Resonance Raman Evidence for a Hydrogen-Bonded Oxo Bridge in the R2 Protein of Ribonucleotide Reductase from Mouse

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The reduction of ribonucleotides to deoxyribonucleotides, precursors in the synthesis of DNA in all living organisms, is catalyzed by several classes of ribonucleotide reductase (RNR).[1−3] Class I enzymes contain a stable neutral tyrosyl radical (Tyr•) and a diiron−oxygen center in the active form of the smaller component protein R2 (RNR−R2).[1−4] Catalytically active Class I RNR consists of a 1:1 complex of the two proteins, R1 and R2, each of which is a homodimer. The crystal structures of *Escherichia coli* proteins R1 and R2 (diferric form without radical) have been determined.[5] Based on the diferric cluster structure in the *E. coli* R2 protein,[5a,b] the activity-dependent Tyr• should be found about 5.2 Å (tyrosyl-oxygen to Fe1) from the diferric cluster. The 3D crystal structure of mouse R2 protein with only Fe2, the iron farthest from Tyr•, occupied is known.[6] The very weak exchange and dipolar couplings between Tyr• and the iron−oxygen cluster[2d] are somewhat different in active R2 proteins from *E. coli*, mouse, herpes simplex virus type 1 (HSV1), and *Salmonella typhimurium*.[7] Recent high-frequency EPR and Q-band MIMS ENDOR studies of mouse and HSV1 R2 have shown that the Tyr• oxygens are hydrogen bonded to D_2O exchangeable protons, possibly on a water bound to Fe1.[8] In this study, resonance Raman (rRaman) spectroscopy[9] shows that, in contrast

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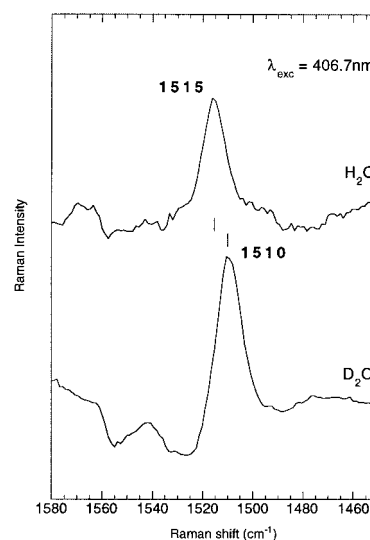
Figure 1. Effect of D_2O on the mouse RNR−R2 protein tyrosyl radical resonance Raman spectrum (λ_{exc} = 406.7 nm, laser power at sample ∼10 mW, 30 accumulations at 4 °C).

to the enzyme from *E. coli*, for active mouse RNR−R2, both Tyr• and the μ-oxo bridge are hydrogen bonded, which can affect the reactivity of the R2 protein.

The higher energy region (1800−1000 cm^{-1}) of the rRaman spectrum of active mouse RNR−R2 protein (with excitation, λ_{exc}, at 406.7 nm, which is near λ_{max} for the Tyr• absorption spectrum) shows one major feature at 1515 cm^{-1} (Figure 1). When changing excitation wavelength to 363.8 nm or in the absence of the radical in the R2 protein, it was not possible to detect this vibration. The vibration is assigned as the tyrosyl ν_{7a} mode (Wilson notation[10]), a carbonyl stretching vibration, which appears at 17 cm^{-1} higher energy than the corresponding vibration in *E. coli* RNR−R2[11] and also 12 cm^{-1} higher than the Tyr$_D$• but similar to the Tyr$_Z$• (two hydrogen-bonded radicals in Photosystem II).[12] When mouse RNR−R2 is reconstituted in a reaction between apoprotein, Fe-(II) ions, and dioxygen in (or exchanged with) D_2O-containing buffer,[13] this vibration for the iron−radical center is observed at 1510 cm^{-1} (Figure 1). This 5 cm^{-1} shift to lower energy reconfirms the presence of a hydrogen bond between an exchangeable proton and the tyrosyl oxygen.[8] This shift is most easily explained as a consequence of the increased mass of the deuterium isotope. The absence of an intense mode (ν_{8a}, a C=C

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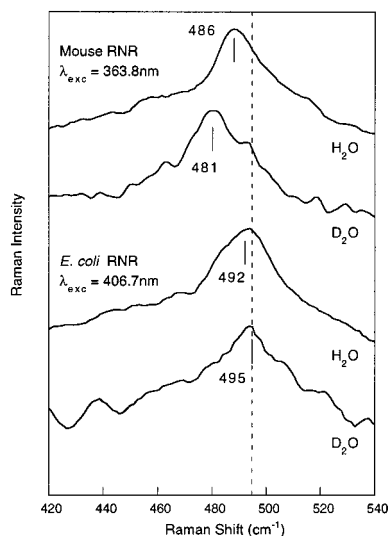
Figure 2. Effect of D_2O on the Raman spectrum of the Fe−O−Fe symmetric stretching vibration in RNR−R2 from mouse and *E. coli* (λ_{exc} = 363.8 nm, laser power at sample ∼10 mW, 30 accumulations at 4 °C).

Table 1. Resonance Raman Parameters and Magnetic Exchange Coupling between the Irons for Fe(III)−O−Fe(III) Protein Sites

protein	ν_s(Fe−O−Fe) (cm^{-1})			J (cm^{-1}) ($H = -2JS_1S_2$)
	ν	$\Delta^{18}O$	ΔD	
Δ^9 stearoyl-ACP desaturase[17]	519	−18	+2	nd[a]
rubrerythrin[18]	514	−18	+2	nd
E. coli RNR−R2 (met and active)[11,16]	493	−13	+4	−92[7]
E. coli RNR−R2 H241A[19]	542	nd	nd	nd
E. coli RNR−R2 E238A[19]	522	−15	−5	nd.
mouse RNR−R2 (active)[b]	486	−13	−5	−77[7b]
HSV1 RNR−R2 (active)[b]	482	nd	nd	−66[7b]

[a] nd, not determined. [b] This work.

ring stretch) around 1600 cm^{-1} confirms[7b] that no strong interaction between the tyrosyl oxygen and the closest iron is present.[15]

The lower energy region (below 1000 cm^{-1}) of the rRaman spectra, taken with λ_{exc} at 363.8 nm (Figure 2) or 406.7 nm, of active mouse RNR−R2, which involves excitation into the oxo-to-Fe(III) charge-transfer transition, shows an enhanced vibration at 486 cm^{-1} not observed in apoprotein or other controls under similar conditions. This feature is sensitive to exchange or reconstitution in $H_2^{18}O$ ($\Delta\nu = -13$ cm^{-1}) and is assigned to the Fe−O−Fe symmetric stretching vibration. It occurs 6 cm^{-1} lower in energy than in *E. coli* RNR−R2.[16] We have been able to observe, with difficulty, a similar feature at 482 cm^{-1} in the spectrum of HSV1 RNR−R2. We have not been able to detect the normally much weaker antisymmetric Fe−O−Fe stretching vibration with certainty in mouse or HSV1 RNR−R2. A comparison of Fe−O−Fe vibrations and exchange coupling constants for different iron−oxygen proteins is given in Table 1. The table also includes data on a *E. coli* RNR−R2 mutant E238A, which also has a very weak antisymmetric mode and in which the μ-oxo bridge was suggested to be hydrogen bonded.[19] The rRaman spectra taken after mouse RNR−R2 was reconstituted in (or exchanged with) D_2O-containing buffer show that this

vibration shifts 5 cm^{-1} to lower energy (Figure 2). In *E. coli* RNR−R2 data from ref 16c (similar results were obtained with the radical containing R2 protein[11]), shown in Figure 2 for comparison, this feature shifts up in energy by about 4 cm^{-1}. We interpret the 5 cm^{-1} downshift of the symmetric mode of Fe−O−Fe in D_2O observed in mouse RNR−R2 as an indication of a hydrogen-bonded μ-oxo bridge. Recent studies of oxy- and deoxyhemerythrin[20] have shown that, while hydrogen bonding does not have as large an influence on the exchange coupling constant (J) as once believed, it does reduce J slightly and has a larger effect on the Fe−O−Fe vibrational frequency. The observed reduction in the J value of mouse RNR−R2 relative to *E. coli* and the 5 cm^{-1} frequency shift are reasonably attributed to a hydrogen-bonding interaction involving the μ-oxo bridge. Thus, the mouse RNR−R2 site appears to have H bonds involving both the Tyr• oxygen and the bridging oxygen. In contrast, *E. coli* RNR−R2 shows no evidence for either hydrogen bond.[7,8,11,16]

The source of the exchangeable proton for the hydrogen bond to the μ-oxo bridge could be either of the water ligands coordinated to each of the irons in mouse R2 protein, based on 2.7−2.9 Å O−O distances in the diferric *E. coli* RNR−R2 structure. The H-bonded water ligands to the iron ions seem to have crucial roles in the formation and structure of the iron−radical site in mouse RNR−R2. The water on Fe1 originates from molecular oxygen in the radical reconstitution reaction, while the water at Fe2 is suggested to have an important function in the conversion of intermediate X to the radical-containing form, as it was postulated to displace a bridging carboxylate oxygen in X.[21] This Fe2 water ligand, which is perturbed in the *E. coli* RNR−R2 mutant E238A[19] and is in a bridging position in the diferric *S. typhimurium* RNR−R2 crystal structure,[22] could contribute the hydrogen bond to the μ-oxo bridge in mouse RNR−R2. The presence of this H bond may help explain how the mouse and the HSV1 R2 proteins can form stable mixed-valence Fe(III)Fe(II) clusters, probably containing hydroxo bridges,[23] at ambient temperature (i.e., the proton involved in the H bond may be transferred to the bridging oxygen), while *E. coli* RNR−R2 lacks this H bond and has not been isolated in the mixed valence form. The reconstitution mechanisms of RNR−R2 from *E. coli* and mouse exhibit different kinetics,[24] with the rate-limiting steps being conversion of intermediate X to the active form and formation of X, respectively, indicating that the radical-forming tyrosine is more strongly interacting with the iron cluster in mouse R2 via the tyrosyl H bond.[7b,8,24] The presence or lack of the two H-bonding interactions in the different RNR−R2 proteins may contribute to this difference in reactivity.

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