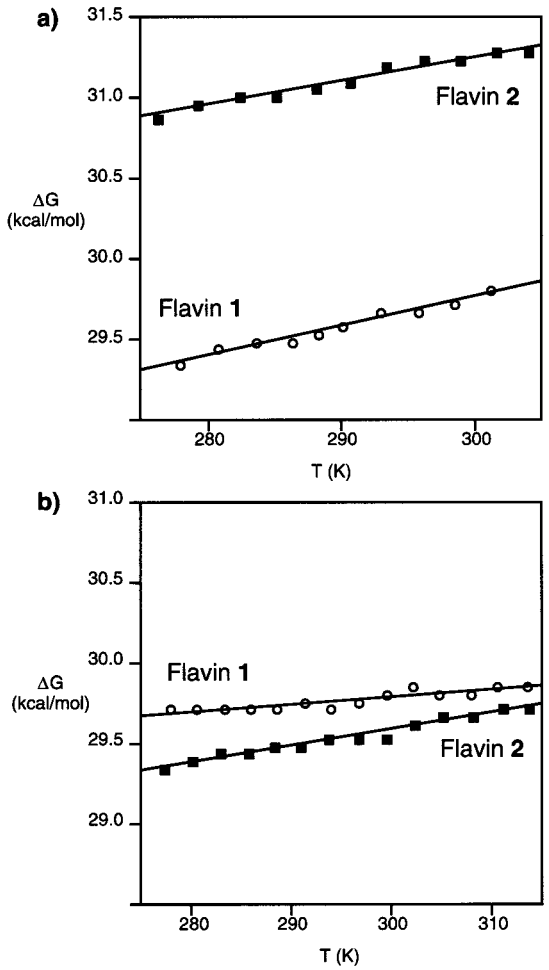

Figure 6. Variable-temperature electrochemistry of **1** (○) and **2** (■) in (a) CH_2Cl_2 and (b) DMF.

Table 2. Thermodynamic Parameters in the Reduction of Flavins **1** and **2**[a]

flavin	solvent	$\Delta H_{Fl_{ox}\rightarrow Fl_{rad}}$[b]	$\Delta S_{Fl_{ox}\rightarrow Fl_{rad}}$[c]
1	CH_2Cl_2	24.2	−18.3
1	DMF	28.4	−4.6
2	CH_2Cl_2	26.7	−15.3
2	DMF	26.6	−10.1

[a] ΔH and ΔS determined through variable-temperature cyclic voltammetry. [b] In kcal/mol. [c] In cal/mol.

bonding to O(2) and O(4) makes the reduction potential of flavin **1** 227 mV (*5.2 kcal/mol*) less negative.

Variable-temperature electrochemical studies of flavins **1** and **2** in dichloromethane and DMF were conducted to provide further insight into the thermodynamics of the N(3)H hydrogen bonding interaction, and to correlate the computationally predicted difference in enthalpy to experimental results (Figure 6, Table 2). The overall change in enthalpy due to hydrogen bonding at N(3) can be determined using flavin **2** as a control in each system, since **2** is insensitive to solvent effects at the N(3) position. The enthalpy difference between **1** and **2** in DMF reflects the difference between the selective stabilization of **1** in the Fl_{ox} state due to the formation of a specific hydrogen bond at the N(3) position and the slightly lesser stabilization of the Fl_{ox} state of **2** due to electron donation by the methyl substituent. The lack of stabilization of the flavin **1** oxidized state in CH_2Cl_2, on the other hand, can be traced to the lack of hydrogen bond acceptors available for the N(3) hydrogen, resulting in an unsatisfied hydrogen bond donor. The difference in enthalpy of reduction between flavin **2** in the two solvents is due to general solvent effects that are the same as those experienced by flavin **1**, and so can be subtracted from the overall enthalpy to yield the stabilization due to the hydrogen bond alone. The enthalpy difference between flavins **1** and **2** in the two solvents is 4.3 kcal/mol, in excellent agreement with the DFT-predicted value of 4.8 kcal/mol.[31]

The redox-state selective recognition of Fl_{ox} can be further demonstrated through comparison of the difference in the entropy of reduction of flavins **1** and **2** in DMF. While reduction of both **1** and **2** is entropically unfavorable, $\Delta\Delta S$ is 5.5 cal/mol more favorable in the flavin **1** reduction than for flavin **2**. On the basis of the increased distance of minimum enthalpy of interaction predicted from Fl_{ox} and Fl_{rad}^{-}, we attribute this increased entropy of **1** to a disordering of the solvent: due to the unfavorable proximity of two electron-rich groups, the DMF molecule constrained in the specific hydrogen bond will be released from the interaction upon flavin reduction to the solvent as a whole. This release mirrors the movement observed in the flavodoxin crystal structures, and may be indicative of a complete breakdown of the N(3)H hydrogen bond in the reduced state of the flavin cofactor in the enzyme.

In summary, the differences in solvent effects on the reduction of flavins **1** and **2** clearly indicate that the N(3) substituents and hydrogen bonding have marked effects on the redox potential of the flavin system. Hydrogen bonding to N(3)H can modulate the reduction potential for the reduction of flavin to the radical anion by at least 80 mV (1.8 kcal/mol), a substantial portion of the total biological range of ∼500 mV. This regulation is comprised in part from an enthalpic effect of 4.3 kcal/mol, arising from selective recognition of oxidized flavin relative to the radical anion. Entropic effects also indicate a favorable process is occurring in flavin **1** reduction that is not present for flavin **2**, which we attribute to the loss of the hydrogen bond and subsequent release of the solvent molecule. Finally, the excellent agreement between biological studies and our electrochemical and computational models indicates that the flavin system within an active site can be modeled effectively using these methodologies. This synergy between model and prototype allows us to explore biological systems in great detail and with high accuracy.

Experimental Section

Materials and General Methods. Solutions were prepared using reagent grade THF dried via distillation over sodium metal, reagent grade CH_2Cl_2 distilled over CaH_2, and DMF fractionally distilled under vacuum. Pyridine was reagent grade and was used without further purification as received from Aldrich. 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. Flavins **1** and **2** were synthesized according to literature procedures.[22]

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 reference electrodes, respectively. A silver wire pseudo reference electrode was used and all potentials are referenced versus the ferrocene/ferrocenium couple.

(30) Due to the electron-donating nature of the amide functions, the N(3)H hydrogen bonding between **1** and **3** would be expected to be stronger than that between **1** and pyridine. As a result, the 6.2 kcal/mol value predicted for the stabilization of **1** through hydrogen bonding at O(2) and O(4) represents a lower bound to this effect.

(31) Some attenuation of the calculated enthalpy derived from this hydrogen bond in the experimental conditions is expected since additional solvent molecules are not considered in the computations.

The sweep rate was 350 mV/s and the studies were run on an argon-purged temperature-controlled cell. Flavin solutions (3.8×10^{-4} M, 0.1 M TBAP) 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 the Supporting Information). For variable-temperature studies, solutions were heated to the maximum temperature and decreased from there in 3 °C steps, allowing 5 min for the system to equilibrate at each step.

Computational Studies. Lumiflavin was selected to model the Fl_{ox}−DMF and Fl_{rad}^{-}−DMF systems. All calculations were run using the Gaussian 98 package.[32] First, a full geometry optimization was run at the ab initio HF/3-21G* level to establish the optimum N(3)−O distance between the lumiflavin Fl_{rad}- and DMF, followed by a coordinate driving constraining the N(3)−O distance. The geometries thus generated were used to run the single point energy calculations at the B3LYP/6-31+G* level to obtain the energy profile shown in Figure 3. For Fl_{ox}, the coordinate driving used the same N(3)−O distance determined for Fl_{rad}^{-}−DMF. Enthalpies reported are relative to unbound species (for further details, see the Supporting Information).

Acknowledgment. This research was supported by the Petroleum Research Fund of the ACS (PRF 33137-AC4,5) and the National Institutes of Health (GM 59249-0). V.R. acknowledges support from the Alfred P. Sloan Foundation, Research Corporation, and the Camille and Henry Dreyfus Foundation. C.M. was supported by NIH Chemistry-Biology Interface Training Grant GM 5-84524. We thank Kulmeet Sandhu for helpful analysis of enzyme structures.

Supporting Information Available: Cyclic and square wave voltammograms for all solvents at room temperature, as well as the $E_{1/2}$ of each square wave used for variable-temperature graphs and calculation details, including the N(3)−O distances used for coordinate driving and the energies obtained are also included (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA994204V

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