Coenzyme Models. IX. Micellar Catalysis of Isoalloxazine (Flavin) Oxidation of Dithiol*

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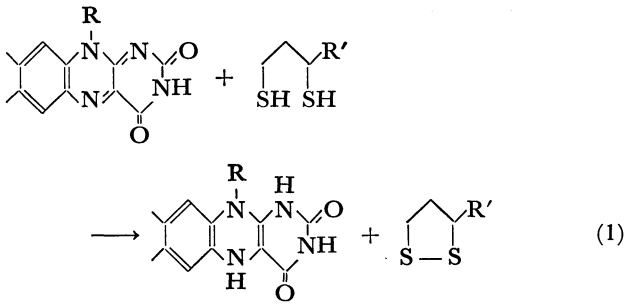
The influence of micelles on the reaction of isoalloxazines and 1,4-butanedithiol(BDT) was studied. The pK_a of BDT was lowered by 0.3 pK unit in the presence of the hexadecyltrimethylammonium bromide(CTAB) micelle, indicating the formation of "hydrophobic ion pairs." The apparent second-order rate constant for the reaction of 3-methyl-10-ethylisoalloxazine and BDT increased by 18-fold on addition of CTAB(3 mM). The UV-visible spectrum of 3-hexadecyl-10-butylisoalloxazine in the presence of the CTAB micelle was similar to that in organic solvents, the rate being enhanced by more than 400-fold as compared with that in a nonmicellar system. Anionic(SDS) and nonionic(Brij-35) micelles suppressed the reaction. The results show that the flavin oxidation of dithiol is facilitated by the environments of the CTAB micelle.

Reduction of flavins by dihydrolipoic acid is one of the interesting oxidation-reduction reactions catalyzed by flavoenzymes(*e.g.*, lipoamide dehydrogenase). Gascoigne and Radda[1] found that dihydrolipoic acid reacts with flavins in the absence of enzyme to produce lipoic acid and reduced flavins(Eq. 1). The subsequent examination established the fact that flavin oxidation of dithiol is of first-order in dithiol concentration and buffer-catalyzed, while that of monothiol is of second-order in monothiol concentration, not being subjected to buffer-catalysis.[2-4]



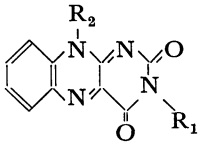
(1)

We have found that the reactivity of some coenzymes and their model compounds are markedly affected by the microenvironments of micelles and polysoaps.[5-7] This is of interest from the viewpoint that the reactivity of coenzymes must a priori be very susceptible to the microenvironments of apoenzymes. In particular, the nucleophilic reactivity of some anions (including thiolate anions such as glutathione and coenzyme A) is drastically enhanced in the presence of the cationic hydrophobic aggregates.[7,8] This unusual activation of anions is conceivably derived from the formation of a "hydrophobic ion pair" between the surfactant cation and the anionic nucleophile.[9,10] Since flavins are reduced by dissociated species of dithiol,[1-3] it occurred to us that the efficiency of this biologically important reaction would be markedly affected by the hydrophobic environment.

In this paper, we wish to report on the micellar effect on the reaction of isoalloxazines and 1,4-butanedithiol(BDT: an analogue of dihydrolipoic acid). Isoalloxazines chosen are 3-methyl-10-ethylisoalloxazine(**1**) and 3-hexadecyl-10-butylisoalloxazine(**2**).

* Contribution No. 418 from this department.

Isoalloxazine:



(**1**) $R_1=CH_3-$, $R_2=CH_3CH_2-$

(**2**) $R_1=CH_3(CH_2)_{15}-$, $R_2=CH_3(CH_2)_3-$

Surfactant:

CTAB, $CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$

SDS, $CH_3(CH_2)_{11}OSO_3Na$

Brij-35, $CH_3(CH_2)_{11}(CH_2CH_2O)_{23}OH$

Experimental

Materials. 3-Methyl-10-ethylisoalloxazine(**1**) and 10-butylisoalloxazine were supplied by Professor F. Yoneda(for preparation, *cf.* Ref. 11). 3-Hexadecyl-10-butylisoalloxazine was obtained by treating 10-butylisoalloxazine(130 mg: 0.5 mmol) with hexadecyl iodide(176 mg: 0.5 mmol) at room temperature in *N,N*-dimethylformamide containing excess powdered K_2CO_3(500 mg). The progress of the reaction was monitored by the TLC method (silica gel–ethyl acetate). The insoluble parts were filtered off after two days, and the solvent was evaporated *in vacuo*. The residual brown oil was extracted with carbon tetrachloride, the extract being washed with an aqueous solution of 0.1 M NaOH and water, dried over anhydrous K_2SO_4. The reaction mixture was concentrated to dryness, and the yellow residue was recrystallized from ethanol–acetonitrile; mp 77—80 °C. Found: C, 72.15; H, 9.44; N, 10.98%. Calcd for $C_{30}H_{46}N_4O_2$: C, 72.83; H, 9.37; N, 11.33%.

1,4-Butanedithiol was distilled under N_2 stream before use: bp 98—102 °C/17 mmHg (lit,[12] bp 74.5 °C/10 mmHg). Hexadecyltrimethylammonium bromide was recrystallized from ethanol before use, and other surfactants(sodium dodecylsulfate, Brij-35) were used without further purification.

Titration of 1,4-Butanedithiol. The spectrophotometric titration was carried out under anaerobic conditions with a Thunberg cuvette. Absorbance at 240 nm(thiolate anion) was chosen. The detailed procedure has been described.[7,13]

Kinetics. All the kinetic measurements were carried out anaerobically at 30±0.1 °C at a calculated ionic strength (μ=0.06 with KCl) unless otherwise stated. The reactions were followed spectrophotometrically by monitoring the reduction of isoalloxazine (λ_{max}, 433 nm for **1** and 440 nm for **2**). The stock solution of BDT was prepared in ethanol just before the experiment. Since excess BDT was present in all the cases, the pseudo first-order behavior was observed. The pH of the reaction mixture was confirmed

not to vary from pH measurements (TOA Digital pH Meter, Model HM-15A) before and after the reaction.

Results

Spectrophotometric Titration of 1,4-Butaneditiol(BDT). Prior to the kinetic measurements, the equilibrium constants for the –SH ionization(K_a) were estimated in the absence and presence of the CTAB micelle. Since the critical micelle concentration (CMC) for CTAB is estimated to be about 8×10^{-4} M under the present conditions,[14] the titration for the latter system was conducted in the presence of 3 mM CTAB. The results are shown in Fig. 1.

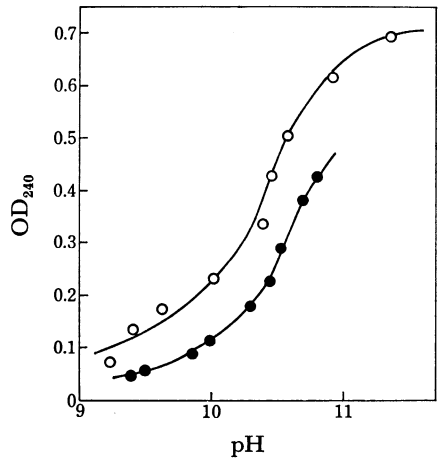


Fig. 1. Spectrophotometric titration of 1,4-butanedithiol. [1,4-Butanedithiol]$=2.20\times10^{-4}$ M, $\mu=0.06$ with KCl.
(○), [CTAB]$=3.0\times10^{-3}$ M; (●), CTAB was not added.

Under the anaerobic conditions at pH 9.2—11.4, only one inflection point was observed. The titration curve monitored by the absorbance(OD) at 240 nm(thiolate anion) is expressed by Eq. 2, where ε is the molar absorption coefficient of thiolate anion. The linear correlation between a_H and $1/OD_{240}$ is indicated by Eq. 3.

$$OD_{240} = \varepsilon[BDT]\left(\frac{K_a}{K_a+a_H}\right) \quad (2)$$

$$\frac{1}{OD_{240}} = \frac{1}{\varepsilon[BDT]} + \frac{a_H}{\varepsilon[BDT]K_a} \quad (3)$$

Good linear relationships($r>0.99$) were observed for the treatment of the experimental data by Eq. 3. From the slope and the intercept, K_a and ε were determined: in the presence of CTAB (3 mM), $pK_a=10.4$, $\varepsilon=3.41\times10^3$ $M^{-1}cm^{-1}$; in the absence of CTAB, $pK_a=10.7$, $\varepsilon=3.30\times10^3$ $M^{-1}cm^{-1}$. According to Benesch and Benesch,[15a] the molar absorption coefficients for aliphatic thiolate anions are $(4—6)\times10^3$ $M^{-1}cm^{-1}$. The observed inflections may correspond to the first dissociation(K_{a1}) of two SH groups.[15b] The pK_{a1} value was lowered in the presence of the CTAB micelle by *ca.* 0.3 pK unit.

Absorption Spectra of Isoalloxazines. Figure 2 shows absorption spectra of oxidized and reduced forms of isoalloxazines. The isoalloxazine **1** shows an absorption maximum at 433 nm($\varepsilon=14200$ $M^{-1}cm^{-1}$). the reduction by BDT giving a tight isosbestic point at 323 nm. The spectra were hardly affected by addition of CTAB above CMC. At the completion of the reaction, admittance of O_2 regenerated **1** quantitatively. In the presence of the CTAB micelle(λ_{max} 440 nm, $\varepsilon=11500$ $M^{-1}cm^{-1}$), the spectrum of **2** shows distinct shoulders at 420 nm and 460—470 nm(Fig. 2), isosbestic points for the reduction by BDT appearing at 278, 324, 367, and 385 nm. Since similar shoulders are observable in organic solvents(acetonitrile, dioxane), the isoalloxazine ring of **2** should be present in the hydrophobic region of the micelle.

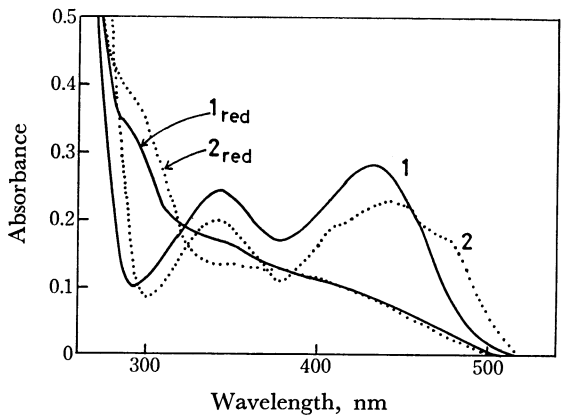


Fig. 2. Spectra of oxidized and reduced forms of isoalloxaznes. [isoalloxazine]$=2.0\times10^{-5}$ M, [CTAB]$=3.0\times10^{-3}$ M, pH 10.05.
——, **1** and reduced **1** ($\mathbf{1}_{red}$); ----, **2** and reduced **2** ($\mathbf{2}_{red}$). Reduction was performed with 1.04×10^{-3} M 1,4-butanedithiol.

Rate Measurements. With excess BDT, isoalloxazine concentration as a function of time gave good pseudo

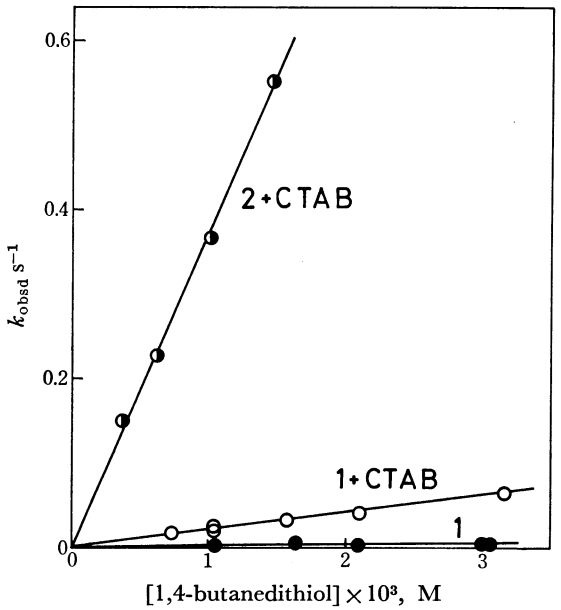


Fig. 3. Pseudo-first order rate constant (k_{obsd}) *vs.* [1,4-butanedithiol].
[Isoalloxazine]$=(1—3)\times10^{-5}$ M, [CTAB]$=3.0\times10^{-3}$ M, pH 10.05+0.02.

first-order plots up to 3 half-lives under all reaction conditions in the present work. When the concentration of BDT was varied, plots of pseudo first-order rate constants(k_{obsd}) against the BDT concentration showed linearity(Fig. 3), indicating that the reaction is of first-order with respect to the concentrations of isoalloxazine and BDT. The apparent second-order rate constants(k_2') can be obtained by dividing k_{obsd} by the total concentration of BDT.

The apparent second-order rate constants at pH 10.05+0.02 are given in Table 1. At this pH, the rate constant for the reaction of **1** and BDT was accelerated by a factor of 23 by the CTAB micelle. The rate for the reaction of **2** and BDT is further accelerated, being more than 400 times greater than that of **1** and BDT in a nonmicellar system. The results suggest that the cationic environments remarkably improve the electrophilicity of isoalloxazine ring and/or the reductive activity of the thiolate anion.

The apparent second-order rate constants for the reaction of **1** and BDT are plotted against the concentration of CTAB in Fig. 4. The plots give a sigmoidal curve: around the CMC, the rate constants rose rapidly with increasing the CTAB concentration. At optimal CTAB concentration(*ca.* 5 mM), 35 times rate augmentation was observed. In contrast, addition of anionic (SDS) and nonionic(Brij-35) surfactants suppressed the reaction above their CMC (Fig. 5). Thus,

TABLE 1. APPARENT SECOND-ORDER RATE CONSTANTS AND pH_{max}

Isoalloxazine	CTAB mM	k_2' at pH 10.05 $M^{-1}s^{-1}$	$k_{2'max}$ $M^{-1}s^{-1}$	pH_{max}
1	0	0.904	1.78	10.9
1	3.0	21.0	32.0	10.4
2	3.0	375	447	10.3

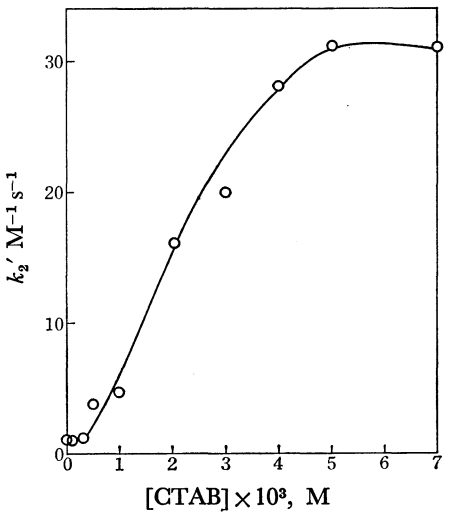


Fig. 4. Apparent second-order rate constants for the reaction of **1** and 1,4-butanedithiol plotted as a function of CTAB.
[**1**]=3.01×10⁻⁵ M, [1,4-butanedithiol]=1.05×10⁻³ M, pH 10.05±0.02, μ=0.06 with KCl.

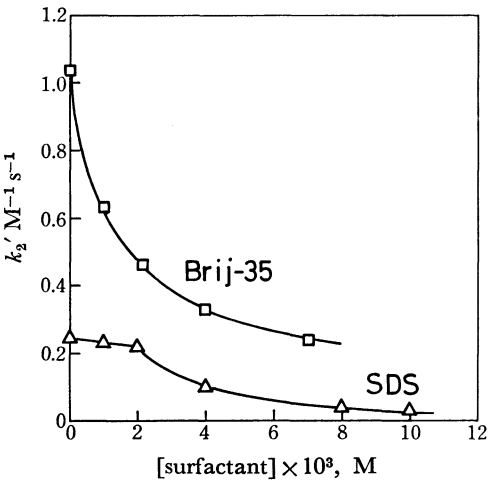


Fig. 5. Apparent second-order rate constants for the reaction of **1** and 1,4-butanedithiol plotted as a function of anionic (SDS) and nonionic (Brij-35) surfactants. (△), SDS, pH 9,10±0.03; (□), Brji-35, pH 10.05± 0.02. Other reaction conditions are recorded under Fig. 4.

only the cationic micelle acts as a catalyst for the reaction of **1** and BDT.

Effects of pH, Salt Concentration, and Buffer Concentration. The logarithm of the apparent second-order rate constant is plotted as a function of pH in Fig. 6. Plots of log k_2' *vs.* pH give bell-shaped curves.[3,4] The pH values(pH_{max}) and the rate constants($K_{2'max}$) at the rate maxima are summarized in Table 1. We see that the pH_{max} values are lowered by 0.5—0.6 pK unit on addition of CTAB(3 mM). The maximal rate constant for the reaction of **1** and BDT is enhanced 18 times in the presence of the CTAB micelle and that of **2** 250 times, as compared with that for **1** and BDT in a nonmicellar system.

The rate of micelle-catalyzed system is influenced by

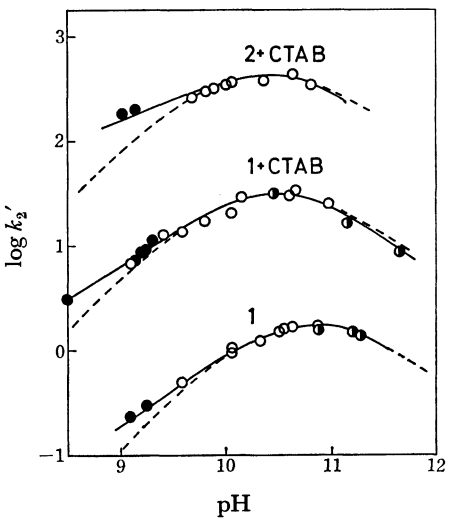


Fig. 6. pH Dependence.
[CTAB]=3.0×10⁻³ M. pH was adjusted with KOH (◑), carbonate (○), and borate (●). Ionic strength was maintained at 0.06 with KCl. Dotted curves (theoretical pH-rate profiles) are obtained from Eq. 6.

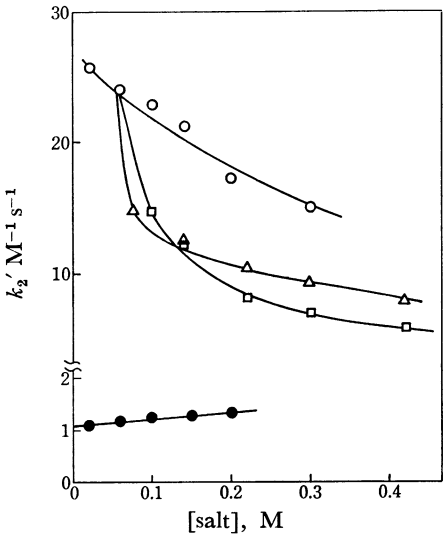


Fig. 7. Effect of ionic strength and buffer concentration on the reaction of **1** and 1,4-butanedithiol.
pH 10.05±0.03, [CTAB]=3.0×10^{-3} M. (○), Carbonate; (●), carbonate without CTAB; (□), KCl; (△), K_2SO_4. pH of the latter two systems was maintained with 0.06 M carbonate.

the salt concentration of the reaction medium. The second-order rate constants for the reaction of **1** and BDT in the presence of the CTAB micelle are suppressed by increase in salt concentration (KCl, K_2SO_4; Fig. 7). Increase in the carbonate buffer($KHCO_3$–K_2CO_3) concentration does not retard the reaction as conspicuously as KCl and K_2SO_4. As shown by Gascoigne and Radda[1] and Loechler and Hollocher,[3] the flavin oxidation of dithiol is subjected to general catalysis. In the present system, the reaction of **1** and BDT in a nonmicellar system was slightly accelerated with increasing carbonate buffer concentration(black circles in Fig. 7); third-order rate constant for general catalysis(*i.e.*, slope for the plots of black circles), 1.3 $M^{-2}s^{-1}$. Supposedly, the micelle-catalyzed reaction is also subjected to general catalysis. The increase in carbonate buffer concentration would provide two opposing effects: the rate deceleration due to the increased ionic strength and the rate acceleration due to the increased local concentration of bicarbonate ion as an acid around the micelle surface. As a result, the rates would become less susceptible to the change in buffer concentration.

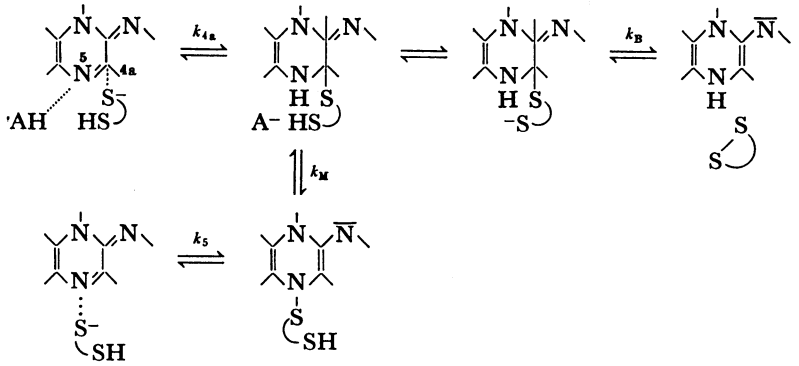
Discussion

Micellar catalysis provides suitable model systems of the enzymatic catalysis.[16,17] Micelles can influence rates and equilibria of biologically important reactions. The micellar environment evaluated with the fluorescent emission is akin to that of the active site of enzymes.[17,18] Thus, it seems significant to assess the influence of the micellar enviroments on the enzyme-like reactions, especially on the coenzyme-dependent reactions.

One of the most significant findings on the micellar catalysis would be the unusual enhancement of nucleophilicity of a variety of anionic species when they are bound to the cationic micellar and polymer micellar phase.[8,10] In studies on nucleophilic reactions of thiolate anions, we found that the thiolate anion bound to the cationic micellar phase is very susceptible to air oxidation.[7,13] This suggests that the formation of the hydrophobic ion pair would enhance not only the nucleophilicity but the reactivity as a reducing agent. This led us to investigate the effect of micellar environments on the oxidation-reduction reactions containing thiol groups.

The most plausible mechanism for the reaction of flavins and dithiols is the formation of adduct followed by the nucleophilic attack of intramolecular thiolate anion. The free radical mechanism can be disregarded on the basis of kinetic evidence.[3,4] Two kinetically equivalent mechanisms were suggested: (i) nucleophilic attack by thiolate anion on C(4a), aided by a general acid catalysis at N(5)(k_{4a} process in Eq. 4), and (ii) nucleophilic attack on N(5)(k_5 process) followed by 5→4a migration of RS^+(k_M process).**

According to Loechler and Hollocher,[3] buffer catalysis is observed only when the 4a-addition(*i.e.*, k_{4a} process) is involved in the rate-limiting step. This is based on the fact that the negative charge on N(5) developed by 4a-addition is energetically unfavorable ($pK_a\approx24$), unless the adduct formation is aided by acid catalysis. The present system is buffer-catalyzed in the absence and probably in the presence of the CTAB micelle(Fig. 7). Therefore, mechanism (i) is not incompatible with the present kinetic situation. 5-Addition(*i.e.*, mechanism (ii)) is presumed not to be buffer-



(4)

** This mechanism was proposed for the reaction of flavins and monothiols.[4]

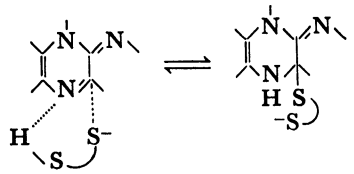
catalyzed, since the anionic charge is developed on N(1), the pK_a of which is much lower ($\approx$6.6).[19] If 5-adduct is formed, 5→4a migration of RS^+ becomes obligatory prior to breakdown(k_B process). Since the reaction which yields the cationic charge in the transition state is extremely suppressed by the cationic micelle,[5,6] the migration should be significantly inhibited by the CTAB micelle. However, the CTAB micelle acts as catalyst for the isoalloxazine oxidation of BDT. Thus, mechanism (ii) is unlikely in the present system.

Similar reaction orders and pH-rate profiles observed for the micellar and nonmicellar systems indicate that the reaction mechanism is not essentially changed by the micelle. The result of spectrophotometric titration of BDT shows that the pK_{a1} is lowered by 0.3 pK unit in the presence of the CTAB micelle. pH_{max} values (Table 1) are lowered by 0.5—0.6 pK unit. The lowering of pK_a is generally observed for anionic species bound to the cationic micelle,[9,13,20] and such anions are greatly activated with no apparent exceptions. For example, the nucleophilic reaction of coenzyme A and *p*-nitrophenyl acetate is accelerated by a factor of 290 in the presence of the CTAB micelle, the pK_a being lowered by 0.5 pK unit as compared with the corresponding value in a nonmicellar system.[7] On the other hand, spectral data(Fig. 2) indicate that **1** is not much concentrated in the micellar phase. Thus, the rate increase for the reaction of **1** and BDT would be attributed to the concentration of BDT in the micellar phase and to the enhanced nucleophilicity of BDT anion therein.

The 18—23 fold rate augmentation in **1** oxidation of BDT seems rather small. It has been shown that **1** oxidation of monothiols shows a remarkable rate enhancement by a factor of 10^2—10^5 times.[21] Some mechanistic difference between monothiol and dithiol may cause the different susceptibility to the micellar catalysis. Possible interpretation might be as follows. (i) The k_B process is presumed to be rate-limiting for flavin oxidation of monothiol.[3,4] Since the k_B process is a simple nucleophilic attack on flavin–S– by RS^-, the rate would directly reflect the enhanced nucleophilicity of RS^-. On the other hand, the k_{4a} process, a rate-limiting step for dithiol,[3] is a nucleophilic attack concerted with acid catalysis. In most cases, the cationic micelle retards acid catalyzed reactions.[5,6,22,23] Therefore the total reaction rate would not be greatly enhanced despite the favorable influence on the nucleophilicity. (ii) The monoanion of BDT is subjected to micellar activation to a smaller extent due to the intramolecular hydrogen bond with the undissociated SH group. There is a precedent that reactivity of naked anion in aprotic environment is efficiently quenched by a single intramolecular hydrogen bonding.[24] Micellar desolvation of anion stabilized by intramolecular hydrogen bonding might be considerably difficult.

Isoalloxazine **2** is scarcely soluble in an aqueous solution. The isoalloxazine ring of **2** resides in relatively hydrophobic region of the micelle(Fig. 2). Both **1**+CTAB and **2**+CTAB systems employ similar pH_{max} (10.4 and 10.3, respectively), indicating that both systems oxidize similar species of BDT. Thus, a mechanistic change in the **2**+CTAB system is hardly expected. The rate increase in this system could be derived from the concentration effect of **2** in the micellar phase and the facilitation of flavin oxidation due to the change in the oxidation potential of **2**. The latter conjecture is supported by the fact that flavin oxidation is more facile in dipolar aprotic solvents[25] to which the cationic micellar environment is said to be akin.[17]

The requirement of a general acid catalysis for 4a-addition is discussed by Bruice *et al.*[4,26,27] in detail on the basis of the libido rule of Jencks.[28] Since H_3O^+ catalysis is *completely* inhibited by the cationic micelle when the substrate is buried in the micellar phase,[6] H_3O^+ dependent processes (k_3 and k_6 in Loechler and Hollocher's scheme) should be disregarded in the cationic micellar system. Thus, acid species present in the cationic micellar solution are H_2O, buffer acid, and intramolecular SH group. When the reaction system is buffered by H_2O–OH^-, intramolecular SH group would play the role of a general acid(Eq. 5).



(5)

If the reaction is of first-order in BDT monoanion as expressed by $v_{obsd}=k_2[HS(CH_2)_4S^-][isoallozaxine]$, the corresponding pH-rate profile is given by

$$k_2' = k_2 a_H K_{a1}/(a_H^2 + a_H K_{a1} + K_{a1}K_{a2}) \quad (6)$$

where k_2 denotes a true second-order rate constant. Theoretical curves(Fig. 6, dotted line) derived from Eq. 6*** show that the experimental data satisfy Eq. 6 at $pH > pH_{max}$, while the observed rate is greater at $pH < pH_{max}$. In the latter pH region, buffer acid would significantly contribute to the observed rate.

In conclusion, the reactivity of BDT as a reducing agent is remarkably enhanced in the presence of the cationic micelle, and the electrophilicity of isoalloxazines can be affected by the micellar environements.

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*** Based on the relationships, $pK_{a2} \approx pK_{a1} + 1.0$[3] and $pH_{max} = 0.5(pK_{a1} + pK_{a2})$, appropriate k_2, K_{a1}, and K_{a2} were chosen by the trial-and-error method. Best fit values are: for **1**, $k_2 = 2.9\ M^{-1}\ s^{-1}$, $pK_{a1} = 10.4$, $pK_{a2} = 11.4$; for **1**+CTAB, $k_2 = 52.2\ M^{-1}\ s^{-1}$, $pK_{a1} = 9.9$, $pK_{a2} = 10.9$; for **2**+CTAB, $k_2 = 730\ M^{-1}\ s^{-1}$, $pK_{a1} = 9.8$, $pK_{a2} = 10.8$.

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