Observation of a Carbonyl Feature for Riboflavin Bound to Riboflavin-Binding Protein in the Red-Excited Raman Spectrum†

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Flavins play a key role as cofactors in biology, particularly in the oxidation of fuel molecules such as glucose and fatty acids. Many of the properties of flavins are modulated by their interaction with proteins, and X-ray crystallographic studies have revealed a variety of modes of flavin–protein binding.[1] Although hampered by high intrinsic fluorescence, several Raman studies have also been undertaken on flavin–protein complexes in order to characterize molecular details of the protein–ligand interaction. The studies include spontaneous resonance Raman (RR), resonance coherent anti-Stokes Raman, resonance inverse Raman, and surface-enhanced resonance Raman scattering spectroscopies.[2,3] We now report high-quality Raman difference data obtained using red (647.1 nm, Kr^+) excitation for riboflavin (RF) (Figure 1) bound to riboflavin-binding protein (apo-RBP) in the micromolar range using a single spectrograph equipped with a charge-coupled device (CCD) and a holographic notch filter.[4] This method enables us to make the first direct comparison of Raman spectra between free and bound RF at low concentration (50–200 μM) both in H_2O and D_2O solutions. Importantly, a band ascribable mainly to the carbonyl stretching of the isoalloxazine ring was observed for the first time for bound RF. The results provide new insight into RBP–isoalloxazine ring interactions involving ring III (Figure 1).

Comparisons of the 647.1-nm excited Raman spectra of free (200 μM) and bound (100 μM) RF for H_2O solution at pH 7.5 in Figure 2 and for D_2O solution at pD 7.5 in Figure 3 indicate that some bands undergo modest shifts upon binding. In H_2O solution, four bands at 1714, 1412, 1259, and 1183 cm^{-1} in Figure 2A shift to 1723, 1408, 1254, and 1178 cm^{-1} in Figure 2B. The band near 1720 cm^{-1}, which has been observed in the deep-UV resonance Raman spectra of free flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) by Copeland and Spiro,[5] can be assigned to a stretching vibration of ring III of the isoalloxazine (Figure 1) that includes a large amount of C4=O stretching motion. This assignment is supported by a normal coordinate analysis of lumiflavin[6,7] and infrared absorption spectra of lumiflavin and RF.[8] Interestingly, the band is upshifted by 9 cm^{-1} upon binding (Figure 2), indicating that the environment around the C4=O carbonyl group in the protein matrix is changed compared to that in aqueous solution. For binding in D_2O solution, the same degree of frequency upshift (from 1701 to 1710 cm^{-1} in Figure 3) is seen for the carbonyl stretching mode. Another point of note is that the intensity of the carbonyl stretching is appreciably enhanced by deuteration of N3H. This observation is reminiscent of the observation in the 266-nm excited RR spectra of the uracil residue of deoxyuridine 5′-monophosphate,[9] where the 1654-cm^{-1} band due to C4=O stretching of the uracil is remarkably enhanced in intensity by deuteration of N3H. This enhancement was ascribed to the removal of the vibrational coupling of the carbonyl stretch with N3H bending,[9] and a similar effect may be operating in the isoalloxazine system. For the unbound RF, the carbonyl frequency is downshifted from 1714

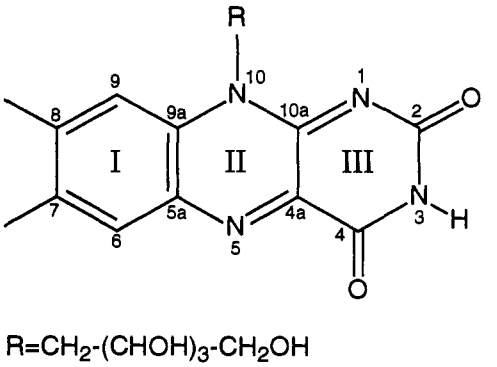

Figure 1. Structure and atomic numbering of riboflavin.

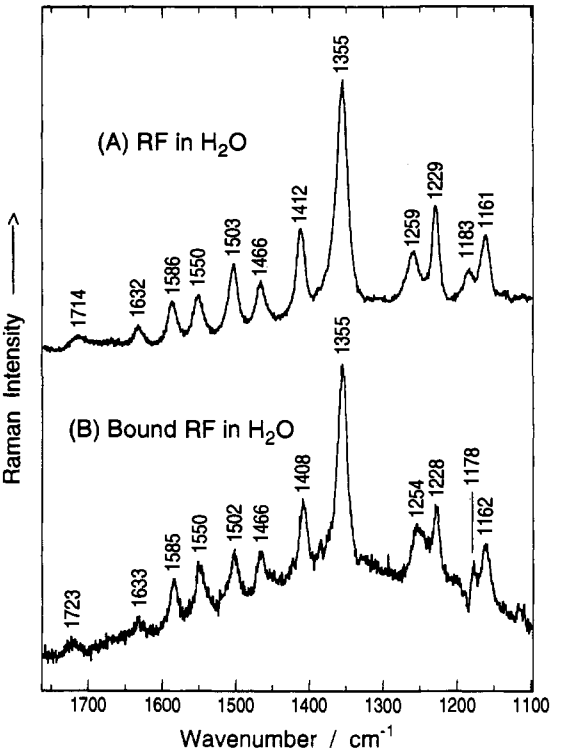

Figure 2. 647.1-nm excited Raman spectra of (A) 200 μM RF in H_2O solution, 50 mM Tris buffer, pH 7.5 (with the buffer solution bands subtracted) and (B) 100 μM RF bound to RBP in H_2O solution, 50 mM Tris buffer, pH 7.5, obtained by subtracting the Raman spectrum of ca. 250 μM RBP in 50 mM Tris buffer, pH 7.5 from the Raman spectrum of mixture of ca. 250 μM RBP and 100 μM RF in the same buffer. Spectral conditions: 400 mW, 647.1 nm, 90° scattering, 8-cm^{-1} spectral slit width, 10-min data acquisition time (30 scans of 20-s exposure). The Raman instrumentation is based on a single polychromator equipped with a CCD and a notch filter (ref 4). For bound RF, typically 10 mg of riboflavin binding protein from chicken egg white, apo form, lyophilized powder (Sigma) was dissolved in 665 μL of 50 mM Tris buffer, pH 7.5 (pD 7.5 for the D_2O solution) and then filtered with Millipore Millex-GV (0.22 μm pore size). The 125-μL aliquot was mixed with 125 μL of 200 μM RF in the same buffer prior to use.

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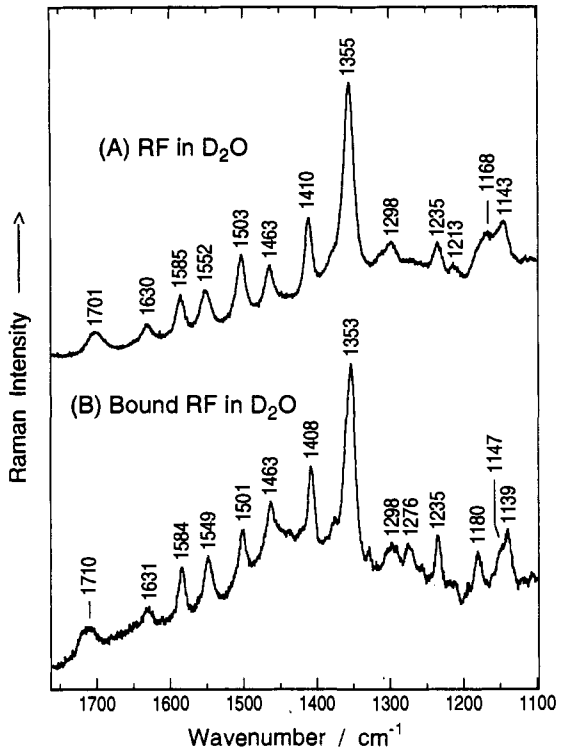

Figure 3. 647.1-nm excited Raman spectra of (A) 200 μM RF in D_2O solution, 50 mM Tris buffer, pD 7.5 (with the buffer solution bands subtracted) and (B) 100 μM RF bound to RBP in D_2O solution, 50 mM Tris buffer, pD 7.5. Conditions similar to those used in Figure 2.

to 1701 cm^{-1} by deuteration of N3H of the isoalloxazine ring (Figures 2 and 3), indicating that the carbonyl stretching mode does indeed include a contribution from N3H bending.[5] With this coupling occurring, it is possible that the C4=O stretch could be shifted for the bound RF in H_2O solution via interactions between N3—H and the protein. However, the possibility is removed by the occurrence of identical C4=O shifts upon binding in both H_2O and D_2O solutions. Thus, we adopt the simple explanation that the observed high-frequency shift for C4=O stretch upon binding is due to a reduction in hydrogen-bonding strength about the carbonyl. Therefore, the hydrogen bonding between the C4=O and the protein is less strong than that between the C4=O and water molecules for RF free in solution.

The observed frequency shifts of bands at 1412 and 1259 cm^{-1} (Figure 2) upon binding are small but reproducible. Previously, Kitagawa and co-workers[10] proposed that the band at 1259 cm^{-1}, which is due to C2—N3—C4 stretching vibrations coupled with a N3—H bending vibration, may serve as a probe of the N3—H···protein interaction. The rationale is that the vibrational coupling between C2—N3—C4 stretching and N3—H bending is modulated by hydrogen bonding at the N3H position, giving rise to a frequency shift of the 1259 cm^{-1} vibrational mode. McFarland and co-workers[11] observed the RR spectra of RF in solution using KI as a fluorescence quenching agent and demonstrated that both bands at 1412 and 1259 cm^{-1} (Figure 2A) shift to lower frequencies when water (a good hydrogen-bond acceptor and donor) is replaced by dimethyl sulfoxide (a weak hydrogen-bond acceptor). On the basis of these earlier studies,[10,11] the observed downshift seen upon binding to RBP in H_2O (from 1412 to 1408 cm^{-1} and from 1259 to 1254 cm^{-1} in Figure 2) can be explained by assuming that the hydrogen bonding between N3H and the protein is weaker than that between N3H and water molecules in aqueous solution.

In summary, the picture emerging from our data is that strong hydrogen-bonding interactions between RBP and the C4N3 fragment of ring III do not exist. The interactions are not as strong as those between the fragment and water molecules for the free RF in solution. In a more general vein, our results demonstrate that it is now possible to compare high-quality Raman data for unbound and protein-bound ligands at micromolar concentrations for samples that were heretofore essentially inaccessible due to sample fluorescence and the need to employ millimolar concentrations.

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