A Model System for Flavoenzyme Activity − Binding of Flavin and Modulation of Its Redox Potentials through Coordination to a Lewis-Acidic Azamacrocyclic Zinc(II) Complex

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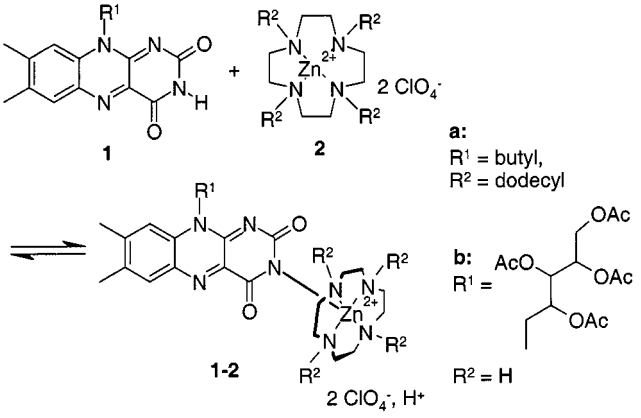
Keywords: Enzyme models / Macrocycles / Electron transfer / Molecular recognition / Electrochemistry

The coordination of flavins to Lewis-acidic zinc(II)cyclen complexes, as a mimic for a metalloprotein binding site, changes their redox properties significantly. The coordination facilitates the electrochemical reduction of the flavin and stabilizes the flavinhydroquinone redox stage, so that a 600 mV more positive potential is necessary for reoxidation. Binding of flavin to the receptor molecule is not restricted to nonpolar solvents and works in methanol. Here, interception of the ECE mechanism of reduction is possible leading to a stabilization of the flavosemiquinone radical anion redox stage, which was proved by its characteristic UV absorption in spectroelectrochemistry. Although it has not yet been observed in flavoproteins, this study shows that flavin binding by coordination to a metal binding site is suitable for cofactor binding and modulation of its redox properties as well as other intermolecular interactions.

Introduction

Flavoproteins containing the flavin cofactors FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide) are frequently encountered redox enzymes involved in an enormous range of biotransformations, signal transductions, and electron-transfer processes.[1−8] To fulfill their specific functions the enzymes mediate one- or two-electron transfer redox events at potentials that vary over a range of more than 500 mV (about 11 kcal/mol).[9] From this it is apparent that the redox properties of flavin as the single redox cofactor must be adapted and controlled by reversible noncovalent interactions between the apoenzyme and the flavin to suit the requirements of the enzyme function. So far influence by hydrogen bonding,[10−12] aromatic stacking,[13−16] dipole interactions[17−19] and steric effects[20] have been observed in biological systems, but the exact contribution to changes in redox properties of each of these intermolecular forces is difficult to determine.[21−26] Therefore model systems have been prepared and studied to reveal the modulation of redox properties by supramolecular interactions.[27] Detailed investigations by Rotello,[28−34] Yang[35−39] and Shinkai[40,41] have confirmed and explained the significant effect of the various interactions on the redox potential of flavins. As shown by earlier work,[42,43] flavins bind various metal ions. Thus, the redox potential changes as electrochemical measurements have shown.[44,45] However, reversible coordination of the flavin to a suitable metal ion-binding site as an alternative conceivable situation for cofactor binding has not been addressed in this context. Even if a direct coordination of a flavin cofactor to a metal binding site is not observed in flavoenzymes, yet, the importance of such interactions for function and structure of biological molecules in other context make this a conceivable scenario.

To predict the feasibility of such a proposed binding motif, we have investigated changes in redox properties of 10-butyl flavin (**1a**) and riboflavin tetraacetate (**1b**),[46] a derivative of vitamin B_2, upon reversible coordination to Lewis-acidic zinc(II) complexes **2**, as very simple models for a metalloprotein coordination site (Scheme 1).[47−52]



Scheme 1. Binding equilibrium of 1,4,7,10-tetraazacyclododecane zinc(II) bisperchlorate complexes (**2**) to flavins (**1**)

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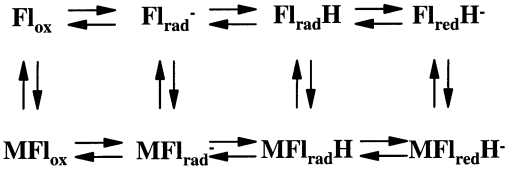
Results and Discussion

Binding Motif

The binding of riboflavin and other imides to Lewis-acidic zinc(II) azamacrocycles has been studied by Kimura and co-workers thoroughly.[47] Several X-ray structures have confirmed that the deprotonated imide nitrogen coordinates to the zinc(II) ion, which is complexed by tetraaza-cyclododecane. The interaction of imide and the metal complex is supported by hydrogen bonds between imide carbonyl oxygen and azamacrocycle N−H, whereby it remains unclear if this is a direct interaction or water molecules bridge the structure.[53,54] The binding motif has been used for the recognition of anions and neutral molecules[55−62] such as riboflavin[47] and riboflavin tetraacetate,[63] in water and organic solvents. In this study we investigate the effect of such binding on the redox properties of the coordinated flavin. Scheme 2 summarizes all possible equilibria in a flavinoid − metal complex system with competing redox processes, protonation/deprotonation and complexation/decomplexation.



Scheme 2. Schematic representation of flavinoid species which equilibrate by redox processes, protonation and metal ion complexation

Binding and Modulation of Flavin Redox Properties in Dichloromethane

The cyclic voltammogram of **1a** shows the typical redox states of a flavin in an aprotic solvent. The observed process of reversible two-electron reduction is explained perfectly by the ECE mechanism, a successive transfer of an electron to the oxidized flavin (Fl_{ox}, flavoquinone), protonation of the radical anion (Fl_{rad}- to $Fl_{rad}H$) and rapid electron transfer to give the flavohydroquinone anion ($Fl_{red}H^-$).[27] Addition of aliquots of **2a**[64] to the solution results in a reduction in voltage of the reduction peak potential by approx. 100 mV. This suggests the formation of an aggregate $Fl_{ox}M$ (M = metal complex receptor),[65] which is reduced to $Fl_{rad(red)}M$. The first oxidation wave belonging to the reoxidation of Fl_{rad}- disappears. More significantly, the second oxidation wave of the reoxidation process, which is assigned to the oxidation of $Fl_{red}H^-$, is shifted by more than +600 mV to positive potential. The coordination of the flavin to the zinc ion complex stabilizes the flavohydroquinone redox state, so that a potential of approx. −0.3 V vs. Fc/Fc^+ is necessary for reoxidation (Figure 1). A similar, but less pronounced effect has been observed with some artificial hydrogen bond receptors[27] and other metal complexes.[66]

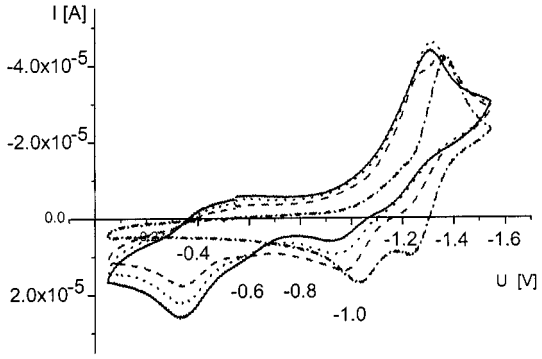


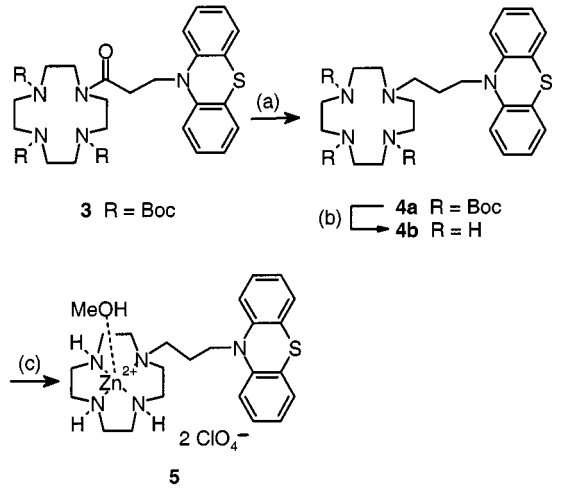
Figure 1. Cyclic voltammogram of **1a** (10^{-3} mol/L) (− · −); **1a** + **2a** (1 equiv.) (− −); **1a** + **2a** (2 equiv.) (···); **1a** + **2a** (3 equiv.) (—) 0.5 V/s in 0.1 mol/L NBu_4 BF_4 in CH_2Cl_2 vs. Fc/Fc+

Binding and Modulation of Flavin Redox Properties in Methanol

All model systems for flavoenzyme activity reported so far are functionally restricted to nonpolar organic solvents, such as dichloromethane, to avoid interception of the usually weak interactions between flavin and receptor by the solvent. Aprotic solvents with low dielectric constants are used as a mimic of the hydrophobic protein interior in which the cofactor might operate. However, a functional artificial receptor for flavin binding under physiological conditions must be able to interact with the binding partner and modulate its redox properties in competitive solvents. The coordinative bond between flavin N(3) and a Lewis-acidic azamacrocyclic zinc(II) complex does provide sufficient binding strength for this task.[67]

To verify the interaction of zinc(II) cyclen (**2b**) with riboflavin tetraacetate (**1b**) in its oxidized flavoquinone form in methanol, the binding constant was determined spectroscopically. To do so, the azamacrocycle was labeled with phenothiazine, which effectively quenches the emission of riboflavin. Starting from the known compound **3** reduction with borane, deprotection and formation of the zinc(II) complex gave **5** (Scheme 3). Fluorescence titration of **1b** with **5** in methanol and fitting the data to a 1:1 binding isotherm gave a binding constant of $K = 38000$ L/mol (Figure 2),[68] which should be a good estimate for the binding constant of **1b** to **2b** under these conditions. We assume that contributions of the phenothiazine label to the binding are small. The stoichiometry of the aggregate was confirmed by a Job's plot analysis (see supporting information).

Cyclic voltammograms were recorded to investigate the effect of coordination of the flavin N(3) to the zinc cyclen complex in methanol. Reduction of pure **1b** is observed at a peak potential of $E_p^{red} = -960$ mV vs. Fc/Fc^+, while reoxidation occurs at a peak potential of $E_p^{ox} = -780$ mV vs. Fc/Fc^+. The redox process is rationalized by the ECE mechanism, whereby the protonation of Fl_{rad}^- to $Fl_{rad}H$ is rapid and $Fl_{rad}H$ is reduced immediately. $Fl_{red}H^-$ is therefore the only product detectable. Chemical and electrochemical steps are fully reversible as shown by repetitive scans.



Scheme 3. Synthesis of **5**; (a) $BH_3{\cdot}SMe_2$, 93%; (b) TFA, CH_2Cl_2, 89%; (c) $Zn(ClO_4)_2{\cdot}6\ H_2O$, MeOH, 100%

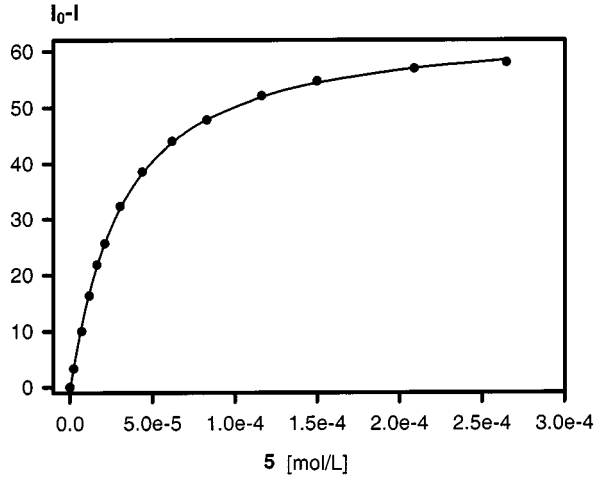
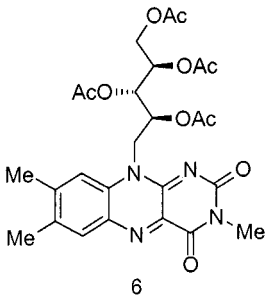


Figure 2. Change in emission intensity of **1b** ($c_0 = 7.35 \times 10^{-5}$ mol/L) upon addition of **5**; the curve was fitted to a 1:1 binding isotherm to yield a binding constant of $K = 38000$ L/mol (error +/−5%); emission intensity given in arbitrary units

The interpretation is supported by spectroelectrochemistry (see supporting information). Upon reduction the characteristic absorption bands of the flavoquinone form of **1b** (maxima at about 450 nm, 375 nm, 260 nm, and 220 nm, respectively) disappear, while the absorption of the flavohydroquinone $Fl_{red}H$ emerges (maxima at about 350 nm, 295 nm, and 255 nm, respectively). The flavosemiquinone redox stage is not detected. Oxidation restores the original absorption spectra of the oxidized flavoquinone form.

For comparison, cyclic voltammogram and spectroelectrochemistry of N(3)-methyl riboflavin tetraacetate (**6**, Scheme 4) in methanol were recorded (see supporting information). The data are identical with **1b** and again in agreement with the ECE mechanism. Obviously, protonation of the Fl_{rad}- species by methanol is so rapid, that its formation or oxidation cannot be detected.

We then investigated the effect of added **2b** on the electrochemistry of **1b** in methanol (Figure 3). Upon addition of



Scheme 4. Structure of compound **6**

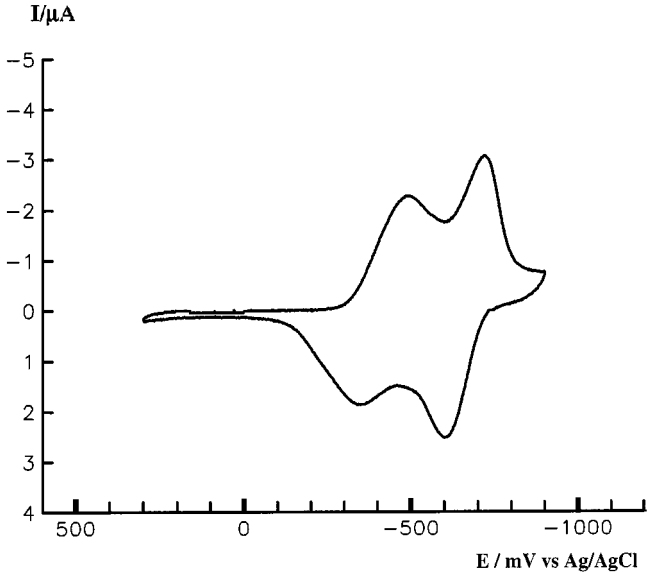


Figure 3. Thin layer cyclic voltammogram of **1b** ($c = 1.3 \times 10^{-3}$ mol/L) and **2b** ($c = 1.3 \times 10^{-3}$ mol/L) in CH_3OH/0.1 M $LiClO_4$ at a scan rate of $v = 25$ mV/s

one equivalent of **2b,** the thin-layer cyclic voltammogram showed well-separated quasireversible waves indicating the involvement of noncoordinated **1b** and coordinated **1b**−**2b** species.[69] Spectroelectrochemistry shows the exclusive formation of $Fl_{red}H^-$ most likely in its complexed form.

In order to shift the equilibrium to more complete coordination we added 10 equivalents of **2b** to **1b** (Figure 4).

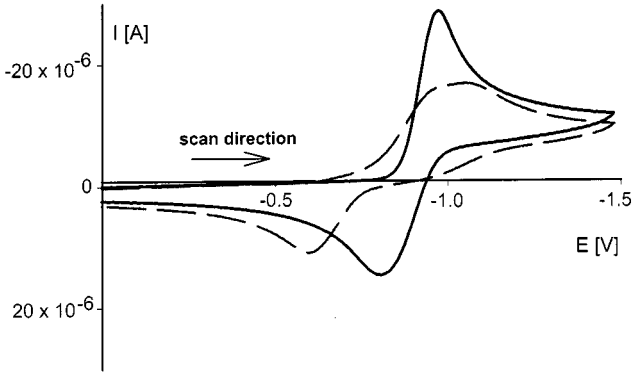


Figure 4. Cyclic voltammogram of a solution of **1b** (10^{-3} mol/L) (—) and **1b** (10^{-3} mol/L) + **2b** (10^{-2} mol/L) (− − −) in methanol (0.1 mol/L $LiClO_4$) at 20 °C and 0.1 V/s; all potentials vs. Fc/Fc+

The cyclic voltammogram of the solution reveals a broad and unresolved reduction wave around $E = -600$ mV to -1050 mV vs. Fc/Fc^+ indicating structural reorganisation during the reduction process. Oxidation takes place at $E_p^{ox} = -550$ mV vs. Fc/Fc^+. The electrochemical cycle is fully reversible, as confirmed by repetitive scans under thin layer conditions.

Spectroelectrochemical characterization reveals a two-step reduction process. The first step is clearly identified as the formation of the flavosemiquinone radical anion of the coordinated flavin peaking at 470, 390, 360, and 260 nm,[70−72] which is also evidenced by differential UV/Vis-spectroelectrochemistry showing a typical positive differential absorption at the long-wavelength-edge of the spectrum at about 500 nm (Figure 5, top). For comparison the changes in absorption of **6** and **2b** in acetonitrile were recorded (Figure 5, middle). In this case, as reported earlier, the formation of the radical anion is observed upon reduction, because no NH-proton is available to initiate the ECE mechanism. Changes in absorption spectra upon reduction are identical with the changes in the case of **1b**−**2b** in methanol, confirming the formation of a radical anion in spite of the polar protic conditions (Figure 5, top).[73−75] It is also interesting to note that upon reduction of **6** and **2b** in acetonitrile a broad absorption centered at about 720 nm emerges (indicated by the arrow in Figure 5, middle), the intensity of which increases on prolonged reduction. We tentatively assign these bands to the radical anion or dianion of biflavin **7** (Scheme 5) which is likely to be formed on reduction.[76,77] Likewise, we have found that the reduction in acetonitrile of **6** also leads to the long-wave length absorption bands (Figure 5, bottom). Obviously, the process of dimerization only occurs in the absence of protic solvents or NH-acidic groups.

A second process follows the radical anion formation at more negative potential (Figure 6, top). From spectroelectrochemical data this process is attributed to the formation of the flavohydroquinone.[78] For comparison the changes in absorption upon reduction of **1b** in methanol, a process that yields exclusively the flavohydroquinone, are given in Figure 6, bottom. To investigate the importance of the imide N(3) binding of flavin to **2b**, to changes in redox properties, cyclic voltammograms of mixtures of **6** and **2b** in methanol were recorded. The cyclic voltammogram of **6** remained virtually unchanged upon the addition of equimolar amounts of the zinc complex (see supporting information).[79] Therefore we come to the conclusion that the formation of the coordinative bond between N(3) imide nitrogen of flavin and the Lewis-acidic metal center is of major importance for the modulation of the flavin redox properties.[80]

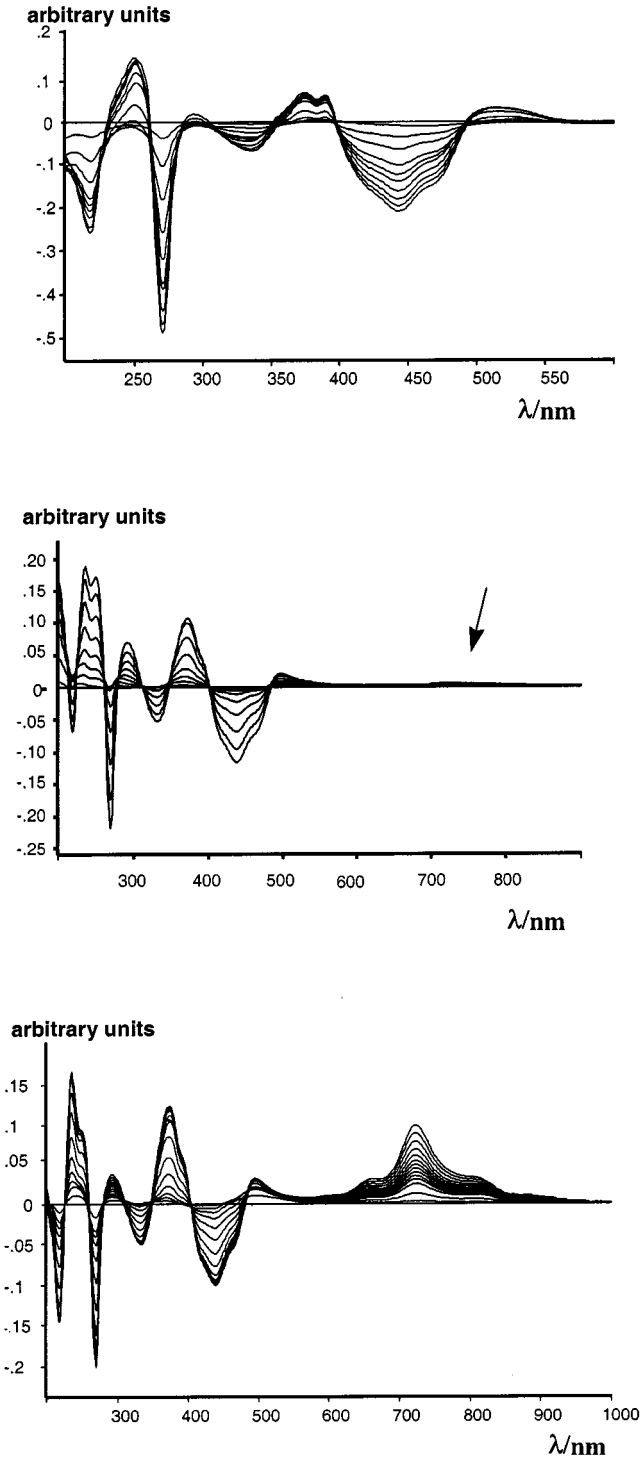
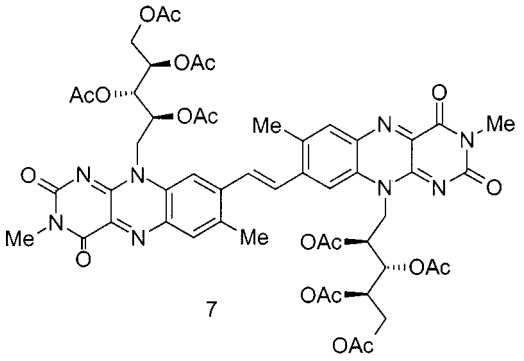


Figure 5. Differential spectroelectrochemical scans upon reduction; top: **1b** ($c = 1.1 \times 10^{-3}$ mol/L) − **2b** ($c = 1.1 \times 10^{-2}$ mol/L) in CH_3OH/0.1 M $LiClO_4$; middle: **6** ($c = 9.5 \times 10^{-4}$ mol/L) + **2b** ($c = 9.5 \times 10^{-4}$ mol/L) in CD_3CN/0.1 M tetra-*n*-butylammonium hexafluorophosphate (TBAHF); bottom: **6** ($c = 1.0 \times 10^{-3}$ mol/L) in CH_3CN/0.1 M TBAHF

Conclusion

The binding of flavin derivatives to Lewis-acidic zinc(II) complexes **2** changes their redox properties significantly. In dichloromethane solution the coordination generally facilitates the electrochemical reduction and significantly stabilizes the flavinhydroquinone form. The potential for the reoxidation is shifted due to coordination to the metal complex by more than + 600 mV. Similar, but less pronounced effects have been achieved previously with artificial hydrogen bonding receptors.[28] However, the coordinative bond



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Scheme 5. Structure of compound **7**

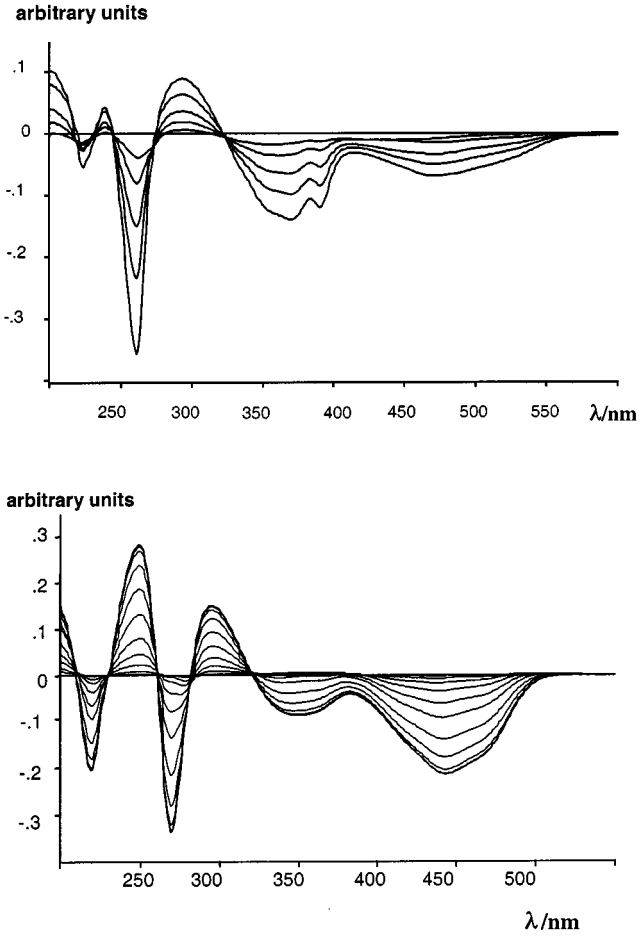

Figure 6. Differential spectroelectrochemical scans upon further reduction of **1b** ($c = 1.1 \times 10^{-3}$ mol/L) − **2b** ($c = 1.1 \times 10^{-2}$ mol/L) in CH_3OH/0.1 M $LiClO_4$, (top) and for comparison reduction of **1b** ($c = 1.2 \times 10^{-3}$ mol/l) in CH_3OH/0.1 M $LiClO_4$ (bottom)

between flavin and the zinc azamacrocycle, which may be regarded as a very simple model for a metalloprotein binding site, works in polar competing solvents too. Measurements in methanol show that the binding motif allows interception of the ECE reduction mechanism and stabilization of a flavosemiquinone radical anion of vitamin B_2 in a polar solvent for the first time. As a consequence the flavin chromophore switches from a two-electron-one-step to a two-step-one-electron-each mediator by mere coordination.[81−85] The formation of the radical anion was confirmed by its characteristic UV absorptions obtained from spectroelectrochemistry. This leads to the conclusion, that reversible flavin binding by kinetically labile, but thermodynamically strong coordinative bonds to Lewis-acidic metal centers is a valuable alternative binding motif for the design of artificial flavin receptors that modulate flavin redox properties. The use of such receptors is not restricted to nonpolar organic solvents and should even be possible under physiological conditions. In addition, it can be concluded that flavin cofactor binding to metalloproteins by coordinative bonds should fulfill the task of reversible cofactor binding and allow the on/off switching of redox properties. The question remains whether this binding motif in flavoproteins may also be operative in natural systems.

Experimental Section

General Remarks: Compounds **1a**,[86,87] **2a**,[64] **2b**[88] and **3**[63] were synthesized according to known procedures. Melting points were taken on a hot-plate microscope apparatus and are not corrected. NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) in [D]chloroform solutions unless otherwise stated. The multiplicity of the ^{13}C signals was determined with the DEPT technique and quoted as: (+) for CH_3 or CH, (−) for CH_2 and (C_{quat}) for quaternary carbons. CC means column chromatography on silica gel. UV- and fluorescence spectra were recorded on HP 8452A, Perkin−Elmer MPF 44 and Hitachi F-4500 spectrometers. Cyclic voltammograms were recorded with Autolab PGSTAT20 on glassy carbon working electrode or with Amel 553 and Amel 5000 on Pt disk electrode. Ferrocen/ferrocenium was used as internal reference. Spectroelectrochemical measurements were performed with Perkin−Elmer Lambda 9 and Polytec spectrometer, and Amel 2053 potentiostat.[89]

Tri-*tert*-butyl 10-(3-Phenothiazin-1-ylpropionyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (4a): A mixture of **3** (325 mg, 0.48 mmol) and $BH_3{\cdot}SMe_2$ (1 mL) (2 mol/L in THF, 2 mmol) in dry THF (15 mL) was refluxed for 2 h under nitrogen. After cooling to room temperature 50 mL of methanol/water (4:1, v:v) was carefully added. When gas evolution ceased, the solvents were evaporated to dryness in vacuo. The crude product was dissolved in CH_2Cl_2, filtered, and the solution was washed with aqueous NaOH (2 mol/L), dried over Na_2SO_4 and the solvent was removed in vacuo to yield 269 mg (93%) of **4a**, as a white solid (m.p. 104 °C). − ^{1}H NMR: δ = 1.36 (s, 27 H), 2.26 (m, 2 H), 2.74−3.55 (m, 20 H), 6.86 (m, 2 H), 7.08 (m, 2 H), 7.07 (m, 2 H), 7.11 (m, 2 H). − ^{13}C NMR: δ = 28.3 (+), 28.6 (+), 33.9 (−), 44.9 (−), 45.3 (−) 50.2 (−), 50.5 (−), 51.0 (−), 51.7 (−), 79.0 (C_{quat}), 80.6 (C_{quat}), 80.6 (C_{quat}), 115.7 (+), 122.7 (+), 125.7 (C_{quat}), 127.4 (+), 127.5 (+), 144.8 (C_{quat}), 156.4 (C_{quat}). − IR (KBr): $\tilde{\nu}$ = 3452, 2979 cm^{-1}. − UV/Vis (CH_3CN): λ_{max} (lg ε): 192 (5.947), 256 (3.307), 308 (0.423). − MS (70 eV, EI): *m/z* = 711 (100) $[M]^+$, 611 (20) $[M - C_5H_9O_2]^+$, 57 (80) [butyl].

3-Phenothiazin-10-yl-1-(1,4,7,10-tetraazacyclododecan-1-yl)propane (4b): A solution of **4a** (250 mg, 0.352 mmol) and of TFA (2.5 mL) in dry CH_2Cl_2 (2.5 mL) was stirred for 1.5 h under nitrogen at room temperature. The reaction mixture was diluted with CH_2Cl_2 (15 mL) and aqueous NaOH (6 M, 2.5 mL), washed twice with aqueous NaOH (2 mol/L), dried over Na_2SO_4 and the solvent was removed in vacuo to give **4b** (129 mg, 89%) as a white solid, m.p. 59 °C. − ^{1}H NMR (400 MHz, [D_6]acetone): δ = 2.75 (m, 6 H),

2.80 (m, 12 H), 2.90 (m, 2 H), 3.50 (m, 2 H), 6.95 (m, 2 H), 7.08 (m, 4 H), 7.20 (m, 2 H). − ^{13}C NMR (100 MHz, [D$_6$]-acetone): δ = 29.9 (−), 44.8 (−), 46.2 (−), 46.9 (−), 48.3 (−), 52.3 (−), 53.2 (−), 117.3 (+), 123.3 (+), 123.3 (C_{quat}), 128.1 (+), 128.4 (+), 146.5 (C_{quat}). − IR (KBr): $\tilde{\nu}$ = 3418, 2926, 1458 cm^{-1}. − UV/Vis (CH_3CN): λ_{max} (lg ε): 192 (4.382), 256 (4.222), 308 (3.324). − MS (70 eV, EI): *m/z* = 411 (15) [M]$^+$, 212 (100) [methyl phenothiazine].

3-Phenothiazin-10-yl-1-(1,4,7,10-tetraazacyclododecan-1-yl)propane Zinc(II) Bisperchlorate (5): $Zn(ClO_4)_2 \cdot 6\ H_2O$ (88 mg, 0.24 mmol) dissolved in methanol (2 mL) and **4b** (100 mg, 0.24 mmol) dissolved in methanol (3 mL) were combined and refluxed for 1 h under nitrogen. Most of the solvent was evaporated, the precipitated product was collected by filtration and dried in vacuo. Yield of **5**: (167 mg, 100%), as a white solid, m.p. 154 °C. − ^{1}H NMR (400 MHz, [D$_4$]MeOH): δ = 2.60−3.60 (m, 22 H), 6.80−7.40 (m, 8 H). − ^{13}C NMR (100 MHz, [D$_4$]-MeOH): δ = 26.5 (−), 34.5 (−), 43.3 (−), 44.7 (−), 45.5 (−), 48.4 (−), 48.6 (−), 48.8 (−), 49.9 (−), 49.2 (−), 49.4 (−), 49.8 (−), 117.1 (+), 123.9 (+), 123.9 (+), 126.9 (+), 128.3 (+), 128.6 (+), 144.3 (C_{quat}). − IR (KBr): $\tilde{\nu}$ = 3450, 3444, 2929, 1118 cm^{-1}. − UV/Vis (CH_3CN): λ_{max} (lg ε): 192 (3.160), 256 (2.646), 306 (0.338). − MS (FAB, NBA): *m/z* = 776 (20) [M^{2+} + 3 ClO_4^-] $^-$, 403 (100). − $C_{24}H_{37}N_5O_9SCl_2$: calcd. C 44.93 H 5.77 N 10.92; found C 44.89 H 5.79 N 10.89.

Acknowledgments

This work was supported by the Volkswagen Foundation and the Fonds der Chemischen Industrie. Scholarships from the Land Niedersachsen (MP) are gratefully acknowledged.

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Received December 18, 2000
[O00641]