BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 52 (12), 3606—3610 (1979) [Vol. 52, No. 12

Enhanced Reactivity of a Flavin Bound to Quaternized Poly(4-vinylpyridine) *via* 8-Position. A Model System of a Flavin Coenzyme Covalently-linked to Enzymes†

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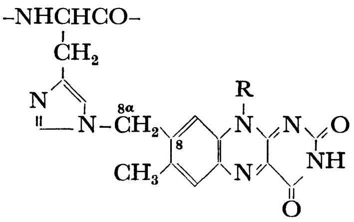
(Received May 15, 1979)

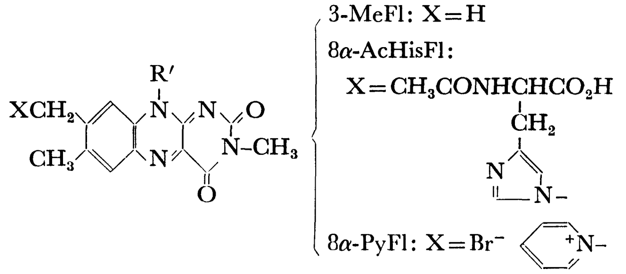
The rate constants for the reaction of the title flavin and the monomeric analogues with 1-benzyl-1,4-dihydronicotinamide (BzlNH), 1-nitroethanide ion, glutathione (GSH), and thiophenol (PhSH) were determined at 30 °C. Although the reactivity of 8α-AcHisFl is slightly improved as compared with that of unmodified flavin, the rate constants for 8α-PyFl which is expected to mimic the reactivity of protonated 8α-AcHisFl were significantly increased. The rate constants for the oxidation of anionic reductants(1-nitroethanide ion, GSH, and PhSH) were further increased by immobilization of the 8α-PyFl structure in quaternized 4VP polymers. The kinetic results implicate that (1) with regard to the action of a flavin covalently-linked to enzymes *via* histidine residue, the flavin must be more reactive in acidic pH region due to protonation of the histidine than in basic pH region, (2) the cationic polyelectrolyte environment accelerates selectively the oxidation of anionic reductants, and (3) the title flavin serves as a strong, selective oxidizing agent under ambient reaction conditions.

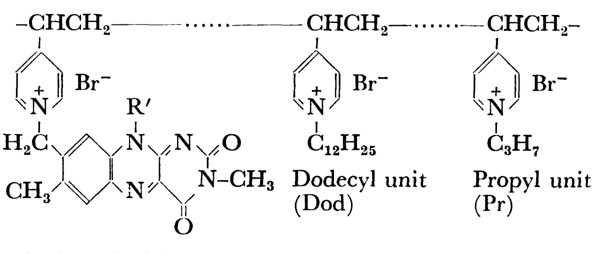
Suggestion has been made that a fourth flavin exists in nature in addition to riboflavin, FMN, and FAD. In 1955, Kearney and Singer[1)] presented unequivocal evidence that succinate dehydrogenase contains covalently-linked FAD which is not extracted from tissues by the conventional denaturation methods. The claim has been further supported by their subsequent studies.[2–4)] In the last five years, it has been established that no less than ten flavoenzymes contain the covalently-linked flavin in the active sites and that the covalent linkage occurs in most cases at $C^{8\alpha}$ position, *e.g.*, *via* the imidazole group of the histidine residue of the enzymes.[5)]



The influence of 8-substituents on the reactivity of the isoalloxazine ring has been estimated in detail by several groups,[6–10)] but studies on 8α-substitution are very limited. Edmondson *et al.*[11,12)] reported, on the basis of flash photolysis and oxidation-reduction potentials, that 8α-substitution by histidine or cysteine gives rise to the electron-withdrawing influence on the isoalloxazine ring system. Except for their study, little is known with certainty about the influence of 8α-substitution on the reactivity of flavin despite its importance in connection with the behavior of covalently-linked flavin.

The rate constant for the reaction of sulfite ion with 8α-(N_1-histidinyl)flavin is greater by a factor of only 1.4 than that with unmodified flavin.[12)] This suggests that the 8α-(N_1-histidinyl) group may slightly affect the reactivity of the isoalloxazine ring. However, two important effects, reaction environment and protonation of the imidazole group, remain to be clarified in order to generalize the conclusion with respect to the covalently-linked flavin systems. Both effects should cause a significant change in the reactivity of covalently-linked flavin. With these objects in view, we have synthesized the following flavins and estimated the oxidative reactivity toward 1-benzyl-1,4-dihydronicotinamide (BzlNH), 1-nitroethanide ion ($CH_3\bar{C}HNO_2$), glutathione (GSH), and thiophenol (PhSH) (R′=tetra-*O*-acetylribityl group).





Flavin unit (Fl)

4VP-Fl-Dod-0: Fl, 6 mol%; Dod, 0 mol%; Pr, 88 mol%.
4VP-Fl-Dod-20: Fl, 6 mol%; Dod, 20 mol%; Pr, 67 mol%.

Results

Oxidation of 1-Benzyl-1,4-dihydronicotinamide. Flavin oxidation of BzlNH (Eq. 1) is a typical model

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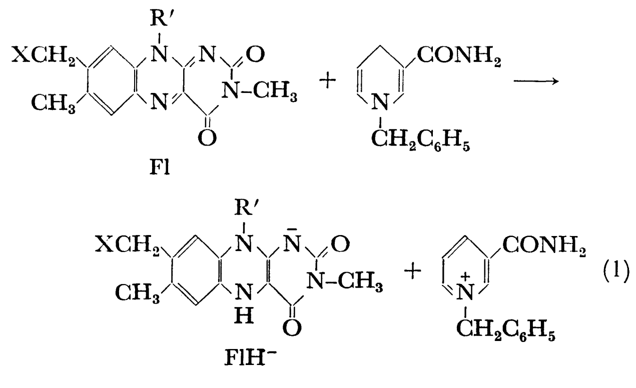
† Coenzyme Models 22. Abbreviations employed are: GSH, glutathione; PhSH, thiophenol; BzlNH, 1-benzyl-1,4-dihydronicotinamide; 3-MeFl, 3-methyltetra-*O*-acetylriboflavin; 8α-AcHiFl, 3-methyl-8α-[N_α-acetyl-(N_1-histidinyl)]-tetra-*O*-acetylriboflavin; 8α-PyFl, 3-methyl-8α-(1-pyridinio)-tetra-*O*-acetylriboflavin bromide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; 4VP, 4-vinylpyridine.

reaction for the inter-coenzyme hydrogen transfer between NADH and flavin, the rate constants being in line with the polarographic half-wave potentials of flavins.[13,14] The second-order rate constant (k_2) for the oxidation by 8α-AcHisFl is 1.5 times greater than that by unmodified flavin, 3-MeFl (Table 1). The small rate augmentation is in line with the observation of Edmondson *et al.*[12] that the 8α-(N_1-histidinyl) group behaves as a weak electron-withdrawing substituent.

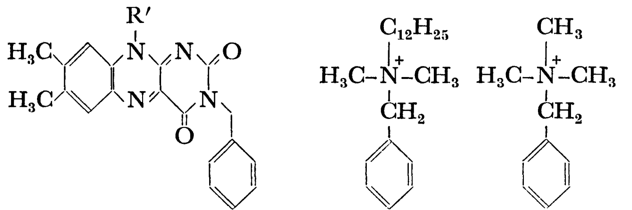


(1)

Table 1. Rate constants for the oxidation by monomeric and polymer-bound flavins

Flavin	k_2/M^{-1} s^{-1}		k_3'/M^{-2} s^{-1}	
	BzlNH[a)]	$CH_3\bar{C}HNO_2$[b)]	GSH[c)]	PhSH[b)]
3-MeFl	32.4	($<10^{-7}$)	1.98	(<1)
8α-AcHisFl	48.1	($<10^{-7}$)	2.59	(<1)
8α-PyFl	363	4.8×10^{-4}	4.29	152
4VP-Fl-Dod-0	277	0.642	9500	31100
4VP-Fl-Dod-20	301	1.31	56100	110000
St-Fl-Dod-22[f)]	201	0.501	833[d)]	6240

a) pH 9.00, $\mu=0.02$ with KCl. b) pH 8.60, $\mu=0.05$ with KCl, $[CH_3\bar{C}HNO_2]=0.01$—0.05 M. c) pH 8.65, $\mu=0.02$ with KCl, [GSH]$=1.00\times10^{-3}$ M. d) pH 8.80, $\mu=0.02$ with KCl : cited from Ref. 26. e) pH 6.70, 40 vol% ethanol, $\mu=0.02$ with KCl, [PhSH]$=1.00\times10^{-3}$ M. f) The preparation of this polymer is described in Ref. 26.



Scheme 1.

NADH analogues decompose rapidly into tetrahydronicotinamides in acidic pH region where imidazole is protonated,[15] making it impossible to evaluate directly the rate constant for the reaction of BzlNH with protonated 8α-AcHisFl. We thus employed 8α-PyFl as an analogue of protonated 8α-AcHisFl. 8α-PyFl oxidizes BzlNH 11.2 times faster than 3-MeFl. On the other hand, immobilization of the 8α-PyFl unit in the quaternized 4VP polymers is insignificant as regards the enhancement of the oxidative reactivity of flavin toward BzlNH. The reaction is presumably accelerated by protonation of 8α-histidyl group and almost unaffected by polyelectrolyte reaction environments.

Oxidation of 1-Nitroethanide Ion. Although D-amino acid oxidase (flavoenzyme) rapidly oxidizes 1-nitro-1-alkanide ion to the corresponding aldehydes and nitrite ion (Eq. 2),[16] no corresponding nonenzymatic reaction is considered to occur under ambient reaction conditions.[6] Neither 3-MeFl nor 8α-AcHisFl can oxidize

$$\mathrm{Fl} + CH_3\bar{C}HNO_2 \xrightarrow{OH^-} \mathrm{FlH}^- + CH_3CHO + NO_2^- \quad (2)$$

1-nitroethanide ion under the present reaction conditions, the second-order rate constant being presumed to be smaller than 10^{-7} M^{-1} s^{-1} (Table 1). On the other hand, oxidation of 1-nitroethanide ion by 8α-PyFl was easily detected under the same reaction conditions, the rate constant being augmented by more than three orders of magnitude relative to that of 3-MeFl. Yokoe and Bruice[6] reported that 3,10-dimethyl-8-cyanoisoalloxazine, an electron-deficient isoalloxazine with a positively-shifted poralographic half-wave potential, is able to oxidize 1-nitro-1-alkanide ion with $k_2=(2$—$5)\times10^{-3}$ M^{-1} s^{-1}. The k_2 value for 8α-PyFl (4.8×10^{-4} M^{-1} s^{-1}) is thus smaller by only one order of magnitude than that for 3,10-dimethyl-8-cyanoisoalloxazine.

The second-order rate constants were further enhanced by immobilization of the 8α-PyFl unit in the quaternized 4VP polymers. For example, the k_2 value for 4VP-Fl-Dod-20 is augmentated by 10^7 fold and 3×10^3 fold in comparison to those of 3-MeFl and 8α-PyFl, respectively, exceeding even that of 3,10-dimethyl-8-cyanoisoalloxazine by two to three orders of magnitude. The result is in contrast to the trend observed in flavin oxidation of BzlNH. Since the reaction rate for polymer-bound flavin was first-order in flavin and 1-nitroethanide ion under the employed reaction conditions (footnotes, Table 1), the reaction route is not apparently changed by immobilization of the 8α-PyFl unit in the cationic polymers.

Oxidation of Thiols. The oxidation of thiols by the conventional flavins (Eq. 3) is first-order in flavin and second-order in thiols.[6,17] The reaction of the

$$\mathrm{Fl} + 2\mathrm{RSH} \xrightarrow{-H^+} \mathrm{FlH}^- + \mathrm{RSSR} \quad (3)$$

monomeric flavins with GSH was confirmed to be of second-order in GSH (Fig. 1), the apparent third-order rate constants ($k_3'=v_{\mathrm{obsd}}/[\mathrm{flavin}][\mathrm{RSH}]^2_{\mathrm{total}}$) increasing in the order, 3-MeFl<8α-AcHisFl<8α-PyFl. Although aliphatic thiols such as GSH and 2-mercaptoethanol are slowly oxidized by flavin, thiophenol is not oxidized under ambient reaction conditions unless the electron-deficient isoalloxazine is employed.[6] As expected, oxidation of PhSH by 3-MeFl and 8α-AcHisFl could not be detected under the present reaction conditions, but that by 8α-PyFl was detected accurately to give $k_3'=152$ M^{-2} s^{-1}. The k_3' value for 3,10-dimethyl-8-cyanoisoalloxazine determined under the comparable reaction conditions is 5×10^4 M^{-2} s^{-1},[6] so that the k_3' value for 8α-PyFl is smaller by about two orders of magnitude than that for the electron-deficient iso-

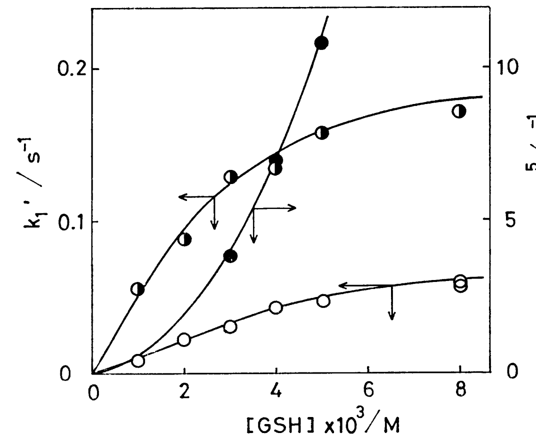


Fig. 1. Plots of k_1' as a function of GSH concentration. (●) 8α-PyFl; (○) 4VP-Dod-0; (◑) 4VP-Fl-Dod-20.

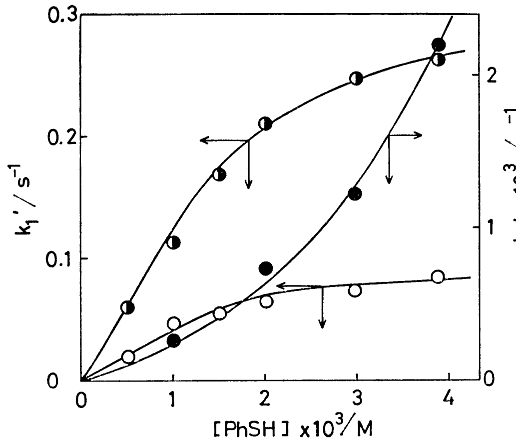


Fig. 2. Plots of k_1' as a function of PhSH concentration. (●) 8α-PyFl; (○) 4VP-Fl-Dod-0; (◑) 4VP-Fl-Dod-20.

alloxazine with an 8-cyano group.

Plots of pseudo first-order rate constant ($k_1' = v_{obsd}$/[flavin]) vs. thiol concentration are given in Figs. 1 and 2. In contrast to the second-order dependence of conventional flavin on thiol concentrations, the pseudo first-order rate constants (k_1') for 4VP-Fl-Dod-0 and 4VP-Fl-Dod-20 showed a saturation phenomenon which is essentially approximated by sigmoid curves. The result indicates that the oxidation by polymer-bound flavins (polymer-Fl) proceeds *via* complexation as shown by

$$\text{polymer-Fl} + 2\text{RSH} \underset{K_m}{\rightleftharpoons} \text{polymer-Fl}\cdot(\text{RSH})_2 \xrightarrow{k_{cat}} \text{polymer-FlH}^- + \text{RSSR} \quad (4)$$

where K_m is the equilibrium constant for dissociation of polymer-Fl·RSH_2 complex ($=$[polymer-Fl]$[RSH]^2_{total}$/[polymer-Fl·$(RSH)_2$]) and k_{cat} the first-order intracomplex reaction rate constant. If we assume that [flavin]$\ll[RSH]_{total}$, the corresponding rate equation can be expressed by

$$v_{obsd} = k_1'[\text{polymer-Fl}] = \frac{k_{cat}[\text{polymer-Fl}][\text{RSH}]^2_{total}}{K_m + [\text{RSH}]^2_{total}}. \quad (5)$$

The theoretical plot of k_1' *vs.* $[RSH]_{total}$ gives a sigmoid curve. Following the derivation of the Lineweaver-Burk equation from the Michaelis-Menten equation, Eq. 5 can be re-written as follows which gives the linear relation between $1/k_1'$ and $1/[RSH]^2$.

$$\frac{1}{k_1'} = \frac{1}{k_{cat}} + \frac{K_m}{k_{cat}} \cdot \frac{1}{[\text{RSH}]^2_{total}} \quad (6)$$

The plots (Figs. 3 and 4) show fairly good linearity ($r<0.98$). The slope (K_m/k_{cat}) and the intercept ($1/k_{cat}$) were determined by least-squares computation. The k_{cat} and K_m values are summarized in Table 2. The apparent third-order rate constants ($k_3' = v_{obsd}$/[Polymer-Fl]$[RSH]^2_{total}$) at $[RSH]_{total} = 1.00 \times 10^{-3}$M are given in Table 1.

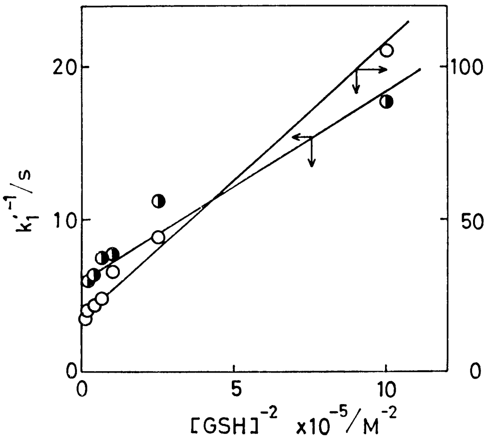


Fig. 3. Plots of $k_1'^{-1}$ *versus* $[GSH]^{-2}$. (○) 4VP-Fl-Dod-0; (◑) 4VP-Fl-Dod-20.

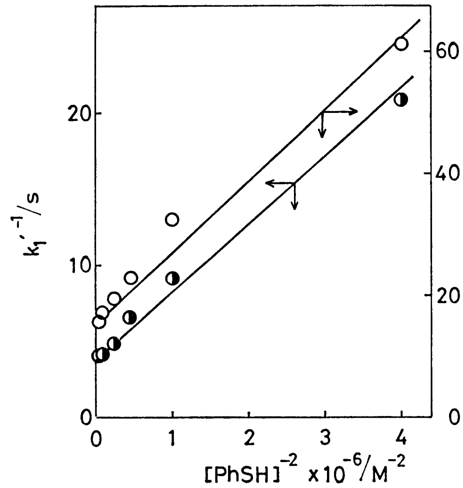


Fig. 4. Plots of $k_1'^{-1}$ *versus* $[PhSH]^{-2}$. (○) 4VP-Fl-Dod-0; (◑) 4VP-Fl-Dod-20.

Examination of the apparent third-order rate constant (k_3') reveals that (1) the rate augmentation of 10^3—10^4 fold is achieved by immobilization of 8α-PyFl unit in the quaternized 4VP polymers and (2) the rate constants increase with increase in the dodecyl group content.

Flavin immobilized in the quaternized 4VP polymers

Table 2. Kinetic parameters for the oxidation of GSH and PhSH by polymer-bound flavins

Flavin	GSH k_{cat} / s^{-1}	GSH K_m / M^2	GSH k_{cat}/K_m / $M^{-2}\,s^{-1}$	PhSH k_{cat} / s^{-1}	PhSH K_m / M^2	PhSH k_{cat}/K_m / $M^{-2}\,s^{-1}$
4VP-Fl-Dod-0	0.0606	5.36×10^{-6}	1.14×10^{4}	0.0645	8.00×10^{-7}	8.06×10^{4}
4VP-Fl-Dod-20	0.169	2.15×10^{-6}	8.05×10^{4}	0.263	1.13×10^{-6}	2.33×10^{5}
St-Fl-Dod-22			833[a]			6.24×10^{3} [b]

a) k_3' at pH 8.80: cited from Ref. 18. b) k_3' at pH 6.70: cited from Ref. 19.

via 8α-position gives rate constants greater than flavin immobilized in cationic polystyrene *via* 3-position.[18] Since oxidation of GSH and PhSH by flavin immobilized in cationic polystyrene shows a simple second-order dependence upon GSH and PhSH under the comparable reaction conditions,[18,19] the improved reactivity of 8α-PyFl unit in 4VP polymers should be rationalized in terms of the efficient complexation with thiol substrates. Kinetic parameters (Table 2) indicate that 4VP-Fl-Dod-20 which is classified as a more hydrophobic polymer possesses k_{cat} values greater than 4VP-Fl-Dod-0 by factors of 3—4. On the other hand, no significant difference could be found for K_m values. This suggests that the hydrophobicity of 4VP polymer is related to the efficiency of intra-complex reaction and not to the binding efficiency.

Discussion

The oxidative reactivity of flavin was not largely improved by introduction of histidine into 8α-position.[12] The present study established that 8α-PyFl employed as an analogue of protonated 8α-AcHisFl exhibits enhanced reactivities toward several substrates. This indicates that the reactivity of flavin covalently-linked to enzymes *via* the histidine residue can also be enhanced in acidic pH region (pK_a of histidine linked to the 8α-position is estimated to be 6.9).[20]

Shinkai *et al.* reported the micellar effects on flavin oxidation.[21—23] In general, the reaction of flavin and NADH analogue is affected to a smaller extent by surfactant micelles,[21] whereas flavin oxidation of anionic species such as carbanions and thiolates is remarkably facilitated by cationic micelles.[22,23] The marked rate acceleration is rationalized in terms of enhanced local concentration of anions by interaction with micellar cationic surface and activation of anions by desolvation in micellar hydrophobic environment.[24,25] The results (Table 1) indicate that oxidation of 1-nitroethanide ion, GSH, and PhSH is facilitated considerably by immobilization of 8α-PyFl structure in the cationic 4VP polymer, the k_2 value for flavin oxidation of BzlNH being hardly improved. The trend is in line with the micellar effects. The marked rate acceleration might be caused by the high cationic charge density along the polymer chain which concentrates anionic reactant species and by polymeric hydrophobic environment.

Cationic polystyrene polymers which immobilize flavin *via* 3-position were synthesized (Scheme 1).[26] The reaction of NADH (polyanionic species) with the immobilized flavin proceeded according to Michaelis-Menten kinetics.[26] However, oxidation of 1-nitroethanide ion, GSH, and PhSH showed no saturation phenomenon.[18,19,27] This indicates that the reaction proceeds according to a simple second-order path, or the dissociation constant (K_m) is greater than the employed reactant concentrations even if the reaction occurs *via* complexation. In contrast, oxidation of GSH and PhSH by flavin immobilized in the cationic 4VP polymers gave distinct saturation curves under comparable reaction conditions (Figs. 1 and 2). It is not clear why quaternized 4VP polymers adsorb GSH and PhSH more efficiently than cationic polystyrene polymers. A possible explanation is that the flavin unit in the 4VP polymers is immobilized as a cationic unit and that in the polystyrene polymers as a neutral unit. The flavin unit in cationic polystyrenes may exist, due to the hydrophobicity, in a hydrophobic domain of the polymer which is isolated from a cationic, substrate-binding site, the enhanced substrate concentration at the cationic site thus not being reflected advantageously by the subsequent flavin oxidation process.

The results can be summarized as follows: (1) the reactivity of isoalloxazine ring is enhanced by protonation of 8α-(N_1-histidinyl) group, (2) the oxidation of carbanion and thiols is facilitated by cationic polymer environment, and (3) as a result of combination of (1) and (2), flavin immobilized in cationic 4VP polymers *via* 8α-position acts as an excellent oxidizing agent for anionic species.

Experimental

Materials. Preparation of BzlNH and 3-MeFl was reported.[26] 8-Methyl group of 3-MeFl was brominated accoding to the method of Walker *et al.*[20] The content of 3-methyl-8α-bromotetra-*O*-acetylriboflavin was estimated from the decrease of the $\underline{C}(8)$–CH_3 peak in the NMR spectrum[20] (δ=2.58 ppm in $CDCl_3$) to be 64%. The mixture was used without further purification for the preparation of 8α-PyFl, 8α-AcHisFl, and 4VP-bound flavins.

In a 100 ml flask covered with aluminium foil were placed 0.3 g of brominated flavin mixture containing 3.2×10^{-4} mol of 3-methyl-8α-bromotetra-*O*-acetylriboflavin and 0.4 ml (4.8×10^{-4} mol) of pyridine which were dissolved in 60 ml of ethanol. The mixture was then heated under reflux and the progress of the reaction was monitored by TLC method (silica gel and ethyl acetate). The spot for 3-methyl-8α-bromotetra-*O*-acetylriboflavin(R_f=0.20) disappeared and a new spot attributed to 8α-PyFl appeared at the origin. After 6 h, the spot for 3-methyl-8α-bromotetra-*O*-acetylriboflavin became undetectable. Solvent ethanol was evaporated *in vacuo*, and the yellow residue was recrystallized three times from ethanol-diisopropyl ether; mp 85—97 °C (hygroscopic), yield 0.3 g. NMR (Me_2SO-d_6): $COCH_3$, 1.60 ppm (3H), 2.40 ppm (3H), 2.20 ppm (6H); 7-CH_3, 2.52 ppm, 3H; $\underline{N}$(3)–CH_3, 3.39 ppm, 3H; $\underline{N}$(10)–CH_2, 4.40 ppm, 2H; CH_2 in ribityl group, 4.95 ppm, 2H; CH in ribityl group, around 5.4 ppm, 3H; 8-CH_2, 6.24 ppm, 2H; 6-H, 7.78 ppm, 1H; 9-H, 8.14 ppm, 1H; pyridine, 8.3—9.2 ppm, 5H. Found: C, 48.50; H, 4.98; N, 9.02%. Calcd for $C_{31}H_{34}N_5O_{10}Br$: 2.84 H_2O: C, 48.50; H, 4.84; N, 9.12%.

The preparation of 8α-AcHisFl was carried out according to the method of Walker *et al.*[20] 0.20 g (1.0×10^{-3} mol) of N_α-acetyl-L-histidine, and 1.28 g of brominated flavin mixture containing 1.3×10^{-3} mol of 3-methyl-8α-bromotetra-*O*-acetylriboflavin were dissolved in 30 mol of *N,N*-dimethylformamide, and the mixture was stirred at 50 °C in the presence of 1.38 g (1.0×10^{-2} mol) of powdered potassium carbonate. The progress of the reaction was monitored by TLC method (silica gel and ethyl acetate). The spot of 3-methyl-8α-bromotetra-*O*-acetylriboflavin became undetectable after 48 h. The solvent was evaporated *in vacuo*, the orange residue being taken with 100 ml of chloroform. The chloroform layer separated was washed with 50 ml of 0.5 M phosphate buffer solution to extract 8α-AcHisFl and unreacted N_α-acetyl-L-histidine. The aqueous layer was then adjusted to pH 4 with acetic acid, saturated with ammonium sulfate, and then extracted four times with 100 ml of 1-butanol. The 1-butanol solution was dried over sodium sulfate for one day. The solvent was evaporated *in vavuo*, the residue being recrystallized twice from ethanol–diisopropyl ether; mp 244—247 °C, yield 0.45 g. Found: C, 49.97; H, 5.43; N, 12.02%. Calcd for $C_{34}H_{39}N_7O_9\cdot3H_2O$: C, 50.62; H, 5.25; N, 12.15%.

Polymer-bound flavins (4VP-Fl-Dod-0 and 4VP-Fl-Dod-20) were prepared from 3-methyl-8α-bromotetra-*O*-acetylriboflavin and poly(4-vinylpyridine) (MW 100000; Ko-ei Kagaku Kogyo Co.). Two grams of poly(4-vinylpyridine) were dissolved in 10 ml of *N,N*-dimethylformamide and mixed with 1.8 g of brominated flavin mixture containing 1.9×10^{-3} mol of 3-methyl-8α-bromotetra-*O*-acetylriboflavin. The reaction mixture was heated under reflux for 4 h and then poured into diisopropyl ether. The recovered polymer became orange plates when dried under reduced pressure. The composition of flavin unit was determined by the absorption spectrum method, assuming that the molar absorbancy index is equal to that of 8α-PyFl(ε= 10040 in *N,N*-dimethylformamide). The flavin content thus determined was 6 mol %. The polymer was further quaternized by propyl bromide and dodecyl bromide according to the method employed for the preparation of polysoaps.[28] The quaternized polymers were purified by extensive dialysis and the composition was estimated by means of NMR for the dodecyl unit[28] and potentiometric titration for the propyl unit. The results are given in terms of the structure of polymer-bound flavins.

Kinetics. The rates for flavin oxidation of BzlNH were estimated under aerobic conditions by following the decrease in the absorption band of BzlNH at 357 nm. The other flavin oxidation reactions were performed under anaerobic (N_2) conditions by following that of flavins at *ca.* 445 nm. Details of the methods were described in previous papers.[18,26,27] All the reactions were carried out at 30 °C (Tables 1 and 2).

As reported earlier,[6,13,22,26] flavin oxidation of BzlNH and 1-nitroethanide ion is first-order in flavin and substrate, while that of GSH and PhSH is second-order in thiol.[18] On the other hand, plots of oxidation rate *versus* thiol concentration for 4VP polymer-bound flavins show sigmoidal curves (Figs. 1 and 2).

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