Tetrahedron Vol. 44, No. 18, pp. 5669 to 5674, 1988
Printed in Great Britain.

0040-4020/88 $3.00+.00

SPONTANEOUS FORMATION OF FLAVIN RADICALS IN AQUEOUS SOLUTION BY COMPROPORTIONATION OF A FLAVINIUM CATION AND A FLAVIN PSEUDOBASE

Humphrey I. X. Mager‡ and Shiao-Chun Tu*

Department of Biochemical and Biophysical Sciences, University of Houston.
Houston, Texas 77204-5500, U.S.A.

(*Received in US* 10 *May* 1988)

ABSTRACT – 5-Ethyl-3-methyllumiflavin cation **I** and 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin pseudobase **II** (X = OH) are in a rapid equilibrium in aqueous solution at room temperature. A spontaneous intermolecular one-electron transfer between **I** and **II** resulted in an accumulation of 5-ethyl-3-methyllumiflavin semiquinone **III** *in the absence of any additional electron donor*. 5-Ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin radical cation **IV** (X = OH) was also formed along with **III** in a 1:1 molar ratio. Due to subsequent decompositions, **IV** was not quantitatively accumulated. The rate of this intermolecular one-electron transfer was pH-dependent. The observed pH-rate profile shows an excellent agreement with the theoretical curve calculated on the basis of the apparent pK_a of the $\mathbf{I} + H_2O \rightleftharpoons \mathbf{II} + H^+$ equilibrium, thus providing unambiguous evidence for the proposed mechanism. The yield of **III** was also pH-dependent. At pH > 5, **III** was accumulated in yields which did not exceed the theoretical value of 50%. Ring contractions of **IV** to non-flavin products were the predominant decompositions. At pH < 5, **III** could be obtained in 60-95% yields. Apparently additional **III** was formed from decompositions of **IV**. No formation of O_2 or H_2O_2 was found at pH 2-7. The main decomposition of **IV** is assumed to generate **I** and hydroxyl radicals. This could account for the buffer and solvent effects on the yield of **III**. 3-Methyl-lumiflavin **VIII** was also detected as a minor decomposition product in 1-6% yields, and a mechanism is proposed for this N^5-dealkylation pathway.

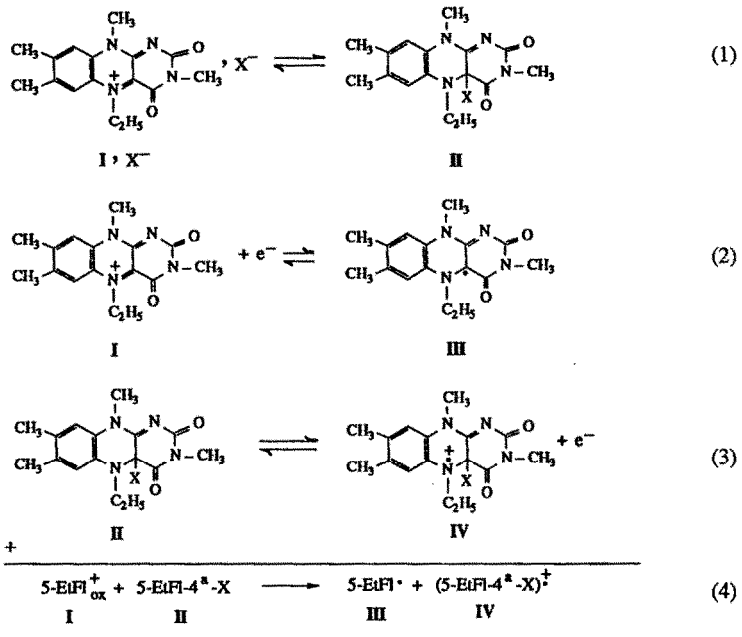
The intermediacy of flavin-4a-adducts in flavoprotein external monooxygenase catalysis has been well established.[1-6] However, much remains to be learned about the chemical reactivities of this type of flavins. As a continuation of a series of studies to elucidate the chemistry of 4a-substituted dihydroflavins,[7-9] our more recent work[10,11] has been particularly directed to the delineation of the functional role of 4a-substituted flavin intermediates in bacterial luciferase (a flavo-monooxygenase) catalysis. Schuster[12] first speculated that bacterial bioluminescence may follow a *Chemically Induced Electron Exchange Luminescent* (CIEEL) mechanism. Subsequently, Mager and Addink[8] have postulated the first detailed CIEEL mechanism for this bioluminescent reaction which involves a key step of one-electron rearrangement converting the dihydroflavin-4a-peroxyhemiacetal intermediate to a novel 4a-hydroxyflavin radical cation.

In order to test this hypothesis, our initial efforts were directed to the formulation of a model system in which the formation of a stabilized 4a-hydroxyflavin radical cation could be detected. Such studies indeed led to the demonstration of the generation of 5-ethyl-4a-hydroxy(or methoxy)-3-methyl-4a,5-dihydrolumiflavin radical cation **IV** (X = OH; OMe) in an intermolecular one-electron transfer from 5-ethyl-4a-hydroxy (or methoxy)-3-methyl-4a,5-dihydrolumiflavin pseudobase **II** (X = OH; OMe) to 5-ethyl-3-methyllumiflavin cation **I** [7-9] (Eq. 4). 5-Ethyl-3-methyllumiflavin semiquinone **III** was also formed along with **IV** in a 1:1 molar ratio. The mechanism for such a reaction has been proposed as shown in Scheme 1. With either **I** or **II** as the starting reactant, the key steps involve an equilibrium between **I**,X^- and **II** (Eq. 1) and an electron transfer from **II** to **I** (Eqs. 2, 3).

More recently, the reversible electrochemical generation of 4a,5-substituted flavin radical cations **IV** (X = OH; OMe) from the flavin adducts **II** (X = OH; OMe) in acetonitrile has been demonstrated.[11] Both the intermediates and the final products of electrooxidation of **II** (X = OH; OMe) were spectrally and electrochemically identified.

These earlier studies were all carried out in low-polarity organic solvents. In contrast, this work focuses on the elucidation of the chemistry of flavinium cation and 4a-hydroxyflavin pseudobase in aqueous media. Reactions of **I** and **II** in aqueous media are expected to be more complicated. For example, **IV** formed in the reaction can react with proton to generate protonated flavin semiquinone cation (5-EtFlH$^+$) and other product(s).[8] Furthermore, when **IV** was formed in benzene and then reacted as a 1-electron acceptor with dibenzoylperoxide, chemiluminescence was observed (unpublished results).This chemiluminescence, however, could be nullified by the addition of small amounts of water, suggesting an instability of **IV** in aqueous solution at room temperature. In order to advance our chemical knowledge of 4a-derivatized flavins and to better correlate the information of model studies with flavo-monooxygenase catalysis, it is important to examine the chemistry of the

‡ On sabbatical leave from September 1986 until September 1987. Permanent address: 34 de Ruyterstraat, 2266 KZ LEIDSCHENDAM, The Netherlands.



Scheme 1

flavinium cation **I** and the corresponding 4a-hydroxyflavin pseudobase **II** (X = OH) in aqueous media.

RESULTS AND DISCUSSION

In agreement with the literature,[13] a rapid equilibrium between **I** and **II** (X = OH) was found to exist in H_2O:

$$5\text{-EtFl}^{+}_{ox} + H_2O \rightleftharpoons 5\text{-EtFl-4}^{a}\text{-OH} + H^{+} \quad (5)$$

When the flavinium salt and the 4a-hydroxyflavin pseudobase were each dissolved in an aqueous solution at a given pH, the final solutions exhibited identical spectra. The effect of pH on spectra is illustrated in Fig. 1. At pH 7.00 (curve **a**), the equilibrium was shifted to the right forming 5-EtFl-4a-OH essentially as the sole flavin species. In contrast, a practically complete shift of the equilibrium to 5-EtFl^{+}_{ox} was obtained in strongly acidic solution (curve **b**). At pH 3.00 to 7.00, both flavin species appeared. In 0.1 M citrate, pH 4.15 (= apparent pK'_a), both species existed in a 1:1 ratio (curve **c**).

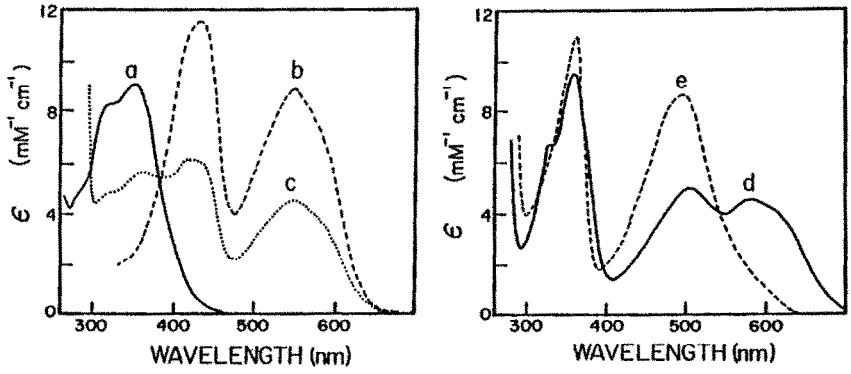


Fig. 1 (Left). Absorption spectra of (**a**) 5-EtFl-4a-OH in 0.1 M phosphate buffer, pH = 7.00; (**b**) 5-EtFl^{+}_{ox} in 0.2 N HCl; (**c**) 5-EtFl-4a-OH / 5-EtFl^{+}_{ox} (1:1) in 0.1 M citrate buffer, pH = 4.15.

Fig. 2 (Right). Absorption spectra of (**d**) 5-EtFl· in 0.1 M phosphate buffer, pH = 7.00; (**e**) $5\text{-EtFlH}^{\ddagger}$ in 0.2 N HCl.

Fig. 2 shows the reference spectra of the flavosemiquinone **III** at pH 7.00 (curve **d**) and the protonated form of **III** ($5\text{-EtFlH}^{\ddagger}$; curve **e**) in a strongly acidic solution. New convenient methods were developed to obtain these reference solutions. 5-EtFl-4a-OH or a mixture of this and 5-EtFl^{+}_{ox} (cf. curve **c**) can be rapidly converted to 5-EtFl· (cf. curve **d**) using hydroxylamine as an electron donor. Subsequent acidification changed curve **d** to curve **e**. Alternatively, 5-EtFl^{+}_{ox} in 0.2 N HCl (cf. curve **b**) was readily reduced to $5\text{-EtFlH}^{\ddagger}$ (curve **e**) using sodium sulfite. Reoxidation of $5\text{-EtFlH}^{\ddagger}$ to 5-EtFl^{+}_{ox} in

0.1-0.2 N HCl (curve **e**→**b**) can also be easily accomplished by adding $NaNO_2$. These methods were routinely used for the determination of concentrations or molecular extinctions of the radical preparations.

All the experiments described in this paper, including the spectral measurements, were carried out at 23°C under strictly anaerobic conditions. The aqueous solutions described in Fig. 1 showed *spontaneous* productions of the flavosemiquinone in the dark. These reactions were indicated by gradual spectral changes finally leading to spectra identical with either the curve **d** or **e** (Fig. 2) or **d** superimposed upon **e** depending upon the pH of the sample solutions. No accumulation of flavin radical cation **IV** was found. Starting from solutions at pH 7.00 and in 0.2 N HCl (cf. curves **a** and **b**, Fig. 1) the changes occurred very slowly, taking 4 and 8 days, respectively, for a practically complete disappearance of the starting compound. However, on adjusting the pH of the reaction mixture to a value between pH 3.00 to 5.00 the time required for the flavosemiquinone accumulation was reduced to a couple of hours. We believe that these flavin radical formation reactions can be described by Scheme 1 (X = OH). The present finding on the flavin radical formation in aqueous solutions enables us to formulate a quantitative kinetic test for the proposed mechanism as discussed below.

In H_2O, the electron transfer (Eq. 4; X = OH) is much slower in comparison with the equilibrium (Eq. 5) and the decompositions of **IV**. For example, the rate constant for the electron transfer, as measured by the appearance of UV-absorption of 5-EtFl•, was found to be 935 M^{-1} min^{-1} at pH 3.52. When the equilibrium (Eq. 5) was shifted either to the right or the left, one of the two reactants for Eq. 4 would be present in a trace amount leading to a very slow radical formation. Consequently, increases of the reaction rate, with a maximum at the apparent pK'_a, can be expected if the pH of the reaction was adjusted to be closer to the pK'_a.

On defining: R = [5-EtFl-4^a-OH] / [5-EtFl$^+_{ox}$] and [Fl_{tot}] = [5-EtFl$^+_{ox}$] + [5-EtFl-4^a-OH] at time = t, the rate of the disappearance of 5-EtFl$^+_{ox}$ (Eq. 6) can be represented by Eq. 7:

$$-d[5\text{-EtFl}^+_{ox}] / dt = k\cdot[5\text{-EtFl}^+_{ox}]\cdot[5\text{-EtFl-4}^a\text{-OH}] \quad (6)$$

$$-d[5\text{-EtFl}^+_{ox}] / dt = k\cdot[Fl_{tot}]^2\cdot[R / (1+R)^2] \quad (7)$$

The initial rate of the disappearance of 5-EtFl$^+_{ox}$ (and, similarly, of 5-EtFl-4^a-OH) is proportional to $R/(1+R)^2$. Based on the apparent equilibrium constant K'= [H$^+$]•[5-EtFl-4^a-OH] / [5-EtFl$^+_{ox}$] (Eq. 5) and using the value determined for the pK'_a (= 4.15) in 0.1 M citrate buffer, a theoretical pH-profile was calculated (Fig. 3) showing the relative initial rate ($V_{pH}/V_{pK'a}$) versus the pH with a maximal ratio of 1 at pH = pK'_a. The excellent agreement of the observed values with the theoretical pH-profile (Fig. 3) provides unambiguous evidence for the mechanism proposed for the process (Eq. 4).

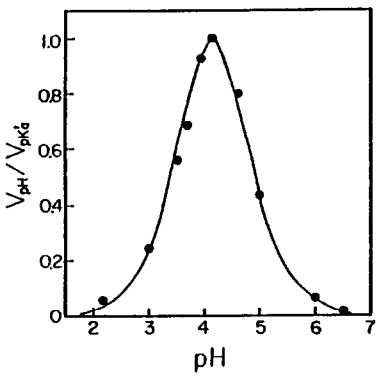


Fig. 3.: Effect of pH on the relative initial rate ($V_{pH}/V_{pk'a}$) of one-electron transfer (Eq. 4; X = OH) in aqueous solution. Theoretical profile, —; observed values, ●.

The yields of flavin products obtained after the spontaneous electron transfer (Eq. 4) had taken place at different pH's are summarized in Table I. Column 3 shows the total production of 5-EtFl• plus 5-EtFlH$^+$. In 0.1 M citric acid, a pK'_a = 2.55 was found for the equilibrium as shown in Eq. 8. Subsequent acidification of the reaction mixtures produced additional

$$5\text{-EtFlH}^+ \rightleftharpoons 5\text{-EtFl}\cdot + H^+ \quad (8)$$

5-EtFlH$^+$ in an amount corresponding exactly with the amount of 5-EtFl• present before acidification; no other effects were found. This proved that no accumulation of **IV** had occurred in aqueous solution at room temperature, confirming the indication from preliminary studies on the instability of **IV** in H_2O.

The nature of the decomposition of **IV** requires further classification: (**1**) As appeared from Table I, at pH > 5, yields of 5-EtFl• plus 5-EtFlH$^+$ did not exceed the theoretical value of 50% (Eq. 4). Under such conditions, decompositions of **IV** have mainly led to ring-contracted (non-flavin) products, identical to the ones described previously.[14] (**2**) At pH < 5, the formation of non-flavin derivatives decreased while the accumulation of 5-EtFl• plus 5-EtFlH$^+$ increased to 60-95% yields. Apparently conversions of **IV** to form additional **III** had occurred. Reactions that might be responsible for such conversions

are shown by Eqs. 9-13. In cases of Eqs. (9 + 10) and Eq. 11, these reaction(s) together with the reactions shown in Scheme 1 can form a cyclic process for the conversion of **IV** to **III**.

Reaction medium	pH	5-EtFl• and/or 5-EtFlH‡• (%)	3-Me-lumi-flavin (%)	Total flavin recovery (%)
0.1 M citrate	6.00	40	2	42
0.1 M citrate	5.00	52	2	54
0.1 M citrate	4.64	60	2	62
0.1 M citrate	4.15	76	4	80
0.1 M citrate	3.95	81	4	85
0.1 M citrate	3.70	85	4	89
0.1 M citrate	3.52	94	5	99
0.1 M citrate	3.00	86	5	91
0.1 M citric acid	2.15	95	6	101
0.1 M phosphate	7.00	29	1	30
0.1 M acetate	4.30	53	2	55
0.1 N acetic acid		75	6	81
0.2 N HCl		76		

Table I: Effects of the buffer type and pH of the reaction medium on the yields of flavin products generated by the spontaneous electron transfer (Eq. 4) at 23° in the dark.

$$(5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} \longrightarrow (5\text{-EtFl-}4^{a}\text{-OH})^{++} + e^{-} \quad (9)$$

$$(5\text{-EtFl-}4^{a}\text{-OH})^{++} \longrightarrow 5\text{-EtFl}^{+}_{ox} + H^{+} + [O] \quad (10)$$

$$(5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} \longrightarrow 5\text{-EtFl}^{+}_{ox} + [HO\bullet] \quad (11)$$

$$(5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} \xrightarrow{(H^{+})} 5\text{-EtFl}\bullet + H^{+} + [O] \quad (12)$$

$$(5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} + H_2O \longrightarrow 5\text{-EtFl}\bullet + H^{+} + H_2O_2 \quad (13)$$

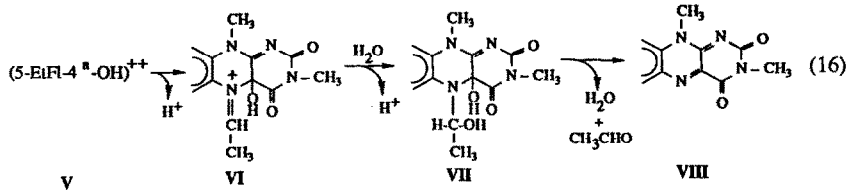
No accumulation of O_2 or H_2O_2 was detected during or at the end of the radical formation process performed in aqueous solution in the pH 2-7 range. A sensitive bioluminescent assay for O_2 was applied based on the light emission to be obtained on introducing O_2-containing samples to an anaerobic suspension of *Vibrio harveyi* cells. Considering the high sensitivity of this assay (1 nmol of O_2 per sample can be easily detected) and the slow O_2-consumption by 5-EtFlH‡•, particularly at lower pH, any O_2 formed in a decomposition of **IV** (X = OH) as given by Eq. 12 would not have escaped this detection. The absence of H_2O_2 was also indicated by applying a luminol/hemoglobin chemiluminescent assay. Moreover, no effects were found when parallel experiments in 0.1 M citrate buffers were carried out in the presence of the enzyme catalase.

It is concluded that reactions shown as Eqs. 12 and 13 are insignificant in aqueous solution at pH 2-7. Consequently, the main decomposition of **IV** in H_2O is believed to result in the formation of 5-EtFl$^{+}_{ox}$ according to Eqs. 9 + 10 or Eq. 11. This is fully consistent with the chemistry in acetonitrile as recently revealed from electrochemical investigations.[11] In earlier chemical studies,[7,9] the one-electron oxidation of **IV** (Eq. 9) has been formulated to proceed through a disproportionation (Eq. 14). On the same level of oxidation, a secondary intermolecular one-electron transfer (Eq. 15) might also occur.

$$2\ (5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} \longrightarrow 5\text{-EtFl-}4^{a}\text{-OH} + (5\text{-EtFl-}4^{a}\text{-OH})^{++} \quad (14)$$

$$(5\text{-EtFl-}4^{a}\text{-OH})^{\ddagger\bullet} + 5\text{-EtFl}^{+}_{ox} \longrightarrow (5\text{-EtFl-}4^{a}\text{-OH})^{++} + 5\text{-EtFl}\bullet \quad (15)$$

Apart from decomposition according to Eq. 10, N^5-dealkylation of the flavin di-cation transient **V** to form 3-methyl-lumiflavin **VIII** (Eq. 16, Scheme 2) should also be considered. As based on the sum of Eqs. 4 + 14 (or 15) + 16, a complete decomposition according to Scheme 2 would result in yields of 66.7% for 5-EtFl• and 33.3% for 3-methyllumiflavin. However, from the results mentioned in Table I, the N^5-dealkylation process (Scheme 2) has proven to be a minor pathway.



(16)

Scheme 2

Since no generation of O_2 was found, it is now assumed that the main decomposition of **IV** to **I** is coupled with the formation of oxygen species on the level of hydroxyl radical (Eq. 11). This could also account for the effects on the flavin radical accumulation shown by different buffers and solvents. For example: the yields of **III** in citrate buffers at pH 4.15 and 4.64 were higher than the yield in acetate buffer at pH 4.30 (cf. Table I); a reasonable stability of **IV** was observed in

benzene[8,10] whereas **IV** was unstable in chloroform or acetonitrile. The reversal of Eq. 11, i.e. the addition of a free radical to **I** leading to a flavin radical on the level of **IV**, will be described in a subsequent paper.

Concerning the spontaneous formation of flavin radicals, attention was also given to an alternative mechanism (Eq. 17) formulated in an analogy with an earlierly proposed mechanism for the conversion of 5-methylated flavins.[15] According to Eq. 17, solvolysis of the starting compound might give the N^5-dealkylated dihydroflavin (FlH_2) which would subsequently reduce two molecules of the N^5-alkylated flavinium cation to give a theoretical yield of 33.3% for Fl_{ox} (= 3-methyllumiflavin) and 66.7% for 5-EtFl•. However, our findings both on the kinetics (Fig. 3) and the flavin material balance (Table I) excluded this possibility.

$$5\text{-EtFl}^+_{ox} \underset{H^+}{\overset{H_2O}{\rightleftharpoons}} 5\text{-}(CH_3CHOH)\text{-Fl-1-H} \underset{CH_3CHO}{\rightleftharpoons} FlH_2 \xrightarrow{2\ (5\text{-EtFl}^+_{ox})} Fl_{ox} + 2H^+ + 2\ (5\text{-EtFl}\bullet) \quad (17)$$

CONCLUSION

A rapid equilibrium between the flavinium cation **I** and the derived 4^a-hydroxyflavin pseudobase **II** (X = OH) in aqueous solution and the subsequent slow intermolecular one-electron transfer to generate the flavin radicals **III** and **IV** (Eq. 4) have been established. Structurally, the flavin radical cation **IV** is closely related to a postulated key-intermediate in bacterial bioluminescence. It was found that **IV** was rather unstable in aqueous media. The main decomposition of **IV** has been proposed to lead to the formation of **I** and HO• radicals (Eq. 11).

EXPERIMENTAL

Materials and methods

Citric acid, trisodium citrate, $(NH_2OH)_2{\cdot}H_2SO_4$, $Na_2S_2O_4$, Na_2SO_3, MeCN, acetic acid, and conc. HCl were reagent grade products from Fisher. Citrate buffers (0.1 M) were made by mixing 0.1 M citric acid with 0.1 M trisodium citrate.

5-Ethyl-3-methyllumiflavinium perchlorate (I, ClO^-_4) and 5-ethyl-4^a-hydroxy-3-methyl-4^a,5-dihydrolumiflavin (II, X = OH) were prepared as described in the literature.[9,13] A fresh solution of 5-EtFl-4^a-OH in 0.1 M phosphate buffer pH 7.00 (curve **a**; Fig. 1) showed the following λ_{max} in nm (ε in $M^{-1}cm^{-1}$): 348 (9500); 316^{sh} (8800); 286^{sh} (5400); 222 (37000). A fresh solution of 5-EtFl^+_{ox} in 0.2 N HCl (curve **b**; Fig. 1) gave the following λ_{max} in nm (ε in $M^{-1}cm^{-1}$): 545 (9000); 430 (11800); 282 (49000); 222 (37000). Fresh stock solutions of **I**,ClO^-_4 or **II** (X = OH) in MeCN were used. The concentrations of the stock solutions (4.00 - 5.00 x 10^{-3} M) were determined by adding 30-µl samples to 3.00 ml of 0.2 N HCl and measuring the absorbance at 545 nm (ε = 9000 $M^{-1}cm^{-1}$). In general, the stock solutions of **I**,ClO^-_4 or **II** (X = OH) were injected to deaerated buffers in amounts giving final concentrations in the range of 3.00 - 5.00 x 10^{-5} M.

Nitrogen was purified over a BASF R3-11 catalyst. Anaerobic experimentts were carried out by using a special apparatus made in this laboratory. It consisted of a 10-mm quartz cuvette fused to one or two separate compartments, provided with rotaflo valves, allowing the reactants to be mixed at any moment. In general, the buffer (6-10 ml) was first introduced into the apparatus after which N_2 was flushed through for 3-24 h. Stock solutions of the flavins and the reactants were injected and all outlets of the apparatus were immediately closed. The volumes of the reaction mixtures were derived from their net weights. Spontaneous accumulation of 5-EtFl• and/or $5\text{-EtFlH}^{\cdot}$ occurred on keeping the anaerobic reaction mixtures at 23°C in the dark.

UV- and fluorescence-spectra were recorded using a Perkin-Elmer 553 absorption spectrophotometer and a Perkin Elmer MPF-44A fluorescence spectrophotometer, respectively.

Kinetic measurements of the decrease of **I** and **II** (X = OH) were taken immediately after the addition of the starting compound by monitoring changes in absorption at λ_{max} = 545 and 348 nm, respectively. No irradiation effects were observed during the time scans as indicated from comparisons with reaction mixtures kept in darkness. The yields of the flavosemiquinone accumulation were derived from the absorbances at the characteristic maxima. Subsequently, results were checked by adding conc. HCl to a final concentration of 0.1-0.2 N HCl to generate the protonated radical ($5\text{-EtFlH}^{\cdot}$) and, finally, by adding $NaNO_2$ to reoxidize $5\text{-EtFlH}^{\cdot}$ to 5-EtFl^+_{ox}. These determinations gave very consistent results.

The 3-methyllumiflavin formation was determined by subjecting the reaction mixtures (before the acidification mentioned above) to fluorometric analysis ($\lambda_{excitation}$= 466 nm; $\lambda_{emission}$= 520 nm) and relating the results to a calibration curve given by the authentic compound. In strongly acidic solutions, the presence of $5\text{-EtFlH}^{\cdot}$ or 5-EtFl^+_{ox} may affect the analysis.

The pK' of 5-EtFl^+_{ox} (= 4.15; Eq. 5) was derived from the absorbances shown by mixtures of 5-EtFl^+_{ox} and 5-EtFl-4^a-OH at 545 nm in 0.1 M citrate buffers at different pH's and from the increase of the absorbances at 545 nm obtained on adding conc. HCl to a final concentration of 0.1-0.2 N HCl.

The pK'_a for the protonated flavosemiquinone (= 2.55; Eq. 8) was calculated from the absorbance at 492 nm, shown by a mixture of the neutral and protonated radical species in 0.1 M citric acid and from the total absorbance following the addition

of conc. HCl to a final concentration of 0.1-0.2 N HCl. $[5\text{-EtFl}^{\bullet}] = \Delta A_{492}/\Delta\varepsilon_{492}$ (ΔA_{492} = increase of the absorbance at 492 nm following acidification; $\Delta\varepsilon_{492} = \varepsilon_{5\text{-EtFlH}^{\ddagger}} - \varepsilon_{5\text{-EtFl}^{\bullet}} = 3550\ M^{-1}cm^{-1}$ at 492 nm).

Reference spectra of 5-EtFl$^{\bullet}$ and 5-EtFlH‡ (Fig. 2) were obtained as described below. A deaerated 0.3 M solution of NH_2OH in 0.1 M phosphate buffer pH 7.00 (25 µl) was injected into an anaerobic 3.00 - 3.50 x 10^{-5} M solution of 5-EtFl-4a-OH in 0.1 M phosphate buffer pH 7.00 (6.00 ml; cf. curve **a**, Fig. 1) immediately giving a solution of 5-EtFl$^{\bullet}$ (cf. curve **d**, Fig. 2). This sample showed the following λ_{max} in nm (ε in $M^{-1}cm^{-1}$): 576 (4600); 501 (5100); 356 (9800); 327 (7100). Addition of conc. HCl (200 µl) immediately gave 5-EtFlH‡ (cf. curve **e**, Fig. 2) with λ_{max} in nm (ε in $M^{-1}cm^{-1}$): 492 (8800); 358 (11000). The molecular extinction coefficients were calculated from the recovery of $5\text{-EtFl}^{+}{}_{ox}$ obtained on reoxidation of 5-EtFlH‡ by $NaNO_2$. A reference solution of 5-EtFlH‡ (curve **e**, Fig. 2) was also obtained by adding 100 µl of 0.3 M of Na_2SO_3 in deaerated H_2O to a 5.00 x 10^{-5} M anaerobic solution of $5\text{-EtFl}^{+}{}_{ox}$ in 0.2 N HCl (6.00 ml; cf. curve **b**, Fig. 1). The reaction required about 25 min at 23°C to complete.

H_2O_2 determinations were carried out using a luminol chemiluminescence method.[16] A luminol working solution was freshly prepared daily by adding 1 part of the stock solution (4 mg luminol in 200 ml 0.01 N NaOH) to 10 parts of buffer (0.1 M glycine, 0.1 M NaCl, pH 11.0). A hemoglobin solution in water was prepared by adjusting the absorbance at 414 nm to 0.7. A 10-µl aliquot of the hemoglobin solution was mixed with 1 ml of luminol working solution. To this, 1-20 µl of a H_2O_2 standard or sample was added and the peak light intensity of chemiluminescence was recorded. In a typical run, 10 µl of 10^{-5} M H_2O_2 added to an assay solution produced a chemiluminescence peak intensity of 36 x 10^9 q/s. A linear response was obtained for up to at least 20 µl of 10^{-5} M H_2O_2 addition. The addition of 20 µl of flavin samples (total flavin concentration > 2 x 10^{-4} M) to the assay solution gave no light. However, a subsequent addition of H_2O_2 standard to the same assay solution produced more light than solutions containing the same amount of H_2O_2 but without the flavin sample. In conclusion, the flavin samples contained no H_2O_2 but somehow could enhance the light production by H_2O_2.

Determination of O_2 concentrations was achieved by the use of luminous *Vibrio harveyi* cells. *V. harveyi* cells were grown to the late log phase in a nutrient medium.[17] The cell suspension was transferred, by using a syringe, to a vial (20.6 ml total volume; 2.5 cm diameter; ca 4 cm height) with a long narrow neck (5 cm length; ca 1.5 mm diameter for opening; 7 mm outer diameter) and kept at room temperature. The O_2 in the medium was exhausted by the cellular metabolism in < 1 min, as judged by the lack of bioluminescence of the cell suspension. Using a Hamilton gas-tight syringe with a 4-inch length needle, 5- to 50-µl aliquots of air saturated H_2O ($[O_2] = 2.5 \times 10^{-4}$ M at 23°C) were injected. The peak bioluminescence intensities following each injection were recorded for the construction of a standard curve.

For the preparation of flavin sample solutions, a vial (covered with a rubber cap) containing 2.6 ml of 0.1 M citrate buffer (cf. Table I) was bubbled with N_2 for about 1h to remove O_2. Then, 60 - 100 µl of control MeCN or MeCN containing 1.2 - 2.1 x 10^{-2} M 5-EtFl-4a-OH were added by a gas-tight Hamilton syringe. Immediately, several other gas-tight syringes (100 µl; 4-inch length needles) were inserted through the rubber cap. Each was rapidly washed with the solution under N_2 for > 6 times, then filled to the 70 µl mark and kept in the dark with the tip of each needle kept immersed in the solution remaining in the vial for the reaction time required while the N_2-bubbling was continued. For the analysis, the solution in each syringe was pushed out a little until 50 µl was left, the syringe was inserted into the measuring cell suspension, the 50-µl content injected and light production measured. The same procedure was carried out with anaerobic and aerobic control solutions. No O_2 formation was detected in flavin containing reaction solutions.

ACKNOWLEDGEMENT

This work was supported by a grant from the Texas Advanced Technology Research Program to S.-C.T. through the University of Houston, and by grants E-1030 from the Robert A. Welch Foundation and GM25953 from NIH.

REFERENCES

1. Hastings, J. W., Balny, C., LePeuch, C., and Douzou, P. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3468-3472.
2. Massey, V., and Hemmerich, P. (1975) *Enzymes* (3rd Ed.) *12*, 191-252.
3. Kurfürst, M., Ghisla, S., and Hastings, J. W. (1984) *Proc. Natl. Acad. Sci. USA 81*, 2990-2994.
4. Ballou, D. P. (1984)) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., and Mayhew, S. G., Eds.) pp 605-618, de Gruyter, Berlin.
5. Bruice, T. C. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., and Mayhew, S. G., Eds.) pp 45-55, de Gruyter, Berlin.
6. Tu, S.-C. (1986) *Methods Enzymol. 133*, 128-139.
7. Addink, R. and Mager, H. I. X. (1984) in *Flavins and Flavoproteins* (Bray, R.C., Engel, P.C., and Mayhew, S.G., Eds.), pp 29-32, de Gruyter, Berlin.
8. Mager, H. I. X., and Addink, R. (1984) in *Flavins and Flavoproteins* (Bray, R. C. , Engel, P. C., and Mayhew, S.G., Eds.), pp 37-40, de Gruyter, Berlin.
9. Mager, H.I.X. and Addink, R. (1985) *Tetrahedron 41*, 183-190.
10. Mager, H. I. X. and Tu, S.-C. (1988) in *Flavins and Flavoproteins* (Edmondson, E. E. and McCormick, D.B., Eds.), pp 583-592, de Gruyter, Berlin.
11. Mager, H. I. X., Sazou,D., Liu, Y. H., Tu, S.-C., and Kadish, K. M. (1988) *J. Am. Chem. Soc.* (in press).
12. Schuster, G.B. (1979) *Acc. Chem. Res. 12*, 366-373.
13. Ghisla, S., Hartmann, U., Hemmerich, P., and Müller, F. (1973) *Ann. Chem.* 1388-1415.
14. Mager, H.I.X.(1979) *Tetrahedron Letters*, 3549-3552.
15. Kemal, C. and Bruice, T.C.(1976) *J. Am. Chem. Soc. 98*, 3955-3964.
16. White, E. H., and Brundrett, R. B. (1973) in *Chemiluminescence and Bioluminescence* (Cormier, M. J., Hercules, D. M., and Lee, J., Eds.), pp 231-244, Plenum, New York.
17. Nealson, K. (1978) *Methods Enzymol. 57*, 153-166.