Time-Resolved Resonance Raman Observation of Protein-Free Riboflavin Semiquinone Radicals†

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Abstract: This paper reports the first time-resolved resonance Raman observation of riboflavin semiquinone anion, neutral, and cation radicals in aqueous solution. These species are short-lived in their unbound state. Therefore, their detection by conventional CW laser excitation, used in previous attempts, was not possible. Information on vibrational frequencies, provided by this work, is essential for probing chemical interactions, such as protein and ligand interactions, in the redox intermediate states of flavin molecules using Raman spectroscopy.

Flavoenzymes act as cocatalysts for a host of redox reactions in living cells. These reactions proceed via intermediary formation of semiquinones produced by one-electron reduction of oxidized flavins. Over the years, resonance Raman (RR) spectroscopy has been used extensively to study the vibrational structures of flavins and flavoproteins.[1-3] Recently, several Raman studies on semiquinone intermediates have been reported. The neutral form of semiquinone radical stabilized in riboflavin-binding protein,[4] *Clostridium MP* flavodoxin,[5] adrenodoxin reductase,[6] and cytochrome P-450 reductase[7] has been examined. The surface-enhanced RR signals of this radical adsorbed on silver electrodes have also been detected.[8] The anionic form of the radical, which has been more elusive, has been observed recently in D-amino acid oxidase,[9] glucose oxidase,[10] and bovine liver monoamine oxidase.[10] In spite of these numerous studies, information on the vibrational frequencies of free flavin semiquinones in solution, which is essential for probing the effects of protein and ligand interactions on vibrational spectra and structure, is not available. This work provides this information which has been sought unsuccessfully for many years.

In their unbound state in solution the flavin semiquinones are short-lived and can be observed only by time-resolved techniques. They react by disproportionation, the rate constant ($2k$) for which has been measured as 1.2×10^9 M^{-1} s^{-1} for the neutral and 7×10^8 M^{-1} s^{-1} for the anionic riboflavin semiquinone in aqueous solution.[11] Therefore, the CW laser excitation technique used in previous attempts was inadequate to detect the RR signals of these species. Here we report the time-resolved resonance Raman observation of riboflavin semiquinone anion, neutral, and cation radicals in aqueous solution.

Experimental Section

The semiquinone (SQ) radicals were prepared by reduction of riboflavin (Rf) by solvated electron and $CO_2^{\bullet-}$ radicals, produced by pulse radiolysis in aqueous solution, as described in ref 11. Radiolysis of deaerated water produces e_{aq}^- and OH• radicals. The OH• radical was scavenged by adding excess *tert*-butyl alcohol in N_2-saturated solutions. The $CO_2^{\bullet-}$ radical was prepared by reaction of OH• radical with formate ion in N_2O-saturated solution in which e_{aq}^- also converts to OH• radical on the nanosecond time scale. The e_{aq}^- reacts with flavin with a rate constant of $\sim 1.7 \times 10^{10}$ M^{-1} s^{-1}.[11] Therefore, at the ~0.2 mM Rf concentration used in these studies, the reaction is complete within ~0.3 μs after the electron pulse. The $CO_2^{\bullet-}$ radical reaction is slower ($\sim 1.4 \times 10^9$ M^{-1} s^{-1})[11] and takes about 3 μs for its completion. In both reactions the semiquinone radicals can be observed within a few microseconds after the electron pulse.

The expermental setup and measurement procedures used in this laboratory for recording the RR spectra of transient species have been described in detail elsewhere.[12] A 2 MeV, ~100-ns electron pulse delivered by a Van de Graaff accelerator is used for radiolysis. An excimer pumped dye laser (~10-ns pulses) is used for probing Raman scattering and an optical multichannel analyzer (OMA) system employing an intensified gated (~20 ns) diode array for detection. In the present study, the spectra were averaged over ~10 000 pulses, by synchronous operation of the electron and laser beams at 7.5 Hz. A flow system was used to refresh solution between consecutive pulses to avoid product accumulation in the Raman cell. The Raman peak positions were measured by reference to the known bands of ethanol and cyclohexane and are accurate within ±2 cm^{-1}.

Results and Discussion

The RR spectrum of RfSQ anion radical, prepared by reaction of electron with Rf^- at pH ~11 and observed 1 μs after the electron pulse, is depicted in Figure 1a. Excitation was at 365 nm where the anionic form of RfSQ absorbs strongly.[11] The spectra are displayed after subtraction of solvent and Rf^- bands from the raw data. An identical spectrum was obtained when Rf^- was reduced by $CO_2^{\bullet-}$ at pH ~11. The resonance Raman spectrum of neutral RfSQ ($pK_a \sim 8.3$) was obtained at pH ~5 (Figure 1b), and that of RfSQ cation ($pK_a \sim 2.3$) at pH ~0.6 (Figure 1c), following similar experimental procedures and employing ~343-nm excitation.[11] Vibrational frequencies of the three forms of radical are also presented in Figure 1.

The vibrational frequencies of RfSQ anion radical observed here are in qualitative agreement with those of D-amino acid oxidase (DAO) semiquinone anion complexed with picolinate.[9] In this flavoprotein, the free semiquinone anion bands generally shift to lower frequencies, except for the 1498-cm^{-1} band which

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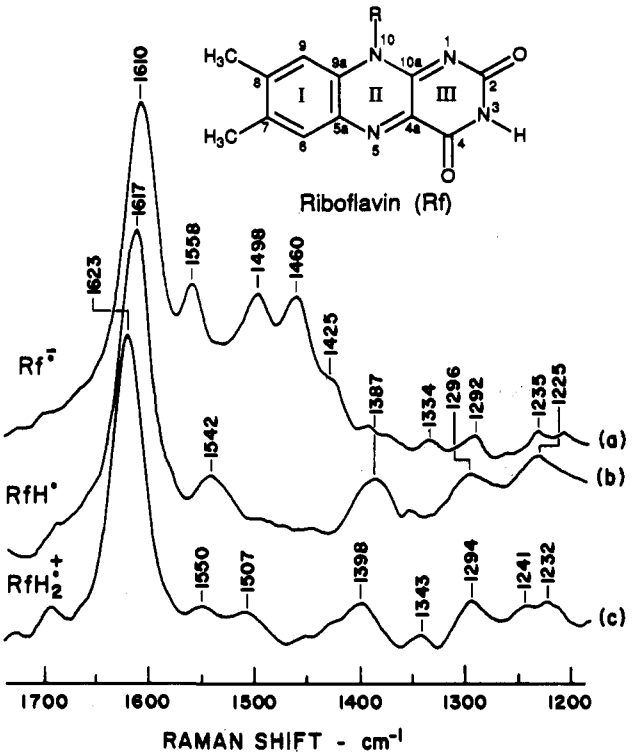

Figure 1. Resonance Raman spectra of semiquinone radicals prepared by electron pulse irradiation of aqueous solutions containing 2×10^{-4} M riboflavin. (a) Anion radical, 365-nm excitation, 0.5 μs after electron pulse. (b) Neutral radical, 343-nm excitation, 3 μs after electron pulse. (c) Cation radical, 343-nm excitation, 3 μs after electron pulse. Background spectra taken without electron pulse have been subtracted. For spectrum in Figure 1a, riboflavin was reduced by hydrated electron in a N_2-saturated solution containing 0.5 M *tert*-butyl alcohol at pH ~11. The spectra in Figure 1b,c were obtained by reducing riboflavin by $CO_2^{\bullet-}$ radical in N_2O-saturated solutions containing 0.5–1 M sodium formate at pH ~5 (Figure 1b) and pH ~0.6 (Figure 1c).

shifts to a higher frequency by ~18 cm^{-1}. There is little resemblance, however, with the RR frequencies and intensity pattern of the species attributed to semiquinone anion in monoamine oxidase B (MAO B).[10] The Raman frequencies of neutral RfSQ closely resemble those observed for the radical adsorbed on Ag electrodes[8] or when bound to various proteins.[4-7] However, the intensity patterns are often different, as higher excitation wavelengths have been used in earlier studies. The RfSQ cation frequencies of the radical bound to protein or metal surfaces are not available for comparison.

The bond properties of the semiquinone state are generally intermediate between those of fully oxidized and reduced states.[12-15] For example, the vibrational frequency of the redox-sensitive CO stretching mode in *p*-benzosemiquinone anion radical, 1435 cm^{-1},[13] is 230 cm^{-1} lower than in *p*-benzoquinone (oxidized state) and 171 cm^{-1} higher than in hydroquinone (reduced state).[15] In neutral *p*-benzosemiquinone radical, the CO stretching frequency shifts upward by 76 cm^{-1} with respect to the anion radical, as the two CO bonds become nonequivalent on protonation at one of the oxygen atoms.[14] Thus, the gain or loss of an electron can drastically change the vibrational frequencies of the redox-sensitive bonds.[12] In flavin system, the redox-sensitive bonds can be readily identified as $C_{4a}N_5$, $C_{4a}C_{10a}$, and N_1C_{10a} by structural comparison between the fully oxidized and reduced forms.[1,2] In the semiquinone state, the properties of these bonds are expected to be intermediate. Therefore, the common procedure of one-to-one correlation between the nearest semiquinone and oxidized flavin frequencies is not very useful in vibrational assignments. In the following discussion reference is made to Figure 1 for atomic numbering of the isoalloxazine (flavin) ring.

The oxidized flavin frequencies in the 1500–1600-cm^{-1} region are sensitive to the $^{13}C_{4a}$ and $^{15}N_5$ substitutions,[2] placing the stretching frequency of the isolated $C_{4a}N_5$ bond near 1550 cm^{-1}. In DAO SQ anion radical,[9] a similar isotopic sensitivity is seen in the vibrational frequencies in the ~1330-cm^{-1} region. A drop of ~250 (±50) cm^{-1} in frequency provides spectroscopic evidence for drastically reduced $C_{4a}N_5$ bond strength in flavin semiquinone anion radical. Because of near equivalence of flavin $C_{4a}N_5$ and N_1C_{10a} bonds, charge and spin distributions on N_1 and N_5 positions in the anion radical are expected to be similar, implying comparable $C_{4a}N_5$ and N_1C_{10a} bond strengths, and, as a result, the $C_{4a}C_{10a}$ bond must approach a double bond. The molecular structure of the cation radical is expected to be qualitatively similar to that of the anion radical, although the N_1C_2 bond (ring III bonds in general) should be relatively weaker, as there is no delocalization of electronic charge on ring III in this case. The near equivalence of the $C_{4a}N_5$ and N_1C_{10a} bonds is lost on protonation only at N_5 (or N_1) in the neutral radical, thus transferring the spin density on C_{4a} position which leads to weaker $C_{4a}C_{10a}$ and stronger N_1C_{10a} bonds than in anion or cation radicals. This simple description of flavin semiquinone structures, which is intermediate between the oxidized and reduced flavin structures, has been well recognized.[1-11] However, it has seldom been used for making vibrational assignments. It is very useful, when combined with the observed effects of protonations on the RR spectra, to identify the bonds which make major contribution to a particular frequency.

In flavoprotein semiquinones, assignment of the highest frequency RR band in the ~1600-cm^{-1} region, which is also the most intense band, has been uncertain. This band has been assigned either to the Rf mode 1584 cm^{-1} (ring II, CC and CN stretch) or to 1630 cm^{-1} (ring I CC stretch). It is fairly certain that this frequency cannot be ascribed to ring II CN stretch, as it shifts very little on $^{13}C_{4a}$ and $^{15}N_5$ substitutions.[9] Similarly, the upward shift in the 1610-cm^{-1} frequency of the RfSQ anion radical by 7 cm^{-1} in neutral and 13 cm^{-1} in cation radical, observed here, suggests that the ring I C=C bonds cannot be the principal contributors. Therefore, the most probable assignment of this band is to a mode involving significant contribution from the $C_{5a}C_{9a}$ stretching motion. Protonation at the N_5 position should slightly weaken the N_5C_{5a} bond and strengthen the $C_{5a}C_{9a}$ bond, consistent with a slight increase in frequency. The 1542–1558-cm^{-1} frequency in the RfSQ radicals is observed at 1555 cm^{-1} in DAO SQ anion radical where it undergoes negligible shifts on isotopic substitutions at the 1, 2, 3, 4, 4a, 5, and 10a isoalloxazine ring positions. Therefore, we tentatively attribute this vibration to ring I and II. It seems to involve some $C_{4a}C_{10a}$ stretching component as the frequency in the neutral radical shifts downward by 16 cm^{-1} with respect to that in the anion radical. The RfSQ anion radical band at 1498 cm^{-1} (1507 cm^{-1} in cation radical) in all probability represents a vibrational mode with significant $C_{4a}C_{10a}$ stretching component, as the corresponding band in DAO SQ anion at 1516 cm^{-1} is very sensitive to the $^{13}C_{4a}$ and $^{13}C_{10a}$ isotopic substitutions.[9] In cation radical, the frequency of this vibration is expected to be slightly higher, as protonations at N_1 and N_5 sites strengthen the $C_{4a}C_{10a}$ bond, as observed. This assignment is further supported by the absence of any RR band in the 1400–1500-cm^{-1} region in neutral radical, as the $C_{4a}C_{10a}$ bond is expected to be much weaker. The 1460-cm^{-1} frequency

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of the RfSQ anion radical, which is not observed in neutral and cation radicals, suggests its association with the N_1C_2 bond, as this bond becomes much weaker on N_1 as well N_5 protonation. Assignment of this vibration to a mode involving N_1C_2 stretch with some $C_{4a}C_{10a}$ stretching component is also indicated by the $^{13}C_2$ and $^{13}C_{4a}$ shifts in the DAO SQ anion radical. The weakly enhanced 1425-cm^{-1} vibration in the RfSQ anion also involves some $C_{4a}C_{10a}$ stretching component, along with contributions from the CN and CC bonds in ring III, as suggested by the isotopic shifts in the 1422-cm^{-1} band of DAO SQ anion. It is not clear if the prominent bands at 1398 cm^{-1} in the RfSQ cation and at 1387 cm^{-1} in the neutral radical correlate with the 1460- or the 1425-cm^{-1} band of the anion radical. We prefer the former correlation on intensity considerations, although the 1387-cm^{-1} frequency in neutral radical is lower than in cation radical which suggests larger $C_{4a}C_{10a}$ stretching component. Thus, the 1387-cm^{-1} vibration of the neutral radical correlates with the 1498-cm^{-1} vibration of the RfSQ anion radical also. Those vibrational modes which have little involvement of the $C_{4a}N_5$, $C_{4a}C_{10a}$, N_1C_{10a}, and N_1C_2 bonds should be similar in oxidized flavin and flavin semiquinones.

The availability of the RR frequencies of protein-free flavin semiquinone radicals makes it possible to investigate the effects of chemical interactions on the structure and bonding. For example, from the structural inferences and vibrational assignments discussed above, the 1617-cm^{-1} frequency of the neutral radical should be sensitive to N_5H bonding, but not to N_3H bonding. As this frequency remains unchanged in the radical adsorbed on Ag electrodes,[8] structural stabilization by N_3H–metal binding is indicated. In *Clostridium MP* flavodoxin,[5] this frequency drops by $\sim$6 cm^{-1}, indicating a N_5H-protein hydrogen bond interaction much stronger than that with water. In the flavin semiquinone anion radical, strong N_5-protein bonding will increase the 1610-cm^{-1} frequency. Since this frequency decreases by $\sim$8 cm^{-1} in DAO,[9] protein interaction at the N_5 site must be small or negligible, as compared to water interaction. The protein–hydrogen bonding at N_1, if stronger than in water, should increase the 1498-cm^{-1} frequency ($C_{4a}C_{10a}$ bond) and decrease the 1460-cm^{-1} (N_1C_2 bond) frequency of the anion radical. This is the case in DAO SQ anion[9] where these frequency shifts are fairly large (18 and 12 cm^{-1}, respectively), indicating very strong protein interaction at the N_1 site. These examples illustrate that the semiquinone frequencies are much more sensitive to the site and strength of the protein interaction than those of the parent flavin.

In summary, we have demonstrated here that by employing time-resolved technique with near-UV excitation, the RR spectra of flavin semiquinone radicals in solution are readily observable. Comparison of the vibrational frequencies of anion, neutral, and cation radicals is extremely useful for making vibrational assignments.