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Flavin Receptors. Effect of the Acidity of Melamine Derivatives Bearing a 2-Arylguanidinium Ion on 6-Azaflavin Binding in Chloroform

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The pK_a's of melamine derivatives bearing a 2-arylguanidinium ion and their binding constants for 6-aza-10-dodecylisoalloxazine (6-azaflavin) were nicely correlated with the Hammett σ of the substituents. However, the rates of the oxidations of *N*-benzyl-1,4-dihydronicotinamide (BNAH) and dithiothreitol (DTT) by 6-azaflavin were not affected by the substituents of the receptors in $CHCl_3$.

Molecular assemblies due to hydrogen bonds have attracted considerable attention from the viewpoints of molecular recognition and supramolecular architecture.[1] The number and arrangement of hydrogen-bonding sites[2] and the acidity of the H-donors and the basicity of the H-acceptors[3] are significant factors that control the binding ability. Meanwhile, hydrogen bonds are recognized to be one of the important factors that regulate the functions of a flavin, based on enzymatic,[4] theoretical,[5] and model[6] investigations. Flavin receptors using hydrogen bonds of the guanidinium ion are quite useful for understanding of the roles of H-bonds on the redox properties of flavin, because the guanidinium group is an arginine residue that plays an important role in arginine-dependent enzymes;[7a] more importantly, the effect of hydrogen bonds on base-promoted flavin oxidation can be examined due to a higher pK_a (> 10) of the guanidinium ion.[7]

We have reported that a melamine derivative bearing a guanidinium ion (**1**) strongly binds 6-azaflavin through five hydrogen bonds ($K = 1.4\times10^5$ M^{-1} in $CHCl_3$; 1 M = 1 mol dm^{-3}), compared with the corresponding 2-phenyl guanidinium ion (**2a**) ($K = 5.3\times10^3$ M^{-1} in $CHCl_3$). This unusual phenomenon is ascribed to a steric hindrance of the 2-phenyl group on complex formation based on a ^1H NMR study.[6a] It could be said, as a matter of course, that complexation due to hydrogen bonds is strengthened by a more acidic H-donor in the case of a similar steric hindrance.

In this paper, we describe the synthesis of melamine derivatives bearing a 2-arylguanidinium ion, the determination of the pK_a's of the guanidinium ions in H_2O–MeCN (30% v/v) and the binding constants (K) for 6-azaflavin and 10-dodecylisoalloxazine (Fl), and the substituent effect of the receptors on the reactivities of 6-azaflavin for BNAH and DTT oxidations in $CHCl_3$. The structure of the H-bonded complex of 6-azaflavin and the employed compounds were shown in Chart 1.

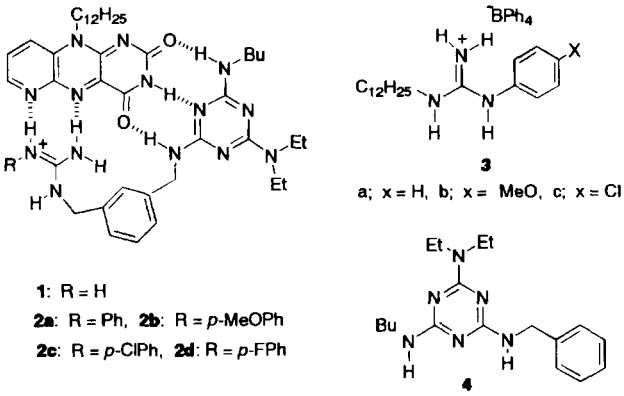

Chart 1. Complex of 6-azaflavin with **1** or **2** and related compounds.

Results and Discussion

Synthesis of Receptors. Melamine derivatives bearing the 2-arylguanidinium ion (**2**) were synthesized by procedures similar to those of **2a**, as previously described.[6a] Namely, **2** and **3** were prepared from 2-[3-(aminomethyl)-benzylamino]-4-butylamino-6-diethylamino-*s*-triazine[6a] and dodecylamine with *N*-aryl-*S*-methylisothiouronium iodides[8] in ethanol, respectively. In cases where the guanidinium iodide was hard to crystallize, the iodide anion was exchanged by BPh_4^- or ClO_4^-.

Determination of pK_a of the Guanidinium Ions. The pK_a values of the guanidinium ion of **2** were determined by spectroscopic pH-titration in buffer solutions (30% MeCN). After the absorption changes of **2** and **3a** were confirmed to be pH-reversible between pH ca. 8 and 13, they were plotted against the pH to give sigmoid curves[6a] for all of the guanidinium ions examined. The thus-obtained pK_a values are listed in Table 1. Since the pK_a values of conventional guanidinium ions are known to be in the range of 12—13,[7] the 2-arylguanidinium ions are more acidic by 2—3 pK_a units. The larger pK_a value of **3a** than that of **2a** is due to

Table 1. The pK_a Values, Binding Constants, and Free Energy Changes

Receptor	pK_a[a]	6-Azaflavin		Fl	
		K/M^{-1}	$-\Delta G$/kcal mol^{-1}	K/M^{-1}	$-\Delta G$/kcal mol^{-1}
2a	10.7 ± 0.1	5.3 ± 0.3 × 10^3[b]	5.1	4.5 ± 0.0 × 10^2[c]	3.6
2b	11.2 ± 0.1	2.7 ± 0.1 × 10^3[d]	4.7	4.6 ± 0.1 × 10^2[c]	3.6
2c	10.2 ± 0.1	1.1 ± 0.1 × 10^4[d]	5.5	n.d	
2d	10.5 ± 0.1	6.4 ± 0.1 × 10^3[d]	5.2	4.3 ± 0.1 × 10^2[c]	3.5
3a	11.2 ± 0.1	2.2 ± 0.1 × 10^2[e]	3.1	6.0 × 10[e]	2.4
3b	n.d[f]	2.1 ± 0.0 × 10^2[e]	3.1	n.d	–
3c	n.d	2.4 ± 0.0 × 10^2[e]	3.2	n.d	–
4	–	1.4 ± 0.0 × 10^2[b]	2.9	1.5 ± 0.2 × 10^2[b]	3.0

a) In H_2O–MeCN (30% v/v), 25 °C. b) From our previous study (Ref. 6a). C) Fluorescence spectroscopy: [Fl] = 4.0×10^{-5} M, [**2**] = $0-1.25\times10^{-3}$ M, 20 °C. d) UV-vis spectroscopy; [6-azaflavin] = 5.0×10^{-5} M, [**2**] = $0-4.5\times10^{-4}$ M, 25 °C e) Fluorescence specroscopy; [3-Me-6-azaflavin] = 5.0×10^{-5} M, [**3**] = $0-1.25\times10^{-3}$ M, 20 °C. f) Not determined.

the electron-donating property of the dodecyl group of **3a**. The pK_a values were nicely correlated with the Hammett σ to give $\rho = +2.0$ (correlation coefficient = 0.994), which is in good agreement with $\rho = +2.18$ for the pK_a values of the 2-aryl-1,1,3,3-tetramethylguanidinium ions.[7d]

Binding Constants. The binding constants of **2** and **3** for 6-azaflavin and 10-dodecylisoalloxazine (Fl) were determined by UV-vis or fluorescence spectroscopy in $CHCl_3$, as previously described.[6a] The results are complied in Table 1. For 6-azaflavin binding, the log K values of **2** were nicely correlated with the Hammett σ to give $\rho = +1.2$ (correlation coefficient = 0.994), indicating that more acidic H-donors exhibit larger K values. We have shown previously that the free-energy change of 6-azaflavin·**1** (five H-bonds) is larger than that of the sum of each component (two H-bonds of guanidinium **3** and three H-bonds of melamine **4**), due to a preorganization to decrease the entropic loss for complexation.[6a] However, the free-energy change of **2a** ($-\Delta G$ = 5.1 kcal mol^{-1}), for example, is smaller than the sum of the each component (**3a** and **4**, $-\Delta G = 3.1+2.9 = 6.0$ kcal mol^{-1}), implying that the steric hindrance is operative for the complexation of 6-azaflavin·**2**. For Fl, the larger K values of **2** than those of **4** indicate that the guanidinium ion of **2** is involved in H-bonds at the N(1)- or N(5)-position of the isoalloxazine ring. However, the binding constants of **2** for Fl are little affected by the substituents. This may be explained by the fact that the binding of Fl·**2** is mainly controlled by three H-bonds of the melamine moiety of **2** (Fl·**2** vs. Fl·**4**).

Substituent Effect of the Receptor on Oxidation Activity. We have already shown that N(1)-hydrogen bonding facilitates the reaction occurring at the N(5)-position, and N(5)-hydrogen bonding accelerates the reactions proceeding via a nucleophilic attack at the C(4a)-position.[6a] By employing **2**, the substituent effect was kinetically examined for the oxidations of BNAH and DTT by 6-azaflavin in $CHCl_3$. The pseudo-first-order rate constants were determined by following the absorption decreases of 6-azaflavin at 440 nm under anaerobic conditions. For BNAH oxidation, the rates increased slightly with an increase of [**2**], and no substituent effect was observed. For DTT oxidation, although the rates increased with an increase of [**2**], no substituent effect of the receptors was observed. Typical kinetic data are listed in Table 2. No substituent effect may be explained by the fact that the range of the pK_a change by the aryl substituents is too small to affect the oxidation activity of 6-azaflavin.

Table 2. Pseudo-First-Order Rate Constants and Relative Rates[a]

Receptor	BNAH[b]		DTT[c]	
	k_{obs}/s^{-1}	Rel rates	k_{obs}/s^{-1}	Rel rates
none	5.48 × 10^{-3}	1.0	4.07 × 10^{-3}	1.0
2a	1.14 × 10^{-2}	2.1	2.45 × 10^{-1}	60
2b	1.08 × 10^{-2}	2.0	2.55 × 10^{-1}	63
2c	1.13 × 10^{-2}	2.1	2.31 × 10^{-1}	57
2d	1.10 × 10^{-2}	2.0	2.45 × 10^{-1}	30

a) [6-Azaflavin] = 5.0×10^{-5} M, $CHCl_3$, N_2, 25 °C.
b) [BNAH] = 2.50×10^{-3} M, [**2**] = 4.0×10^{-4} M.
c) [DTT] = [Bu_3N] = 1.00×10^{-3} M, [**2**] = 3.0×10^{-4} M.

In summary, we have demonstrated that the binding constants of melamine derivatives bearing a 2-arylguanidinium ion for 6-azaflavin are influenced by the substituents of the aryl groups, and that the oxidation activities of 6-azaflavin for BNAH and DTT are insensitive to the substituents.

Experimental

^{1}H NMR spectra were recorded on a Varian Gemini-200 (200 MHz) or a JOEL JAM α-500 (500 MHz) instrument with chemical shifts from tetramethylsilane. The electronic absorption spectra were recorded on a JASCO Ubset-560 or a Shimadzu UV-2200A spectrophotometer. Fluorescence spectra were recorded on a Hitachi 850 fluorescence spectrophotometer. Fast atom bombardment (FAB) mass spectroscopy was performed on a Shimadzu/Kratos Concept 1S spectrometer using *m*-nitrobenzyl alcohol as the matrix. Stopped-flow rate measurements were performed with an Otsuka Electronics RA-401 spectrophotometer. Flash column chromatography was performed by using a Wakogel C-200 (70—150 μm, Wako Pure Chemical Co.). Elemental analyses were performed at the Center of Instrumental Analysis of Gunma University. The melting points are uncorrected. Special-grade chloroform (Kanto Chemicals) was used without further purification. Acetonitrile and DMF were purified by distillation from calcium hydride.

N-Aryl-S-methylisothiouronium Iodides: A mixture of *N*-arylthiourea (40 mmol) and MeI (50 mmol) in MeOH (80 ml) was stirred overnight at room temperature. After evaporating MeOH and excess MeI under reduced pressure, the residue was washed with diethyl ether (80 ml×5) and recrystallized from EtOH–diethyl ether.

p-MeO: Yield 47%, mp 159.5—160 °C (MeOH–diethyl ether) (Ref. 8b, 160—163 °C). *p*-Cl: Yield 93%, mp 163—164 °C (EtOH-diethyl ether)(Ref. 8b, 165—168 °C). *p*-F: Yield 96%, mp 142.5—143.5 °C (EtOH–diethyl ether)(Ref. 8b 149.5—150.5 °C).

Receptors **2b**, **2c**, and **2d** were prepared in a similar to **2a**.[6a] Namely, a mixture of 2-[3-(aminomethyl)benzylamino]-4-butylamino-6-diethylamino-*s*-triazine[6a] (3.4 mmol) and *N*-aryl-*S*-methylisothiouronium iodide (3.4 mmol) in EtOH (40 ml) was refluxed for 3 d. After the solvent was evaporated in vacuo, the residue was washed with diethyl ether, and purified by recrystallization.

2b: Yield 30%, mp 152—153 °C (THF–hexane). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 0.91 (3H, t, J = 7.36 Hz, $-(CH_2)_3C\underline{H}_3$), 1.13 (6H, m, $NCH_2C\underline{H}_3$), 1.33—1.38 (2H, m, $N(CH_2)_2C\underline{H}_2CH_3$), 1.50—1.53 (2H, m, $NCH_2C\underline{H}_2CH_2CH_3$), 3.33 (2H, t, J = 7.00 Hz, $NC\underline{H}_2(CH_2)_2CH_3$), 3.49—3.53 (4H, m, $NC\underline{H}_2CH_3$), 3.80 (3H, s, $OC\underline{H}_3$), 4.50 (2H, s, $C_6H_4C\underline{H}_2NHC(NH_2)$=$N^+H$–), 4.57 (2H, s, triazine$NHC\underline{H}_2C_6H_4$), 6.90, 7.12 (4H, d, J = 6.68 Hz, $C_6H_4OCH_3$), 7.26—7.38 (8H, m). Found: C, 51.42; H, 6.42; N, 19.47%. Calcd for $C_{27}H_{40}IN_9O$: C, 51.19; H, 6.36; N, 19.90%

2c: Yield 32%, mp 194—196 °C (EtOH–diethyl ether). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 0.91 (3H, t, J = 7.35 Hz, $-(CH_2)_3C\underline{H}_3$), 1.12 (6H, m, $NCH_2C\underline{H}_3$), 1.32—1.37 (2H, m, $N(CH_2)_2C\underline{H}_2CH_3$), 1.49—1.52 (2H, m, $NCH_2C\underline{H}_2CH_2CH_3$), 3.31 (2H, t, J = 7.00 Hz, $NC\underline{H}_2(CH_2)_2CH_3$), 3.51—3.53 (4H, m, $NC\underline{H}_2CH_3$), 4.53 (2H, s, $C_6H_4C\underline{H}_2NHC(NH_2)$=$N^+H$–), 4.56 (2H, s, triazine$NHC\underline{H}_2C_6H_4$), 7.06—7.40 (12H, m, Ar–H, guanidino NH). MS(FAB): Found: *m/z* 622. Calcd for $C_{26}H_{37}FIN_9$: (M+1), 622.

2d: After the residue was dissolved in H_2O–MeOH, excess $NaClO_4$ was added under stirring. The formed precipitates were collected and recrystallized. Yield 40%, mp 179—180 °C (MeCN–diethyl ether). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 0.92 (3H, t, J = 7.45 Hz, $-(CH_2)_3C\underline{H}_3$), 1.14 (6H, m, $NCH_2C\underline{H}_3$), 1.26 (2H, m, $N(CH_2)_2C\underline{H}_2CH_3$), 1.33—1.38 (2H, m, $NCH_2C\underline{H}_2CH_2CH_3$), 3.33 (2H, t, J = 7.00 Hz, $NC\underline{H}_2(CH_2)_2CH_3$), 3.49—3.55 (4H, m, $NC\underline{H}_2CH_3$), 4.53 (2H, s, $C_6H_4C\underline{H}_2NHC(NH_2)$=$N^+H$–), 4.57 (2H, s, triazine$NHC\underline{H}_2C_6H_4$), 7.06—7.40 (12H, m, Ar–H, guanidino NH). Found: C, 50.98; H, 5.97; N, 20.37%. Calcd for $C_{26}H_{37}Cl_2N_9O_4$: C, 51.17; H, 6.11; N, 20.65%.

Receptors **3** were similarly prepared from dodecylamine and *N*-aryl-*S*-methylisothiouronium iodide in EtOH. After evaporating the solvent, the residue was subjected to column chromatography (silica gel, $CHCl_3$–MeOH, 30 : 1 v/v). An oily product was dissolved in MeOH–H_2O and excess $NaBPh_4$ was added under stirring. The formed precipitate was collected and recrystallized.

3a: Yield 20%, mp 84—85 °C (EtOH–diethyl ether). Found: C, 81.52; H, 8.75; N, 6.18%. Calcd for $C_{43}H_{64}BN_3 \cdot 0.7H_2O$: C, 81.16; H, 8.78; N, 6.60%.

3b: Yield 25%, mp 92—93 °C (EtOH–diethyl ether). Found: C, 80.19; H, 8.79; N, 6.55%. Calcd for $C_{44}H_{56}BN_3O \cdot 1/3H_2O$: C, 80.10; H, 8.66; N, 6.55%.

3c: Yield 20%, mp 94—95 °C (EtOH–diethyl ether). Found: C, 75.38; H, 8.07; N, 6.18%. Calcd for $C_{43}H_{53}BClN_3 \cdot 1.5H_2O$: C, 75.38; H, 8.24, N, 6.13%.

6-Aza-10-dodecylisoalloxazine (6-azaflavin), 6-aza-3-methyl-10-dodecylisoalloxazine, and 10-dodecylisoalloxazine were supplied from previous study.[6a]

Determination of pK_a. The pK_a's were spectrophotometrically determined by pH-titration in a buffer solution containing MeCN (30% v/v) (0.1 M phosphate or borate buffer, I = 0.3 with KCl). The absorption spectra of **2** (1.0×10^{-4} M) were recorded at different pH's (pH 8.6—13.2). The wavelengths used for pH-titration were 290 nm for **2a** and **3a**, 290 nm for **2b**, 285 nm for **2c**, and 275 nm for **2d**.

Determination of Binding Constants. The binding constants were determined by absorption or fluorescence spectroscopy, as described in previous papers.[6a]

Rate Measurements. (a) BNAH oxidation. In a Thunberg cuvette, 60 μl of a 6-azaflavin stock solution (2.5×10^{-3} M in $CHCl_3$) and a suitable amount of the receptor (1.0×10^{-2} M or 5.0×10^{-3} M in $CHCl_3$) were added into the cell part with $CHCl_3$, and 30 μl of BNAH (2.5×10^{-1} M in $CHCl_3$) was placed in the upper part of a cuvette. The total volume of the contents in the cuvette was adjusted to 3 ml by adding $CHCl_3$. Both solutions were bubbled with $CHCl_3$-prehumidified O_2-free N_2 obtained by passing through a vanadium(II) sulfate solution, H_2O, paraffin, NaOH pellets, and $CHCl_3$ for 20 min. The reaction was initiated by mixing. Pseudo-first-order rate constants were determined by following the absorption decreases of 6-azaflavin at 440 nm. (b) DTT oxidation. The rate constants were determined by a stopped-flow technique. In one reservoir, 120 μl of 6-azaflavin (2.5×10^{-3} M in $CHCl_3$), 60 μl of Bu_3N (0.1 M in $CHCl_3$), and a suitable amount of the receptor (1.0×10^{-2} M or 5.0×10^{-3} M in $CHCl_3$) were placed with CHC_3 (3 ml); 30 μl of DTT (0.1 M in $CHCl_3$) was placed in another reservoir with $CHCl_3$ (3 ml). After N_2 bubbling for 20 min and quick mixing, the absorption decreases at 440 nm were followed.

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