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Remarkable stabilization of the anionic semiquinone radical of 6-azaflavin by hydrogen bonding with a receptor in chloroform

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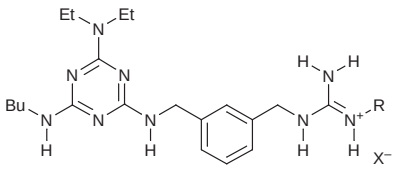
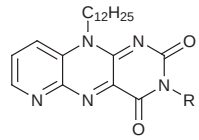
An anionic semiquinone radical of 6-azaflavin (6-AzaFl) was found to be stabilized by hydrogen bonding of a melamine derivative bearing an *N*-phenylguanidinium ion in $CHCl_3$, but not by the correponding *N*-unsubstituted guanidinium ion.

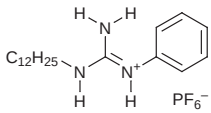
Flavin conenzymes such as FMN and FAD exhibit diverse functions through interactions with apoproteins, in which hydrogen bondings play important roles in the regulation of redox prperties.[1] Flavin semiquinone radicals are known to be stable when bound to apoproteins, whereas non-bound semiquinone radicals are unstable due to disproportionation.[2] Yoneda *et al*. reported that the anionic semiquinone radical of flavin 6-carboxylate is stabilized by intramolecular hydrogen bonding of the 6-CO_2H group at the N(5) position even in aqueous solution.[3] This suggests that the hydrogen bonding to the N(5) position is essential for stabilization of an anionic semiquinone radical of flavin. This was tested by employing 6-AzaFl and a melamine derivative bearing an *N*-phenylguanidinium ion **1** in $CHCl_3$. We report herein that receptor **1** is able to stabilize the anionic semiquinone radical of 6-AzaFl in $CHCl_3$, whereas receptor **2** is unable to stabilize it.



1 R = Ph, X = I
2 R = H, X = ClO_4



6-AzaFl R = H
6-Aza-MeFl R = Me



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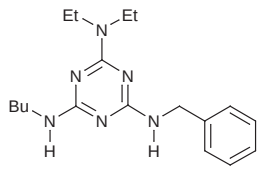

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Receptor **1** was prepared by reaction of 2-butylamino-4-diethylamino-6-(3-aminomethyl-benzylamino)-*s*-triazine[4] with *S*-methyl-*N*-phenylisothiouronium iodide[5] in EtOH, and **3** was prepared from dodecylamine and *S*-methyl-*N*-phenylisothiouronium iodide, followed by counteranion exchange with KPF_6.† The pK_a value of the guanidinium hydrogen of **1** was determined to be 10.7 by spectroscopic pH titration at 280 nm in buffer solutions containing 20% MeCN,‡ which is considered to be lower than that of **2** by at least 1–2 pK_a units.[6]§ The binding constant of 6-AzaFl·**1** was determined spectrophotometrically [K = (5.3 ± 0.3) × 10^3 dm^3 mol^{-1} in $CHCl_3$] as described previously.[4] Despite of more acidic guanidinium hydrogen of **1**, the K value of 6-AzaFl·**1** is smaller than that of 6-AzaFl·**2** [K = (1.4 ± 0.1) × 10^5 dm^3 mol^{-1} in $CHCl_3$].[4] This requires an explanation, since a more acidic H-donor is known to give a larger binding constant for H-bonded complexation.[7] The thermodynamic parameters for the complex formation (ΔH and $T\Delta S_{298}$: −27 and −6.0 kJ mol^{-1} for 6-AzaFl·**1**; −34 and −5.0 kJ mol^{-1} for 6-AzaFl·**2**)¶ indicated that the complex formation is mainly controlled by the enthalpy term. The ^{1}H NMR study of the complexes implied steric hindrance for complexation of 6-AzaFl and **1**. Namely, as shown in Fig. 1, the larger upfield shifts of C(7)-H of 6-AzaFl upon addition of **1** rather than **2** suggest that C(7)-H is situated in a position close enough to feel the ring current of the *N*-phenyl ring of **1** due to the steric hindrance between C(7)-H and the *ortho*-H of the *N*-phenyl ring.

Redox potentials of 6-AzaFl were determined by cyclic voltammetry in CH_2Cl_2.[8]‖ In the absence of the receptors, 6-AzaFl showed a reversible redox couple ($E_{1/2}$ = −971 mV *vs*. ferrocene/ferrcenium). Upon increasing the concentration of the receptors, the redox potentials shifted in a positive direction in both receptors, finally leading to fixed potentials; $E_{1/2}$ = −738 mV for **1** (5 equiv.), and −767 mV for **2** (3 equiv.). The shifts of the potentials due to the receptors ($\Delta E_{1/2}$) are 233 mV for **1** and 204 mV for **2**, corresponding to stabilization of the 6-AzaFl radical anion by 22 and 20 kJ mol^{-1}, respectively. It should be noted that the cyclic voltammogram of 6-Aza-MeFl was not affected by addition of **1**.

Formation of a semiquinone radical of 6-AzaFl was detected spectrophotometrically by employing the oxidation of dithiothreitol (DTT) in $CHCl_3$ under anaerobic conditions as shown in Fig. 2. In the presence of **2** or a mixture of **3** (K = 180 ± 2 dm^3 mol^{-1}) and **4** (K = 150 ± 6 dm^3 mol^{-1}),[4] the absorption spectrum of 6-AzaFl [Fig. 2(*a*)] was changed to that of 2e-reduced 6-AzaFl [Fig. 2(*b*)]. On the other hand, in the presence of **1**, the spectrum shown in Fig. 2(*c*) was observed, suggesting formation of the anionic semiquinone radical of 6-AzaFl,[2,9] which was confirmed to be stable for at least 48 h. With a large

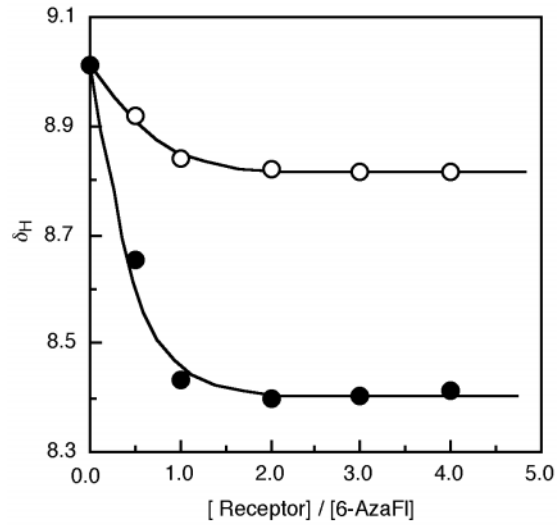

Fig. 1 Changes of chemical shifts of C(7)H in $CDCl_3$ upon addition of the receptors at 25 °C: (●) **1**, (○) **2**.

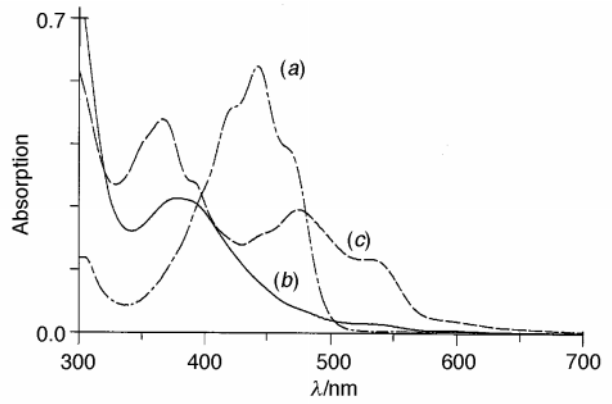

Fig. 2 Absorption spectra of 6-AzaFl in the reaction with DTT. [6-AzaFl] = 5.0×10^{-5} mol dm^{-3}, [DTT] = [Bu_3N] = 5.0×10^{-4} mol dm^{-3} in the presence of **1** or **2** (1.0×10^{-4} mol dm^{-3}) in $CHCl_3$ at 25 °C under N_2; (*a*) oxidized form, (*b*) reduced form, and (*c*) anionic semiquinone radical.

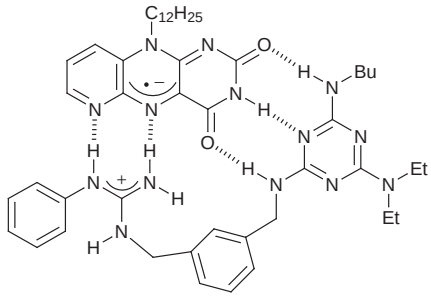

Fig. 3 Structure of 6-AzaFl$^{\cdot-}$ **1**.

excess of DTT, the spectrum shown in Fig. 2(*c*) changed to that shown in Fig. 2(*b*). The spectrum shown in Fig. 2(*b*) was found to give that in Fig. 2(*c*) after O_2 bubbling only with the receptor **1**, suggesting formation of the radical by coproportionation of reduced 6-AzaFl and oxidized 6-AzaFl, or direct electron transfer from the reduced 6-AzaFl to O_2.[2b] Plots of the amount of the anion radical (absorption at 525 nm) *vs*. [**1**] allowed us to calculate the binding constant as 7.7×10^5 dm^3 mol^{-1} which is much larger than that of 6-AzaFl·**1** due to stronger hydrogen acceptability of the anionic radical (6-AzaFl$^{\cdot-}$) as shown in Fig. 3.

Formation of 6-AzaFl radical anion in the presence of **1** was also confirmed by EPR spectroscopy in $CHCl_3$ under anaerobic conditions (Fig. 4). Although hyperfine lines could not be obtained, a *g* value of 2.0040 is in reasonable agreement with those obtained for other flavin radicals.

In summary, we have demonstrated that the acidity of a H-donor of a receptor molecule plays a crucial role in the stabilization of the anionic semiquinone radical of 6-azaflavin. This is the first example showing that intermolecular hydrogen bonds are able to stabilize the anionic semiquinone radical. The receptor molecule could be regarded as an apoprotein model. Furthermore the present results are of use for understanding the functional groups at the active sites of flavoenzymes which give a stable anionic semiquinone radical.

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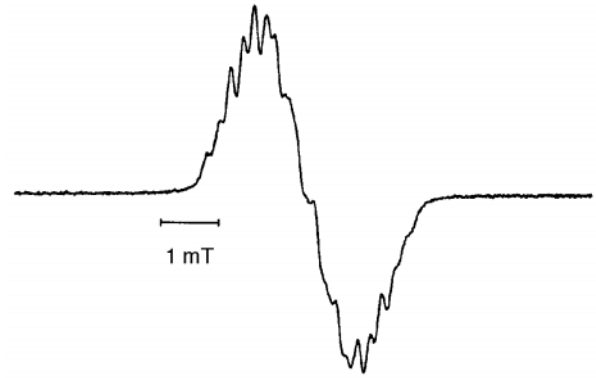

Fig. 4 EPR spectrum of the radical generated by reaction of 6-AzaFl (5.0×10^{-3} mol dm^{-3}) with DTT (5.0×10^{-3} mol dm^{-3}) and Bu_3N (5.0×10^{-3} mol dm^{-3}) in the presence of **1** (5.0×10^{-3} mol dm^{-3}) in $CHCl_3$ at 25 °C under N_2.

Notes and references

† Compound **1**: Yield 54%, mp 178–179 °C (EtOH–diethyl ether). Satisfactory elemental analyses and ^{1}H NMR data were obtained. Compound **3**: Yield 70%, mp 63–65 °C. Receptors **2** and **4**, and 6-azaflavins were supplied from our previous study (ref. 4).

‡ MeCN was added to improve the solubility of **1**.

§ The pK_a for **2** could not be determined by spectroscopic pH titration because of the lack of noticeable absorption changes, but was estimated to be 12–13 (ref. 6).

¶ The thermodynamic parameters were calculated from the following data: 6-AzaFl·**1**; 9.1×10^3 dm^3 mol^{-1} (10 °C), 4.2×10^3 (20), 3.9×10^3 (30), 2.8×10^3 (40). 6-AzaFl·**2**; 1.5×10^5 dm^3 mol^{-1}(20 °C), 1.1×10^5 (30), 7.2×10^4 (40), 5.0×10^4 (50).

‖ To compare the potentials, we used conditions similar to those of ref. 8. [6-AzaFl] = 1.0×10^{-3} mol dm^{-3}, [$Bu_4N^+ClO_4^-$] = 0.1 mol dm^{-3}, 25 °C. Scan rate: 100 mV s^{-1}.

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