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Vincent Sichula
Ying Hu
Ekaterina Mirzakulova
Samuel F. Manzer
Shubham Vyas

See next page for additional authors

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Author(s)
Vincent Sichula, Ying Hu, Ekaterina Mirzakulova, Samuel F. Manzer, Shubham Vyas, Christopher M. Hadad, and Ksenija D. Glusac

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Mechanism of N(5)-Ethyl-flavinium Cation Formation Upon Electrochemical Oxidation of N(5)-Ethyl-4a-hydroxyflavin Pseudobase

Vincent Sichula, Ying Hu, Ekaterina Mirzakulova, Samuel F. Manzer, Shubham Vyas, Christopher M. Hadad, and Ksenija D. Glusac*

Department of Chemistry, Bowling Green State University, Bowling Green, Ohio 43403, and Department of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

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We investigated the oxidation behavior of 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin (pseudobase Et-FlOH) in acetonitrile with the aim of determining if the two-electron oxidized Et-FlOH2+ undergoes a release of hydroxyl cation and the production of 5-ethyl-3methyllumiflavinium cation (Et-Fl+). The focus of this work is to investigate the possibility of using Et-FlOH as a catalyst for water oxidation. The cyclic voltammetry demonstrates that Et-FlOH exhibits two one-electron oxidation potentials at +0.95 and +1.4 V versus normal hydrogen electrode (NHE), with the second oxidation potential being irreversible. The production of Et-Fl+ is observed in the cyclic voltammetry of Et-FlOH and has been previously assigned to the release of OH· from the two-electron oxidized Et-FlOH2+. The results of our study show that this is not the case: (i) we performed bulk electrolysis of the electrolyte solution at +2 V and then added Et-FlOH to the electrolyzed solution. We found that Et-Fl+ is produced from this solution, even though Et-FlOH itself was not oxidized; (ii) reactions of Et-FlOH with chemical oxidants (ceric ammonium nitrate, nitrilosyl tetrafluoroborate, and tetrabutyllumonium persulfate) demonstrate that Et-Fl+ production occurs only in the presence of strong Lewis acids, such as Ce4+ and NO2+ ions. On the basis of these results, we propose that the production of Et-Fl+ in the electrochemistry of Et-FlOH occurs because of the shift in the Et-FlOH/Et-Fl+ acid–base equilibrium in the presence of protons released during anodic oxidation. We identified two sources of protons: (i) oxidation of traces of water present in the acetonitrile releases oxygen and protons and (ii) two-electron oxidized Et-FlOH2+ releases protons located on the N(5)-alkyl chain. The release of protons from Et-FlOH2+ was confirmed by cyclic voltammetry of Et-FlOH in the presence of pyridine as a base. The first oxidation peak of Et-FlOH at +0.95 V is reversible in the absence of pyridine. The addition of pyridine leads to the shift of the oxidation potential to a less positive value, which is consistent with a proton-coupled electron transfer (PCET). Furthermore, the anodic current increases, and the cathodic peak becomes irreversible, giving rise to two additional reduction peaks at −0.2 and −1 V. The same reduction peaks were observed in the high scan rate cyclic voltammogram of Et-FlOH in the absence of pyridine, implying that the release of protons indeed occurs from Et-FlOH2+. To determine which functional group of Et-FlOH2+ is the source of protons, we performed DFT calculations at the B3LYP/6-311++G** level of theory for a reaction of Et-FlOH2+ with pyridine and identified two proton sources: (i) the >N–CH2– group of the N(5) alkyl chain and (ii) the –OH group in the 4a-position of the radical cation. Because the appearance of new reduction peaks at −0.2 and −1.0 V occurs in the model compound that lacks –OH protons (Et-FIOMe), we conclude that the proton removal occurs predominantly from the >N–CH2– moiety.

Introduction

Flavin-dependent monooxygenases represent a class of flavoproteins that catalyze the incorporation of atomic oxygen into organic substrates. The catalysis involves a reaction between molecular oxygen and the reduced flavin cofactor to produce a 4a-hydroperoxy derivative. The flavin moiety of the hydroperoxide derivative polarizes the O–O bond, which enables the release of atomic oxygen from the flavin to the organic substrate. A special class of monooxygenases is bacterial luciferases, which cause bioluminescence. In the absence of the protein framework, the flavin cofactor reacts with oxygen to produce 4a-hydroperoxy derivative, but it does not act as a catalyst for insertion of atomic oxygen because of the fast release of hydrogen peroxide. However, N(5)-ethyl flavininium perchlorate (Et-Fl+, Figure 1) has been found to catalyze a range of oxidation reactions and has been used as a model system for mechanistic studies of bacterial luminescence.

Our interest in Et-Fl+ was stimulated by previous reports suggesting that it may perform catalytic water oxidation. Two previously reported experiments suggested that such catalysis could occur: (i) Et-Fl+ reacts with water to produce its pseudobase Et-FlOH23 and (ii) two-electron electrochemical oxidation of Et-FlOH leads to the production of Et-Fl+, possibly by releasing a hydroxyl cation.20 The first reaction can be readily monitored by UV/vis absorption spectroscopy, as illustrated in Figure 1a. The pKa value for the pseudobase formation is 3.6, which suggests that the reaction between Et-Fl+ and hydroxide anion is thermodynamically favored (ΔG ≈ −15 kcal/mol). Figure 1b illustrates the second reaction. The cyclic voltammogram of Et-Fl+ exhibits two reversible peaks at +0.17 and +0.79 V versus normal hydrogen electrode (NHE), with the second oxidation potential being irreversible.
Electrochemical Oxidation of a Flavin Pseudobase


Figure 1. (a) pH-dependent absorption spectra of Et-Fl⁺ in aqueous phosphate buffer (pH 0.7, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 5.7, 6.0, 6.5, and 7.0). (b) Cyclic voltammograms of Et-Fl⁺ and Et-FIOH in acetonitrile (supporting electrolyte: 0.1 M Bu4NClO4, working electrode: platinum, scan rate: 0.1 V/s). (c) Thermodynamic cycle demonstrating the possibility of the OH⁻ release from Et-FIOH²⁺.

−0.52 V versus normal hydrogen electrode (NHE), which correspond to the reduction of Et-Fl⁺ to produce semiquinone (Et-Fl⁻) and hydroquinone (Et-Fl⁻), respectively. The cyclic voltammogram of Et-FIOH consists of a reversible peak at $E_{1/2} = +0.95$ V due to one-electron oxidation of Et-FIOH and a second irreversible peak at $E = +1.4$ V that arises because of another one-electron oxidation to produce dication, Et-FIOH²⁺. When the voltage is scanned back to negative potentials, we observe a growth of peaks at +0.17 and −0.52 V that correspond to Et-Fl⁺. These results suggest that the two-electron oxidation of Et-FIOH leads to the recovery of Et-Fl⁺, possibly by creating a hydroxyl cation. As shown in Figure 1c, the release of OH⁻ from Et-FIOH leads to the recovery of Et-Fl⁺, through the release of OH⁻. When the voltage is scanned back to negative potentials, we observe a growth of peaks at +0.17 and −0.52 V that correspond to Et-Fl⁺.

These previously published results suggest that Et-Fl⁺ could be used to oxidize water catalytically, which would be an exciting avenue for the development of a fully organic water oxidation catalyst as an addition to the currently known organometallic catalysts. To investigate the oxidation behavior of Et-FIOH in more detail, we performed a series of electrochemical oxidation experiments on Et-FIOH and detailed supplemental density functional theory (DFT) calculations, which are presented in this article. These results suggest that the production of Et-Fl⁺ from oxidized Et-FIOH²⁺ does not involve a release of an OH⁻ cation, but instead a shift in the Et-FIOH/Et-Fl⁺ acid–base equilibrium due to the release of protons during the anodic oxidation of Et-FIOH.

Experimental Section

Syntheses. General Methods. All starting chemicals were purchased from Sigma-Aldrich chemicals and used as received. NMR spectra were recorded on a Bruker 300 MHz spectrometer. Electron impact ionization (EI) and MALDI mass spectra were measured on a Shimadzu QP5050A and a Brucker Daltonics Omnimass spectrometer. For reactions that required an inert atmosphere, argon of standard quality was used.

Neutral-4,5-dimethyl-2-nitrofluoracetanilide (1). Compound 1 was prepared according to the literature. Trifluoroacetic acid anhydride (12.6 g, 60 mmol) was added dropwise to a stirred solution of 1 (4.98 g, 30 mmol) in methylene chloride (60 mL) cooled in ice bath. Triethylamine (9 mL) was added dropwise to the vigorously stirred solution. The reaction mixture was warmed to room temperature and stirred for 1 h. The reaction mixture was transferred to a separatory funnel and washed with 2 M HCl, NaHCO₃, and brine. The aqueous layer was extracted with dichloromethane. Combined organic extracts were dried over anhydrous Na₂SO₄, filtered, evaporated, and dried over vacuum to give 7.58 g (96% yield) as a yellow solid.

N-Ethyl-4,5-dimethyl-2-nitrobenzenamine (2). Compound 3 was prepared by the modified literature procedure. Trifluoroacetic acid anhydride (12.6 g, 60 mmol) and K₂CO₃ (17.25 g, 125 mmol) in methylene chloride (60 mL) was added dropwise and refluxed overnight. DMF was vacuum-evaporated, and the residue was dissolved in water and extracted with dichloromethane. Combined organic extracts were dried over anhydrous Na₂SO₄, filtered, concentrated in vacuum to yield a yellow-red residue. The residue was subjected to flash chromatography using dichloromethane as an eluent to give 4.51 g (93% yield) as an orange solid. ¹H NMR (300 MHz, CDCl₃): 7.93 (s, 1H), 7.80 (s, 1H), 6.62 (s, 1H), 3.33 (m, 2H), 2.97 (s, 3H), 2.18 (s, 3H). EI-MS: m/z 262 (M⁺).
N1-Ethyl-4,5-dimethyl-1,2-benzenediamine (4). Compound 3 (0.04 g, 26 mmol) was dissolved in 150 mL of concentrated HCl in a two-necked round-bottomed flask and purged with Ar. Excess tin foil (15.53 g, 131 mmol) was added to the reaction mixture and heated at 100 °C for 3 h under an inert atmosphere. The flask was cooled in an ice—water bath. Sodium hydroxide solution (∼400 mL, 3.5 M) was added until the pH reached 10. This caused a white precipitation of SnO and the product. The reaction flask was heated to boil. The color of the precipitation changed to gray. The precipitate was filtered out and rinsed with 300 mL of boiling water. The filtrate was cooled to precipitate the reaction mixture was stirred overnight and then acidified with glacial acetic acid (pH 3 to 4). Ethanol was removed under reduced pressure, and an orange precipitate formed while the remaining watery phase was cooled. The precipitate was filtered, washed with dithionate-containing water, and dissolved in dithionate containing 5 M ammonia. The insoluble residue was filtered out, and the filtrate was acidified with glacial acetic acid. The resulting precipitate was collected, washed with dithionate-containing water, and dried under vacuum to give 0.270 g of orange compound (yield 85%). 1H NMR (300 MHz, CDCl3) overlaid with D2O/Na2S2O4): 6.68 (s, 1H), 6.44 (s, 1H), 3.75 (q, J = 8 Hz, 2H), 3.37 (q, J = 8 Hz, 2H), 3.28 (s, 3H), 2.14 (s, 3H), 2.10 (s, 3H), 1.28 (t, J = 8 Hz, 3H), 1.07 (t, J = 8 Hz, 3H).

5-Ethyl-3-methyl-lumiflavinium Perchlorate (Et-Fl+). Et-Fl+ was synthesized according to the published procedure.3 Compound 7 (0.314 g, 1 mmol) was dissolved in 10 mL of dithionate containing ammonia and filtered. The filtrate was added to 15 mL of 70% HClO4. Then, NaNO2 (0.50 g, 7 mmol) and NaClO4 (0.50 g, 4 mmol) were added to the reaction mixture. The reaction mixture was stirred at 10 °C for 2 h. The resulting purple precipitate was filtered and washed with water, MeOH, ether, and CHCl3 to yield 0.264 g (yield 75%) of the product. 1H NMR (CD3CN, 300 MHz): δ: 1.00 (3H, t, J = 6.9 Hz, CH3−CH2), 1.25 (3H, t, J = 6.8 Hz, CH2−CH3), 2.30 (3H, s, −CH3 arom.), 2.32 (3H, s, −CH3 arom.), 3.21 (3H, s, N−CH3), 3.43 (2H, q, J = 7 Hz, CH2−CH3), 4.45 (2H, q, J = 7.2 Hz, CH2−CH3), 7.09 (1H, s, C−H arom.), 7.20 (1H, s, C−H arom.). MALDI-MS: m/z 314 (M+).

5,10-Diethyl-4a,5-dihydro-4a-hydroxy-3,7,8-trimethylbenz[g]pteridine-2,4(3H,10H)-dione (Et-FlOH). Et-FlOH was prepared according to published procedure.25 Et-Fl+ (200 mg) was dissolved in 2 mL of water/acetonitrile mixture (1:10), and 10 mL of 0.1 M phosphate buffer at pH 7 was added to the solution. The mixture was stirred for 10 min, and the resulting light-yellow precipitate was filtered and crystallized from acetone/water. Et-FlOH was obtained in 63% yield (94 mg). 1H NMR (300 MHz, CDCl3): 7.06 (s, 2H), 4.37 (m, 2H), 3.40 (m, 5H), 2.91 (s, 1H), 2.31 (s, 3H), 1.27 (t, J = 8 Hz, 3H), 0.98 (t, J = 8 Hz, 3H). MALDI-MS: m/z 330 (M+).

5,10-Diethyl-4a,5-dihydro-4a-methoxy-3,7,8-trimethylbenz[g]pteridine-2,4(3H,10H)-dione (Et-FlOMe). Et-FlOMe was synthesized according to published procedure.37 Et-Fl+ (0.11 g, 2.7 mmol) was dissolved in 3 mL of methanol, and sodium metal (0.014 g, 6.1 mmol) was added. The reaction mixture was stirred for 30 min at room temperature. The precipitate was filtered off, washed with methanol to give 62 mg (yield 67%) as a light-yellow solid. 1H NMR (300 MHz, CDCl3): 7.19 (s, 1H), 7.12 (s, 1H), 4.31 (m, J = 3 Hz, 2H), 3.58 (m, J = 7.2 Hz, 2H), 3.26 (s, 3H), 3.04 (s, 3H), 1.30 (d, J = 6.9 Hz, 3H), 1.08 (t, J = 6.9 Hz, 3H). MALDI: m/e 344 (M+).

**Electrochemistry.** All electrochemical measurements were done in acetonitrile using tetrabutyl-ammonium perchlorate (TBAP) as a supporting electrolyte. Acetonitrile was purchased from Sigma-Aldrich (Anhydrous, 99.8%). The solvent was refluxed over CaH2 for 8 h and then distilled. Anhydrous pyridine was purchased from Sigma-Aldrich. Pyridine (20 mL) was left to stand overnight over 4 g of NaOH pellets. Pyridine was filtered off, refluxed over BaO for 4 h, and then distilled and stored over 4 Å molecular sieves. TBAP was purchased from Fluka, recrystallized from methanol, and dried under vacuum. Cyclic voltammetry and bulk electrolysis were done using an EC Epsilon potentiostat (Bioanalytical Systems). Cyclic voltammetry was performed in a cell (MF-1056, Bioanalytical Systems) equipped with reticulated vitreous...
The concentration of Et-FlOH was 10^{-5} M in all experiments.

Reactions of Et-FIOH with Chemical Oxidants. Chemical oxidation was performed in acetoneitrile with three oxidants: (i) ceric ammonium nitrate (CAN, 99.99%, Sigma-Aldrich), (ii) nitrosyl tetrafluoroborate (NO, 95%, Sigma-Aldrich), and (iii) TBAPS. The syntheses of TBAPS were achieved from tetrabutylammonium hydrogen sulfate (Sigma-Aldrich) and potassium persulfate (Acros Organics) according to the published procedure. The changes in the UV/vis absorption spectra were monitored using Agilent 8453 UV spectrometer in a 2 mm quartz cell. UV/vis absorption spectra were collected as follows: using freshly distilled acetoneitrile (black); 0.5 h after distillation (red); 4 h after distillation (blue).

Results and Discussion

Syntheses. The flavinium salt Et-Fl^{+} and its pseudobase Et-FIOH were synthesized according to Scheme 1. The first two reactions were conducted with a goal of converting 4,5-dimethyl-2-nitroaniline (1) to monoalkylated compound 3. To avoid dialkylation of the amine, compound 1 was initially reacted with trifluoroacetic anhydride to produce amide 2. Further alkylation of the amine 2 using ethyl iodide, followed by hydrolysis yielded monoalkylated compound 3 in excellent yield. A similar method was used previously to obtain a 2-methoxyethyl amino derivative. The subsequent reduction of nitro-group using Sn/HCl afforded bis-amine 4, which was found to be unstable in the presence of oxygen. To avoid large losses, compound 4 was immediately reacted with alloxane using a condensation method developed by Kuhn. The N3 position of product 5 was methylated using methyl iodide to afford model compound 6. The conversion of 6 to flavinium salt Et-Fl^{+} was achieved in a two-step reaction using a procedure developed by Müller. In the initial step, 6 was reduced to its hydroquinone form 7 using sodium dithionate, then ethylated using ethyl iodide to form EtFIH. Compound EtFIH reacts with oxygen to produce 4a-hydroperoxy derivative Et-FIOOH. To avoid product losses due to this reaction, we kept the product under an inert atmosphere and immediately reacted in the next step, which involved the oxidation using NaNO2 and HClO4 to produce the final product Et-Fl^{+}. The formation of Et-FIOH pseudobase from Et-Fl^{+} was achieved using acetoneitrile/water mixture at pH 7.

Electrochemistry. We performed bulk electrolysis of the electrolyte solution (0.1 M TBAP in acetoneitrile) at +2 V. After 10 min of electrolysis, we added a small amount of solid Et-FIOH to the solution and measured the UV/vis absorption spectrum. Figure 2a demonstrates the conversion of Et-FIOH (absorbs at 355 nm) to Et-Fl^{+} (absorbs at 420 and 550 nm), even if Et-FlOH itself was not oxidized. The amount of produced Et-Fl^{+} increases with the amount of water present in the electrolyte solution, which is evident from the spectra obtained at different time intervals after acetoneitrile distillation. These results suggest that the oxidation of water at +2 V leads to a release of molecular oxygen and solvated protons. The solvated protons decrease the pH of the solution and lead to the production of Et-Fl^{+}. Further evidence of the water oxidation can be seen from Figure 2b, which shows the cyclic voltammograms of Et-FIOH in acetoneitrile in the absence and presence of 4 Å molecular sieves. The amount of Et-Fl^{+} produced is smaller in the presence of molecular sieves, as judged by the current intensities at +0.17 and -0.52 V (reduction peaks of
Et-Fl\(^+\)). It is important to note that even small traces of water will cause a strong effect because the reaction of protons with Et-FIOH creates new equivalents of water that can repeat the cycle (inset scheme in Figure 2).

The pH decrease due to water oxidation could account for Et-Fl\(^+\) formation in the electrochemical oxidation of Et-FIOH. Figure 3 represents the effect of switching potential on the electrochemical behavior of Et-FIOH. As the switching potential is increased, more current is observed in return scans at potentials for Et-Fl\(^+\) at +0.17 and −0.52 V. Figure 3b shows the dependence of current at +0.09 V (Et-Fl\(^+\) reduction peak) as a function of switching potential. It can be seen that Et-Fl\(^+\) becomes produced once the switching potential exceeds the value of +1.2 V and increases for higher switching potentials. Figure 3c shows how the presence of water influences the cyclic voltammogram of 0.1 M TBAP in acetonitrile. We can see that water oxidation becomes significant at potentials above +1.2 V, the same potential at which Et-Fl\(^+\) becomes produced, suggesting that the water oxidation mechanism can account for the production of Et-Fl\(^+\) from Et-FIOH.

Another electrochemical process that could lead to a pH decrease is the oxidation of the electrolyte (TBAP). Perchlorate anion undergoes one-electron oxidation at +2.3 V,\(^{49}\) and the produced perchlorate radicals are known to abstract hydrogen atoms from acetonitrile to produce perchloric acid.\(^{50}\) To investigate the role of electrolyte, we obtained cyclic voltammograms of Et-FIOH at different concentrations of TBAP, and we plotted the resulting current at 0.09 V as a function of switching potential (Figure 4). The amount of Et-Fl\(^+\) produced remained unaffected by TBAP concentrations, and thus the pH decrease in aforementioned experiments is not due to TBAP oxidation.

**Reactions with Chemical Oxidants.** Further confirmation that Et-Fl\(^+\) is not produced as a result of the release of a hydroxyl cation from Et-FIOH\(^2+\) comes from the reactions of Et-FIOH with chemical oxidants (Figure 5). We investigated the reaction of Et-FIOH with three oxidants: (i) CAN, (ii) nitrosyl tetrafluoroborate (NOBF\(_4\)), and (iii) TBAPS. The progress of each reaction was monitored by UV/vis absorption spectroscopy (Figure 5). The production of Et-Fl\(^+\) (absorbs at 420 and 550 nm) was observed in the presence of CAN and NOBF\(_4\). The production of Et-Fl\(^+\) in these cases is instantaneous and almost quantitative: In the presence of NOBF\(_4\), Et-FIOH converts to Et-Fl\(^+\) with ∼100% efficiency. The effect of CAN on Et-FIOH is dual: it converts Et-FIOH to Et-Fl\(^+\); then, it reacts with Et-Fl\(^+\) (as confirmed by mixing Et-Fl\(^+\) and CAN and monitoring the changes in the UV/vis absorption, data not shown). Therefore, the smaller yield of Et-Fl\(^+\) produced in the reaction of CAN with Et-FIOH is explained by the decomposition of Et-Fl\(^+\) in the presence of CAN. On the contrary, the production of Et-Fl\(^+\) in the presence of persulfate ions was not observed. Instead, the oxidation leads to the slower increase in the oxidation product at 310 nm.

Table 1 lists the oxidation potentials of the compounds used in this study. The reported values for NOBF\(_4\) and CAN were obtained in acetonitrile,\(^{31,52}\) whereas the reported potential for TBAPS was obtained in water.\(^{53}\) The TBAPS potential might be different in acetonitrile, but it can be safely assumed that it remains higher than the two-electron potential of Et-FIOH. The potentials presented in Table 1 demonstrate that NOBF\(_4\) and TBAPS can be used to achieve the two-electron oxidation of Et-FIOH, whereas CAN is not a strong enough oxidant in acetonitrile to produce Et-FIOH\(^2+\). Even though persulfate ion is the strongest oxidizing agent in Table 1, the production of Et-Fl\(^+\) was not observed when Et-FIOH was treated with TBAPS. At the same time, the production of Et-Fl\(^+\) was almost quantitative in the presence of NOBF\(_4\), which barely has enough oxidizing power to perform a two-electron oxidation of Et-FIOH. Furthermore, the formation of Et-Fl\(^+\) was observed in the presence of CAN, which cannot perform a two-electron oxidation of Et-FIOH. This result led us to conclude that the production of Et-Fl\(^+\) is not associated with the oxidation of Et-FIOH. For example, the oxidation of Et-FIOH seems to occur only in the presence of TBAPS, and the oxidized product does not produce as a result of the release of a hydroxyl cation from Et-FIOH\(^2+\).
not exhibit any absorption at 420 and 550 nm, where Et-Fl+ absorbs. This result suggests that the oxidation of Et-FlOH leads to the product that absorbs at 310 nm. Instead, Et-Fl+ production was observed only in the presence of strong Lewis acids Ce4+ and NO+, suggesting that the reaction involves a transfer of OH− from Et-FlOH to ceric and nitrosyl cations. Table 1 lists the equilibrium constants for reaction of cations involved in this study with OH− ions. Even though the reported acid/base constants were measured in water, it is safe to assume a linear dependence of the equilibrium constants going from water to acetonitrile [pK\textsubscript{a}(CH\textsubscript{3}CN) = 5.2 ± 1.313pK\textsubscript{a}(H\textsubscript{2}O)]. From these data, we can see that the transfer of OH− from Et-FlOH to Ce4+ or NO+ is a thermodynamically favored process, whereas the transfer of OH− from Et-FlOH to Bu\textsubscript{4}N+ is not. These calculations fully support the mechanism in which the Et-Fl+ formation is associated with a Lewis-type acid–base reaction between Et-FlOH and Lewis acids Ce4+ and NO+.

**Effect of Pyridine.** The data presented so far demonstrate that the formation of Et-Fl+ occurs because of the reaction of Et-FlOH with Bronsted or Lewis acids present in the solution. We demonstrated that the anodic oxidation of water decreases the solution pH, leading to the production of Et-Fl+ from Et-FlOH. However, these results do not address the role of two-electron oxidized Et-FlOH2+ in Et-Fl+ formation. We postulated that Et-FlOH2+ could also contribute to the decrease in the solution pH by a reaction: Et-FlOH2+ → Et-FlO− + + H+. To investigate this possibility, we analyzed the electrochemical oxidation of Et-FlOH in the presence of pyridine as a base. As can be seen from Figure 6a, the reversible one-electron oxidation potential of Et-FlOH shifts slightly to the less positive value as the pyridine concentration is increased. We interpret this result in terms of proton-coupled electron transfer (PCET): One-electron oxidation of Et-FlOH leads to the formation of radical cation, Et-FlOH+. Because of its positive charge, Et-FlOH+
is acidic, and it loses a proton in the presence of pyridine to produce Et(-H)\textsuperscript{+} radical (Figure 6c). PCET generally occurs by either coupled CEP process (electron and proton are transferred in a single step) or consecutive ET/PT or PT/ET process. On the basis of the fact that the initial anodic peak of Et-FIOH does not shift significantly to the lower potential (Figure 6a), we postulate that the consecutive ET/PT mechanism takes place. The consecutive ET/PT process is also consistent with the thermodynamic cycle presented in Figure S3 of the Supporting Information. Previous pulse radiolysis experiments suggest that the loss of proton occurs upon one-electron oxidation of Et-FIOH.\textsuperscript{56} The pK\textsubscript{a} value of Et-FIOH\textsuperscript{+} was estimated to be 3.7, whereas the pK\textsubscript{a} value of Et-FIOH is \textasciitilde 10. The change in the pK\textsubscript{a} value upon oxidation is rather modest in comparison with systems in which the coupled CEP process occurs. (For example, pK\textsubscript{a} value of dihydroanthracene drops from +41 to \textasciitilde 15 upon oxidation.)\textsuperscript{29} Using this value, we estimated the \(\Delta G\) values for coupled CEP and consecutive ET/PT processes (Figure S3). The results show that \(\Delta G^\text{ET} < \Delta G^\text{EP}\). Assuming that the activation barriers depend linearly on \(\Delta G\), we can conclude that the consecutive mechanism is more likely to occur. The proton is most probably removed from the \(-\text{CH}_2\) moiety of the N(5)-ethyl group, as will be discussed in the following paragraph. In addition to the shift of the oxidation potential, we observed an increase in the anodic current at increasing pyridine concentrations. We assign this increase to an additional one-electron oxidation following initial PCET, which converts the neutral Et(-H)\textsuperscript{+} radical to Et(-H\textsuperscript{+})\textsuperscript{+} iminium ion (Figure 6c). Additional oxidation frequently accompanies initial PCET processes\textsuperscript{60-62} because charge neutralization achieved by proton removal facilitates the elimination of the second electron from neutral radical, in our case Et(-H\textsuperscript{+})\textsuperscript{+}. Therefore, the second oxidation peak of Et-FIOH moves from \textasciitilde +1.4 V in the absence of pyridine to \textasciitilde +0.95 V in the presence of base, and the overall oxidation process that occurs at +0.95 V in the presence of pyridine involves a net transfer of two electrons and a proton, producing an iminium ion, Et(-H\textsuperscript{+})\textsuperscript{+}FIOH\textsuperscript{+}, as illustrated in Figure 6c.

The remaining question is which proton is being removed from Et-FIOH\textsuperscript{+} radical cation. In the case of neutral Et-FIOH, the most acidic proton is located on the \(-\text{OH}\) group, with a pK\textsubscript{a} value of 9.2.\textsuperscript{10} Upon formation of Et-FIOH\textsuperscript{+}, it is reasonable to expect a pK\textsubscript{a} shift to a value lower than 9.2 and that the deprotonation would lead to the formation of Et-FIO\textsuperscript{+}. To determine if this is the case, we prepared 4\textalpha\textendash methoxyflavin derivative (Et-FIOMe) and investigated its oxidation behavior in the presence of pyridine. Because Et-FIOMe lacks acidic O\textendash H proton, we anticipated that the oxidation behavior of Et-FIOMe would remain unaffected by the presence of pyridine. To our surprise, we found that this is not the case. Figure 6b demonstrates that Et-FIOMe undergoes qualitatively the same PCET process as Et-FIOH, suggesting that the proton is not being removed the \(-\text{OH}\) moiety. Because both Et-FIOH and Et-FIOMe undergo a similar reaction with pyridine, we propose that the source of protons is not the \(-\text{OH}\) moiety but instead the N(5)-ethyl group. It is well known that amine radical ions are acidic and that they loose protons from \(\alpha\)-carbons.\textsuperscript{63,64} In the case of Et-FIOH\textsuperscript{+}, the positive charge is mostly localized on the N(5)-atom, making the protons located on neighboring \(-\text{CH}_2\)\textendash group the most probable candidates for deprotonation. This conclusion is further supported by previous studies on Et-FI\textsuperscript{+} chemistry,\textsuperscript{17} showing that Et-FI\textsuperscript{+} undergoes base-catalyzed decomposition to lumiflavin, the process being initiated by a loss of proton from the N(5)-\text{CH}_2\textendash group.

It is interesting to note that the effect of pyridine is stronger in the case of Et-FIOH, as can be seen by a more drastic decrease in the cathodic current at +0.95 V as a function of pyridine concentration (Figure 6c). These results suggest the possibility that the proton removal from Et-FIOH\textsuperscript{+} in the presence of pyridine occurs from either the \(-\text{OH}\) or the \(-\text{N}\textendash \text{CH}_2\)\textendash group, whereas the proton removal from Et-FIOMe occurs only from the \(-\text{N}\textendash \text{CH}_2\)\textendash group. To investigate the acidities of \(-\text{OH}\) and \(-\text{N}\textendash \text{CH}_2\)\textendash protons in Et-FIOH\textsuperscript{+}, we optimized the Et-FIOH\textsuperscript{+} structure using the DFT/B3LYP method and calculated the atomic charges using NPA analysis. The computed NPA charge densities indicated that the hydroxyl H-atom is the most positively charged H-atom in the molecule with a positive charge of 0.514, whereas the H-atoms on the two \(-\text{N}\textendash \text{CH}_2\) groups carry the charge density of 0.230. These results suggest that the acidic proton is localized on the \(-\text{OH}\) group of Et-FIOH\textsuperscript{+}.

To understand the effect of pyridine in the cyclic voltammetry of Et-FIOH, we computed the thermochemistry of the two reactions: (a) Et-FIOH\textsuperscript{+} + PYR \rightarrow Et(-H\textsuperscript{+})FIOH\textsuperscript{+} + PYRH\textsuperscript{+} and (b) Et-FIOH\textsuperscript{+} + PYR \rightarrow Et-FIO\textsuperscript{+} + PYRH\textsuperscript{+}. In the gas phase, both of the reactions are endothermic by 24 kcal/mol at the B3LYP/6-311+G**/B3LYP/6-31G* level of theory. However, when these two sets of calculations were carried using the PCM model (implicit solvation) of acetonitrile, both reactions became exothermic by 24 kcal/mol at the same level of theory. Therefore, the removal of proton from Et-FIOH\textsuperscript{+} can occur from either the \(-\text{OH}\) or the \(-\text{N}\textendash \text{CH}_2\)\textendash moiety. In the following text, we will consider only the removal from the \(-\text{N}\textendash \text{CH}_2\)\textendash group because this reaction explains the formation of electrochemically active products with reduction potentials at \textasciitilde 0.20 and \textasciitilde 1.0 V. (See the further text.)

So far, we have demonstrated that the one-electron oxidized Et-FIOH becomes acidic enough to lose protons in the presence of pyridine. In the absence of pyridine (pK\textsubscript{a} of pyridinium ion is 5.2),\textsuperscript{65} neutral Et-FIOH (pK\textsubscript{a} of the pseudobase is 3.6) can serve as a base. Because Et-FIOH is a weaker base, it cannot remove protons from one-electron oxidized Et-FIOH\textsuperscript{+} radical cation, as demonstrated by reversible one-electron oxidation peak in Figure 1b. However, Et-FIOH could abstract protons from more acidic Et-FIOH\textsuperscript{+}, which would explain the appearance of Et-FI\textsuperscript{+} during anodic oxidation of Et-FIOH. If this was the case, then the two-electron oxidation of Et-FIOH in the absence of pyridine would give rise to the same products as

![Figure 7](image)

Figure 7. (a) Cyclic voltammograms of Et-FIOMe in acetonitrile in the presence of varying concentrations of pyridine (0–3 mM). Supporting electrolyte: TBA\textsuperscript{+}, sweep rate: 0.1 V/s. (b) Variable scan rate, background-subtracted cyclic voltammograms of Et-FIOH in 0.1 M TBA\textsuperscript{+}. Sweep rates: 1 (black), 5 (red), and 10 V/s (blue).
one-electron oxidation of Et-FIOH in the presence of pyridine. The following experiments are aimed at comparing the two processes.

Figure 7a demonstrates that one-electron oxidation of Et-FIOMe leads to the appearance of two additional peaks in the cathodic scan: at −0.2 and −1.0 V. The same results were obtained in the case of Et-FIOH (data not shown). We assign the two peaks to decomposition products obtained from iminium ion, Et(-H+)-FIOMe+. It is interesting to note that the same two reduction peaks were obtained in variable scan-rate cyclic voltammograms of Et-FIOH in the absence of pyridine. Whereas we did not observe these peaks at 0.1 V/s scan rates, an increase in the scan rate from 0.1 to 25 V/s lead to the growth of peaks at −0.2 and −1.0 V. These results strongly suggest that the two-electron oxidation of Et-FIOH in the absence of pyridine leads to the same intermediates as one-electron oxidation of Et-FIOH/Et-FIOMe+ in the presence of pyridine. On the basis of these results, we propose the mechanism for Et-FI+ formation during anodic oxidation of Et-FIOH in Scheme 2. One-electron oxidation of Et-FIOH at +0.95 V forms Et-FIOH+ radical cation, which is stable in the absence of pyridine. Subsequent one-electron oxidation at ~+1.4 V creates a dication, Et-FIOH2+, which loses a proton to produce iminium ion Et(-H+)-FIOH+. The base responsible for proton abstraction is Et-FIOH, which leads to the formation of Et-FI+ and water.

The further decomposition of iminium ion Et(-H+)-FIOH+ is not clear. One possible pathway involves the cleavage of N-alkyl chain to produce methyl-lumiflavin (Me-Lf) and acetaldehyde. Me-Lf exhibits one-electron reduction peak at ~−1.0 V,66 which coincides with the peak we observe upon two-electron oxidation of Et-FIOH. To investigate this mechanism, we performed UV/vis spectropolarimetric measurements on Et-FIOH, and we found no evidence of Me-Lf formation, which exhibits strong absorption at 443 nm66 (Figure 8). The UV/vis spectra were obtained for two samples: (i) Et-FIOH in the presence of pyridine using +1.1 V of applied voltage and (ii) Et-FIOH in the absence of pyridine using a potential of +1.4 V. In the absence of pyridine, the disappearance of 350 nm absorption band that corresponds to Et-FIOH is concurrent with the growth of three absorption peaks in the visible range at 414, 485, and 554 nm (Figure 8d). The peak at 485 nm grows during the initial 45 s but disappears at longer times and is assigned to the absorption of Et-FIOH+.20 The bands at 414 and 554 nm are assigned to Et-FI+, whose visible absorption spectrum is shown in Figure 8f. The presence of Et-FIOH+ and Et-FI+ absorption bands is consistent with the mechanism proposed in the Scheme 2. The absorption band corresponding to Me-Lf at 443 nm was not observed, suggesting a different decomposition pathway for Et(-H+)-FIOH+ iminium ion. Because no additional bands were observed in the visible range, we conclude that the product of Et(-H+)-FIOH+ decomposition does not absorb in the visible range. Figure 8a shows the changes that occur in the UV range during electrolysis of Et-FIOH solution at +1.4 V. After 10 min of electrolysis, the absorption band of Et-FIOH at 350 nm fully disappears, and four new absorption bands appear at 300, 286, 222, and 194 nm. Absorption bands at 222 and 286 nm partially originate from Et-FI+, whereas the rest of the absorption bands are assigned to the absorption of the product(s) of Et(-H+)-FIOH+ decomposition. The fact that the product absorbs in the UV region (below 300 nm) suggests that the decomposition of Et(-H+)-FIOH+ does not lead to the Me-Lf formation but possibly to the cleavage of the isaloxazine ring. Figure 8b,e shows the spectral changes during +1.1 V electrolysis of Et-FIOH in the presence of pyridine. In this case, the absorption bands from Et-FI+ at 222, 286, 414, and 554 nm are much weaker. This is consistent with the fact that in the presence of pyridine Et-FIOH does not abstract protons from oxidized Et-FIOH to produce Et-FI+ and water. After 10 min
of electrolysis, the spectrum consists of weak absorption bands due to Et-Fl' and additional bands at 184, 256, and 300 nm. The band at 256 nm appears in the same spectral range as the pyridine absorption (Figure 8c), and it might arise because of the absorption of pyridinium ion formed during PCET. Again, the rest of the absorption bands are assigned to the absorption of Et(+H)-FIOH decomposition products. In is interesting to note that the absorption spectrum obtained upon chemical oxidation of Et-FIOH with persulfate ions exhibits absorption at 300 nm (Figure 5), possibly due to same oxidation products. Therefore, we conclude that the decomposition of Et(+H)-FIOH gives rise to the product with longest the wavelength absorption at 300 nm, suggesting a cleavage of the isoalloxazine ring.

Given the results presented in this manuscript, we conclude that the production of Et-Fl' during the anodic oxidation of Et-FIOH does not involve the formation of OH-, as previously hypothesized.20 Instead, we propose that the formation of Et-Fl' occurs via a shift in the Et-FIOH/ Et-Fl' acid–base equilibrium due to the formation of a strong Bronsted acid, Et-FIOHg+. Because this pathway does not lead to catalytic water oxidation, we are currently developing pseudobase model compounds that lack α H-atoms on the N-alkyl chain.

Conclusions

This article describes the oxidation behavior of Et-FIOH, a flavin-based pseudobase derived from N(5)-ethyl-flavinium perchlorate (Et-Fl'). Previous experiments involving cyclic voltammetry of Et-FIOH20 suggested that the two-electron oxidation of Et-FIOH produces a dication Et-FIOH2+ that releases hydroxyl cation and Et-Fl+. Because such a reaction could lead to the development of a purely organic water oxidation catalyst, we investigated the process in more detail. Our findings suggest that the release of hydroxyl cation does not occur in the electrolysis of Et-FIOH. Instead, we find that the production of Et-Fl' is associated with the shift in the Et-FIOH/ Et-Fl' acid–base equilibrium due to the formation of Bronsted and Lewis acids during anodic oxidation of Et-FIOH.

We present several experiments that support this assignment: (i) We electrolyzed the electrolyte solution (0.1 M TBAP in acetonitrile) at +2 V, after which we added Et-FIOH to the solution. We observed the production of Et-Fl' even though Et-FIOH was not oxidized. The amount of generated Et-Fl' correlated with the amount of water present in the acetonitrile solution. These results suggest that the anodic oxidation of water leads to a pH decrease in the electrolyte solution, which in turn leads to the conversion of Et-FIOH to Et-Fl'. (ii) We investigated the reactions between Et-FIOH and a series of chemical oxidants and found that the formation of Et-Fl' does not correlate with the oxidizing power of the reagent used. Instead, the formation of Et-Fl' correlates with Lewis acidities of oxidizing agents used in the study. (iii) The anodic oxidation of Et-FIOH in the presence of pyridine as a base demonstrates that the PCET from Et-FIOH radical cation occurs. The proton loss occurs from the N(5)-CH2 moiety of the molecule, as demonstrated by oxidation experiments involving Et-FIOHMe derivative. However, DFT calculations suggest that the proton loss in Et-FIOH2+ can occur from either >N–CH2– or –OH moiety of the molecule. Upon PCET in Et-FIOH and Et-FIOHMe, two additional reduction peaks were observed at ~0.2 and ~1.0 V. The same products were observed upon the two-electron oxidation of Et-FIOH, suggesting that Et-FIOH2+ releases protons in the absence of pyridine. The released protons then shift the Et-FIOH/ Et-Fl' equilibrium, giving rise to the formation of Et-Fl' reduction peaks observed in the cyclic voltammetry of Et-FIOH.

These findings provide valuable information for studies of possible catalytic water oxidation by pseudobase derivatives. We identified that the loss of a proton from N(5) alkyl chain leads to the decomposition of the flavin pseudobase via an iminium ion formation. For a design of a catalytic system, this process needs to be deactivated by a preparation of pseudobases that lack α H-atoms at the N(5) R group.

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Supporting Information Available: Discussion of possible water oxidation catalysis by Et-FIOH and accompanying DFT calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes
