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Resonance Raman Spectra and Structure of Flavins Bound to $(NH_3)_4Ru^{II}$

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Resonance Raman (RR) spectra are reported for $(NH_3)_4Ru^{II}$ complexes of riboflavin (RBF) and 10-methylisoalloxazine (10-MeIAlo), excited at 647.1 nm, in resonance with a strong absorption band that is assigned to a $Ru^{II} \rightarrow$ flavin charge transfer. RR bands II and III, which are associated with the pyrazine ring, shift down on complexation of RBF to $(NH_3)_4Ru^{II}$, similar to the shifts in flavin derivatives with electron-donating C8 substituents, which stabilize a "quinoid" resonance structure, but opposite to the shift observed on semiquinone formation. The downshift is interpreted as a consequence of $Ru^{II} \rightarrow$ flavin back-bonding, which stabilizes a resonance form in which the N5–C4a bond is lengthened. The Ru^{II} complexes show especially strong enhancements of bands in the 1150–1350-cm^{-1} region, associated with pyrazine and uracil modes, which are plausibly involved in the molecular distortion of the charge-transfer excited state. Rich spectra are also observed below 1000 cm^{-1}, involving in-plane deformation modes, several of which involve large uracil contributions, as evidenced by shifts on H/D exchange, ionization, and methylation at N3. A strong band of $(NH_3)_4Ru^{II}$(RBF) at 275 cm^{-1} is tentatively identified as the Ru–N5 stretching mode, although in $(NH)_4Ru^{II}$(10-MeIAlo) this band is replaced by a 285–310-cm^{-1} doublet. There are a number of RR spectral differences between the RBF and 10-MeIAlo complexes that are not understood.

Introduction

The flavin chromophore has been studied extensively via resonance Raman (RR) spectroscopy.[1-8] The bands of oxidized flavin have been cataloged and analyzed with the aid of a normal-coordinate calculation.[9] Detailed spectra have also become available for the semiquinone form.[10] Recently synthesized Ru^{II}–flavin complexes,[11,12] in which Ru^{II} is bound to the flavin N5 and O4 atoms and to four ammonia ligands (see Figures 1 and 2), are of interest as possible models for metal–flavin interactions in biology. In these complexes, the oxidation level of the flavin is uncertain. Structural and spectroscopic data indicated extensive $Ru^{II} \rightarrow$ flavin electron donation and led to the suggestion that the complexes might best be described as containing flavin, semiquinone, and Ru^{III}.[11,12] RR spectroscopy is capable of distinguishing oxidized from semiquinone forms of flavin and is sensitive, in general, to the electronic structure of the chromophore. We find that these complexes give excellent RR spectra, free of flavin fluorescence. The ring-mode frequencies are shifted appreciably relative to those of oxidized flavin, but not in the direction observed for semiquinone.[10] The intensity pattern is also different from either oxidized or semiquinone forms, bands between 1100 and 1300 cm^{-1} becoming especially strong. Many bands are seen below 1000 cm^{-1} that are sensitive to N3 deuteration or ionization and to changes in the flavin substituents.

Experimental Section

The complexes $[(10\text{-MeIAlo})(NH_3)_4Ru](PF_6)_2 \cdot H_2O$, $[(3,10\text{-}Me_2Alo)(NH_3)_4Ru](PF_6)_2 \cdot H_2O$, and $[(RBF)(NH_3)_4Ru]Cl_2 \cdot H_2O$ were prepared as previously described.[11] Samples were dissolved in 0.1 M sodium phosphate buffer to a concentration of 1 mM. The pH (pD) was adjusted to 2 or 12 to protonate (deuterate) or deprotonate the N3 position (pK_a = 7.4).[11] The N3-methylated complex was dissolved in pH 7 phosphate buffer.

Raman spectra were obtained with 647.1-Å Kr^+ laser (Spectra Physics 170) excitation (150 mW), via 135° back-scattering from spinning NMR tubes, and a Spex 1401 double monochromator (spectral slit width 7 cm^{-1}) equipped with a photomultiplier and photon-counting electronics. The data were collected digitally by a MINC minicomputer in 0.5-cm^{-1} increments at 2 s/point and smoothed by Fourier filtering.

Results and Discussion

Bands I, II, and III and the Flavin Structure. Figure 1 shows the chemical structure and atom numbering for flavins. The *o*-xylene, pyrazine, and uracil rings are labeled I, II, and III, respectively. Riboflavin (RBF) has methyl substituents at C8 and C7 and a ribityl group at N10. 10-Methylisoalloxazine (10-MeIAlo) has a methyl group at N10 and hydrogen atoms at C8 and C7.

Figure 2 shows the structure of the Ru^{II}–flavin complexes,[11,12] while Figure 3 compares their absorption spectrum with that of oxidized flavin, which has characteristic bands at 450 and 370 nm. In the Ru^{II} complexes, these are still seen, with slight wavelength shifts, and in addition there is a strong new band near 620 nm.[11] Neutral flavin semiquinones also absorb at long wavelengths but have two bands, at ~580 and ~520 nm, that are understood to correspond to the two oxidized flavin transitions (π–π^*), strongly red-shifted due to the open-shell structure.[18] Thus, the absorption spectrum of the complex (which has a closed shell) is best attributed to a somewhat perturbed oxidized flavin with a long-wavelength $Ru^{II} \rightarrow$ flavin charge-transfer (CT) band.[11,12] Figure 4 compares the 488.0-nm RR spectrum of oxidized riboflavin bound to riboflavin-binding protein (RBP), which quenches the flavin fluorescence,[2] with the 647.1-nm RR spectrum of the Ru^{II}–riboflavin (Ru–RBF) complex, in its N3–H, N3–D, and $N3^-$ forms (N3 is the nitrogen of the uracil ring that contains the only ionizable proton of flavin). When the N3-ionized form was prepared in D_2O (pD 12) there were no changes in the spectrum, either above or below (vide infra) 1000 cm^{-1}, showing that none of the RR features are due the coordinated

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Table I. Flavin RR Frequency (cm^{-1}) Correlations above 1000 cm^{-1}

band label[a]	RBP	N5-Me (FMN semiquinone)[b]	$(NH_3)_4Ru^{II}$(RBF) pH 2	pD 2	pH 12	$(NH_3)_4Ru^{II}$(10-MeIAlo) pH 2	pD 2	pH 12	N3-Me
			1714	1710	1730	1700		1703	1714
I	1631		1614	1613	1621	1611			
II	1584	1613	1566	1567	1567	1579	1578	1581	1593
III	1548	1590	1528	1523		1528	1527	1548	1528
IV	1503	1516				1505	1500	1516	1502
V	1465	1443			1456				
VI	1407	1376	1406						
VII	1355	1334	1345	1344	1341	1342	1342	1340	1343
VIII	1302		1295	1295	1287	1300	1301	1309	1301
IX	1282	1282	1270	1270	1266	1265	1267	1288	1266
X	1252	1224	1234	1234	1233	1233	1245	1260	1248
XI	1229	1179	1186	1201	1186	1208	1210	1195	1222
XII	1170		1166	1157			1156	1173	1190
XIII	1161		1131	1131	1129	1129	1129	1128	1165
			1019	1022	1022	1031	1041	1044	1132
									1109
									1066

[a] Oxidized flavin mode numbering as in ref 9. Correlations of Ru-flavin bands below 1300 cm^{-1} with modes IX-XIII are questionable because of different isotope shift patterns (see text). [b] From ref 10.

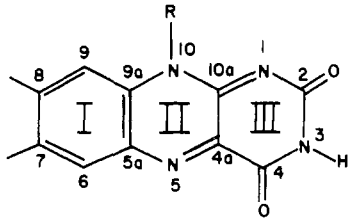

Figure 1. Flavin ring and atom numbering.

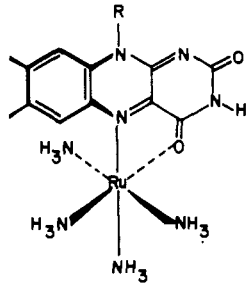

Figure 2. Structure of flavin complexes with $(NH_3)_4Ru^{II}$. In the $(NH_3)_4Ru^{II}$(10-MeIAlo) crystal structure,[11] the halves of the flavin ring are folded by 9.9° about the N5–N10 axis.

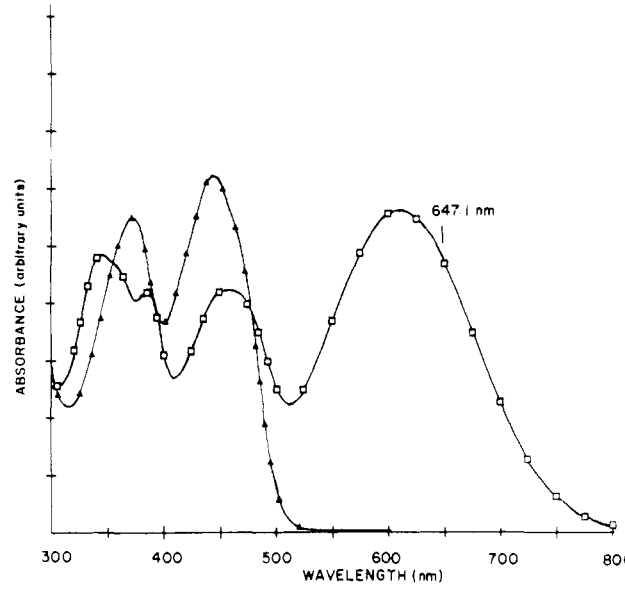

Figure 3. Absorption spectra of riboflavin (▲) in 0.1 M sodium acetate buffer, pH 5 and of $(NH_3)_4Ru^{II}$(RBF) (□) in 0.1 M sodium phosphate buffer, pH 2. The position of the 647.1-nm excitation line is marked.

NH_3 molecules (since H/D exchange at NH_3 does take place under these conditions[11]).

The RR frequencies of RBP above 1300 cm^{-1} can readily be correlated with those of Ru–RBF, as shown in Table I. The high-frequency bands, 1631, 1584, and 1548 cm^{-1}, labeled I, II, and III,[9] all shift down by ~20 cm^{-1}. In flavin semiquinone, however, the 1631-cm^{-1} band is not seen, while the 1584- and 1548-cm^{-1} bands shift *up* to 1613 and 1590 cm^{-1}.[10] This upshift is believed to be associated with protonation (or methylation) of the pyrazine ring N atom (N5), in a basically unperturbed flavin structure,[12] since protonation of pyrazine itself is known to produce a nearly identical shift.[14] The binding of Ru^{II} to N5 should have a similar effect, and the downshifts observed instead must reflect a substantial perturbation of the flavin structure.

The downshifts in bands II and III are similar to those observed for flavins with electron-donating substituents at the C8 position[6,7] (e.g., 8-$(CH_3)_2$N–RBF (1558 and 1519 cm^{-1}), which stabilize the "quinoid" resonance structure of flavin (Figure 6B). While this structure is not itself supported by Ru^{II} complexation, a related one, shown in Figure 6D, can be produced via back-bonding from Ru^{II} to the flavin π system. Support for back-bonding comes from the crystal structure of the 10-methylisoalloxazine (10-MeIAlo) complex,[11] which has a short (1.98 Å) Ru–N5 bond distance, N5–C4a and C4–O bonds that are significantly (0.05 Å) longer than those of oxidized flavins, and a C4–C4a bond that is significantly (0.07 Å) shorter. The altered conjugation pathway reflected in the lengthened N5–C4a bond may account for the lowered frequencies in bands II and III in the Ru^{II} complex and also in 8-substituted flavins favoring the quinoid structure. As might be expected, the parallelism breaks down when band I, which is localized on the *o*-xylene ring,[9] is compared. Its frequency is 20 cm^{-1} lower in Ru^{II}–RBF than in RBP, whereas for 8-substituted flavins its frequency initially rises with increased quinoid contribution but decreases when the quinoid contribution is dominant,[6] as reflected in very low values of the band II and III frequencies (e.g., 1614, 1536, and 1504 cm^{-1} for bands I, II, and III of lactate oxidase substituted with 8-S^-–FMN[6]).

Figure 5 shows RR spectra of the 10-MeIAlo complex and its N3-deuterated, -ionized, and -methylated forms. The

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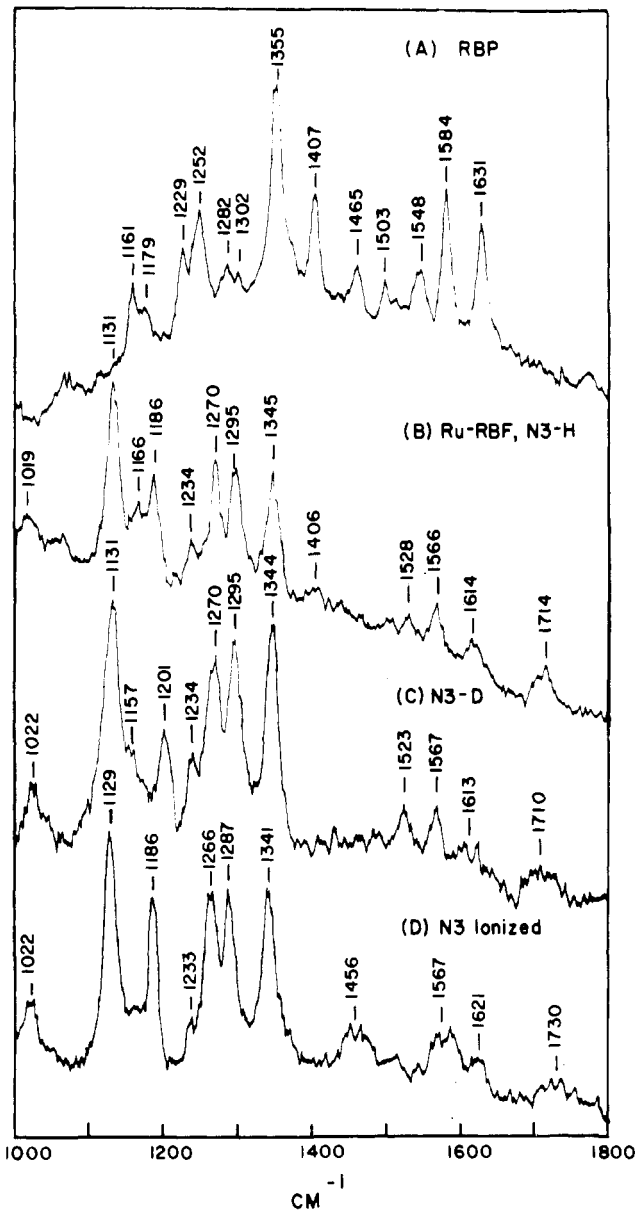

Figure 4. RR spectra, above 1000 cm^{-1}): RBP (A) (488.0 nm, 100 mW, 10-cm^{-1} slit width); $(NH_3)_4Ru$(RBF) at pH 2 (N3–H form) (B), pD 2 (N3–D form) (C), and pH 12 (N3-ionized form) (D) (647.1 nm, 150 mW, 7-cm^{-1} slit width).

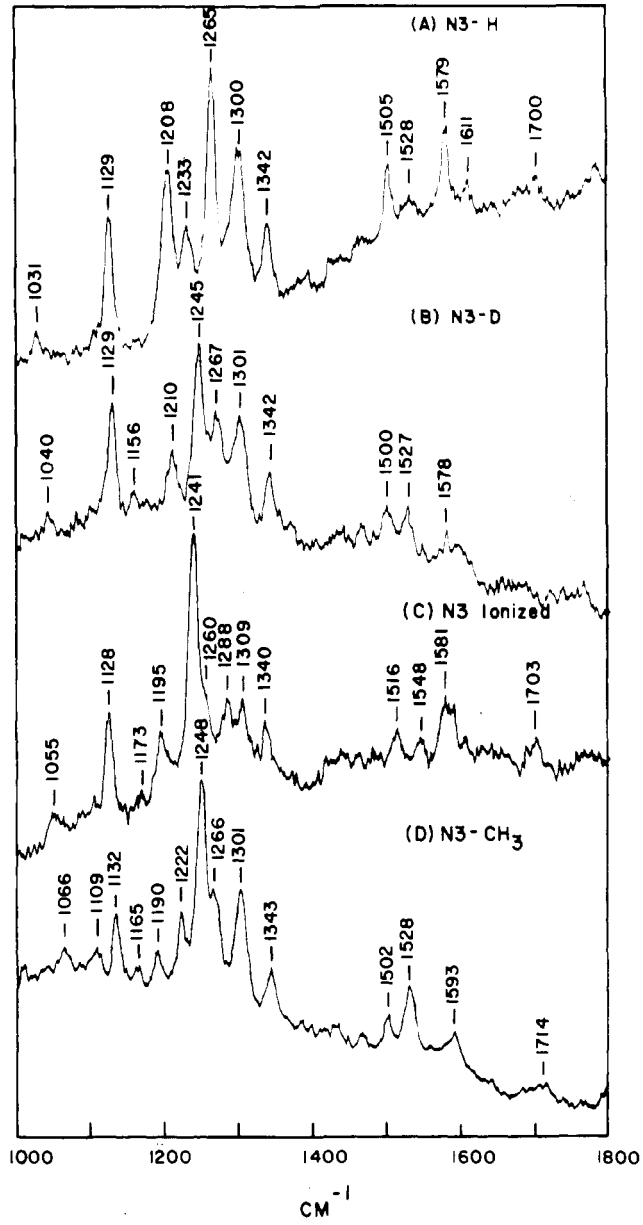

Figure 5. RR spectra, above 1000 cm^{-1}: $(NH_3)_4Ru$(10-MeIAlo) at pH 2 (N3–H form) (A), pD 2 (N3–D form) (B), and pH 12 (N3-ionized form) (C) and the N3-methylated complex (D) (647.1 nm, 150 mW, 7-cm^{-1} slit width).

frequencies of bands I and III are the same as those of Ru^{II}–RBF, but the band II frequency is 13 cm^{-1} higher (1579 vs. 1566 cm^{-1}). Thus, the 7- and 8-methyl groups appear to have some effect, presumably via their electron-donating propensity, on the structure of the pyrazine ring of the bound flavin.

Other High-Frequency Modes. Resonance Enhancement. Resonance enhancement depends on the extent of the geometric distortion in the resonant excited state along the normal modes of vibration.[15] In contrast to RBP, the Ru^{II}–flavin complexes show weak enhancement for bands above 1400 cm^{-1} but strong enhancement for bands between 1100 and 1350 cm^{-1}. Most of these correspond to modes that are calculated[9] to have major contributions from the pyrazine and uracil rings. If, as suggested above, the long-wavelength band of the complexes is due to a $Ru^{II} \rightarrow$ flavin charge transfer, the excited state would involve the lowest flavin π orbital mainly, with some Ru d_π character mixed in. Indeed, the resonance structure shown in Figure 6D is probably a better description of the excited state than of the ground state. The dominance of RR bands arising from modes with large ring II and III contributions can readily be understood on the basis of the likely distortion of the flavin molecule in the charge-transfer excited state (lengthening of the N5–C4a and C4–O bonds and shortening of the C4–C4a bond). Better calculations are needed to determine whether the eigenvectors of the enhanced modes are actually in conformity with the expected distortion.

The correlation of Ru^{II}–flavin bands below 1300 cm^{-1} with bands IX–XIII of oxidized flavin[9] (Table I) is questionable because of appreciable shifts in frequencies, and also changes in the normal-mode compositions, as evidenced by altered isotope shift patterns. Thus, N3 deuteration shifts the 1186-cm^{-1} Ru^{II}–RBF band *up* to 1201 cm^{-1}, whereas in metal-free flavin it is the 1252-cm^{-1} band X that shifts up to 1290 cm^{-1}. Thus, band X actually seems to be lowered from 1252 to 1186 cm^{-1} upon Ru^{II} complexation, and the N3–D upshift is attenuated. This upshift, also observed for the 1236-cm^{-1} mode of uracil, is calculated to result from a change in the relative contributions of C=O bending, C–N stretching,

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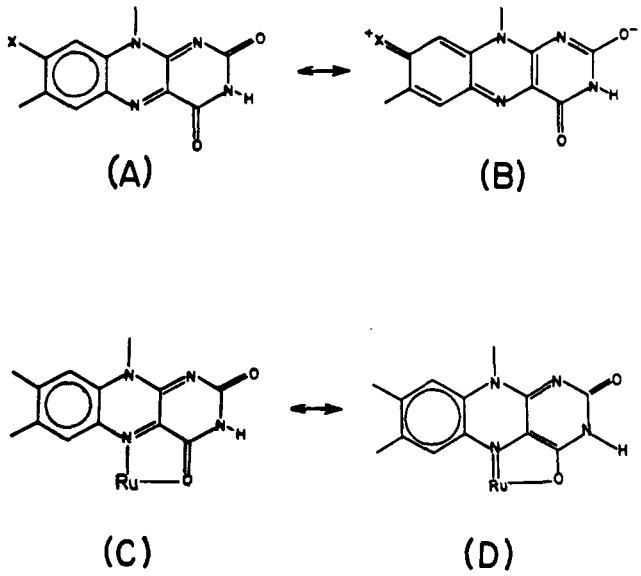

Figure 6. Flavin resonance forms: (A) predominant form for oxidized flavin; (B) quinoid form, stabilized by electron-donating groups, X, at C8; (C) form for metal bound to N5 and O4 of oxidized flavin; (D) form stabilized by Ru $d_\pi \rightarrow$ flavin π^* back-donation.

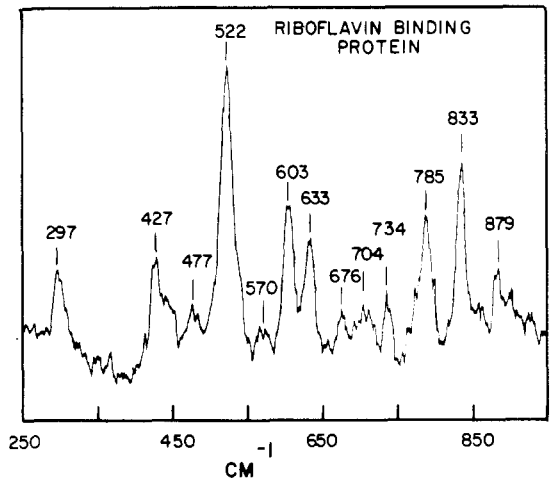

Figure 7. RR spectrum of RBP, below 1000 cm^{-1}. Conditions are as in Figure 4A.

and N–H(D) bending in the normal mode.[16] Thus, binding of O4 by Ru^{II} might well shift the mode and change its composition. Band X in metal-free flavin shows considerable variability among various proteins and has been suggested to be responsive to H bonding.[16] However, we observed no change in the Ru–RBF spectrum when the complex was dissolved in dimethylformamide instead of water.

Surprisingly, the Ru^{II}–10-MeIAlo frequencies in the 1100–1350-cm^{-1} region show appreciable differences from those of Ru^{II}–RBF. A band at 1233 cm^{-1} now appears to shift up, but only to 1245 cm^{-1}, on N3 deuteration. Moreover, the 1233-cm^{-1} band is of moderate intensity, while the 1245-cm^{-1} band of N3–D in Ru^{II}–10-MeIAlo and the corresponding 1241- and 1248-cm^{-1} bands of the N3-ionized and N3–CH_3 analogues are the strongest bands in the spectra. These alternations are not understood; however, the RR spectrum of lumiflavin (in which the ribityl group of RBF is replaced with a methyl group) also differs appreciably from that of RBF in the 1200–1300-cm^{-1} region.[8]

It is of interest that both Ru^{II}–RBF and Ru^{II}–10-MeIAlo show a RR band near 1700 cm^{-1} (the frequency of this weak and broad band is hard to determine with precision) that is absent in RBP. It is plausibly assigned to stretching of the

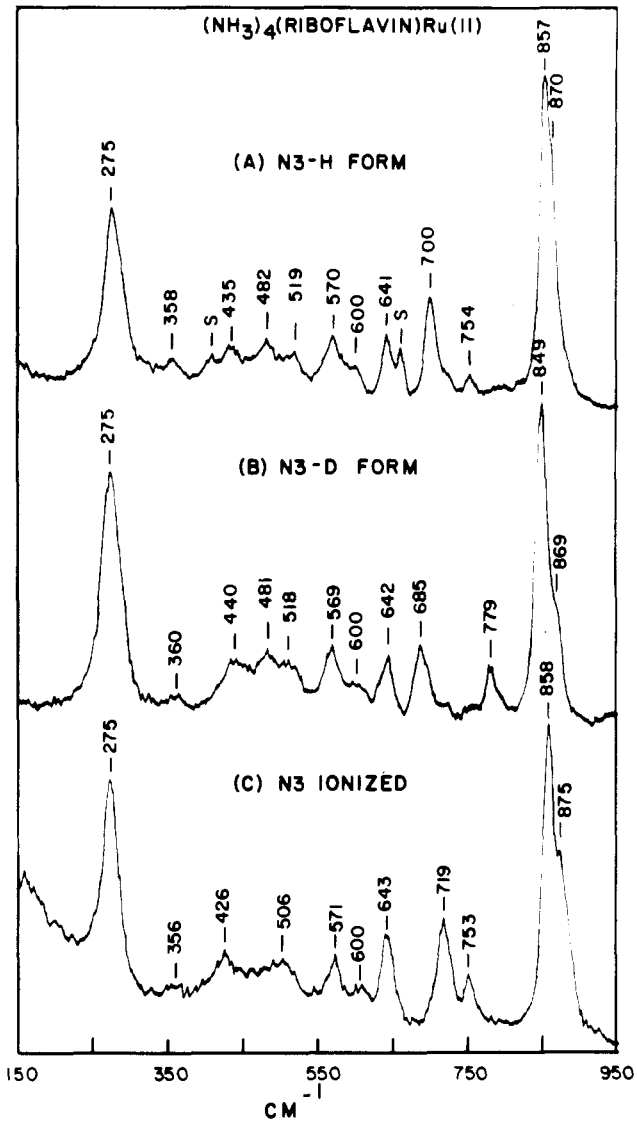

Figure 8. RR spectra, (below 1000 cm^{-1}): $(NH_3)_4Ru(RBF)$ in dimethylformamide (N3–H form) (A) and in aqueous buffer at pD 2 (N3–D form) (B) and pH 12 (N3-ionized form) (C). Bands marked S are due to solvents, and conditions are as in Figure 4B–D.

Table II. Flavin RR Frequency (cm^{-1}) Correlations below 1000 cm^{-1}

	$(NH_3)_4Ru(RBF)$			$(NH_3)_4Ru(10\text{-}MeIAlo)$			
RBP	pH 2	pD 2	pH 12	pH 2	pD 2	pH 12	N3-Me
					943		
879	870	869	875	905	893	903	902
833	857	849	858	875	866	874	810
785				797	797	796	786
734	754	779	753	729		754	733
704	700	685	719				
676				666	666	669	658
633	641	642	643	637	638	640	626
603	600	600	600	601	603	601	602
570	570	569	571	572	571	579	560
522	519	518	506	521	519	521	521
477	482	481		480	473	478	484
427	435	440	426	441	442	443	438
				412	412	414	
	358	360	356				358
297				310	310	305	326
	275	275	275	285	286	285	287
					231		
					181		

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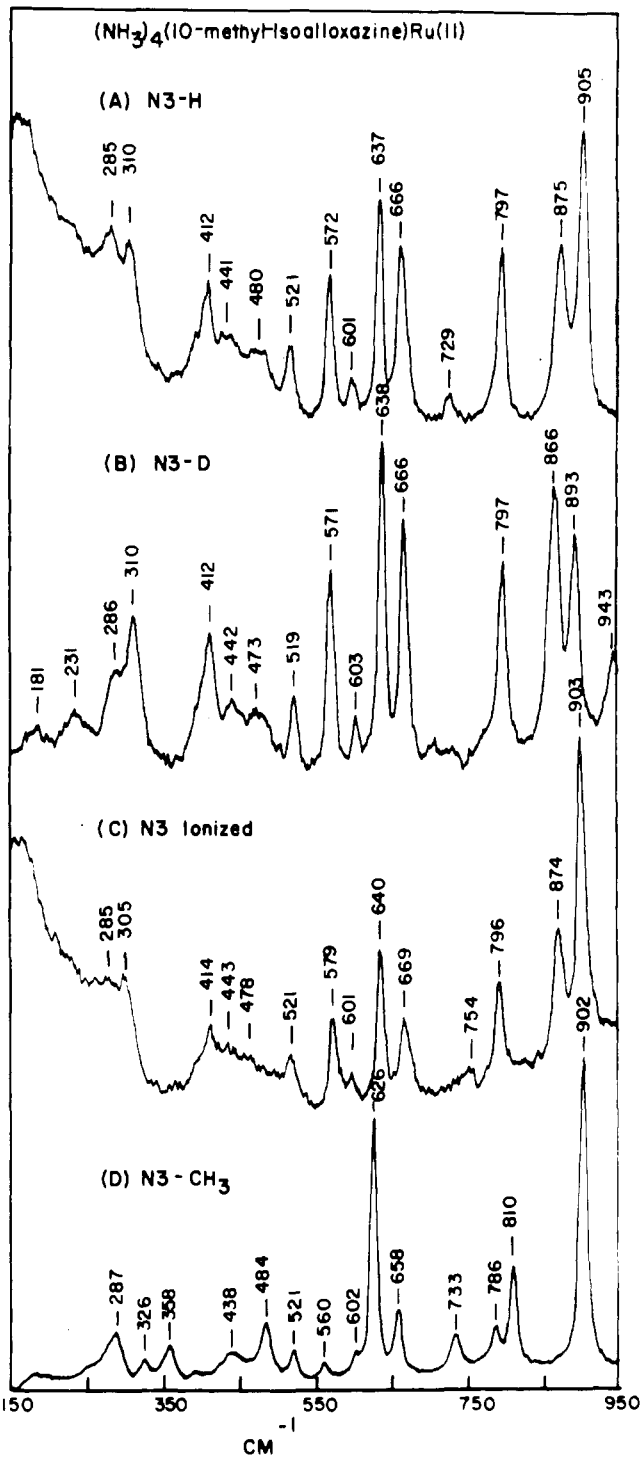

Figure 9. RR spectra of $(NH_3)_4Ru(10\text{-MeIAlo})$, below 1000 cm^{-1}. Conditions are as in Figure 5.

C4=O bond (calculated at 1714 cm^{-1} in lumiflavin)[9], activated by coordination to Ru^{II}.

Low-Frequency RR Spectra. Figures 7–9 show RR spectra for RBP and for the Ru^{II} complexes below 1000 cm^{-1}. While the spectra are very rich, it is possible to make reasonable correlations among them, as shown in Table II. Twenty-two in-plane flavin modes are calculated to occur below 1050 cm^{-1}.[9] These could account for all of the observed bands, since there are 19 listings in Table II. Out-of-plane C–H bends are expected near 850 cm^{-1}, while the remaining out-of-plane modes should be below 600 cm^{-1}.[17] These modes are unlikely to have significant intensity, since the resonant electronic transitions (π–π^* for RBP, MLCT for the Ru^{II} complexes) are polarized in the flavin plane. The slight out-of-planarity observed for Ru^{II}–10-MeIAlo[11] (the coordinated flavin is bent by 9.9° about the N1–N10 axis) might induce some activity into out-of-plane modes, particularly those involving significant displacement of N1 or N10. However, most of the observed RR bands probably arise from ring breathing (the breathing mode of benzene is at 922 cm^{-1}) and in-plane deformation modes.

A large number of frequency shifts are observed upon H/D exchange, ionization, or methylation at N3. Indeed all of the bands down to 600 cm^{-1} seem to be sensitive to one or another of these changes. The modes observed therefore appear to involve substantial contributions from the uracil ring.

There are further surprising differences between Ru^{II}–RBF and Ru^{II}–10-MeIAlo in this region. Thus, the strong 857–870-cm^{-1} doublet of Ru^{II}–RBF is shifted to 875–905 cm^{-1} for Ru^{II}–10-MeIAlo. The latter complex shows strong bands at 666 and 797 cm^{-1} that shift down 10 cm^{-1} on N3 methylation but are unshifted upon N3 ionization or H/D exchange; these do not appear in the Ru^{II}–RBF spectra, but Ru^{II}–RBF has a H/D- and ionization-sensitive band at 700 cm^{-1} that is not seen for Ru^{II}–10-MeIAlo. It is far from obvious how the change in peripheral methylation can produce such marked spectral differences.

Modes involving the stretching of the Ru–N5 and Ru–O4 bonds are expected below 500 cm^{-1}. It is tempting to assign the strong Ru^{II}–RBF band at 275 cm^{-1} to Ru–N5 stretching, since metal–heterocycle bond stretches are usually found in this region,[18] and substantial enhancement of this mode would be anticipated via charge-transfer resonance. However, Ru^{II}–10-MeIAlo does not show this band, but rather a moderately strong doublet at 285–310 cm^{-1}. Although the doublet might be due to Ru–O4, as well as Ru–N5 stretching, it is again unclear why the two complexes should differ so markedly. RBP itself has a moderately strong RR band at 297 cm^{-1}, so that ring modes cannot be ruled out as the source of the Ru^{II}–flavin bands in this region.

Conclusions

While Ru^{II}–flavin complexes can formally be written as Ru^{II}–Fl_{ox} or as Ru^{III}–$Fl_{sq}\cdot$, the RR spectra show that neither oxidized (Fl_{ox}) nor semiquinone ($Fl_{sq}\cdot$) forms of flavin are adequate descriptions of the complexed flavin. These spectra are sensitive to the specific effects of d_π back-donation into the π^* orbitals of Fl_{ox} and are consistent with the resonance form in Figure 6D, which is supported by structural data as the dominant form in the complex. Thus, metal complexation to the flavin N5 and O4 atoms is expected to have a specific RR signature, provided that d_π back-donation is significant.

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Registry No. $(NH_3)_4Ru^{II}$(RBF), 78591-57-8; $(NH_3)_4R^{II}$(10-MeIAlo), 69290-17-1.

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