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A Flavoenzyme Model: Facile Oxidation of Thiols by a Flavin Immobilized in Cationic Polyelectrolytes[1]

SEIJI SHINKAI, SHINJI YAMADA, REIKO ANDO, AND TOYOKI KUNITAKE

Department of Organic Synthesis, Faculty of Engineering, Kyushu University, Fukuoka 812, Japan

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The reactions of a polymer-bound flavin with thiols (2-mercaptoethanol, glutathione, thiophenol, and 1,4-butanedithiol) are remarkably accelerated, when compared with that of a monomeric flavin. The rate enhancements observed were 30- to 6000-fold. In particular, thiophenol which had been believed not to be oxidized by flavin in nonenzymatic systems was oxidized most rapidly among the monothiols examined. The reaction rates were improved by incorporation of a dodecyl group into the flavin-containing polymer. Therefore, the hydrophobic nature of the cationic polymer matrix was concluded to be responsible for the large rate enhancement among other factors.

INTRODUCTION

The interconversion of thiols and disulfides is one of the most important reactions that involve flavoenzymes, as exemplified by dihydrolipoyl dehydrogenase (EC 1.6.4.3) and glutathione dehydrogenase (EC 1.8.5.1). The mechanism by which flavoenzymes perform oxidation–reduction reactions has been studied in nonenzymatic systems by using appropriate model compounds mostly in simple aqueous systems (*1–4*). However, most of the coenzyme binding sites are located in relatively hydrophobic regions of apoenzymes (*5*), and in those regions, the flavin moiety may display reactivities (and mechanism) which are different from those in simple aqueous solutions.

We recently found that flavin oxidation of nitroethane and thiols is surprisingly facilitated in the presence of a cationic micelle and a cationic polysoap (*6–9*). These micellar environments are frequently suggested to be analogous to those of enzymes (*10, 11*), and these relatively simple, synthetic hydrophobic aggregates may be thought as apoenzyme models. As an extension of these studies, we synthesized the following flavin-containing polymers and used them for the oxidation of thiols (Eq. [1]: Ac_4Rib = tetra-*O*-acetylribityl). 3-Methyl-tetra-*O*-acetylriboflavin (3-MeF) was used as the monomeric analog. Thiols employed are 2-mercaptoethanol, glutathione (GSH), thiophenol (PhSH), and 1,4-butanedithiol. The reaction of these flavin polymers and NADH has been reported elsewhere (*12*).

[1] Coenzyme Models 11. Contribution No. 487 from this department. Part 10: S. SHINKAI, S. YAMADA, AND T. KUNITAKE, *Macromolecules* 11, 65 (1978).

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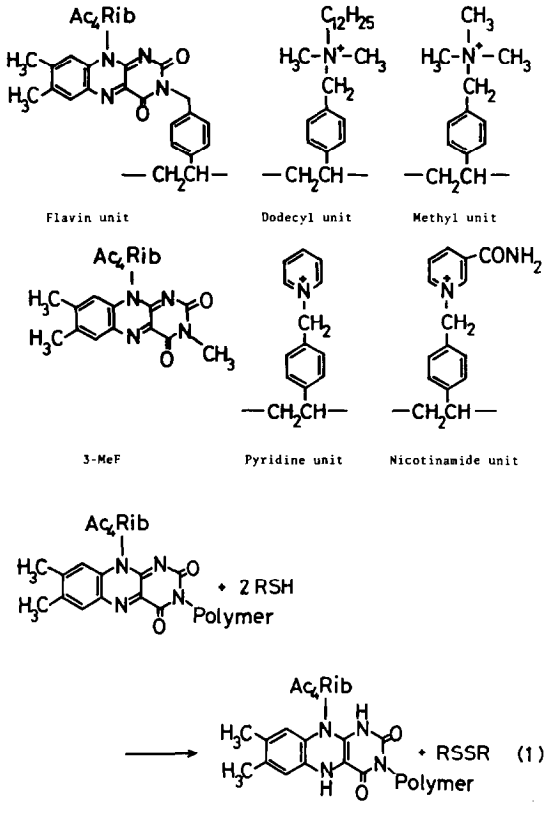

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EXPERIMENTAL

Materials

Preparations of flavin-containing polymers and 3-methyl-tetra-*O*-acetylriboflavin (3-MeF) have been described (*12*). The polymer compositions are summarized in Table 1 together with their abbreviations. The following thiols were distilled under N_2 stream before use: 2-mercaptoethanol, bp 154–156°C (lit. (*13*), 157°C); thiophenol, bp 70–72°C at 29 mm Hg (lit. (*13*), 169°C); 1,4-butanedithiol, bp 98–102°C at 17 mm Hg (lit. (*14*), 74.5°C at 10 mm Hg). Glutathione (GSH) was purchased from Wako Pure Chem. Ind. and used without further purification.

Kinetics

The kinetic measurements were carried out under anaerobic (N_2) conditions in 3 vol% aqueous ethanol at 30 ± 0.1°C at a calculated ionic strength (μ = 0.02 with KCl) unless otherwise stated. Modified Thunberg cuvettes were used in order to achieve the anaerobic condition. The details of the procedure have been described previously (*8*). The progress of the reactions was followed spectrophotometrically

TABLE 1

COMPOSITION OF FLAVIN-CONTAINING POLYMERS

	Composition (mol%)				
	Flavin	Dodecyl	Methyl	Pyridine	Nicotinamide
F-D-0	5.0	0	82	—	—
F-D-7	5.3	7	86	—	—
F-D-12	5.0	12	70	—	—
F-D-22	5.3	22	68	—	—
F-Py-72	5.2	—	—	72	—
F-Nic-59	5.2	—	—	—	59

by monitoring the reduction of flavin (λ_{max}, 453–460 nm for polymeric flavins and 447 nm for 3-MeF). In all cases, excess thiol was present, so that the pseudo-first-order behavior was observed. Introduction of oxygen into the final reaction mixture regenerated immediately reoxidized flavins quantitatively.

When the flavin oxidation of thiophenol was conducted in 3 vol% aqueous ethanol, the reaction system became turbid due to precipitation of diphenyl disulfide. Thus, in this case, the kinetic measurements were performed in 40 vol% aqueous ethanol, and the reaction mixture remained transparent.

Product Analysis

The oxidation of excess thiophenol by F-D-22 was carried out under anaerobic conditions in the dark in 3 vol% aqueous ethanol at pH 6.9 at room temperature. After 2 hr, diphenyl disulfide was collected as precipitates in 92–96% yield. The NMR spectrum (Me_2SO-d_6) agreed with that of the authentic sample. When the same reaction was carried out under aerobic conditions, the yield based on the flavin exceeded 100%. This indicates that flavin acted as a recycling catalyst.

Titration of Thiophenol

The spectrophotometric titration of thiophenol was conducted by using the absorbance of thiophenolate anion (λ_{max} = 264 nm) in the absence of the polymer under the condition identical to the kinetic measurements (40 vol% ethanol): pK_a = 7.6.

RESULTS AND DISCUSSION

Kinetics of the Reaction

In the presence of excess thiols, the absorbance of flavin as a function of time gave satisfactory pseudo-first-order plots under all of the conditions for up to 60–70% completion. Thus, the reaction is first order in flavin. The pseudo-first-order rate constants (k_{obsd}) thus determined gave straight lines when plotted against

[RSH (monothiol)]^2 (Fig. 1). On the other hand, k_{obsd} for the oxidation of 1,4-butanedithiol ($0.5–3.0 \times 10^{-3}$ *M*) produced a straight line against [$HS(CH_2)_4SH$]. Therefore, the oxidation of monothiol is second order in monothiol, but that of dithiol is first order in dithiol, as reported previously for the nonmicellar system (*1–3*); that is, for monothiol,

$$v_{obsd} = k_{obsd}[\text{flavin}] = k_3'[\text{flavin}][\text{RSH}]_T^2 \quad [2]$$

and for dithiol,

$$v_{obsd} = k_{obsd}[\text{flavin}] = k_2'[\text{flavin}][\text{HSSH}]_T \quad [3]$$

where k_3' and k_2' are apparent third-order and second-order rate constants, respectively, and $[RSH]_T$ and $[HSSH]_T$ are total concentrations of thiols. k_3' and k_2' values at given pH are summarized in Table 2.

The rate constants with the flavin polymer are enhanced by 30- to 50-fold for 2-mercaptoethanol and 310- to 420-fold for GSH relative to those with 3-MeF. The greater enhancement for GSH relative to that for 2-mercaptoethanol is attributed to the efficient binding of GSH (partially trianionic in the reaction pH) to the polymer. However, the saturation kinetics were not found under the experimental conditions used (Fig. 1A). The association constant of GSH with quaternized poly(4-vinylpyridine), which contains more than 12 mol% dodecyl group, is estimated to be 170–500 M^{-1} (calculated based on the total concentration of the monomer unit) (*15*). Therefore, the rate saturation (if it exists) would occur at [GSH] $> 10^{-2}$ *M*. The rate measurements at high GSH concentrations ($\sim 10^{-2}$ *M*) were conducted with an ionic strength of 0.3. The high salt concentration was required to maintain the ionic strength constant when the GSH concentration was varied. GSH itself was used as a buffer in order to avoid further increases in ionic

TABLE 2

RATE CONSTANTS FOR THE OXIDATION OF MONOTHIOLS (k_3') AND DITHIOL (k_2')[a]

Polymer	$E_{1/2}$ (V)	k_3' (M^{-2} sec^{-1}) 2-Mercaptoethanol (pH 9.00 ± 0.03)	Glutathione (pH 8.80 ± 0.04)	Thiophenol[b] (pH 6.70 ± 0.05)	k_2' (M^{-1} sec^{-1}) 1,4-Butanedithiol (pH 9.06 ± 0.02)
F-D-0	−0.427	15.4	620	4180	66.0
F-D-7	−0.433	17.0	772	—	75.9
F-D-12	−0.438	14.7	718	—	82.9
F-D-22	−0.441	25.1	833	6240	113
F-D-22[c]		11.5	257	2054	—
F-D-22[d]		7.01	142	—	—
F-Py-72		—	429	1860	—
F-Nic-59		—	361	—	—
3-MeF	−0.492	0.50	1.98	no reaction (<1)	0.441

[a] μ = 0.02 with KCl, 3 vol% ethanol. pH was maintained with 0.02 *M* borate or phosphate.
[b] 40 vol% ethanol.
[c] μ = 0.1.
[d] μ = 0.3.

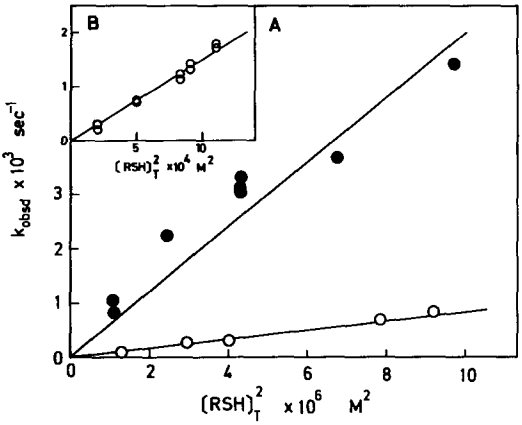


FIG. 1. Pseudo-first-order rate constants for the oxidation of GSH (○) and thiophenol (●) by F-D-22 as a function of $[RSH]_T^2$. A (μ = 0.02): for GSH, pH 8.80 ± 0.04 with 0.02 *M* borate, [flavin] = 2.30 × 10^{-5} *M*; for thiophenol, pH 6.70 ± 0.05 with 0.02 *M* phosphate, 40 vol% ethanol, [flavin] = 2.36 × 10^{-5} *M*. B (μ = 0.3): for GSH, pH 8.80 ± 0.04, [flavin] = 2.29 × 10^{-5} *M*.

strength. The rates were markedly retarded due to the increased salt concentration (see below and Table 2), and the plots of k_{obsd} vs $[GSH]^2$ again provided a linear correlation (Fig. 1B). The complex formation in the present system must have been suppressed by the increase in the salt concentration. It has been noticed that the oxidation of NADH by the immobilized flavin, which proceeds according to the Michaelis–Menten-type reaction path ($K_m \approx 10^{-5}$ *M*) at μ = 0.02, obeys the simple second-order kinetics at μ = 0.3 (12). The remarkable salt inhibition suggests the importance of the electrostatic interaction occurring between GSH and the flavin-containing polymer (see later).

Although flavins were reported to oxidize aliphatic thiols slowly (*2, 4*), oxidation of thiophenol has not been possible in the conventional nonenzymatic system unless a very electron-deficient isoalloxazine, 3,10-dimethyl-8-cyanoisoalloxazine, is employed (*3*). In fact, 3-MeF oxidation of thiophenol was not detected in the present study, and its k_3' value was estimated to be less than 1 M^{-2} sec^{-1}. On the other hand, polymeric flavins readily oxidized thiophenol to diphenyl disulfide (see Experimental). The kinetic study carried out in 40 vol% aqueous ethanol showed that the reaction rate was augmented by more than three orders of magnitude, being much greater than those for aliphatic thiols.

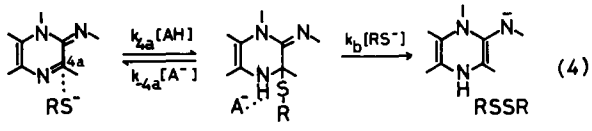
The reaction of 1,4-butanedithiol and the immobilized flavin was similarly accelerated by factors of 150–256 (Table 2).

We recently reported that a cationic micelle (hexadecyltrimethylammonium bromide) serves as catalyst for isoalloxazine oxidation of 1,4-butanedithiol (*9*). The rate increase by the micelle was 18-fold for 3-methyl-10-ethylisoalloxazine and 250-fold for the more hydrophobic 3-hexadecyl-10-butylisoalloxazine. Since the latter isoalloxazine is sparingly soluble in the aqueous solution, the reaction must proceed wholly in the micellar phase. Similarly, large rate enhancements observed for the polymeric flavins in this study imply that the flavin group is located in the relatively hydrophobic domain of polyelectrolytes.

Influence of Ionic Strength

The catalytic effect of polyelectrolytes stems mostly from the enhanced charge density along the polymer chain, and it is frequently influenced by the ionic strength of the medium. In Fig. 2, apparent third-order rate constants, k_3', for the oxidation of 2-mercaptoethanol and GSH are plotted as a function of ionic strength (μ = 0.02–0.6 with KCl). The reactions are suppressed with increasing salt concentrations: the rate decrease is 2- to 5-fold for 2-mercaptoethanol and 5- to 8-fold for GSH. Figure 2 also shows that the oxidation by F-D-22 is more susceptible to the change of ionic strength than that by F-D-0. The magnitude of salt inhibition is comparable to that observed in micellar systems (*7, 9*).

Influence of Buffer Concentration



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The flavin oxidation of dithiols in nonenzymatic systems is subject to general (probably acid) catalysis, while that of monothiols is not (*1–3*). This contrast was attributed to the change in the rate-determining step (*2, 3*); that is, the nucleophilic attack of RS^- on the 4*a* carbon (k_{4a}), which is amenable to general catalysis (*1, 2*), is rate determining for dithiols, since the subsequent breakdown (k_b), the intramolecular process, becomes rate determining for monothiols (*2, 3*). In order to establish the rate-determining step in the cationic matrix, the oxidation rates were measured as a function of buffer concentrations. Since the behavior of boric acid in general acid–base catalysis is frequently anomalous (*16*), barbiturate buffer was adopted. A high ionic strength of 0.3 was employed, though reluctantly, to suppress the influence of the concentration change of ionic species on the reaction rates.

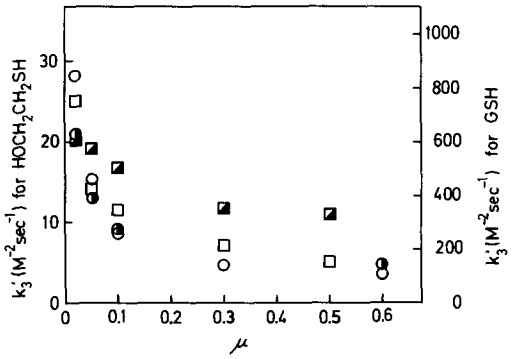

FIG. 2. Apparent third-order rate constants plotted as a function of ionic strength (KCl): (○) F-D-22 + GSH; (◐) F-D-0 + GSH; (□) F-D-22 + 2-mercaptoethanol; (◪) F-D-0 + 2-mercaptoethanol. pH 8.80 ± 0.04 for GSH and 8.90 ± 0.04 for 2-mercaptoethanol.

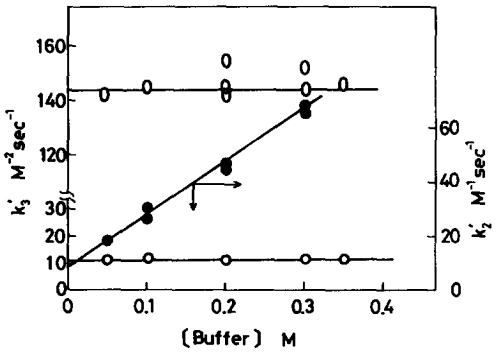

FIG. 3. Rate constants for the oxidation by F-D-22 plotted as a function of buffer (barbituric acid) concentration. μ = 0.3, [flavin] = 2.0–2.4 × 10^{-5} M, pH 8.90 ± 0.03 for 2-mercaptoethanol (O), pH 8.90 for GSH (O), and pH 9.0 for 1,4 butanedithiol (●).

Figure 3 shows the buffer dilution effect in the oxidation of 2-mercaptoethanol, GSH, and 1,4-butanedithiol by F-D-22. Although the oxidation of GSH somewhat lacks in the reproducibility (maximal relative error, 9%), the other systems provided good linear correlations. It is concluded from Fig. 3 that the oxidation of monothiols is not subject to general catalysis, while that of 1,4-butanedithiol is generally catalyzed. The result establishes that the reaction mechanism in the polymer matrix is not essentially different from that in the solution, but one should note that the conclusion is derived from the experiments at high ionic strength where the reactivity of thiols and/or immobilized flavin is considerably suppressed.

pH Dependence

The pH dependence of the oxidation of GSH and 2-mercaptoethanol by F-D-22 is illustrated in Fig. 4. Since the oxidation of monothiols is not subject to general

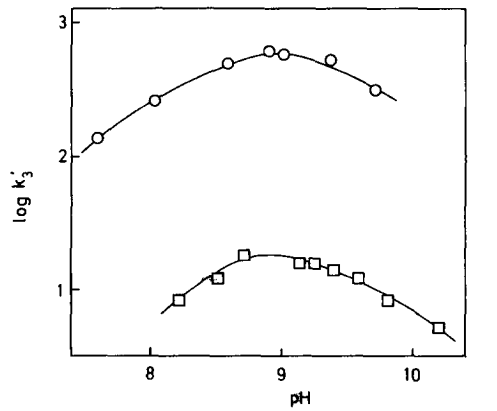

FIG. 4. pH dependence. [Flavin (F-D-22)] = 2.61 × 10^{-5} M; [GSH (O)] = 2.87 × 10^{-3} M, [2-mercaptoethanol (□)] = 1.45 × 10^{-2} M.

catalysis, buffer dilution was not studied. Both systems produced bell-shaped curves. According to Bruice (*17*), the pH–rate profile for the oxidation of monothiols is given by Eq. [5],

$$\begin{aligned} v_{obsd} &= k_3' \,[\text{flavin}][\text{RSH}]_T^2 \\ &= k_s \,[\text{flavin}][\text{RS}^-][\text{RSH}] \\ &= k_s \frac{K_{RSH} a_H [\text{flavin}][\text{RSH}]_T^2}{(K_{RSH} + a_H)^2}, \end{aligned} \qquad [5]$$

where K_{RSH} is the acid dissociation constant of monothiols.

Equation [5] is a typical kinetic equation which provides a bell-shaped pH–rate profile, and the rate maximum is achieved at $a_H = K_{RSH}$. Curve-fitting of Eq. [5] for the data in Fig. 4 provides the k_s and pK_{RSH} values listed in Table 3. The values of kinetically determined pK_{RSH} are slightly smaller (by 0.2–0.3 pK unit) than those of pK_{RSH} determined by titration (in the absence of the polymer). The difference may be rationalized by assuming that a significant fraction of the thiol resides in the polymer micelle volume.

Microenvironmental Effect

It has been established that the logarithm of the rate constant of some flavin-dependent reactions is linearly correlated with polarographic half-wave potentials of flavin ($E_{1/2}$) (*1, 18*), i.e., the positively higher the $E_{1/2}$ value, the greater the reaction rate constant. The $E_{1/2}$ values for polymer-bound flavins (determined at room temperature (*12*)) are listed in Table 2. Although the $E_{1/2}$ values for polymer-bound flavins are higher by 0.05–0.07 V than that of the corresponding monomeric analogue, 3-MeF, such a small difference in $E_{1/2}$ cannot lead to the rate augmentation of 10^2- to 10^4-fold. Furthermore, Table 2 shows that the polymer-bound flavin that has a high $E_{1/2}$ value (e.g., F-D-0) behaves as a *less* reactive species in the oxidation of thiols. This trend is reverse to that in the solution. Therefore, the rate augmentation observed is not primarily associated with the shift of the $E_{1/2}$ value. Instead, the environmental effect should be taken into account as a major source of the rate enhancement.

TABLE 3

DISSOCIATION CONSTANTS (K_{RSH}) AND k_s[a]

	pK_{RSH} (from kinetics)	k_s (M^{-2} sec^{-1})	pK_{RSH} (from titration)
2-Mercaptoethanol[b]	9.0	76.2	9.3[d]
GSH[b]	8.95	2,520	9.1[d]
PhSH[c]	7.3	41,900	7.6

[a] Flavin polymers employed are: F-D-0 (2.40×10^{-5} *M*) for 2-mercaptoethanol and GSH; F-D-22 (2.37×10^{-5} *M*) for PhSH.

[b] 3 vol% ethanol; 30°C.

[c] 40 vol% ethanol; 30°C.

[d] Cited from J. R. WHITAKER, *J. Amer. Chem. Soc.* **84**, 1900 (1962); 29.6°C.

Conceivably, two effects are most important: concentration effect and activation of thiolate anions in the polymer matrix. The concentration effect, which is generally observed in micellar and polymer micellar systems (*10, 19*), requires little comment. The latter factor is associated with the solvent effect on the reactivity of anionic species. The nucleophilicity of anions (including thiolate anions) is much enhanced in the hydrophobic region of cationic micelles and polysoaps (*6–9, 20–27*). Therefore, when the flavin is immobilized in the hydrophobic domain formed by the polystyrene backbone, the activated thiolates will be employed for the oxidation reaction. The absorption band of flavin at around 360 nm is most sensitive to the solvent polarity (*28–30*). For example, the λ_{max} of riboflavin (374 nm, ϵ = 10,800) in an aqueous solution shifts to 344 nm (ϵ = 8400) in a 98% dioxane solution. Similar spectral shifts were observed for polymer-bound flavins: F-D-0, λ_{max} 341 nm and ϵ = 6100; F-D-7, λ_{max} 350 nm and ϵ = 5900; F-D-12, λ_{max} 356 nm and ϵ = 6900; F-D-22, λ_{max} 348 nm and ϵ = 6500, relative to 3-MeF, λ_{max} 362 nm and ϵ = 8800. The data clearly indicate that the flavins reside in the hydrophobic region. The pronounced rate enhancement for thiophenol relative to those of 2-mercaptoethanol and GSH may be attributable to the favorable partitioning of thiophenol in the polymer domain and (probably) to enhanced reactivity of the thiophenol anion in the hydrophobic region (*7, 8, 27*).

CONCLUSIONS

The present study established that the oxidation–reduction reaction between flavin and thiol is markedly accelerated by an immobilized flavin in cationic polymers. The rate acceleration is mainly attributed to the absorption of thiolate anions onto the cationic polymer and to the hydrophobic nature of the reaction site. Since the flavin oxidation of thiols is very sensitive to the microenvironmental effect, future model studies of flavoenzymes must take this fact into account.

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