Model Systems for Flavoenzyme Activity: One- and Two-Electron Reduction of Flavins in Aprotic Hydrophobic Environments

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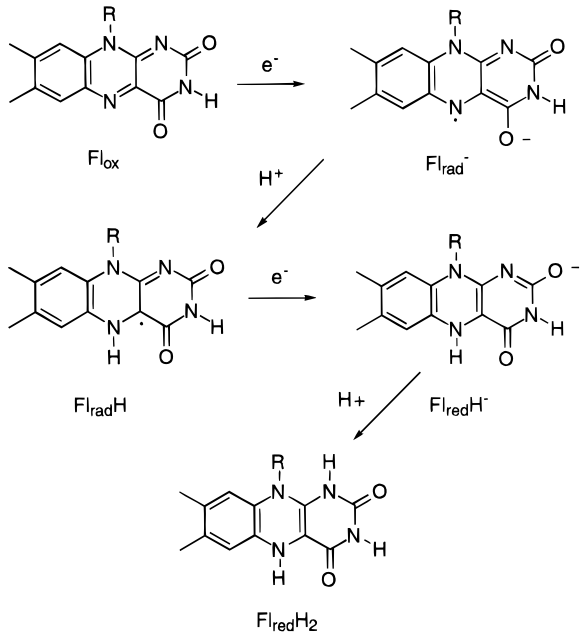
Abstract: The processes occurring during the electrochemically irreversible reduction of flavins in aprotic organic medium have been the cause of considerable controversy. To provide insight into these events we have investigated the reduction of flavins alkylated at N(3). We have demonstrated that the reversible reduction of N(3)-methylated flavin can be converted to the irreversible reduction observed with unalkylated flavins through addition of the weak external proton donor phthalimide. Proton transfer to the flavin radical anion generated at the electrode leads to partial formation of the neutral flavin radical, which is instantaneously further reduced to the fully reduced flavin anion. This mirrors the reduction of unalkylated flavins, where the proton source is the imide proton at N(3) of fully oxidized flavin in bulk solution. Simultaneous electrochemistry and EPR experiments confirm the disappearance of electrogenerated flavin radical anion both methylated and non-methylated at N(3), upon addition of phthalimide. UV/vis spectroelectrochemistry likewise confirmed the generation of the radical anion in the absence of proton donors and fully reduced flavin in the presence of proton donors.

The flavin cofactors FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) are involved in the catalysis of a wide variety of biological redox reactions, including the dehydrogenation of NAD(P)H, lipid esters, and D-amino acids, the oxidation of amines to imines, the formation and cleavage of disulfide bonds, the hydroxylation of aromatic substrates, and the activation of molecular oxygen.[1−4] Flavoenzymes mediate electron transfer processes (e.g., in photosynthesis and oxidative phosphorylation) and are involved in the regulation of neurotransmitters and the detoxification of xenobiotics.[5] Their ability to engage in one- as well as two-electron transfer reactions enables flavins to act as transformers between obligate $2e^-$ donors (e.g., NADH) and obligate $1e^-$ acceptors (e.g. heme Fe).

Analogous to quinones, flavins possess three readily accessible oxidation states (Scheme 1): the fully oxidized flavoquinone (Fl_{ox}); the flavosemiquinone radical, in either the anionic red (Fl_{rad}^-) or neutral blue ($Fl_{rad}H$) form; and the two-electron-reduced flavohydroquinone, which can also exist in either anionic ($Fl_{red}H^-$) or neutral ($Fl_{red}H_2$) form.

The redox properties of flavins in aqueous[6−10] and organic[10−13] media have been studied by potentiometric titration, dc, and ac polarography, cyclic voltammetry (CV), controlled potential coulometry, and simultaneous electrochemistry and EPR (SEEPR). It is well established that in aqueous or protic organic solvents flavins are reduced by a two-electron reduction directly to the flavohydroquinone. While this is relevant to flavoenzymes catalyzing solely two-electron processes, a great number of flavoenzymes are known to operate via the flavin radical,[14] enabling them to act as switches between one- and two-electron processes. An aqueous medium therefore is not appropriate for

Scheme 1[a]



[a] Lumiflavin: R = CH_3. Riboflavin: R = $CH_2(CHOH)_3CH_2OH$. FMN: R = $CH_2(CHOH)_4$-phosphate. FAD: R = $CH_2(CHOH)_4$-pyrophosphate-adenosine. Flavin **1**: R = $CH_2CH(CH_3)_2$.

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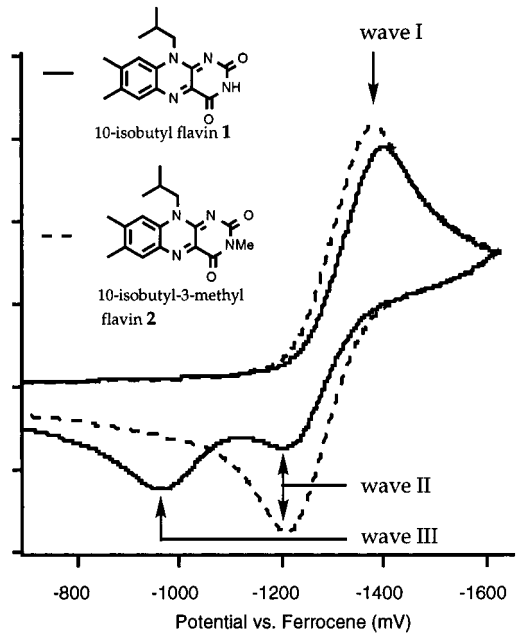

Figure 1. Cyclic voltammogram showing the first reduction wave (wave I) and coupled oxidation waves (waves II and II) of N(10)-isobutyl flavin **1** and N(3)-methyl-N(10)-isobutyl flavin **2** (10^{-3} M) in CH_2Cl_2, TBAP (10^{-1} M), scan rate 500 mV/s.

modeling these systems. This fact is supported by crystal structures of flavoenzymes,[15−17] which reveal that the cofactor is generally buried inside the protein matrix in a hydrophobic environment, allowing only controlled access to protons. Electrochemical studies in an aprotic solvent of low dielectric constant such as methylene chloride therefore provide a better analogy for enzyme-bound flavin and are a valuable tool in the investigation of the complex redox behavior of the cofactor. Previous voltammetric studies[11,12] of flavin reduction in DMSO, DMF, and acetonitrile have indicated the presence of two resolved reduction and three oxidation waves. As shown in Figure 1, the first reduction wave of the N(10)-isobutyl flavin **1** used in our studies was found to be coupled to two oxidation waves, in agreement with previously reported cyclic voltammograms.[11,12]

The interpretation of these observed electrochemical events is up to date controversial. It has been postulated[11] that the flavin radical anion is unstable, decomposing to an unidentified species A and a radical $B^{\bullet -}$. Oxidation of $B^{\bullet -}$ and A would then give rise to wave II and wave III, respectively. It was noted that the CV of riboflavin in DMSO is significantly altered by the addition of the weak proton donor hydroquinone, yet no explanation for the changes was given. In a different study,[12] protonation and one- as well as two-electron reduction was proposed to be responsible for the appearance of new waves in the CV of flavins upon addition of the strong acid $HClO_4$, yet no explanation was given as to why two oxidation waves are coupled to the first reduction wave in neutral solution. In recent research[10] SEEPR has been used to show that a radical species is formed by bulk electrolysis at potentials slightly more negative than the first reduction wave (wave I). The mechanism of formation of this species, however, is uncertain. The question is whether the first redox couple represents a two-electron-reduction step followed by comproportionation of the flavohydroquinone dianion and unreacted flavoquinone to form the radical anion.[10] A second possibility is that a one-electron reduction occurs directly to the radical anion.[11,12] It is also possible that the radical species could arise from a third, unknown, process. To resolve these uncertainties regarding the redox properties of flavin models in hydrophobic media, we have undertaken a combined electrochemical and spectroelectrochemical investigation of flavin reduction. We report here our mechanistic interpretation of the reduction of flavins in aprotic media.

Experimental Section

Materials and General Methods. Solutions were prepared using reagent grade CH_2Cl_2 dried via distillation over CaH_2. Tetrabutylammonium perchlorate (TBAP, obtained from SACHEM, electrometric grade) was recrystallized twice from water and dried for several days under high vacuum. Other chemicals were reagent grade, obtained from Aldrich Chem. Co., Acros Organics, Eastman Organic Chemicals, and EM Science and were used without further purification. 6-Chlorouracil was synthesized according to a literature procedure.[6] Reactions were carried out in flame-dried flasks under argon unless otherwise stated. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Model 1420 IR spectrometer. ^{1}H-NMR spectra were recorded on a Bruker AC200 (200 MHz) spectrometer. Elemental analyses were performed by the University of Massachusetts (Amherst) Microanalysis Service.

Cyclic Voltammetry. All electrochemical and spectroelectrochemical experiments were carried out on an IBM EC/250 voltammetric analyzer equipped with a Houston Instruments HR100 *xy*-recorder. In the CV studies, a 1.6 mm platinum disk and a coiled platinum wire electrode were utilized as working and auxiliary electrodes, respectively. A silver wire pseudo reference electrode was used, and potentials are referenced versus the ferrocene/ferrocenium couple. In all experiments the sweep rate was 300 mV/s unless otherwise stated. The electrochemical cells were dried at 125 °C for several hours and stored in a desiccator prior to use. Solutions of N(3)-methyl-N(10)-isobutyl flavin (10^{-3} M in CH_2Cl_2, 0.1 M TBAP) were degassed by bubbling argon (presaturated with CH_2Cl_2) through them for 4 min. The proton sources, acetanilide, phthalimide, and acetic acid, were prepared as stock solutions (0.1 M in CH_2Cl_2). A total of 3 equiv of the respective proton source was added to the flavin solution in steps of 0.2 equiv, with cyclic voltammograms observed after each addition.

Simultaneous Electrochemistry and EPR. Because of the lossy nature of the samples and because it was desired to minimize perturbation of the microwave field by the working electrode, SEEPR experiments were carried out in a quartz flat cell.[19] A second glass part containing three threaded joints sealed with Teflon ferrules to hold the electrodes and a septum-capped ground-glass joint for degassing and sample injection was connected to the top of the cell. The working electrode, a copper gauze electrode amalgamated by immersion into an aqueous saturated solution of $Hg_2(NO_3)_2$, was inserted into the flat part of the cell. The Ag-wire pseudo reference electrode was positioned directly above the working electrode in order to minimize the *iR*-drop, and the auxiliary electrode, a platinum wire spiral of large surface area, occupied the solvent reservoir above the flat section. The electrode leads were insulated via Teflon heat shrink tubing. After each experiment the working electrode was cleaned in dilute HNO_3 and reamalgamated.

EPR spectra were recorded on an IBM ESP 300 X-band spectrometer equipped with a TE_{104} dual cavity. Flavin solutions (10^{-3} M in CH_2Cl_2, 0.1 M TBAP) were degassed by bubbling argon through them for 5 min and then injected into the cell, which was previously flushed with argon. The cell was mounted within the spectrometer using

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custom-manufactured cell holders, which allow for precise alignment of the cell within the cavity in order to maximize the Q-factor. Bulk electrolysis was carried out simultaneously with signal acquisition (36 scans, 25 kHz field modulation, modulation amplitude 0.098 G).

UV/Vis Spectroelectrochemistry. The cell used in this study was built analogously to the apparatus developed by Salbeck,[20] and has the advantage of being solvent resistant, and capable of carrying out electrolysis under inert gas conditions. The working electrode, an optically transparent thin layer electrode (OTTLE), consists of a gold minigrid (100 lines/in., approximately 70% transparent) sandwiched between two glass slides separated by Teflon spacers (100 mm). A silver wire electrode and a Ni/Au-plated brass disk electrode served as the pseudo reference electrode and the auxiliary electrode, respectively. The main cell body was a cylindrical Pyrex glass cuvette 3 cm in diameter with a ground-glass joint at the top. The rest of the cell consists of a Teflon stopper with holes for degassing, a septum to add solution, and electrode connectors.

Spectra were recorded on a Shimadzu Model 260 UV/vis spectrometer. A reproducible orientation of the cell was achieved by lines engraved on the cell holder, glass cuvette, and Teflon stopper. The cell was fixed by set screws, purged with argon presaturated with CH_2Cl_2, and filled with solution so that the bottom of the OTTLE was just immersed. To account for the absorption characteristics of the cell, a base line spectrum of the cell plus electrolyte (CH_2Cl_2, 0.1 M TBAP) was recorded and subtracted from the other spectra. The potential was stepped up incrementally and the cell allowed to equilibrate (indicated by current decay to a constant value) after each step before spectra were recorded. The Ag wire pseudo reference electrode was calibrated vs ferrocene at the end of the experiment.

Synthesis of Flavin. *N*-Isopropyl-3,4-dimethylaniline (3). To a mixture of 3,4-dimethylaniline (9.69 g, 80 mmol) and isobutyraldehyde (7.27 mL, 5.77 g, 80 mmol) at 0 °C was added titanium isopropoxide[7] (29.76 mL, 28.23 g, 100 mmol) over 30 min. The solution was allowed to warm to room temperature and stirred for 2 h. A solution of sodium cyanoborohydride (3.37 g, 53.6 mmol) in absolute EtOH (80 mL) was then added, followed by stirring for 12 h. Addition of H_2O (16 mL) yielded a thick white suspension, which was stirred open to the atmosphere until HCN evolution ceased. The suspension was then filtered and washed with EtOH, and the filtrate was concentrated using a rotary evaporator. The residue was dissolved in diethyl ether (60 mL), washed with saturated aqueous $NaHCO_3$, water, and saturated aqueous NaCl (~10 mL each), and then dried over Na_2SO_4. The solvent was removed *in vacuo*. Vacuum distillation yielded 9.59 g (68%) of the alkylated aniline as a clear liquid: bp 96−97 °C (1.5 Torr); ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.92 (1H, d, J = 8.3 Hz), 6.44−6.34 (2H, m), 2.89 (2H, d, J = 6.5 Hz), 2.18 (3H, s), 2.14 (3H, s), 1.87 (1H, m, J = 6.5 Hz), 0.96 (6H, d, J = 6.5 Hz); IR (film) 3410, 3010−2880, 1620, 1580, 1505, 1465, 1385, 1315, 1320, 1260, 1215, 1170, 1150, 1120 cm^{-1}. Anal. Calcd for $C_{12}H_{19}N$: C, 81.36; H, 10.73; N, 7.91. Found: C, 81.34; H, 10.89; N, 7.95.

6-(*N*-Isopropyl-3,4-dimethylanilino)uracil (4). A mixture of 6-chlorouracil (2.19 g, 15 mmol) and *N*-isobutyl-3,4-dimethylaniline (5.32 g, 30 mmol) were heated at 150 °C for 24 h.[8,9] After cooling to room temperature, the pale yellow solid was suspended in EtOH/hexane (1:2, 80 mL), precipitating the product as a white powder. The crude product was filtered and washed with hexane. Recrystallization from 1:1 EtOH/EtOAc yielded 4.31 g (60%) of white crystals: mp 258 °C; ^{1}H NMR (200 MHz, $CDCL_3$) δ 7.95 (1H, bs), 7.26−7.21 (1H, m), 6.97−6.92 (3H, m), 4.94 (1H, bs), 3.36 (2H, d, J = 6.9 Hz), 2.30 (s, 6H), 2.08 (1H, m, J = 6.9 Hz), 0.93 (6H, d, J = 6.9 Hz); IR (KBr) 3400−2800, 2360, 1720−1550 (broad), 1500, 1470, 1440, 1400, 1380, 1340, 1250, 1225, 1170, 1145, 1070 cm^{-1}. Anal. Calcd for $C_{16}H_{21}N_3O_2$: C, 66.91; H, 7.32; N, 14.63. Found: C, 66.95; H, 7.43; N, 14.58.

N(10)-Isobutyl flavin N(5)-Oxide 5. To a solution of 6-(*N*-isopropyl-3,4-dimethylanilino)uracil (1.149 g, 4.0 mmol) in AcOH (16 mL) was added in portions $NaNO_2$ (828 mg, 12 mmol) with cooling on ice, providing an orange suspension. After stirring at room temperature for 1.5 h, the product was filtered and washed with EtOH. Trituration with EtOH yielded 936 mg (75%) of orange crystals: mp 287−290 °C dec; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.03 (1H, bs), 8.09 (1H, s), 7.83 (1H, s), 4.43 (2H, bd, J = 6.5 Hz), 2.46 (3H, s), 2.38 (3H, s), 2.30 (1H, m, J = 6.5 Hz), 0.96 (6H, d, J = 6.5 Hz); IR (KBr) 3200−2800, 2360, 1700, 1655, 1535, 1437, 1400, 1240, 1225 cm^{-1}; the material was used without further purification.

N(10)-Isobutyl flavin 1. To a suspension of N(10)-isobutyl flavin N(5)-oxide **5** (942.6 mg, 3.0 mmol) in H_2O/EtOH (1:1, 20 mL) was added $Na_2S_2O_4$ (1.045 g, 6.0 mmol), and the mixture was stirred open to the atmosphere until the initial dark green color changed to bright yellow (several hours). The crude product was filtered, washed with H_2O, dissolved in CH_2Cl_2, and filtered again. After drying over Na_2SO_4, the solvent was removed *in vacuo*. Recrystallization from EtOH/CH_2Cl_2 yielded 729.7 mg (82 %) of bright yellow needles: mp 302−305 °C dec; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.43 (1H, s), 8.06 (1H, s), 7.41 (1H, s), 4.63 (2H, bs), 2.56 (3H, s), 2.45 (3H, s), 2.53−2.35 (1H, m, J = 6.9 Hz), 1.05 (6H, d, J = 6.9 Hz); IR (KBr) 3200−2800, 2360, 1715, 1655, 1580, 1540, 1505, 1450, 1400, 1250 cm^{-1}. Anal. Calcd for $C_{16}H_{18}N_4O_2$: C, 64.44; H, 6.04, N, 18.78. Found: C, 64.15; H, 5.59; N, 18.70.

N(3)-Methyl-N(10)-isobutyl flavin 2. To a suspension of N(10)-isobutyl flavin **1** (59.6 mg, 0.2 mmol) and Na_2CO_3 (212 mg, 2.0 mmol) in dry dimethylformamide (3 mL) was added methyl iodide (1.25 mL, 283.9 mg, 2.0 mmol), and the mixture was stirred at room temperature for 12 h. After removal of the solvent via Kugelrohr distillation under vacuum, the residue was suspended in 10 mL of H_2O, precipitating the product as yellow solid. The solution was then neutralized with 1 M HCl and filtered, and the crude product was washed with H_2O. Recrystallization from EtOH/H_2O yielded 52 mg (82%) of N(3)-methylated flavin as fine yellow needles: mp 239−241 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.08 (1H, s), 7.39 (1H, s), 4.64 (2H, bs), 3.52 (3H, s), 2.54 (3H, s), 2.44 (3H, s), 2.51−2.33 (1H, m, J = 6.9 Hz), 1.04 (6H, d, J = 6.9 Hz): IR (KBr) 2940, 1700, 1650, 1580, 1540, 1455, 1280, 1240 cm^{-1}. Anal. Calcd for $C_{17}H_{20}N_4O_2$: C, 65.40; H, 6.41; N, 17.94. Found: C, 65.21; H, 6.21; N, 17.80.

Results and Discussion

To provide a flavin with sufficient solubility in nonpolar media we have synthesized N(10)-isobutyl flavin **1**, in which the isobutyl group at N(10) causes an enhancement in solubility of 2 orders of magnitude compared to lumiflavin (Scheme 2).[24] Alkylation of flavin **1** with methyl iodide then provided the N(10)-isobutyl-N(3)-methyl flavin **2** used in this research.

As previously mentioned, the CV of N(10)-isobutyl flavin **1** in CH_2Cl_2 exhibits both waves II and III during the oxidative sweep (Figure 1). Surprisingly, the CV of N(10)-isobutyl-N(3)-methyl flavin **2** in dry CH_2Cl_2 is electrochemically reversible, showing only wave II.[25] This finding suggests the involvement of the imide proton at N(3) in the formation of the species that is reoxidized during wave III, either via intramolecular tautomerization of one-electron-reduced flavin or via intermolecular proton transfer from flavoquinone in bulk solution to one-electron-reduced flavosemiquinone in the electrochemical double layer. To test these two hypotheses, we carried out a series of CV titrations in which acetanilide, phthalimide, and acetic acid were added to flavin **2**.

As shown in Figure 2, addition of both phthalimide and acetic acid renders the reduction of flavin **2** irreversible, causing the appearance of wave III and a decrease in the peak current of wave II. Addition of these proton donors therefore replicates

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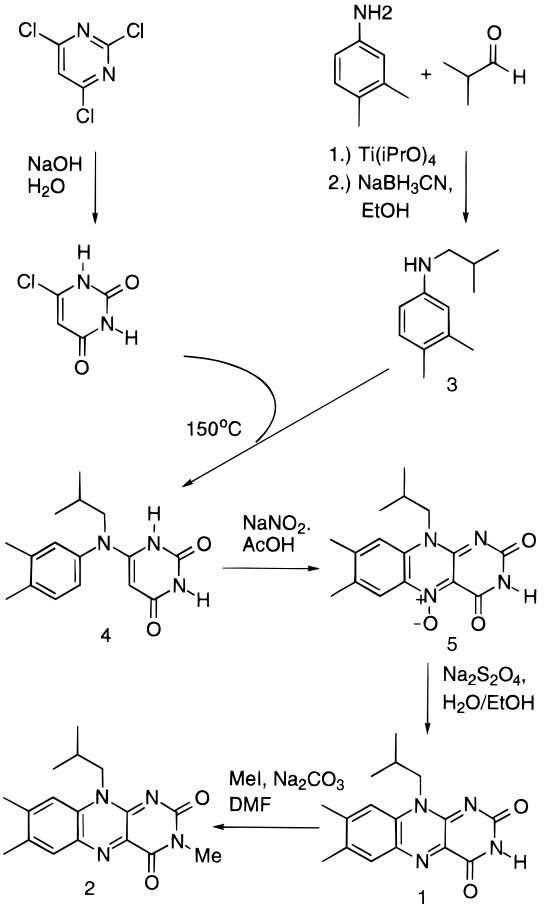
(25) Previously published cyclic voltammograms of N(3)-methyl lumiflavin in DMSO, DMF, and acetonitrile exhibit both wave II and wave III,[12] which may be attributed to residual water content in these solvents, or adsorbtion effects on the electrodes.

Scheme 2. Synthesis of N(10)-Isobutyl Flavin **1** and N(10)-Isobutyl-N(3)-methyl flavin **2** Following the Procedure of Yoneda et al.[22,23]



the unusual oxidative sweep observed in the CV of flavin **1**. Acetanilide, in contrast, has no effect of the CV of flavin **2**, even when added in 3-fold excess. Addition of a proton source of suitable acidity to flavin **2** which is not capable of tautomerization mimics the electrochemical behavior of flavin **1**, supporting the intermolecular proton transfer hypothesis (Scheme 3). While there is not a direct correlation between acidities in differing media, the acidities of aqueous phthalimide and acetanilide can be used as guidelines for estimating their proton donor properties. Acetanilide ($pK_a = 13.39$)[26] is not acidic enough to protonate the flavosemiquinone radical anion; however, phthalimide ($pK_a = 9.90$) is a sufficiently strong proton donor to effect this process. This finding agrees with the reported pK_a of N(3)−H of flavoquinone ($pK_a = 10$),[4,27] which acts as the proton donor in the case of nonalkylated flavin.

A second observation supporting the proposed proton transfer mediated process is an increase in current response upon addition of proton donor. The integrals of wave I in the CV's of flavin **2** alone and of flavin **2** plus 3 equiv of phthalimide shows a 2-fold increase in the charge consumed during the reductive sweep. The increase marks the transition from a one-electron to a two-electron reduction. In the absence of a proton source the reduction of Fl_{rad}^- to the unstable Fl_{red}^{2-} is a rather unfavorable process with a more negative standard reduction potential ($E°$) than the reduction of Fl_{ox} to Fl_{rad}^-. In the presence of a weak acid, Fl_{rad}^- is protonated at N(5), rendering the neutral radical $Fl_{rad}H$. Since the $E°$ for the reduction of $Fl_{rad}H$ to $Fl_{red}H^-$ is less negative than the $E°$ for the reduction of Fl_{ox} to Fl_{rad}^-, $Fl_{rad}H$ is instantly reduced to $Fl_{red}H^-$. This second one-electron reduction can occur either at the electron (ece process) or via disproportionation of $Fl_{rad}H$ with Fl_{rad}^-, forming $Fl_{red}H^-$ and Fl_{ox} (disp1 process, assuming that the protonation of Fl_{rad} is the rate-limiting step) (Scheme 3.)[28]

The rate of the protonation step, first order in the concentration of proton donor, determines the ratio of Fl_{rad}^- to $Fl_{red}H^-$ present at the electrode after the reductive sweep in cyclic voltammetry, hence the gradual increase of wave III and decrease of wave II during the titration of flavin **2** with external

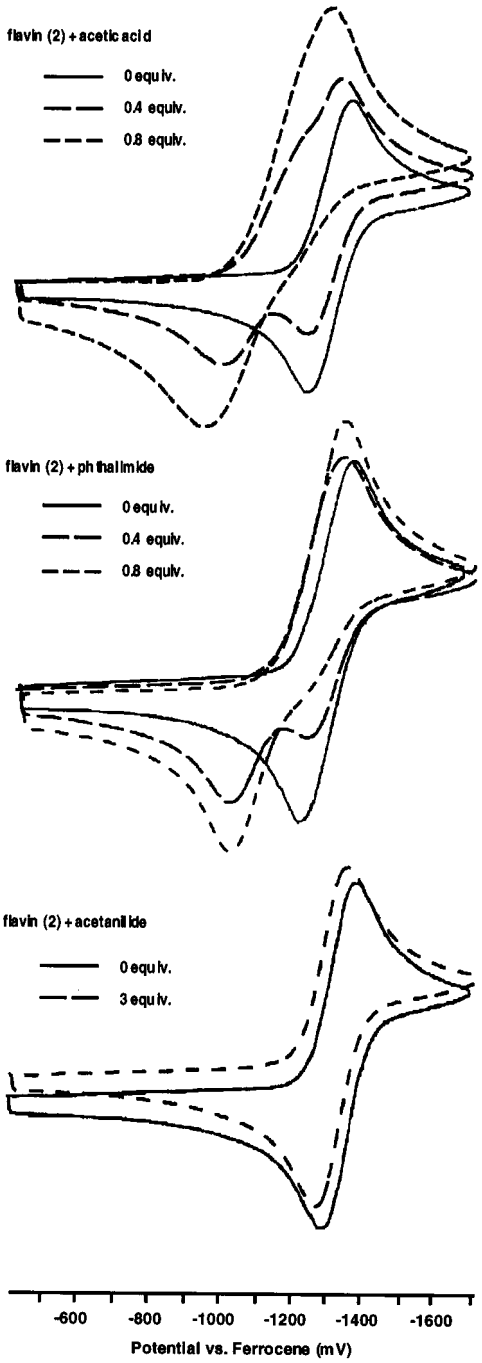


Figure 2. Change in the CV of N(10)-isobutyl-N(3)-methyl flavin **2** upon addition of proton donors. Solutions of acetanilide, phthalimide, and acetic acid (10^{-1} M in CH_2Cl_2) were titrated into the solution of N(3)-methyl-N(10)-isobutyl flavin (10^{-3} M in CH_2Cl_2, 0.1 M TBAP).

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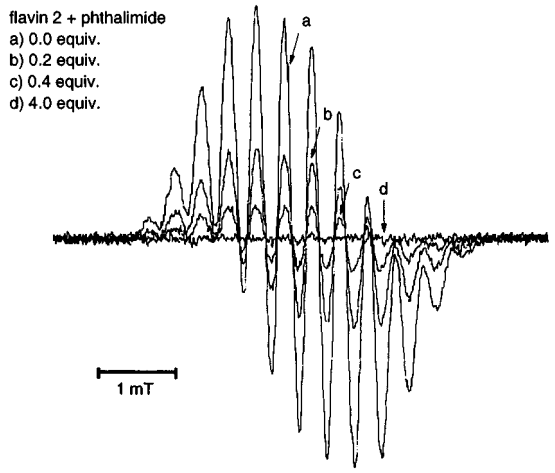
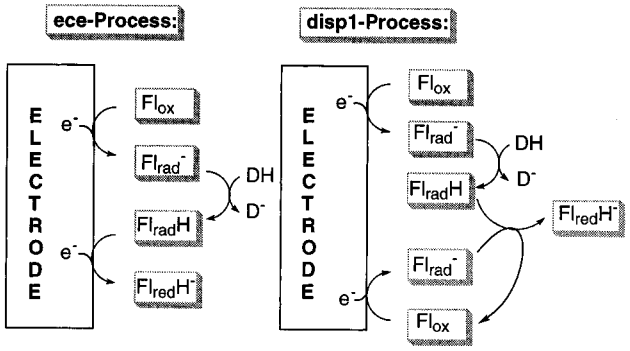


Figure 3. Change in the SEEPR spectrum of N(10)-isobutyl-N(3)-methyl flavin **2** (10^{-3} M in CH_2Cl_2, 0.1 M TBAP) upon addition of phthalimide.

Scheme 3. The Two Possible Mechanisms for the Redox Behavior of Flavin in the Presence of a Proton Donor DH[a]



[a] If the flavin is not alkylated at N(3), DH represents Fl_{ox} in bulk solution.

proton donors. Since the protonation and second one-electron reduction succeed the first one-electron reduction (two unresolved one-electron reductions rather than a two-electron reduction), the position of the reduction wave is only slightly affected. In the case of acetic acid, however, a prewave is observed. Once again, aqueous acidities can be used as benchmarks to provide insight into this behavior. Because the pK_a of acetic acid (pK_a = 4.75)[26] is lower than the pK_a of fully reduced flavin (pK_a = 6.7),[4] the neutral fully reduced flavin $Fl_{red}H_2$ is formed, which has a much lower solubility in methylene chloride[29] due to additional hydrogen-bond interactions. The observed prewave is caused by adsorption of $Fl_{red}H_2$ onto the working electrode. It is well-known that flavins tend to adsorb onto mercury working electrodes in aqueous acidic media, giving rise to a prewave observed in both polarography and cyclic voltammetry.[6,9]

To provide further evidence for our proposed flavin reduction mechanism, we investigated the reduction of flavin **2** using simultaneous electrochemistry and EPR. Bulk electrolysis of a solution of flavin **2** in CH_2Cl_2 within the EPR cavity generated a strong signal corresponding to the flavin radical anion. SEEPR of flavin **2** in the presence of increasing amounts of the external proton donor phthalimide led to a gradual decrease of the EPR signal intensity (Figure 3). A plot of the double integral of the EPR signal versus equivalents of phthalimide added shows a roughly exponential relationship. In the presence of excess

(29) Catalytic hydrogenation of N(10)-isobutyl flavin **1** over palladium on carbon in methylene chloride causes the fully reduced neutral flavin $Fl_{red}H_2$ to precipitate out as a dull yellow solid.

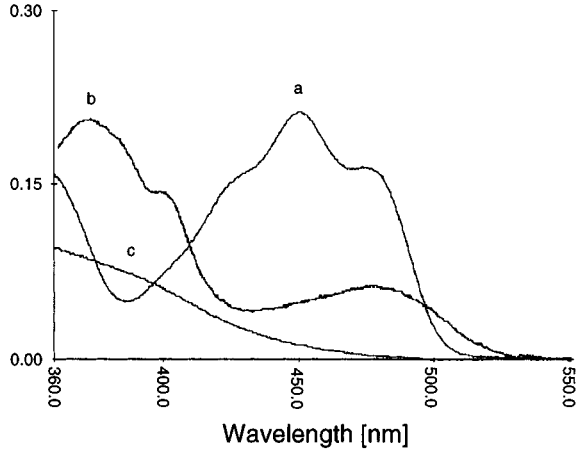


Figure 4. UV/vis spectra of N(10)-isobutyl-N(3)-methyl flavin **2**: (a) fully oxidized (Fl_{ox}), (b) flavin radical anion (Fl_{rad}^-) generated electrochemically in the absence of phthalimide, (c) fully reduced flavin ($Fl_{red}H^-$) generated electrochemically in the presence of 3 equiv of phthalimide.

phthalimide no signal was observable. The hyperfine splitting of the flavin radical signal remained unchanged during the addition of phthalimide, indicating the essentially instantaneous further reduction of the neutral radical to the fully reduced flavin anion (the neutral radical of flavin **2**, which was chemically generated following the procedure of Mueller et al.,[30] exhibits a markedly different EPR spectrum than the anion radical).

SEEPR of flavin **1** also results in the spectrum of the radical anion Fl_{rad}^-, yet bulk electrolysis has to proceed for a longer time before the spectrum is observable, and the signal is of lower intensity than the EPR signal of the radical anion of flavin **2**. During the bulk electrolysis of flavin **1**, Fl_{ox} acts as both the electroactive species and the proton donor. This contrasts with the bulk electrolysis of flavin **2** in the presence of phthalimide, where an excess of external proton donor is available. As the bulk electrolysis proceeds, Fl_{ox} becomes the limiting reagent, and deprotonated Fl_{ox}^- and $Fl_{red}H^-$ accumulate in solution. The equilibrium of deprotonated Fl_{ox}^- and $Fl_{red}H^-$ undergoing comproportionation to two molecules of Fl_{rad}^- under those conditions is shifted toward the radical product, leading to the observable EPR signal. This explains the n_{app} of 1 determined by previous researchers for the reduction of flavin not alkylated at N(3) in aprotic media.[10,11] In cyclic voltammetry, on the other hand, only the small fraction of flavin **1** adjacent to the electrode is reduced, and a large excess of Fl_{ox} is available in bulk solution. As a result, comproportionation does not become a significant process. This interpretation is backed up by SEEPR of nonalkylated flavin **1** in the presence of 3 equiv of phthalimide. As expected, no EPR signal was observable after bulk electrolysis.

To enable spectroscopic determination of the nature of the two-electron-reduced species, we carried out a series of UV/vis spectroelectrochemical experiments. For these studies bulk electrolysis was carried out within an optically transparent thin layer electrode (OTTLE).[31] Since the light beam only crosses the thin layer part and not the bulk solution, the spectrum of the electrogenerated species can be recorded without convolution by the spectrum of the parent species. Electrolysis of a solution of flavin **2** in CH_2Cl_2 resulted in the formation of the red radical anion Fl_{rad}^-, verified by characteristic absorption maxima at 375

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(31) Heineman, W. R.; Hawkridge, F. M.; Blount, H. N. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1984; Vol. 13, pp 1−104.

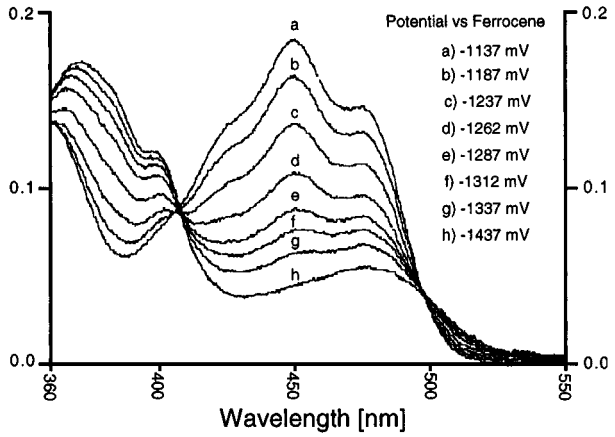

Figure 5. Spectroelectrochemical conversion of N(10)-isobutyl-N(3)-methyl flavin **2** (10^{-3} M in CH_2Cl_2, 0.1 M TBAP) from the fully oxidized form (Fl_{ox}) to the radical anion (Fl_{rad}^-).

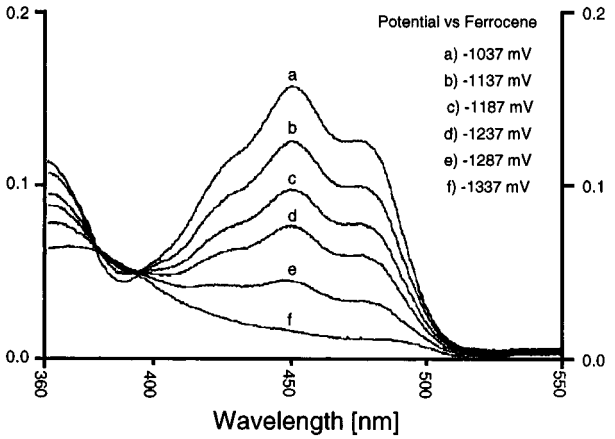

Figure 6. Spectroelectrochemical conversion of N(10)-isobutyl-N(3)-methyl flavin **2** (10^{-3} M in CH_2Cl_2, 0.1 M TBAP, 3 equiv of phthalimide) from the fully oxidized from (Fl_{ox}) to the fully reduced form ($Fl_{red}H^-$).

and 478 nm.[32] The same experiment carried out in the presence of 3 equiv of phthalimide leads to the generation of the dull yellow and nonfluorescent, fully reduced flavin $Fl_{red}H^-$, possessing only shoulder absorption in the visible region (Figure 4).[33] The spectra of the conversion of Fl_{ox} to both Fl_{rad}^- and $Fl_{red}H^-$ possessed isosbestic points (Figure 5 and 6). By stepping the potential down both reactions were reversed, and after full reoxidation the spectrum of Fl_{ox} was regained. Several reduction and oxidation cycles of the solution are possible without changes in the spectra obtained for the different species, verifying that the unusual redox behavior is not a consequence of the formation of degradation products.

The same experiment carried out with a solution of flavin **1** leads to the spectrum of Fl_{rad}^- if no external proton donor is present. Since the solution in the thin layer section is exhaustively electrolyzed, the radical anion is generated via comproportionation as described above. Again, UV/vis spectroelectrochemistry of flavin **1** in the presence of 3 equiv of phthalimide leads to the spectrum of $Fl_{red}H^-$.

In summary, the ability of the radical anion Fl_{rad}^- of flavin **1** and flavin **2** to abstract the imide proton of phthalimide indicates a similar process taking place in flavins unalkylated at N(3). In this case the N(3)−H imide proton of the flavin acts as a proton source causing protonation of Fl_{rad}^-. The neutral radical $Fl_{rad}H$ is instantly reduced to $Fl_{red}H^-$. The availability of protons therefore marks the transition from one-electron to two-electron flavin reduction. A detailed understanding of the redox processes taking place during the electrochemical reduction of flavin is of fundamental value for future model studies of flavoenzyme activity using electrochemical methods. We have already shown that noncovalent interaction between a synthetic receptor and flavin **1**, mimicking the hydrogen-bonding pattern observed in flavoenzymes to C(2)−O, N(3)−H and C(4)−O of the flavin cofactor, not only lowers the standard reduction potential but also causes a relative increase in wave II and a decrease in wave III observed in the cyclic voltammogram of flavin **1**.[24] With the results presented in this paper, these changes upon addition of receptor can be interpreted as selective stabilization of the flavin radical anion Fl_{rad}^-, making one-electron reduction the preferred process. This finding can be explained through the increase in pK_a of N(3)−H of flavin **1** in the hydrogen-bond complex, disfavoring intermolecular proton transfer, which is a prerequisite for the second one-electron reduction. As mentioned previously, the ability of flavoenzymes to selectively carry out one- vs two-electron-redox processes is one of the key features responsible for their catalytic diversity. The model system presented here is a first step in understanding the modification of flavin redox processes on a molecular level. Further studies on flavin host−guest complexes using more sophisticated receptors combining hydrogen bonding and π-stacking interactions are underway and will be reported in due course.

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