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Forum Review

Artificial Flavin Receptors: Effects of Hydrogen Bonding on Redox Properties of a Flavin Mimic

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ABSTRACT

This review describes the roles of hydrogen bonding on the redox properties of a flavin mimic by using artificial flavin receptors. The receptors exploited are melamine derivatives bearing guanidinium ion(s) that strongly bind 6-azaflavin through five or seven hydrogen bonds involving N(1), C(2)=O, N(3)-H, C(4)=O, N(5), and N(6) positions in $CHCl_3$ and $CHCl_3$-acetonitrile. It has been shown that receptors are quite useful for examination of the hydrogen bonding effects on the redox potential, stability of the anionic semiquinone radical, and the oxidation activity of 6-azaflavin. The functionalized receptors have a substrate- or a metal-binding site have been shown to facilitate the reactions by forming the noncovalent assemblies. A possibility as an apoprotein model of the receptors has been mentioned also. Antioxid. Redox Signal. 3, 899–909.

INTRODUCTION

HYDROGEN BONDING is a fundamental force in molecular interactions in biological systems as seen in nucleic acid–base pairing and protein structures. Flavoenzymes exhibit diverse functions, such as dehydrogenation, electron transfer, activation of molecular oxygen, and repair of damaged DNA, in spite of the common catalytic group in flavin coenzymes such as flavin mononucleotide and flavin adenine dinucleotide. Undoubtedly, hydrogen bonds from apoproteins to the isoalloxazine ring are one of the factors that modulate the functions of flavin coenzymes. In fact, x-ray crystallographic data reveal a characteristic hydrogen-bonding network for each of the flavoenzymes (5, 16, 29, 30, 51, 53). If a specific mutation is possible, a role of the hydrogen bonding might be elucidated by comparing wild-type and mutant flavoenzymes (45). Meanwhile, the importance of hydrogen bonds on flavin reactivity has been pointed out from theoretical and experimental viewpoints. Molecular orbital calculations indicate that the hydrogen bonding involving N(1), C(2)=O, N(3)-H, C(4)=O, and N(5) is the most effective in lowering lowest unoccupied molecular orbital energy levels (36, 37). Recently, Nishimoto *et al*. have attempted to show, based on *ab initio* calculations, that the hydrogen bond at C(2)=O is crucial for lowering the activation energy for amino acid oxidation (38). Massey *et al*. have proposed to classify flavoenzymes into two categories according to the position of hydrogen bonds: (a) N(1)-hydrogen bonding activates the N(5) and 10(a) positions of an isoalloxazine ring and stabilizes the anionic semiquinone radical, and (b) N(5)-hydrogen bonding activates the C(4a) position, and is responsible for stabilization of the neutral radical (27, 28). The model investigations support some part of the

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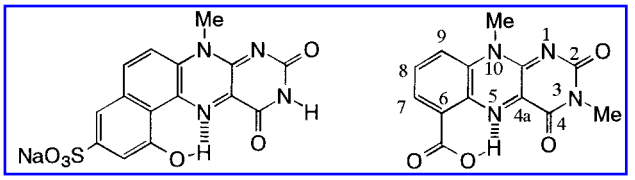


FIG. 1. Intramolecularly N(5)-hydrogen bonded flavin models.

proposals (1, 47). As shown in Fig. 1, those intramolecularly hydrogen-bonded flavin models facilitate the reactions proceeding via nucleophilic attack at the C(4a) position, and the anionic semiquinone radical of a flavin-6-carboxylate is stabilized by intramolecular N(5)-hydrogen bonding even in aqueous solution (47).

Artificial flavin receptors using hydrogen bonds may be promising for examination of intermolecular hydrogen bonding on the redox properties of a flavin. However, it is fairly difficult to design receptors that tightly hold a flavin through regiospecific hydrogen bonding at the heteroatoms of the isoalloxzazine ring. The flavin receptors are also of interest from the viewpoint of an apoprotein model, because the functionalized receptors could offer the functional groups in the vicinity of the bound flavin. It should be noted that introduction of the aproprotein functions into the catalytic systems is inevitable for construction of artificial enzymes (4, 33).

This review presents the effects of intermolecular hydrogen bonding on the redox properties of the oxidation-active flavin models (redox potential, stabilization of anionic semiquinone radical, and oxidation activity) by using flavin-receptor molecules in $CHCl_3$ or $CHCl_3$-acetonitrile (MeCN), and also the usefulness of functional flavin receptors as an aproprotein model from the viewpoint of biomimetic chemistry.

DESIGN OF FLAVIN RECEPTOR MOLECULES

It is known that 2,6-diamidopyridine derivatives bind barbiturates, glutarmides, hydantoins, and thymines through three hydrogen bonds (2, 10, 13, 14). 2,6-Diamidopyridine derivatives are also used as a flavin receptor as shown in Fig. 2. The binding constants (K_a) due to the three hydrogen bonds are in the order of $\sim 10^2\ M^{-1}$ in $CHCl_3$ (3, 49, 58) due to the secondary electrostatic interactions in the alternate three hydrogen bonds (18, 43, 61, 62). Furthermore, the binding constants are highly dependent on the substituent R in 2,6-diamidopyridine derivatives. For example, 2,6-dibenzoylaminopyridine is unable to bind a flavin due to steric hindrance of the phenyl group, which is caused by the planarity requirement of the amide bond (49). This may be a drawback of 2,6-diamidopyridine derivatives as a scaffold for functionalized flavin receptors.

Although the three hydrogen bonds at C(2)=O, N(3)-H, and C(4)=O are known to shift the redox potential in a positive direction (3), the oxidation activity of the flavin is little affected by these hydrogen bonds in $CHCl_3$ (49), probably because of no hydrogen bonding at the N(1) and N(5) positions. As protons are involved at both positions during the redox process, it is desirable that the receptor molecules should involve the N(1) and/or N(5) positions as the hydrogen-bonding sites. It is known that diamino-*s*-triazine and melamine derivatives form three hydrogen bonds with an imide group in a similar fashion to 2,6-diamidopyridine derivatives (23, 24, 54). Melamine scaffolds are quite useful for introduction of plural functional groups due to their synthetic simplicity (19). It should be noted that oxidation-active flavin mimics are required for the kinetic study of flavin-mediated oxidation reactions in aprotic organic solvents. We have exploited oxidation-active flavin mimics by

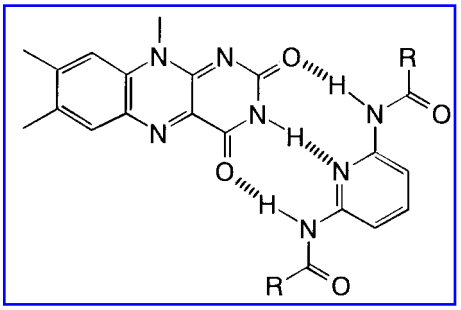


FIG. 2. Hydrogen-bonded complex of flavin and 2,6-diamidopyridine.

chemical modification of an isoalloxazine ring. Ring nitrogen atoms and a long conjugative system have been used as electron-withdrawing moieties. 6-Azaflavin (6-AzaFl) provides not only a high oxidation activity, but also another hydrogen-accepting site. Benzo-dipteridine (BDP), which possesses two isoalloxazine rings fused to a common benzo moiety, has been confirmed to act as a flavin model. These flavin mimics show remarkably high oxidation activity (10^6–10^7-fold) compared with the conventional flavin model (56, 57, 59).

BINDING CONSTANTS AND HYDROGEN-BONDED COMPLEXES

As a guanidinium ion forms AA·DD hydrogen bonds at the N(5) and N(6) positions of 6-AzaFl, we have designed melamine derivatives bearing guanidinium ion(s) that form a complex with 6-AzaFl through five or seven hydrogen bonds. In terms of binding, the most suitable spacer between the melamine and guanidinium moieties has been confirmed to be a *m*-xylylene unit (22). Evaluation of the K_a values and ^{1}H NMR study allows us to depict the structures of the hydrogen-bonded complexes as shown in Fig. 3 (20–22, 31, 50). Although 6-AzaFl·**1** and 6-AzaFl·**2** are isolated as powders, which are confirmed to be of 1:1 stoichiometry by electron spray ionization mass spectroscopy and elemental analysis, single crystals for x-ray crystallographic analysis have not yet been obtained.

From the data in Fig. 3, the following are obvious: (a) K_a values decrease remarkably with increasing MeCN content in $CHCl_3$; (b) the binding ability of **4** is weaker than that of **1** despite having the more acidic hydrogen donor; and (c) the larger K_a values of Fl·**1** and BDP·**1** compared with that of Fl·**4** suggest that the N(1) or N(5) position of Fl and BDP is involved in the hydrogen-bonding site, supported by the fact that K_a of 5-DeazaFl·**1** is larger than that of 5-DeazaFl·**4.**

The pK_a of guanidinium ion of **3** has been determined to be 10.7 (31), smaller by 2–3 pK_a units than that of unsubstituted guanidinium ions, such as **1** (40, 44). As it is known that more acidic hydrogen donors form stronger hydrogen-bonded complexes in the cases of the similar steric hindrance (6, 7, 34, 55), the smaller K_a value of 6-AzaFl·**3** compared with that of 6-AzaFl·**1** requires explanation. ^{1}H NMR study has shown that the chemical shifts of the C(7)-H of 6-AzaFl shift to upfield upon addition of **3,** indicating that the C(7)-H is situated in a position close enough to feel the ring current of the *N*-phenyl ring of **3** due to steric hindrance of the C(7)-H and *o*-H of the *N*-phenyl ring (22).

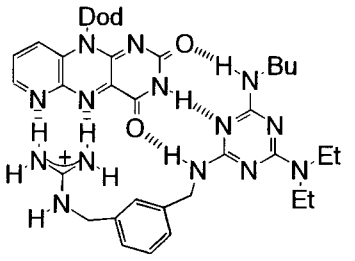
EFFECT OF HYDROGEN BONDING ON THE REDOX POTENTIAL OF 6-AzaFl

As hydrogen bonding is substantially electrostatic in nature, the receptors using hydrogen bonds bind more strongly to a reduced species than a neutral one, shifting the redox potential in a positive direction. In fact, the redox potentials of organic compounds such as 9,10-phenanthrenequinone (11) and 1,8-naphthalimide (12) both shift in a positive direction when involved in hydrogen bonding. Rotello *et al*. have reported that the redox potential of the conventional flavin model shifts in a positive direction (+155 mV) when forming three hydrogen bonds at the imide moiety of the flavin (3, 35). Therefore, it is interesting to examine the positive shifts induced via hydrogen bonding involving the N(1) and N(5) positions of 6-AzaFl.

The cyclic voltammogram of 6-AzaFl shows a reversible one-electron couple in the absence of the receptor in CH_2Cl_2-MeCN (20%). The reduction and oxidation waves shift in a positive direction to reach fixed values with increasing receptor concentrations (**1, 2,** and **3**), representing the redox potentials of the fully hydrogen-bonded complexes (6-AzaFl·**1,** 6-AzaFl·**2,** and 6-AzaFl·**3**). The fixed redox potentials are shown in Table 1 (22).

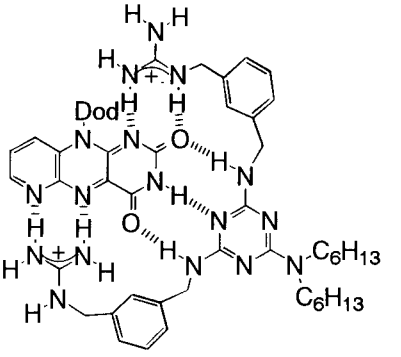
STABILIZATION OF ANIONIC SEMIQUINONE FLAVIN RADICAL

A free flavin radical is known to be quite unstable with respect to disproportionation to the



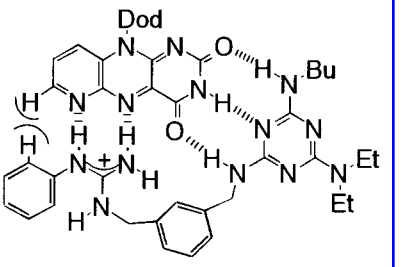
6-AzaFl·**1**

Ka = 140,000 $M^{-1}(CHCl_3)$
Ka = 18.000M^{-1} (10% MeCN)



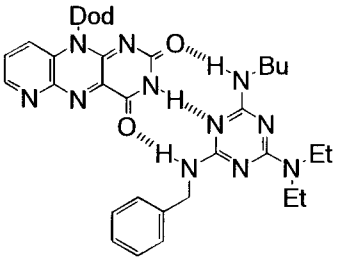
6-AzaFl·**2**

Ka = 38,000 M^{-1} (10 % MeCN)



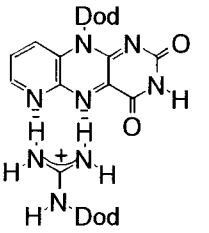
6-AzaFl·**3**

Ka = 53,000 $M^{-1}(CHCl_3)$



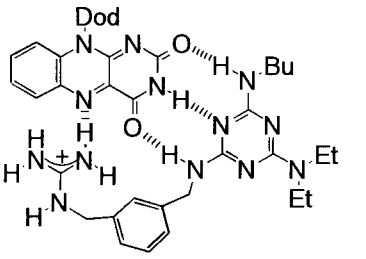
6-AzaFl·**4**

Ka = 140 $M^{-1}(CHCl_3)$



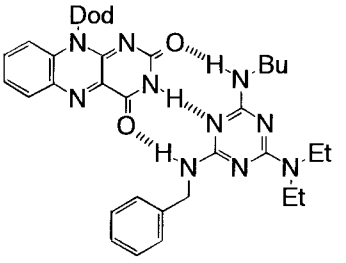
6-AzaFl·**5**

Ka = 100 $M^{-1}(CHCl_3)$



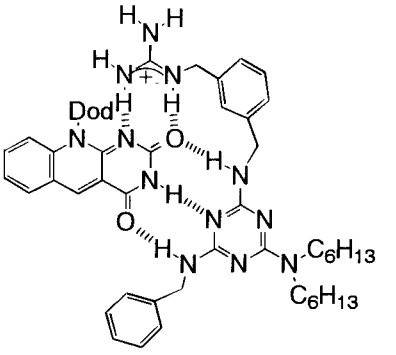
Fl·**1**

Ka = 2,000 M^{-1} $(CHCl_3)$



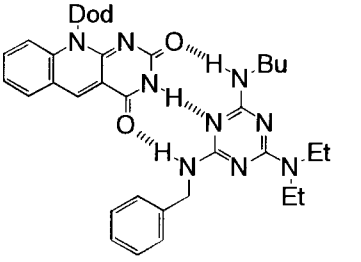
Fl·**4**

Ka = 150 $M^{-1}(CHCl_3)$



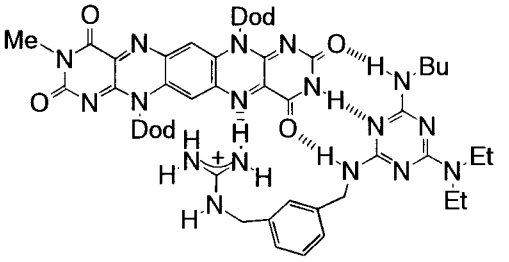
5-DeazaFl·**1**

Ka = 840 M^{-1} $(CHCl_3)$



5-DeazaFl·**4**

Ka = 130 M^{-1} $(CHCl_3)$



BDP·**1**

Ka = 1,700 M^{-1} $(CHCl_3)$

FIG. 3. Hydrogen-bonded complexes and binding constants (K_a).

TABLE 1. REDOX POTENTIALS OF 6-AzaFl IN THE PRESENCE OF THE RECEPTORS IN CH_2Cl_2-MeCN (20%, VOL/VOL)*

Receptor	$E_{1/2}$ *(mV)*	$\Delta E_{1/2}$ *(m/V)*	$\Delta\Delta G$ *(kcal mol^{-1})*
None	−926	0	0
1	−706	+220	−5.2
2	−609	+317	−7.3
3	−674	+252	−5.8

*[6-AzaFl] = 1.0×10^{-3} M, [TBAP] = 0.1 *M* (vs. ferrocene) (TBAP = tetrabutylammonium perchlorate).

oxidized and reduced flavins in aqueous solution (9). As seen in flavodoxins, however, the radicals are generally stable when bound to apoproteins (8). Experimental and calculated spin densities of an anionic semiquinone of flavin indicate that the highest spin density is located at the N(5), and little or no density is located at the N(1) position (8, 32, 52). This suggests that the hydrogen bonding at the N(5) is responsible for stabilization of the anionic semiquinone radicals (32). In fact, Yoneda *et al.* have shown that an anionic semiquinone radical is stabilized by intramolecular hydrogen bonding at the N(5) position even in aqueous solution (47).

As shown in Table 1, the anionic semiquinone radical (6-AzaF$^{\cdot -}$) is considerably stabilized by hydrogen bonding to the receptors in the order of **2** > **3** > **1.** This suggests that the H-bonding at the N(1) position is also important for stabilization of the anionic semiquinone radical as shown in Fig. 4.

The binding constants (K_{rad}) of 6-AzaFl$^{\cdot -}$·**1,** 6-AzaFl$^{\cdot -}$·**2,** and 6-AzaFl$^{\cdot -}$·**3** are determined from ΔG_3 in the thermodynamic cycle as shown in Scheme 1. The K_{rad} values have been calculated to be 7.0×10^7 M^{-1} ($\Delta G_3 = -10.7$ kcal mol^{-1}), 4.8×10^9 M^{-1} ($\Delta G_3 = -13.2$ kcal mol^{-1}), and 1.2×10^8 M^{-1} ($\Delta G_3 = -11.0$ kcal mol^{-1}), which are much larger than the K_a values by factors of 5×10^3, 3×10^5, and 2×10^4, respectively (22).

The anionic semiquinone radical stabilized by the receptor is detected by UV visible and electron paramagnetic resonance spectroscopy (22). Spectroscopic examination of the oxidation of thiols or an NADH model has shown that the anionic semiquinone radical (6-AzaFl$^{\cdot -}$) is considerably stabilized in the presence of the receptors (**2** and **3**) in $CHCl_3$-MeCN (20%) as shown in Fig. 5. In the presence of **2,** 6-AzaFl$^{\cdot -}$ is formed (spectrum c), whereas two-electron-reduced 6-AzaFl is formed in the presence of **1,** or a mixture of **4** and **5.** This indicates that the strength and the position of hydrogen bonds are critical for stabilization of the radical. The radical stability is estimated by the reaction with O_2. From a qualitative O_2-exposure experiment, 6-AzaFl$^{\cdot -}$·**2** is more stable than 6-AzaFl$^{\cdot -}$·**3.** Even in the presence of **2,** the anionic radical has not been observed in ethanol or MeCN, nor when 6-aza-3-methylflavin was used (22).

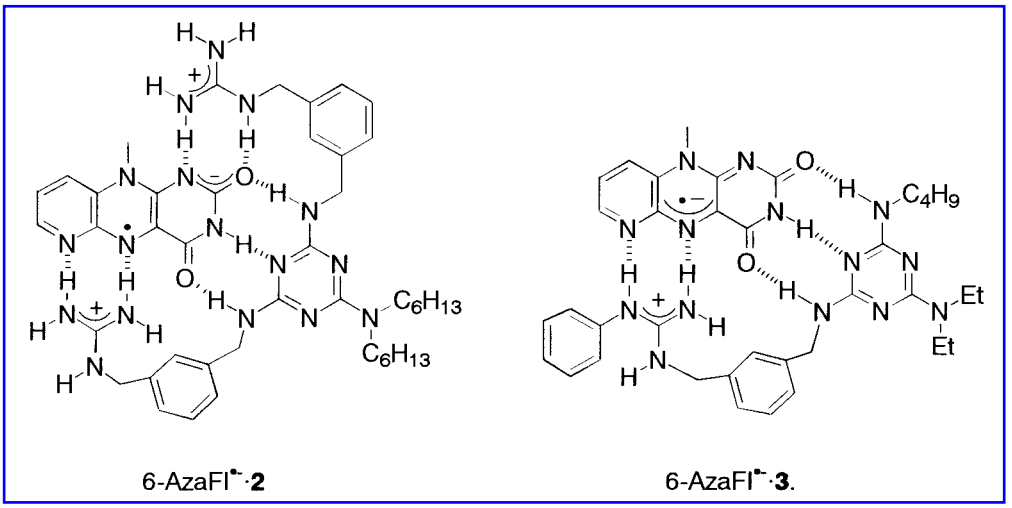
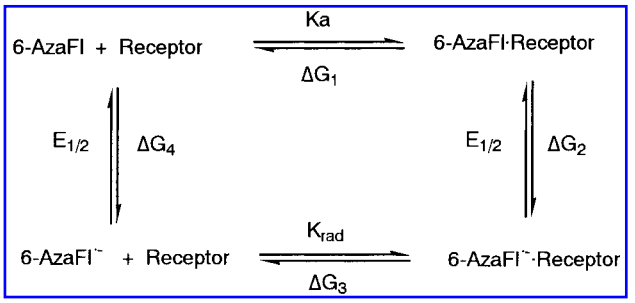


FIG. 4. Hydrogen-bonded complexes of 6-AzaFl$^{\cdot -}$ with 2 and 3.



SCHEME 1. Thermodynamic cycle.

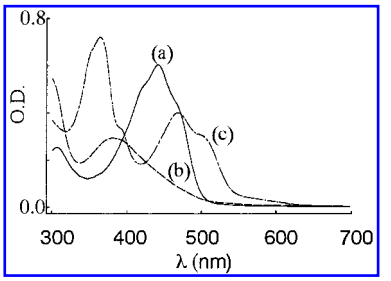
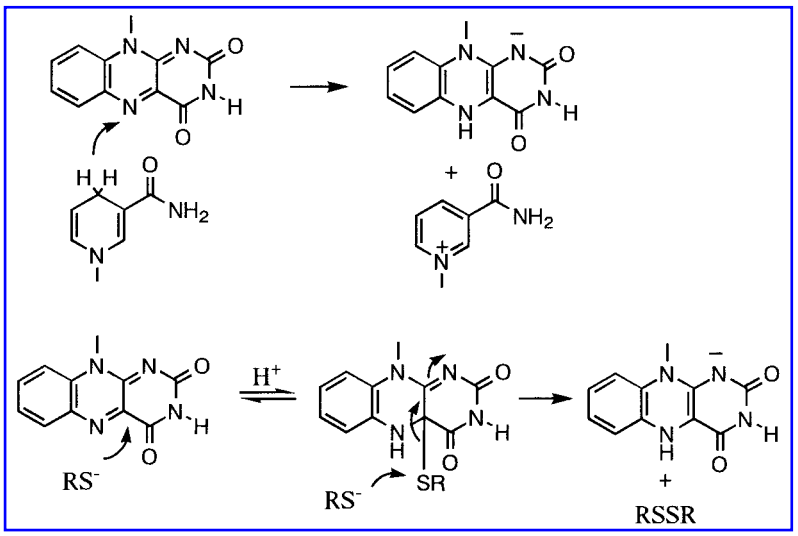


FIG. 5. Spectral changes of the reaction of 6-AzaFl with PhSH in the presence of 2. [6-AzaFl] = 5.0×10^{-5} *M*, [PhSH] = [Bu_3N] = 2.0×10^{-4} *M*, [**2**] = 1.0×10^{-4} *M*, $CHCl_3$-MeCN (20%), N_2, 25°C. Spectrum a, oxidized; spectrum b, two-electron reduced; spectrum c, anionic semiquinone radical.

EFFECT OF HYDROGEN BONDING ON THE OXIDATION ACTIVITY OF FLAVIN

To examine the effects of hydrogen bonding sites on the reactivity of a flavin, it is desirable to study the reactions whose mechanisms have been established. As the oxidation mechanisms for the oxidation of an NADH model and thiols by flavin are well established, they are pertinent for the kinetic study. The former proceeds via a hydride transfer to the N(5) position of an isoalloxazine ring (41, 42, 48), and the latter proceeds via nucleophilic attack of thiolate at the C(4a) position to form an adduct, followed by attack of the second thiolate at the sulfur atom of the adduct to give the corresponding disulfide and reduced flavin (25, 26) as shown in Scheme 2.

The effects of the receptors (**1** and **2**) on the rates of 6-AzaFl are shown in Fig. 6 (22). The rate accelerations for *N*-benzyl-1,4-dihydronicotinamide (BNAH) oxidation are severalfold for **2,** whereas there is almost no effect for **1.** On the other hand, the rate accelerations for thiophenol (PhSH) oxidation are 1.7×10^3-fold for **1** and 7.8×10^3-fold for **2.** As 6-AzaFl·**2** involves the hydrogen bond at the N(1) position, the larger rate acceleration of **2** compared with that of **1** for BNAH oxidation may be ascribed to the N(1)-hydrogen bond. On the other hand, both 6-AzaFl·**1** and 6-AzaFl·**2** involve the N(5)-hydrogen bond, resulting in the large rate accelerations for PhSH oxidation. Meanwhile, it is known that the pK_a of the product is an im-



SCHEME 2. Reaction schemes for the oxidation of an NADH model and a thiol by flavin.

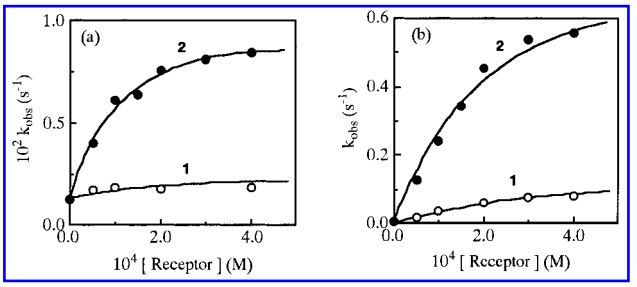


FIG. 6. Effects of the receptors on the rates of the oxidation of BNAH and PhSH. [6-AzaFl] = 5.0×10^{-5} *M*, $CHCl_3$-MeCN (20%), N_2, 25°C. (**a**) [BNAH] = 2.0×10^{-3} *M*. (**b**) [PhSH] = 2.0×10^{-3} *M*, [Bu_3N] = 1.0×10^{-2} *M*.

portant factor in determining rates of acid catalysis (17). As hydrogen bonding stabilizes a negative species, it would be necessary to consider pK_a of the species during the reactions (Scheme 2). The much larger rate accelerations for PhSH oxidation may be explained in part by the fact that formation of the C(4a)-adduct is facilitated by the N(5)-hydrogen bonding due to the larger pK_a of the N(5)-H of the adduct. For BNAH oxidation, the pK_a of the N(1)-H of two-electron-reduced 6-AzaFl (product) is quite small (56).

The results provide experimental evidence to support Massey's proposal that N(1)-hydrogen bonding facilitates the reaction at the N(5) position, and N(5)-hydrogen bonding facilitates the reaction involving nucleophilic attack at the C(4a) position (27, 28).

APPLICATION OF FUNCTIONALIZED FLAVIN RECEPTORS

For construction of more sophisticated model systems, additional functional groups are required, suitably arranged in the vicinity of the reaction site of flavin. Two strategies are conceivable for the functionalization of flavin: covalent and noncovalent functionalizations. In the former, the functional group is introduced into an oxidation-active flavin mimic by covalent bonds (38, 46, 60), and the latter would be achieved by using functionalized flavin receptors. We have designed the receptors (**6** and **7**) bearing a substrate-binding or a metal-binding site as shown in Fig. 7.

The smaller K_a of 6-AzaFl·**6** compared with that of 6-AzaFl·**8** indicates that the second melamine moiety is not involved in the complexation. In other words, the second melamine moiety could be used as a thymine-binding site. If a thymine-linked substrate is used, the reaction would be facilitated due to a proximity effect. The kinetic results indicate that the rates

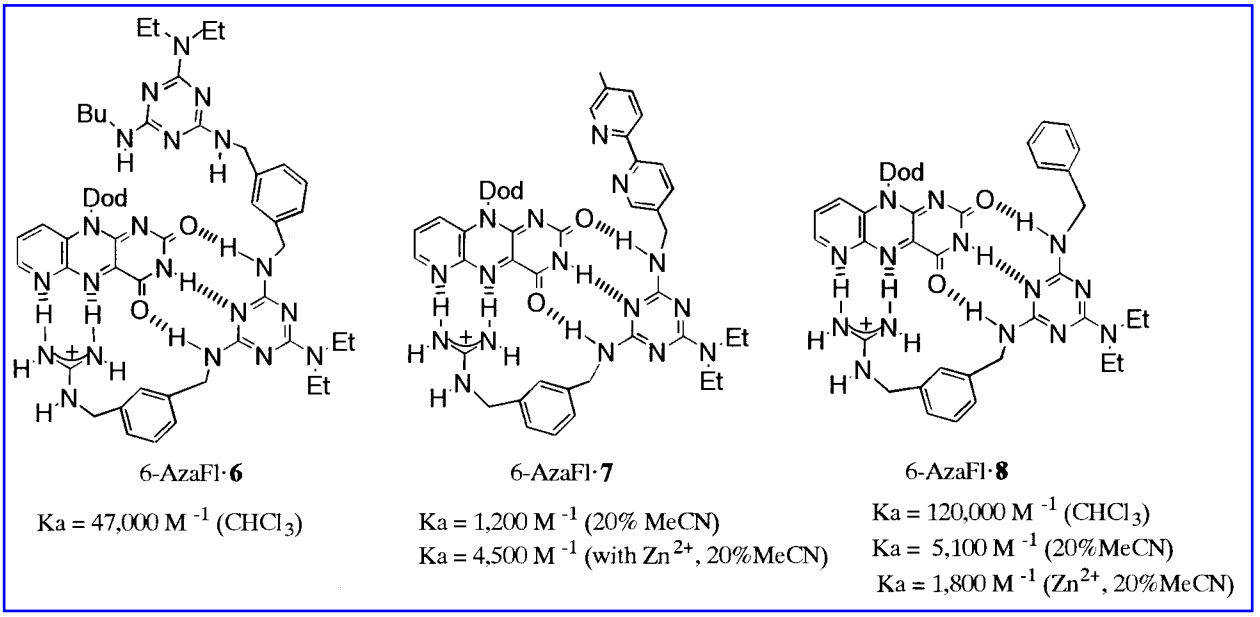


FIG. 7. Hydrogen-bonded complexes of 6-AzaFl and the functionalized receptors and binding constants.

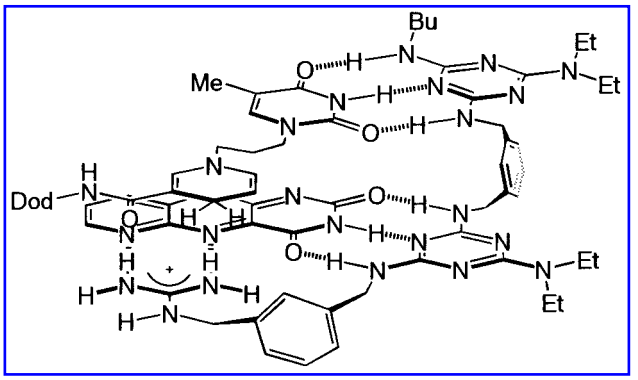


FIG. 8. A possible structure of the ternary complex.

of oxidation of an *N*-(3-thyminylpropyl)-1,4-dihydronicotinamide derivative by 6-AzaFl increase and reach saturation with increasing substrate concentration in the presence of **6** in $CHCl_3$ (15). As shown in Fig. 8, the reaction proceeds via formation of a ternary complex (6-AzaFl·**6**·substrate) to afford 13-fold rate acceleration, whereas no effect is observed in the presence of **8.**

The K_a values of 6-AzaFl·**7** are increased in the presence of Zn^{2+}, whereas the K_a of 6-AzaFl·**8** is decreased in the presence of Zn^{2+}. This suggests that the bound Zn^{2+} at the bipyridine moiety in 6-AzaFl·**7** participates in binding by coordinating to C(2)=O. The rate of the oxidation of BNAH by 6-AzaFl·**7** is increased (40-fold) in the presence of Zn^{2+} in $CHCl_3$/20% MeCN (unpublished observations). The rate acceleration may be explained by the fact that bound Zn^{2+} facilitates the hydride transfer by acting as a Lewis acid as shown in Fig. 9.

The receptor molecules that bind a coenzyme model and a substrate or a metal ion through noncovalent bonds could be viewed as possessing an apoprotein function.

CONCLUSIONS

The investigations in flavin model systems have contributed to the understanding of flavin catalysis at the molecular level. Flavin receptors using hydrogen bonding have been shown to be useful for study of the effects of hydrogen bonding on the redox properties of flavin. Melamine derivatives bearing guanidinium ion(s) bind strongly to 6-AzaFl through five or seven hydrogen bonds involving the N(1), C(2)=O, N(3)-H, C(4)=O, N(5), and N(6) sites, which collectively exert influence on the redox potential, stabilization of the anionic semiquinone radical, and the oxidation activity. The functionalized receptors having a substrate- or metal-binding site form noncovalent assemblies and lead to observable rate accelerations. These receptor molecules are potentially useful as an apoprotein model for construction of sophisticated catalytic systems.

ACKNOWLEDGMENTS

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ABBREVIATIONS

6-AzaFl, 6-azaflavin; BDP, benzo-dipteridine; BNAH, *N*-benzyl-1,4-dihydronicotinamide; MeCN, acetonitrile; PhSH, thiophenol.

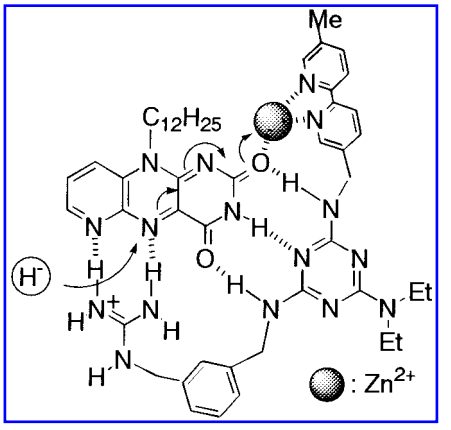

FIG. 9. A plausible role of Zn^{2+} in BNAH oxidation.

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