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Electrochemistry of Metalloflavin Complexes in Dimethylformamide

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Electrochemical studies of a series of stable metalloflavin complexes in nonaqueous media are reported and compared to analogous work in water. While the effects of the nonaqueous media are not consistent, one general result is that both the flavin and metal ion reduction potentials are shifted negatively relative to their values in water. Coordination of Ru(II) at the N-5 site resolves the 1e-reduction processes of the flavin and strongly decreases the potential for addition of the second electron. The most striking result relative to studies performed in aqueous solution is the much larger separation of the two flavin reduction potentials. Results are discussed in light of their biological and catalytic significance.

Introduction

Speculation concerning the importance of metalloflavin chelates in biology dates back more than thirty years [1, 2]. Work by Hemmerich established that the oxidized (Fl_{ox}) and fully reduced (Fl_{red}) flavins are poor chelating agents but that flavosemiquinone anions (Fl·) may exhibit appreciable affinity for some metal ions [3, 4]. Moreover, it was shown that the presence of metal ions marginally stabilized flavosemiquinones by interacting at the N-5 site and so allowed for the study of these species by ESR [5]. Electrochemical studies by Sawyer further suggested a stabilization of the semiquinone form by several metal ions [7, 9]. Alkylation at N-5 provides for nearly quantitative formation of Fl· [10, 11] and implies that the 'blue' form of the flavodoxin electron-transfer protein contains HFl· with the proton residing at N-5.

Since flavins are almost ubiquitous in interfacing between single and double electron transfer involving metalloproteins and organic substrates, it was speculated that this electron-pair splitting and joining function might be facilitated by metal ion chelation [3, 4]. Despite much early enthusiasm for this possibility, only recently have a flavin and metal center been shown to come into sufficiently close proximity in a protein that direct (and possibly inner-sphere) electron-transfer might take place [12]. At the present time only iron sulfur moieties hold any likelihood for direct flavin linkage. In this event, the added ligand field surrounding the iron following flavin coordination should transform it from a high-spin to a low-spin state, so that Ru(II) interactions with flavins provide an adequate model system [13].

Ruthenium(II) complexes constitute the only series of compounds in which the electrochemical consequences of N-5, O-4 flavin chelation can be quantitated as a function of pH. Structural and spectroscopic characterization of these complexes has firmly established the chelation site in both solution and the solid state [13, 14] (see Fig. 1). The intense blue color of these compounds has now been attributed to a metal to ligand charge transfer of the type common for Ru(II) complexes with aromatic heterocycles [13, 15]. Electrochemical and resonance Raman spectroscopic studies have confirmed that their air-stable form involves Ru(II) coordinated to Fl_{ox} [15, 16]. In this communication we report on cyclic voltammetric studies of these compounds in a nonaqueous media (DMF), which was chosen as an approximation to the interior of a protein and compare these results with other studies made in aqueous solution [16, 17].

Experimental Section

Sample solutions consisted of millimolar concentrations of the ruthenium complexes as the chloride or trifluormethylsulfonate salts dissolved in DMF (Burdick and Jackson) adjusted to an ionic strength of 0.1 with tetraethylammonium perchlorate (TEAP). Solutions were purged with Ar or N_2 and were blanketed with the gas while measurements were

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TABLE I. Logarithmic Arguments to Eqn. (1) for Reduction Potentials of Ru–Fl Complexes as a Function of $[H^+]$.

Couple[a]	L_{DMF}	L_{H_2O}
Ru(III)–Fl_{ox}, Ru(II)–Fl_{ox}	$\left[\frac{[H^+]^2 + K_1^{ox}[H^+] + K_1^{ox}K_3^{ox}}{K_1^{ox}[H^+] + K_3^{III}K_1^{ox}}\right]$	Same
Ru–Fl_{ox}, Ru–$Fl^{\cdot}$	$\left[\frac{K_1^{ox}[H^+]^2 + K_1^{ox}K_1^{\cdot}[H^+]}{K_1^{\cdot}[H^+]^2 + K_1^{ox}K_1^{\cdot}[H^+] + K_1^{ox}K_3^{ox}K_1^{\cdot}}\right]$	$\left[\frac{K_1^{ox}[H^+]^2 + K_1^{ox}K_1^{\cdot}[H^+] + K_1^{\cdot}K_3K_1^{ox}}{K_1^{\cdot}[H^+]^2 + K_1^{ox}K_1^{\cdot}[H^+] + K_1^{ox}K_3^{ox}K_1^{\cdot}}\right]^d$
Ru–Fl·, Ru–Fl^{2-}_{red}	$\left[\frac{[H^+] + K_1^{\cdot}}{K_1^{\cdot}}\right]^b$	$\left[\frac{[H^+]^2 + K_3^{red}[H^+]}{[H^+]^2 + K_1^{\cdot}[H^+] + K_1^{\cdot}K_3^{\cdot}}\right]^c$

[a]K values are taken from those listed in Table III. Superscripts denote the oxidation state and subscripts indicate the deprotonation site on the isoalloxazine ring. [b]E in Table III used for this expression is for reduction of Ru–$Fl^{\cdot}$. [c]pK_3^{red} is estimated to be 10.9 from data available in reference 16. [d]m = 0.11 for Riboflavin; 0.088 for 10-MeIAlo; 0.1 for 3,10-Me_2IAlo.

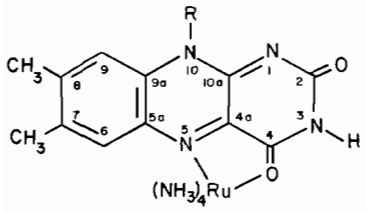

Fig. 1. Structure and numbering system for Ru–Fl complexes. R = ribotyl for riboflavin complex.

made. Cyclic voltammetric scans were normally made on an instrument constructed in this laboratory [18] at scan rates of 125 mV/sec on either a Metrohm HMDE or a Beckman platinum disk as the working electrode and a Pt wire auxiliary electrode. The reference potential was supplied by a double-junction Ag, AgCl reference electrode filled with 0.4 *M* tetraethylammonium chloride in water saturated with silver chloride [19]. Peak potential measurements were internally referenced against those for the ferrocene–ferrocinium couple (0.400 V) [20], but are reported relative to the s.h.e.

Acid concentrations were determined by a pH* scale defined in terms of the response of a Tacussel DMF B-10 glass electrode in conjunction with a DMF C-10 calomel reference electrode, which have been shown to give a Nernstian response to the concentration of hydrogen ion in DMF [21–23]. In the region from pH* = 0 to pH* = 4 the electrode system was calibrated using volumetrically prepared, fresh solutions of trifluormethylsulfonic acid (HTFMS) in DMF. Freshly vacuum distilled HTFMS is nearly anhydrous and completely ionizes in DMF and other non-aqueous solvents [24]. The 1.0 *M* stock solution was standardized by titration with standard, aqueous NaOH. Plots of calibrant pH* values *versus* the potential of the glass electrode yielded straight lines with slopes of 59 ± 5 mV/pH*. Identical behavior was observed using 70% perchloric acid (treated with acetic anhydride) as the calibrant [21–23, 25].

A spectrophotometric technique was devised for calibration of electrode response at higher pH* values using 2,4-dinitrophenol and *p*-nitrophenol, which have pK* values in DMF of 6.34 and 12.19, respectively [25, 26]. In the region of the pK_a, the pH* of the solution may be calculated from the measured absorption of the deprotonated nitrophenol at approximately 430 nm using the following equation:

$$pH^* = pK_a - \log [(A' - A)/A]$$

where A′ is the absorbance of the completely ionized species and A is the absorbance of the buffer solution being calibrated. Typically about 1 mg of the sodium nitrophenolate salt was dissolved in 50 ml of 0.1 *M* TEAP with a very small amount of 25% tetraethylammonium hydroxide (in methanol) added to ensure complete deprotonation. After measuring A′, the pH* was adjusted with 10–50 μL portions of 0.1 *M* HTFMS in DMF and the calibrant value of pH* determined. Response curves yielded linear slopes of 59 mV/pH* within 1.5 pH* units of the pK_a.

Pulse polarographic measurements in aqueous buffers were made using a PARC Model 174A polarographic analyzer in conjunction with a PARC Model 303 static mercury drop electrode (SMDE) and an Ag, AgCl reference electrode. Drop times were typically 1 second with a pulse height of 50 mV and a scan rate of 1–2 mV/sec. In samples requiring a surfactant to minimize spurious polarographic peaks, 1–2 drops of 0.1% Triton X-100 were added. Reduction potentials in aqueous solution were calibrated

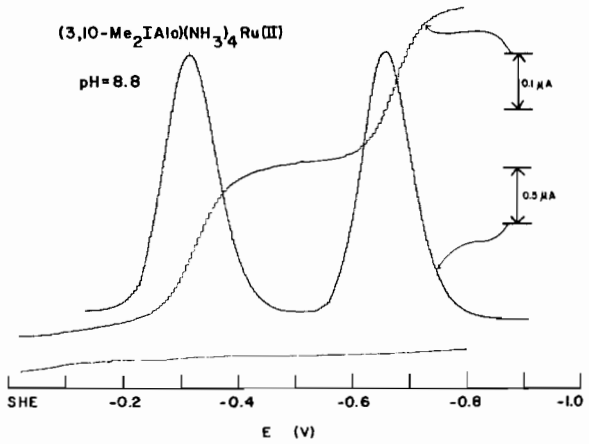

Fig. 2. Differential pulse polarographic scan of the 10-MeIAlo complex in water at pH 7.

using the $[(NH_3)_6Ru]^{3+,2+}$ couple (57 mV) as the internal standard [27].

Electronic spectra were taken at 25 °C on a Perkin-Elmer Model 575 spectrophotometer equipped with a digital background corrector. Semiquinone complexes were prepared for spectrophotometric study by prior reduction with chromous chloride in 0.1 *M* TEAP in DMF added dropwise.

Acid dissociation constants for the Fl_{ox} complexes were measured spectrophotometrically by titrating basic solutions of the metalloflavin complex with standard HFTMS solutions at an ionic strength of 0.1 *M* (TEAP) in DMF. The standard spectrophotometric pK_a equation was employed [28] with pH* measurements made as indicated above. Isosbestic points were observed in all determinations. Values of pK_a for the semiquinone complexes were determined from least squares fits to plots of reduction potential *vs.* pH* using the following general equation [29, 30]:

$$E_h = E + m[\log(L)] \quad (1)$$

where E_h is the reduction potential for the couple at a given pH, E is the pH-independent reduction potential in neutral media, m is the slope in the pH-dependent region (59 mV/pH in water), and the logarithmic argument, L, is given in Table I.

Results

Aqueous Polarography

Pulse and differential pulse polarographic (PP and DPP) measurements made in aqueous media showed two well-resolved reduction processes for the isoalloxazine complexes. Measurements made without

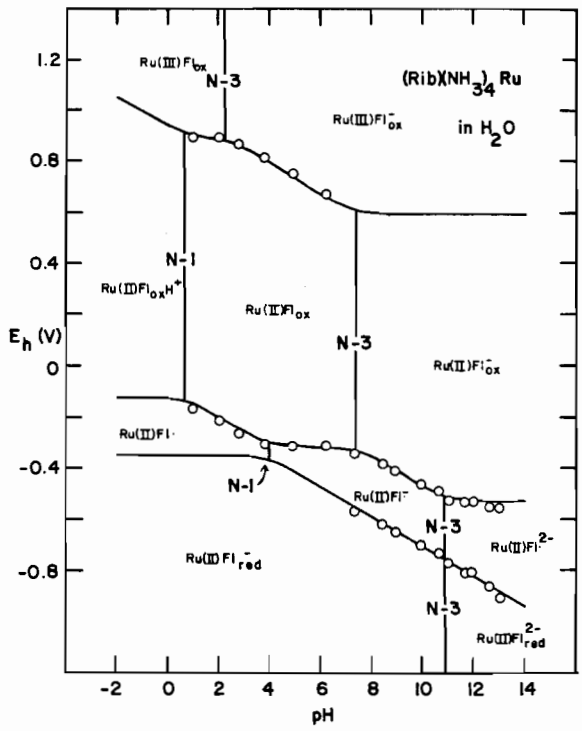

Fig. 3. Plot of E_h *versus* pH for Ru–Rib in water.

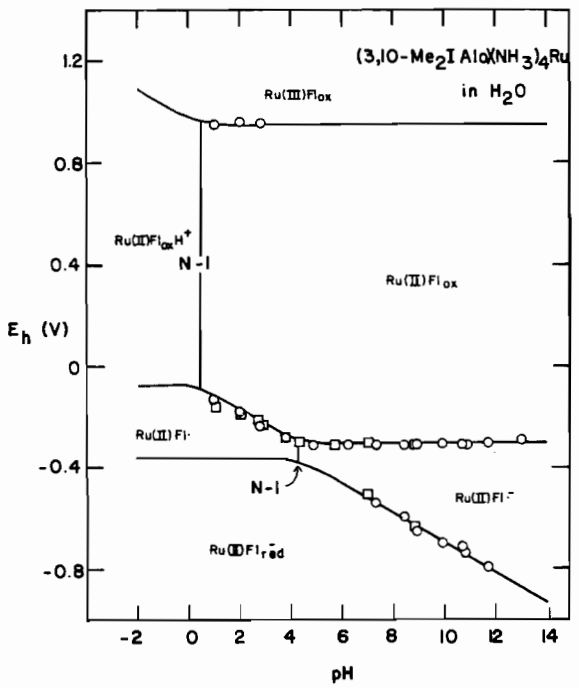

Fig. 4. Plot of E_h for Ru-(3,10Me_2IAlo) in water, circles represent CV points and boxes points obtained by DPP.

TABLE II. Reduction Potentials for Ru–Flavin Complexes in DMF and Water.[a]

Couple	Ligand					
	Rib		10-MeIAlo		3,10-MeIAlo	
	DMF	Water	DMF	Water	DMF	Water
HFl^{+}_{ox}–$HFl^{\cdot}$	–0.34	–0.15	–0.37		–0.35	
Fl_{ox}–$Fl^{\cdot-}$	–0.64	–0.32	–0.62		–0.60	
$HFl^{\cdot}$–HFl^{-}_{red}		–0.35				
$Fl^{\cdot-}$–Fl^{2-}_{red}	–1.41		–1.41		–1.43	
Ru(III)–Fl_{ox}–Ru(II)Fl_{ox}	0.62	0.89	0.59	0.95	0.68	0.94

[a]Values for reduction potentials in water are best values derived from CV data given in reference 16 and PP and DPP data given in this work.

TABLE III. Reduction Potentials for Free Flavins in Aqueous and Nonaqueous Media.

Couple	E(V) in		
	DMF	DMSO	H_2O
$EtFl^{2+}$–$EtFl^{+}_{ox}$	1.04[b]		
$EtFl^{+}_{ox}$–$EtFl^{\cdot}$	0.42[b]		0.43[g,i]
Fl_{ox}–$Fl^{\cdot-}$	–0.55[c,d]	–0.56[h,f]	–0.33[g,e]
H_2Fl^{+}–Fl_{red}	0.14[c,d]		
$HFl^{\cdot-}$–Fl_{red}	–0.30[c,d]		–0.15[g,e]
$EtFl^{\cdot-}$–$EtFl_{red}$	–0.21[b]		–0.01[g,i]
$Fl^{\cdot-}$–Fl^{2-}_{red}		–1.2[a,e] –1.38[a,h,f]	

[a]Cathodic peak potential. [b]5-Ethyl-3-methyllumflavin, see reference 10. [c]Tetraacetyl riboflavin. [d]Taken from references 21 and 22. [e]Riboflavin. [f]See references 8 and 33. [g]See Table II of reference 16 and citations therein. [h]3-Methyllumiflavin. [i]5-Ethyl-7,8,10-trimethylisoalloxazine.

a surfactant (Triton X-100) present, showed large, variable peaks which may be due to streaming effects or to the various time and pH dependent adsorption phenomena known to occur with these complexes [16]. By analogy to earlier cyclic voltammetric (CV) work, the wave centered around –0.3 V in Fig. 2 corresponds to the 1e-electron reduction of $(NH_3)_4$Ru(II)–Fl_{ox} to yield the corresponding semiquinone species, while that centered at –0.64 V is for the addition of a second electron to form Ru–Fl_{red} [16]. Potentials measured for both reductions correlate well with the CV data over the entire pH range (see Figs. 3 and 4).

Below pH 7 the polarographic wave for the reduction of the semiquinone complexes decreased in amplitude with decreasing pH, which is consistent with acid-catalyzed dissociation of Ru–Fl_{red}. The introduction of this chemical reaction following reduction causes the polarographic half-wave potentials to shift negatively so that they do not correlate in a linear fashion with those above pH 7 [31]. Reversible reduction potentials as a function of pH for the 10-methylisoalloxazine (10-MeIAlo) and 3,10-dimethylisoalloxazine (3,10-Me_2IAlo) complexes as determined by CV and DPP are summarized in Table II.

Cyclic Voltammetry in DMF

As illustrated in Fig. 5 cyclic voltammetry of the 10-MeIAlo, 3,10-Me_2IAlo and riboflavin (Rib) complexes in dimethylformamide revealed three well resolved sets of peaks corresponding to those observed in aqueous media [16]. These have been assigned (in order of decreasing reduction potential) to the Ru(III–II), Fl_{ox}–$Fl^{\cdot}$, and $Fl^{\cdot}$–Fl_{red} couples.

Metal Ion Oxidation

Below pH* 2 all three complexes exhibited essentially chemically reversible behavior for the Ru(III–II) couple with pH* independent reduction poten-

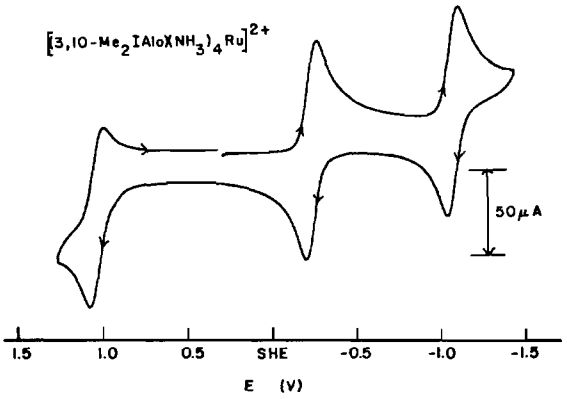

Fig. 5. CV scan of 3,10-Me_2IAlo complex in neutral DMF at an ionic strength of 0.1 adjusted with TEAP.

tials as listed in Table II. In the case of the 3,10-Me_2IAlo complex this behavior held up to pH* 11.2, while for the riboflavin complex such behavior was present only below pH* 5.5. Above these pH* values the cathodic peak decreases markedly and the anodic peak shifts toward more positive potentials. Anodic–cathodic peak separations ($E_{pa} - E_{pc}$) in the quasireversible regions were typically around 95 mV, which is nearly in the range observed for the ferrocene–ferrocinium couple under the same conditions.

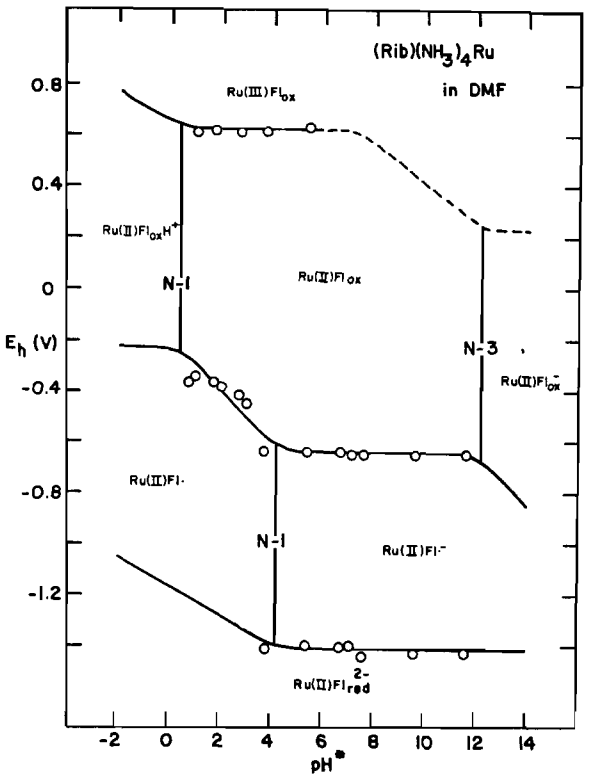

Fig. 6. Plot of E_h *versus* pH* for Ru–Rib in DMF. A pK_a value of approximately 5.5 is estimated for N-3 deprotonation of the Ru(III) complex (see text).

Flavin Reduction

In contrast to the case in water the flavin CV peaks in DMF were manifestly free of adsorption phenomena. Over the pH* region where the CV peaks involving the flavin ligands were determined to be chemically reversible (see below), these couples also approached electrochemical reversibility (at 125 mV/sec) as judged on the basis of anodic and cathodic current peak separations being similar to those exhibited by the ferrocene–ferrocinium internal standard [20].

Reduction potentials for $(Rib)(NH_3)_4Ru(II)$ as a function of pH in water and DMF are graphically presented in Figs. 3 and 6 respectively, and are listed for all complexes in Tables I and II. In all cases the coordinated Fl_{ox}–Fl· couple was found to be chemically reversible over the entire pH* range as determined by equivalent anodic and cathodic peak currents at a scan rate of 125 mV/sec. All three complexes exhibited similar variation in reduction potentials (E_h) as a function of pH* (*cf.* Fig. 6 and Table II). When allowed to vary in a standard least squares method [30], the best values obtained for the logarithmic slope (m in eqn. 1) were significantly higher than 59 mV/pH*. However, other evidence (see discussion section) dictates the addition of a single proton upon 1e-reduction of Ru–Fl_{ox}. Values of pK_a determined spectrophotometrically agreed well with those determined electrochemically.

Since the three complexes showed spectroscopic behavior similar to that exhibited in aqueous solution for similarly protonated forms, proton addition to form coordinated HFl^+_{ox} was determined to occur at N-1 (see Fig. 7). In contrast to the results in aqueous solution, electrochemical behavior deriving from proton loss at the N-3 site, was barely evident for the 10-MeIAlo and Rib complexes owing to solvent interference and possible complex decomposition in the pH* range above 12.5. However, this ionization was readily observed in spectrophotometric titrations, since the solvent decomposition products were not strongly absorbing.

In the pH* range of 4–12, CV showed the coordinated HFl·–Fl_{red} couples to be quasireversible by the criterion given above. Over this broad pH* range both flavin reduction potentials remained constant. Below pH* 4 the anodic peak decreased markedly with respect to the cathodic revealing chemically irreversible behavior in this region, which probably arises from ligand dissociation.

TABLE IV. pKa Values for Ru–Fl Complexes.

Ligand	Ionization Site	Ligand[a]					
		Rib		10-MeIAlo		3,10-Me_2IAlo	
		DMF	Water	DMF	Water	DMF	Water
Fl_{ox}[b]	3		2.3		2.0		
HFl^+_{ox}	1	0.4	0.7	0.4	0.60	0.2	0.47
Fl_{ox}	3	12.2	7.4	12.1	7.37		
HFl·	1	4.2	3.98	4.4	4.1	3.8	4.3
Fl·	3	⩾13[c]	10.9	⩾13[c]	11.3		

[a]Values in aqueous media are from reference 16. [b]Ru(III) complex. [c]Determined to be greater than pH* 13, the range in which the solvent decomposes.

TABLE V. pK_a Values for Free Flavins in DMF and Water.

Flavin	Ionization Site	pK_a	
		DMF[a]	H_2O[b]
Fl_{ox}	3	14.3	10.0
$HFl^{\pm}$	1	3.0	
HFl·	5	11.3	8.3
Fl_{red}	1	10.15	6.7

[a]See references 21 and 22. [b]See citations in reference 16.

Discussion

Metal ion coordination of the flavin profoundly affects both the ionization and electrochemical properties of the isoalloxazine ring resulting in: 1) elimination of the overlap in the ionization equilibria of Fl_{ox}, HFl· and H_2Fl_{red}, so that areas of essentially constant, pH-independent potentials appear, and 2) destabilization of the fully reduced form of the flavin so as to cause a reversal in the order of the two overlapping flavin 1e-reduction potentials and their resolution into two distinct processes. These effects are enhanced in dimethylformamide in ways which could not have been easily predicted. However, one general and understandable result is that all reduction potentials are shifted negatively relative to the comparable couples in water.

Proton Equilibria

The electrostatic effect exerted upon chelation of Ru(II) by the flavin N-5 and O-4 sites increases the acidity of the N-3 site of Fl_{ox} by about two orders of magnitude relative to that observed for free Fl_{ox} in DMF (*cf.* Tables IV and V). In contrast, the ionization constant for coordinated HFl· is about seven orders of magnitude greater than that for the corresponding free ligand. While a small electrostatic effect may be operative, this substantial change is largely due to the metal's forcing the formation of a higher-energy tautomer so that deprotonation is from N-1 rather than N-5 as occurs in the free flavin.

In going from water to DMF some of the solvent effects on proton equilibria are quite similar to those exhibited by free flavins. Reference to Table V reveals that free flavins typically show an increase in their various pK_a values of 3–4 units. This is reflected in the increases in pK_a values for N-3 deprotonation from coordinated Fl_{ox} and Fl· of 3.8 and >2 in transferring from aqueous to nonaqueous media. Such changes can be attributed to the stronger electron pair donor characteristics of DMF, and its decreased hydrogen bonding ability relative to water. These effects tend to localize electron density onto the isoalloxazine ring and thereby decrease its acidity.

The case is different, however, for proton loss from the N-1 sites of coordinated HFl_{ox} and HFl· which exhibit acidity constants similar to those obtained in aqueous media. Stabilization of the deprotonated forms of these complexes may result from the lower overall dielectric constant of DMF favoring the lower charged species so as to offset the usual cation stabilization observed in DMF owing to its relatively higher Lewis basicity [32].

The similarity in the spectra of the various coordinated flavin and flavosemiquinone species (see Fig. 7) with those observed in aqueous solution, whose protonation sites have been definitively assigned [13, 14, 16], confirms their assignment in DMF. Since there is no evidence for proton equilibria involved in the reduction of $Ru–Fl^{\cdot-}$, it must be

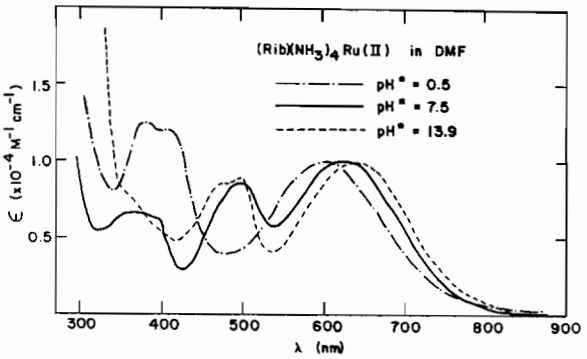

Fig. 7. Spectra of Ru–Rib_{ox} in various protonation forms in DMF.

concluded that coordinated Fl^{2-}_{red} is formed. Reference to Fig. 6 then reveals that the pK_a of coordinated HFl^{-}_{red} can be no greater than 4. This implies an *increase* in the acidity of this species by at least seven orders of magnitude relative to the case in water. Again, it can be inferred that the lower dielectric constant of DMF favors the neutral complex and that specific interactions with solvent lone pairs are of decreased importance.

Reduction Potentials

In contrast to what might be expected for adding an electron to a dipositive rather than monopositive species, the reduction potentials of the 5-metallated flavins are approximately 0.9 V more negatvie than those for 5-alkylated flavins. Even relative to neutral Fl_{ox}, Ru(II)–Fl_{ox} is more difficult to reduce by about 0.1 V. These effects are due to the substantial back donation of electron density onto the isoalloxazine ring from a filled d_π-orbital on the metal capable of interacting with the lowest lying empty π^*-orbital on the ligand. Owing to this extensive retrodative bonding it becomes significantly more difficult to place an additional electron into the same orbital.

In going from water to DMF the coordinated Fl_{ox} reduction potentials shift negatively by approximately the same amount as that of the corresponding free flavin (*cf.* Tables II and III). This destabilization of the more reduced form is in keeping with the solvent characteristics of DMF, which should tend to increase electron density on the solute thereby making it more difficult to reduce.

Failure to form a more protonated form upon addition of an electron accounts for the extremely negative potential for the reduction of Ru–$Fl^{\cdot-}$ relative to the case in aqueous media. The formation of Ru–Fl^{2-}_{red} as the single, stable, fully-reduced species in DMF is primarily responsible for the enhanced separation between the first and second flavin reduction potentials in DMF *versus* water at neutral pH (*cf.* Figs. 3 and 6). In aqueous media this reduction is facilitated by simultaneous proton addition.

Complex Stability

Similar to observations made in aqueous media, both the semiquinone and fully-reduced flavin complexes dissociate by an acid-catalyzed mechanism in DMF; however, owing to the lack of protonation previously mentioned for Ru–Fl^{2-}_{red}, the fully-reduced form persists at higher acid concentrations in the nonaqueous solvent.

Ru(III) Complexes

At low pH it is possible to reversibly oxidize the parent compounds to yield Ru(III) species. Owing to the greater electrostatic effect of this metal ion, which may be enhanced by transfer of flavin π-electron density into the half-vacant metal d_π-orbital, in water the acidity of the N-3 proton in the riboflavin and 10-MeIAlo complexes is enhanced by 12 orders of magnitude relative to the free ligand and 5 relative to the Ru(II) complexes. Ionization at the N-3 site can be definitively assigned since the 3,10-Me_2IAlo complex exhibits no pH dependency for the Ru(III–II) couple in both aqueous and nonaqueous media.

Since irreversible electrochemical behavior appears for the riboflavin and 10-MeIAlo complexes once deprotonation of the Ru(III) complex has been attained, whereas the 3,10-Me_2IAlo complex can be reversibly oxidized at relatively high pH*, N-3 deprotonation is implicated in the degradation of these complexes. This suggests a metal ion induced oxidation of the ligand, since electron transfer should more readily occur from the anionic ligand. The usual solvent characteristics of DMF *versus* water readily account for the lowered potential for the Ru(III–II) couple in the nonaqueous solvent (*cf.* values in Table II).

Conclusion

While an understanding at the molecular-orbital level is still awaited, this study is consistent with recent electrochemical results on N-5 alkylated flavins [10, 33]. It is now fairly certain that 1e-transferring flavins in proteins involve some sort of Lewis acid coordination (usually protonation) at N-5, which serves to stabilize the added electron density that localizes at this site in $Fl^{\cdot}$ [5]. It further implies that, *if* flavin coordination by an iron–sulfur center does occur in a protein, the two 1e-reduction potentials of the flavin should be widely separated and reduction need not be accompanied by simultaneous proton transfer to the flavin. It is expected that the coordinated Fl_{ox}–$Fl^{\cdot}$ couple should be in a biologically accessible range, whereas the fully-

reduced form would probably not be attainable. Moreover, if substantial back donation of electron density onto the flavin occurs, the first flavin reduction potential should be somewhat lower than that of the free flavin and very much lower than that of a flavin protonated or hydrogen bonded at N-5.

The recent report of catalytic oxidation of alcohols and ascorbic acid by a Zr(IV)–Fl complex in nonaqueous media suggests that metalloflavin complexes may become useful in the catalysis of synthetic reactions requiring a 2e–1e interface [34]. In this regard it is important to realize the distinct differences in electrochemical effects exerted on the flavin by coordination of 'hard' *versus* 'soft' (π-donor) metal ions. Both will tend to resolve the two flavin reduction potentials to an extent dependent upon the degree of association at the N-5 site. However, soft metal ions should decrease the values of these potentials, while hard metal ions will induce an opposite effect.

Acknowledgement

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