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Proximity Effect in the Oxidation of Dithiols with 3-Methyllumiflavin

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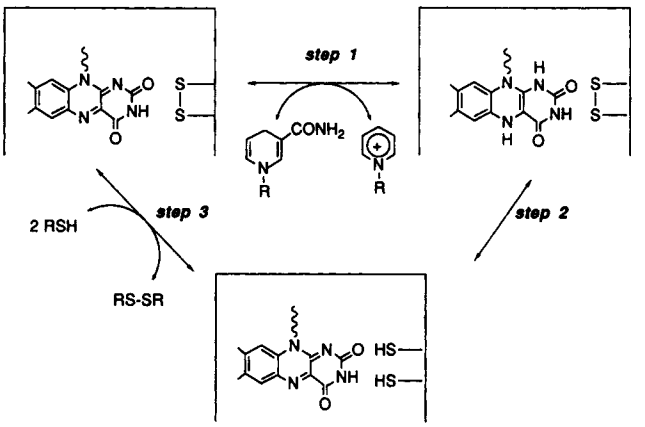
ABSTRACT

Rates of oxidation of various dithiols with 3-methyllumiflavin to afford the corresponding cyclic disulfides have been examined at various pH values in 30% aqueous ethanol at 30°C. 1,3-Propanedithiol, which affords 1,2-dithiolane, has been found to be the most reactive among several dithiols tested in this oxidation. Kinetic results clearly show that highly strained 1,2-dithiethanes are formed as primary products in the oxidation of vic-dithiols with 3-methyllumiflavin.



SCHEME 1

INTRODUCTION

Disulfide reductase, such as glutathione reductase, possesses a disulfide group of a cystine residue together with flavin adenine dinucleotide (FAD) as a prosthetic group [1]. In this enzymatic reaction (Scheme 1), electrons of dihydronicotinamide dinucleotide phosphate (NADPH) are believed to transfer to the FAD situated at the active site at first, affording $FADH_2$ (step 1) which then gives electrons to the disulfide group of the apoprotein to generate two thiol groups at the active site (step 2). The two thiol groups of the apoprotein reduce the substrate disulfide bound to the active site to the corresponding thiols via the thiol-disulfide interchange reaction. The cystine disulfide group is regenerated eventually in this process (step 3).

Mechanisms of oxidation of thiols have been investigated extensively [2]. Concerning the enzymatic reaction mechanism, the mechanism for the oxidation of dithiols by the flavins, i.e., the reverse reaction of step 2 of the enzymatic reaction (Scheme 1), has been investigated in detail using biomimetic systems [3–6]. The enzymatic reaction (Scheme 1) is known to be reversible [1]. The disulfide part of the best catalyst to promote the reaction in Scheme 1 would satisfy the following two factors. First, the disulfide is strained and the strain is released upon conversion to the reduced form, *i.e.*, the dithiol. Second, the dithiol form of the catalyst has a strong reducing ability of substrate disulfides. Thus, the structure of the disulfide part involved in the oxidized form of the catalyst is crucial in the enzymatic reaction. For the understanding of this disulfide-dithiol redox process of the

Dedicated to Professor Huang Yao-Zeng on the occasion of his eightieth birthday.

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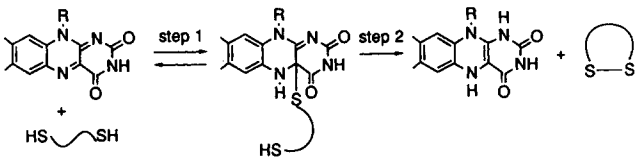


SCHEME 2

apoenzyme, structures and reactivities of dithiols in the oxidation with 3-methyllumiflavin have been investigated, since the mechanism of the reduction of a flavin with a dithiol is quite similar to that of a thiol-disulfide interchange reaction and the kinetic measurement for the reduction of a flavin with a dithiol is experimentally much easier than that for the reduction of a disulfide with dithiols.

The reduction of flavins by dihydrolipoic acid is another interesting example of a redox reaction catalyzed by flavo-enzymes, *i.e.*, lipoamide dehydrogenase. Gascoigne and Radda found that dihydrolipoic acid reacts with flavins in the absence of enzymes to produce lipoic acid and reduced flavin [7]. The oxidation of dithiols with flavin is of the first order with respect to each reactant; however, the oxidation of the monothiol with flavin obeys the third-order kinetic equation, *i.e.*, first order in flavin concentration and second order in the monothiol concentration, and is not general acid-catalyzed. [3,4] Thus, the reaction of flavin with dithiols has been considered to proceed via two steps, as shown in Scheme 2 which involves a thiol-C(4a)flavin adduct.

Other investigations on the proximity effect found in the oxidation of dithiol are the oxidation with molecular oxygen [8,9] and dye [10].

RESULTS AND DISCUSSION

Kinetics studies were carried out in 30% aqueous ethanol (v/v) solution under argon. The rate constants were determined by following the decrease of the absorption of the oxidized form of 3-methyllumiflavin (444 nm) under the pseudo-first-order kinetic conditions. For all cases when dithiols were employed, the oxidized 3-methyllumiflavin was regenerated quantitatively by bubbling O_2 into the cuvette after the reaction, indicating that no side reaction had occurred during the reaction between the flavin and the thiols.

With excess dithiols, 3-methyllumiflavin concentration as a function of time gave good first-order kinetic plots up to two half-lives of the flavin under the reaction conditions (Figures 1 and 3). When the pH of the medium was varied, the plots of the pseudo-first-order rate constant (k_{obs}) against the pH showed a bell-shaped curve (Figures 2 and 4) mainly on the basis of pH_{max} at about the largest rate constant, as reported previously by other investigators. [3,4] When the concentration of the dithiol was varied, plots of k_{obs} against the dithiol concentration fell linearly (Figures 2 and 4), clearly indicating that the reaction is of the first order in the concentration of dithiol. This suggests that dithiols were oxidized intramolecularly to the corresponding disulfide. The apparent second-order rate constant (k'_2) was obtained from the slope of the linear plot of k_{obs} against the total concentration of the dithiol (Equation 1).

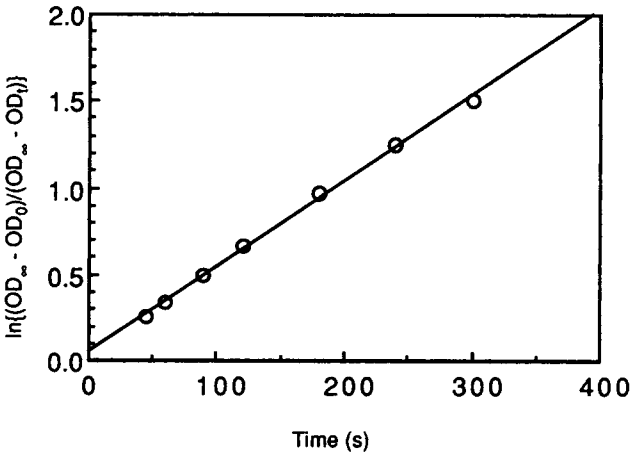


FIGURE 1 First-order kinetic plot of the oxidation of 1,3-propanedithiol (**1**) with 3-methyllumiflavin in 30% aqueous ethanol (pH 10.33, μ = 0.3) at 30°C under argon. [$MeFl_{ox}$] = 5×10^{-5} M; [**1**] = 5×10^{-3} M.

$$-d[Fl_{ox}]/dt = k_{obs}\,[Fl_{ox}] = k'_2\,[\text{dithiol}][Fl_{ox}] \quad (1)$$

$[Fl_{ox}]$ represents the concentration of the oxidized form of 3-methyllumiflavin. Some typical examples of the kinetic results observed in this work are illustrated in Figures 1–5.

In the case of the 2-mercaptoethanol (**9**), a monothiol with 3-methyllumiflavin at pH 10.7 (30% EtOH), the plots of k_{obs} against the square of the concentration of **9** fell on a linear line which did not pass zero point (Figure 6). Hydrolysis of flavin is known to proceed under alkaline conditions [11]. Since the value of the intercept (4.1×10^{-6} s^{-1}) is in good accordance with the rate for the destruction of 3-methyllumiflavin in the control experiment (3.4×10^{-6} s^{-1}) without thiol, the hydrolysis of flavin can compete with such a slow reaction between the flavin and the monothiol.

$$\begin{aligned} -d[Fl_{ox}]/dt &= \{k_{obs} + k_{hydrolysis}\}[Fl_{ox}] \\ &= \{k'^3[\text{2-mercaptoethanol}]^2 \\ &\quad + k_{hydrolysis}\}[Fl_{ox}] \end{aligned} \quad (2)$$

The reaction of *vic*-dithiols with 3-methyllumiflavin is intriguing, since, if the oxidation of a *vic*-dithiol occurs intramolecularly, as in the case of

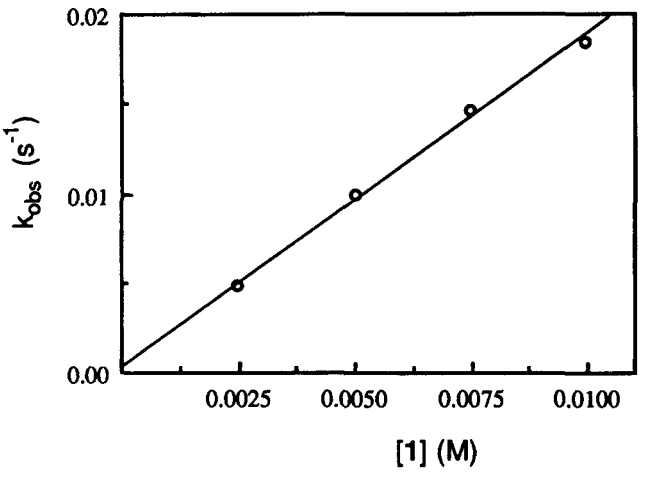


FIGURE 2 Plot of pseudo-first-order rate constant (k_{obs}) for the oxidation of **1** with 3-methyllumiflavin at pH 11.5 against concentration of **1**. $[MeFl_{ox}] = 5 \times 10^{-5}$ M.

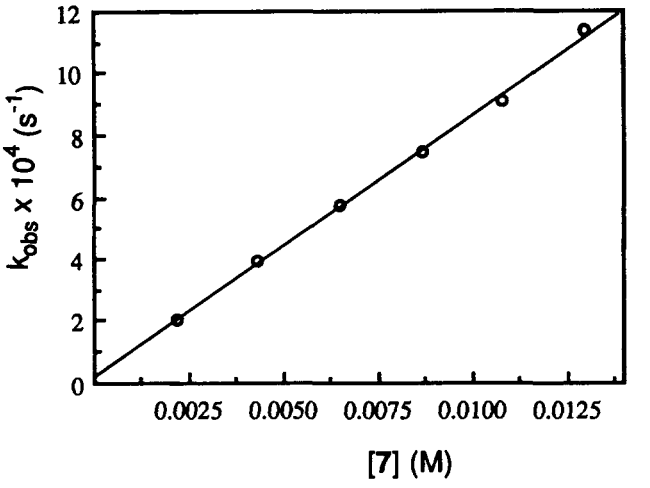


FIGURE 4 Plot of pseudo-first-order rate constant (k_{obs}) for the oxidation of **7** with 3-methyllumiflavin at pH 11.3 against concentration of **7**. $[MeFl_{ox}] = 5 \times 10^{-5}$ M.

other dithiols, a 1,2-dithietane would be formed. Only a special 1,2-dithietane, dithiatopazine, has been known to be an isolable 1,2-dithietane [12]. The reaction of 2,3-dimercapto-1-propanol (**7**) with 3-methyllumiflavin obeyed the rate law of Equation 1 (Figures 3–5). This fact clearly shows that the reaction involves intramolecular S—S bond formation generating 3-hydroxy-1,2-dithietane. When the reaction proceeded to two half-lives of the methyllumiflavin, the initially transparent reaction solution changed to opaque. This is undoubtedly due to the liberation of a polymeric disulfide formed by photo-induced polymerization of the primarily formed 1,2-dithietane derivative (Scheme 3).

Table 1 summarizes the apparent second-order rate constants for dithiols and the apparent third-order rate constant for a monothiol at the optimum pH.

Data in Table 1 are characterized by the following two features.

1. The ability of dithiols to reduce 3-methyllumiflavin follows the same order as the indicated number of methylene group separating the two thiol groups (n) = $5 < 2 < 4 < 3$ when the two thiol groups are linked by a carbon chain which can freely rotate.
2. When two dithiols in which the two thiol

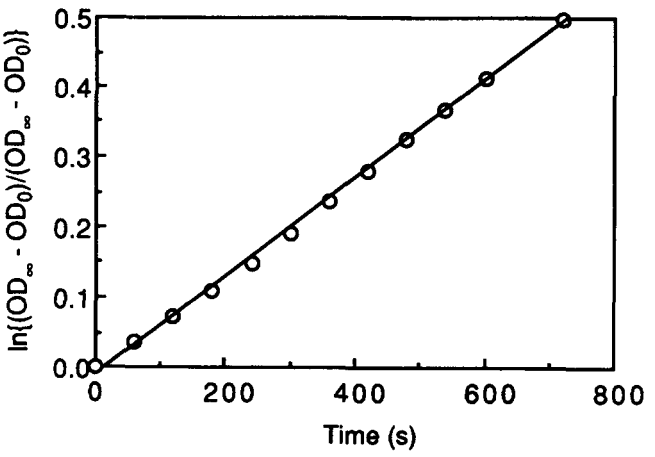


FIGURE 3 Pseudo-first-order kinetic plot of the oxidation of 2,3-dimercaptopropanol (**7**) with 3-methyllumiflavin in 30% aqueous ethanol (pH 11.3, μ = 0.3) at 30°C under argon. $[MeFl_{ox}] = 5 \times 10^{-5}$ M; [**7**] = 5×10^{-3} M.

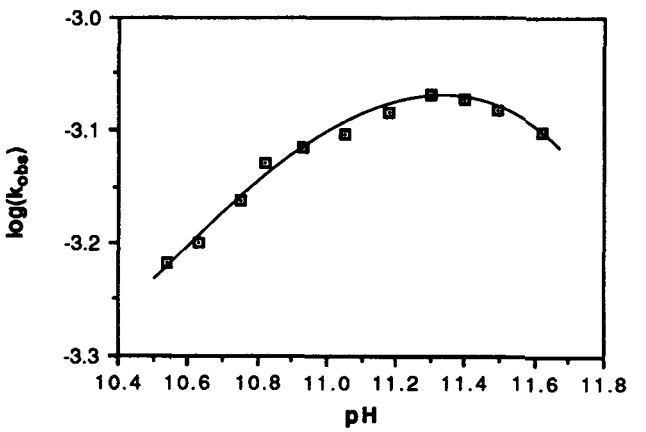


FIGURE 5 Plot of log k_{obs} for the oxidation of **7** with 3-methyllumiflavin against pH. $[MeFl_{ox}] = 5 \times 10^{-5}$ M; [**7**] = 5×10^{-3} M.

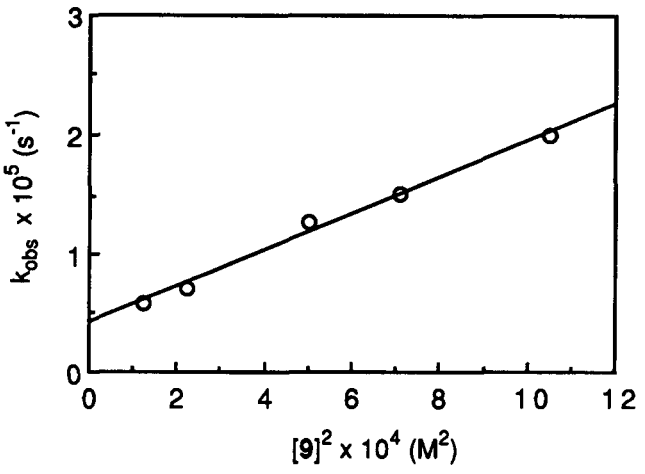


FIGURE 6 Plot of pseudo-first-order rate constant (k_{obs}) for the oxidation of 2-mercaptoethanol (**9**) against square of concentration of **9** in 30% aqueous ethanol (pH 10.7, μ = 0.3) at 30°C under argon. [$MeFl_{ox}$] = 5 × 10^{-5} M; [**9**] = 5 × 10^{-3} M.

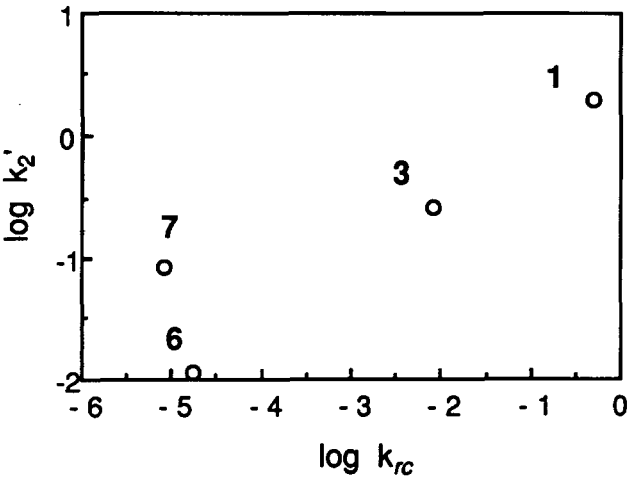
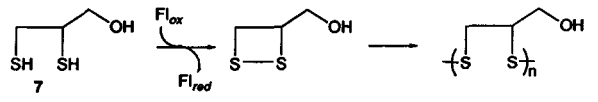


FIGURE 7 Logarithmic plot of k_2' values for the oxidation of dithiols with 3-methyllumiflavin against k_{rc} values for the intramolecular ring closure reaction in Scheme 5.



SCHEME 3

TABLE 1 Kinetic Results for the Oxidation of Dithiols by 3-Methyllumiflavin[a]

Dithiol		pH_{max}	$k_2'(M^{-1}s^{-1})$	Relative rate
HS⌒SH	1	11.5	1.98	7.4
SHSH⌒$CONH_2$	2	11.5	2.21	8.3
HS⌒SH	3	11.6	0.267	1.0
HS⌒OH⌒SH, OH	4	10.6	0.497	1.8
SH, SH	5	10.6	1.72	6.4
HS⌒SH	6	11.6	1.13×10^{-2}	0.042
SH SH OH	7	11.3	8.43×10^{-2}	0.32
SH, SH	8	11.4[b,c]	1.97	>7.4
HS⌒OH	9	10.7[b]	$1.5 \times 10^{-2} M^{-2} s^{-1}$	-

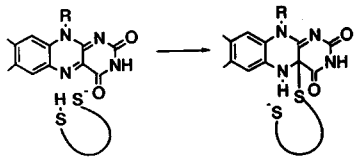
[a]Reaction condition: 0.1 M sodium carbonate buffer, μ = 0.3 (with KCl), 30% EtOH (v/v), 30°C, under argon.
[b]The pH is not optimized.
[c]In 50% EtOH (v/v).

groups are separated by the same number of carbon atoms are considered, the reactivity of the dithiol in which the two thiol groups are fixed in proximity is greater than that of the one in which the two thiol groups are linked by freely rotatable methylene groups, *i.e.*, 1,3-propanedithiol (**3**) and α,α'-dimercapt-*o*-xylene (**5**); **7** and *exo,exo*-bicyclo[2,2,1]heptane-2,3-dithiol (**8**).

Since the mechanism of oxidation of dithiol with flavin, postulated by earlier investigators, [4,5] involved two steps, the structural effect of dithiols observed in this work is concerned with either step 1 or step 2 of Scheme 2. Step 1 is known to be a general acid-catalyzed reaction. Acid species present in the solution are H_3O^+, H_2O, and buffer acids. The intramolecular SH group would act as a general acid catalyst, as shown in Scheme 4.

Such a neighboring SH group participation as a general acid is considered to be greater when the distance between the two thiol groups is shorter. This prediction agrees well with the reactivity order of the dithiols, except for the dithiol **7**.

The second step involves the intramolecular nucleophilic substitution on the sulfur atom attached to the **4a**-carbon in the flavin by the thiol group. The entropy of activation in the reactions



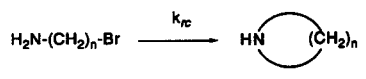
SCHEME 4

of the dithiols should be reduced at the transition state, since they form rigid cyclic disulfides. The magnitude of such an entropy loss should increase with the increase of the freedom of the dithiols. Except for **7**, relative rates are well correlated with the relative magnitude of the expected entropy loss.

The strain energy of the resulting cyclic disulfides formed in step 2 should be partly reflected in the relative enthalpies of activation of reactions of dithiols for step 2. The relative magnitude of the strain energy of a cyclic disulfide can be separated into two terms, *i.e.*, an electronic strain due to an unfavorable lone pair–lone pair interaction between the two vicinal sulfur atoms and the angle strain (Baeyer strain). A dihedral angle around the S—S bond in a strainless linear disulfide is ca. 90°, [13] while that of 1,2-dithiolane is 26° and that of lipoic acid is 35° [14,15]. Among four to seven membered cyclic disulfides, strain energy should be largest in 1,2-dithietane because of its small dihedral angle and the high Baeyer strain. Thus, the strain would increase in the order of dithiothreitol (**4**) > **5** > 1,5-pentanedithiol (**6**) [16]. The slightly lower reactivity of the 1,2-dithiol, **7**, than that expected from the other factors mentioned previously is undoubtedly due to the large strain energy of the reaction product, *i.e.*, the disulfide.

Kinetic data for the similar cyclization reaction as step 2 in the oxidation of dithiol with 3-methyllumiflavin are reported for the reaction shown in Scheme 5 [17].

A logarithmic plot of k_2' values for the reaction shown in Scheme 2 against those (k_{rc}) for the intramolecular cyclization reaction shown in Scheme 5 is shown in Figure 7. Although the rate of formation of the highly strained 1,2-dithiethane deviates from the line, the slope less than one suggests that both step 1 and step 2 in Scheme 2 determine the rate of the reduction of the flavin with dithiols. If step 2 determines the rate of the reaction in Scheme 2, the slope of Figure 7 would be *ca.* 1.



SCHEME 5

In this oxidation, 1,2-dithiolane seems to be the best disulfide to simulate the disulfide involved at the active site of the apoprotein of glutathione reductase (Scheme 1).

EXPERIMENTAL

All melting points were taken on a Yanako instrument and were uncorrected. NMR spectra were recorded on a Hitachi R-600 NMR spectrometer using TMS as an internal standard. UV-visible spectra were taken on a Hitachi 200-20 spectrophotometer. Measurement of pH values was done by use of a Horiba pH meter. 3-Methyllumiflavin was synthesized by a known method [18].

Thiols: **9** and **7** were obtained from Wako and used after distillation under nitrogen at reduced pressure. **4** was from Wako and used without further purification. *Dihydrolipoamide* (**2**) was prepared by the reduction of lipoamide with sodium borohydride according to the literature, mp 64–65° (Ref. [19] 66–67°), yield 90%. 1,3-Propanedithiol (**1**), **2**, **5**, and **6** were prepared by treating the corresponding dibromides with thiourea followed by hydrolysis according to the literature [20] with some modification. A typical procedure is as follows. Thiourea (0.12 mol) was added to 60 ml of ethanol in a flask equipped with a reflux condenser. 1,3-Dibromopropane (0.06 mol) was added and the mixture was refluxed for 6 hours. After cooling, evaporation of solvent under reduced pressure gave white crystals. An aqueous solution containing 45 g of KOH in 150 ml of water was added to the residue, and the whole solution was refluxed for 5 hours under argon. Then the mixture was cooled to room temperature and a solution of 24 ml of conc. H_2SO_4 in 50 ml of water was added dropwise in an ice-water bath. The pH of the solution was adjusted to about 1. The mixture was extracted with $CHCl_3$ three times. The $CHCl_3$ layer was washed with water and dried over $MgSO_4$. After removal of the solvent in vacuo, the residue was distilled under nitrogen at reduced pressure to afford the colorless oil of **1**, yield 68%, bp 71.5–72%/25 mm Hg (Ref. [21] 169–170°/70 mm Hg). **3**: yield 52%, bp 80–81°/20 mm Hg (Ref. [22] 105–106°/30 mm Hg). **6**: yield 49%, bp 118°/31 mm Hg (Ref. [23] 107–108°/15 mm Hg). **5**: crude product was recrystallyzed from $CHCl_3$-hexane. White crystals, yield 55%, mp 44–45°. NMR ($CDCl_3$) d = 1.84 (t, J = 7.0 Hz, 2H, SH), 3.85 (d, J = 7.0 Hz, 4H, CH_2-S), 7.24 (s, 4H, aromatic H). IR (KBr) 2550 cm^{-1} due to SH group. **8** was prepared as follows. *exo*-3,4,5-Trithiatricyclo[5,2,1,0,2,6]-decane (**10**) was prepared by a known method [24]. A suspension of $LiAlH_4$ (1.0 g, 27.7 mmol) in 40 ml dry ether was added slowly at 0°C under argon to a solution of the trisulfide **10** (21.0 mmol) in 30 ml of dry ether. The mixture was brought to room temperature and then stirred for 1 hour. The excess $LiAlH_4$ was de-

stroyed by addition of 10 ml of water, and 5 ml of 10% NaOH was added and the mixture stirred for 20 minutes. The mixture was acidified with dil. HCl. Following extraction with benzene, the organic layer was dried over $MgSO_4$. The solvent was removed under reduced pressure, and the resulting residue was purified by distillation in vacuo to give colorless oil, **8**. Yield 64%, bp. 105–105.5°/5 mm Hg.

Kinetics: All the kinetic measurements were carried out anaerobically at 30°C in 0.1 M carbonate buffer containing 30% ethanol (v/v) at an ionic strength μ = 0.3 (adjusted by addition of KCl). A typical example of the reaction procedure is as follows. A buffer solution (2.1 ml) and 0.6 ml of ethanol containing 1.5×10^{-4} mmol of 3-methylluniflavin were placed in a modified Thunberg cuvette. Ethanol (0.3 ml) containing the appropriate concentration of a dithiol was deposited in the side-arm of the cuvette. Both were deoxygenated for 20 minutes by passing through argon in the dark. The cell was closed and placed in a spectrophotometer equipped with a thermostatted cell-holder. After thermal equilibration at 30°C had been attained, the content of the side-arm was rapidly mixed with the buffer solution. The progress of the reaction was followed by monitoring the decreasing absorption of the oxidized form of the flavin (444 nm).

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