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Identification of a Hydroxide Ligand at the Iron Center of Ribonucleotide Reductase by Resonance Raman Spectroscopy†

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ABSTRACT: The resonance Raman spectrum of protein B2 of ribonucleotide reductase from *Escherichia coli* shows several features related to its oxo-bridged binuclear iron center. A peak at 492 cm^{-1} is assigned to the symmetric stretch of the Fe–O–Fe moiety on the basis of its 13-cm^{-1} shift to lower energy upon ^{18}O substitution. The ^{18}O species shows an additional peak at 731 cm^{-1}, which is a good candidate for the asymmetric stretch of the Fe–O–Fe moiety. Its exact location in the ^{16}O species is obscured by the presence of a protein tryptophan vibration at 758 cm^{-1}. A third resonance-enhanced peak at 598 cm^{-1} is identified as an Fe–OH vibration on the basis of its 24-cm^{-1} shift to lower energy in $H_2{}^{18}O$, its 2-cm^{-1} shift to lower energy in D_2O, and its pH-dependent intensity. A hydrogen-bonded μ-oxo bridge similar to that in hemerythrin is suggested by the unusually low frequency for the Fe–O–Fe symmetric stretch and the 3-cm^{-1} shift to higher energy of ν_s(Fe–O–Fe) in D_2O. From the oxygen isotope dependence of ν_s(Fe–O–Fe), an Fe–O–Fe angle of 138° can be calculated. This small angle suggests that the iron center consists of a tribridged core as in hemerythrin. A model for the binuclear iron center of ribonucleotide reductase is presented in which the hydroxide ligand sites provide an explanation for the half-of-sites reactivity of the enzyme.

Ribonucleotide reductase is the enzyme responsible for a balanced supply of precursors for DNA synthesis in all living organisms. One type of ribonucleotide reductase consists of two nonidentical subunits forming an enzymatically active one-to-one complex (Lammers & Follmann, 1983; Thelander & Reichard, 1979). The small subunit of this type of ribonucleotide reductase contains a unique prosthetic group, a tyrosyl radical stabilized by an adjacent binuclear iron center. In *Escherichia coli* the small subunit of ribonucleotide reductase, which contains two identical 43.5-kDa polypeptide chains, has been denoted protein B2. Over 10 years ago it was observed that the visible absorption spectrum of protein B2 was very similar to that of oxy and hydroxomet forms of the respiratory protein hemerythrin (Atkin et al., 1973; Garbett

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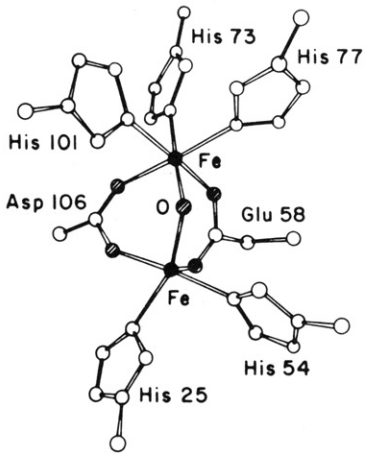
et al., 1969). The similarities between the iron centers of protein B2 and hemerythrin were also evident from Mössbauer spectroscopic (Atkin et al., 1973) and later magnetic susceptibility (Petersson et al., 1980) studies. It was suggested that the iron center of protein B2, like that of hemerythrin, involved two antiferromagnetically coupled ferric irons (Petersson et al., 1980).

In hemerythrin, all ligands of the binuclear iron center are known from crystallographic (Stenkamp et al., 1984, 1985), resonance Raman (Kurtz et al., 1977; Shiemke et al., 1984), and extended X-ray absorption fine structure (EXAFS) (Hendrickson et al., 1982; Elam et al., 1982) studies. The structure of the iron site in methemerythrin on the basis of X-ray crystallography (Stenkamp et al., 1984) is



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In this structure, the two face-sharing octahedral ferric iron atoms are connected via a solvent-derived μ-oxo bridge and two bridging protein carboxylates. Of the remaining six coordination sites, five are occupied by protein imidazoles and one is an exogenous ligand position where hemerythrin expresses its role as an oxygen-transport protein. The natural ligand is hydroperoxide, derived from the reduction of dioxygen during the conversion of diferrous deoxyhemerythrin to diferric oxyhemerythrin. In vitro, other low molecular weight ligands (e.g., OH^-, CN^-, N_3^-, etc.) may occupy this site to give different forms of methemerythrin.

In protein B2 of ribonucleotide reductase the presence of a μ-oxo bridge in the binuclear iron center was recently demonstrated with resonance Raman spectroscopy (Sjöberg et al., 1982). Preliminary crystallographic data have been obtained for protein B2 (Joelson et al., 1984), but since no three-dimensional information is yet available, other ligands of the iron center remain unknown. However, the primary structure of protein B2 from *E. coli* has been deduced from the nucleotide sequence of the corresponding gene (Carlson et al., 1984). Comparison of its protein sequence with those of several other iron-containing ribonucleotide reductases shows that 19 of the ~350 amino acid residues are totally conserved (Sjöberg et al., 1985, 1986b; Thelander & Berg, 1986). One of the highly conserved regions includes an invariant tyrosine residue, which has recently been proved to harbor the tyrosyl radical by use of site-directed mutagenesis (Larsson & Sjöberg, 1986). Since the tyrosyl radical and the iron center are very similar in all species of ribonucleotide reductase studied (Gräslund et al., 1985), it is most likely that ligands of the iron center are also to be found among the conserved glutamate, aspartate, and histidine residues.

In a recent resonance Raman study on oxyhemerythrin and hydroxomethemerythrin, hydrogen bonding between the μ-oxo bridge and the exogenous ligand was clearly demonstrated (Shiemke et al., 1986). In view of the compelling similarities between hemerythrin and protein B2, a similar study was undertaken with the latter protein. The major achievement of this study was the identification of a hitherto unknown hydroxide ligand of the B2 iron center. In addition, hdyrogen bonding of the μ-oxo bridge of protein B2 was established. However, the behavior of the hydrogen-bond donor in B2 is different from that of the hydroxide ligand in hydroxomethemerythrin. A detailed structure of the iron center of protein B2 and a reaction sequence for the formation of the tyrosyl radical are proposed.

Materials and Methods

Homogeneous protein B2 was purified from an overproducing *E. coli* strain carrying the recombinant plasmid pBS1 (Sjöberg et al., 1986a). Hydroxyurea was from Calbiochem-Behring Corp., La Jolla, CA; D_2O (99.8 atom %) was from Norsk Hydro, Oslo, Norway; $H_2{}^{18}O$ (95 atom %) was from Monsanto Co., Miamisburg, OH.

Preparation of Raman Samples. Most Raman studies were performed with the radical-free form of protein B2, obtained by incubating concentrated B2 preparations with 10 mM hydroxyurea for 5 min at 25 °C. All samples were maintained in 25 mM potassium phosphate buffer. Deuterium- or ^{18}O-enriched buffer was prepared by dissolving freeze-dried potassium phosphate buffer in the enriched water. For deuterium enrichment, the procedure was repeated twice. For buffer exchanges, native or radical-free B2 was diluted 10–50 times in the desired buffer and concentrated in a Centricon 10 filtration device (Amicon) at 4 °C for 2 h. This procedure was repeated twice and yielded preparations that were approximately 2 mM in protein B2.

Raman Spectroscopy. Resonance Raman spectra were collected on a computer-interfaced Jarrell-Ash spectrophotometer (Loehr et al., 1979) equipped with a Spectra-Physics 164-01 (Kr) ion laser, an RCA C31034A photomultiplier tube, and an ORTEC Model 9302 amplifier/discriminator. Low-temperature spectra at 90 K were obtained by backscattering from a capillary sample tube held in a copper cold-finger and Dewar cooled with liquid N_2 (Sjöberg et al., 1982). Spectra at 278 K were obtained in the same device cooled with ice. For other temperatures (104–253 K), a Varian E-4540 variable-temperature controller was used and operated with a stream of cold N_2 gas over the sample capillary.

Results and Discussion

Fe–O–Fe and Fe–OH Vibrations. Previous Raman studies of ribonucleotide reductase protein B2 identified the Fe–O–Fe symmetric stretching mode from its 496-cm^{-1} frequency and its 15-cm^{-1} shift to lower energy in $H_2{}^{18}O$ (Sjöberg et al., 1982). These experiments were performed on protein precipitated in 60–80% ammonium sulfate at pH 7.6 and frozen at 90 K. We have now been able to obtain Raman spectra for the protein in solution at 278 K. The data are of markedly improved quality due to the reduced background scatterring of the liquid samples and the removal of vibrational modes due to sulfate. At 278 K, the Fe–O–Fe symmetric stretch occurs at 492 cm^{-1} instead of 496 cm^{-1} (Figure 1A). In addition, two new resonance-enhanced peaks are observed at 598 and 756 cm^{-1}. By comparison with hemerythrin (Table I), the frequency at 598 cm^{-1} agrees well with the value of 565 cm^{-1} for the Fe–OH stretch of the hydroxide ligand in hydroxomethemerythrin, and the one at 756 cm^{-1} is at the correct energy for the asymmetric mode of the Fe–O–Fe vibrations.

Table I: Comparison of Resonance Raman Spectral Properties of Ribonucleotide Reductase, Hemerythrin, and Tribridged Binuclear Iron Model Compounds[a]

sample	ν_s(Fe–O–Fe)			ν_{as}(Fe–O–Fe)		ν(Fe–OH)		
	$H_2{}^{16}O$	Δ in ^{18}O	Δ in D_2O	$H_2{}^{16}O$	Δ in ^{18}O	$H_2{}^{16}O$	Δ in ^{18}O	Δ in D_2O
ribonucleotide reductase								
native B2	493	−15[b]	+5[b]	nd	nd	595	nd	nd
radical-free B2	492	−13	+3	~756[c]	−25[c]	598	−24	−2
hemerythrin[d]								
oxy	486	−14	+4	753	−36			
hydroxomet (cis)	492[e]	−15	+26[f]	782	−42	565	−27	−5
tribridged models								
$[HBpz_3]$[g]	528	−17	nd	751	−30			
[tacn][h]	540	−17	nd	749	−33			

[a] Peak frequencies and isotope shifts in cm^{-1}. Protein spectra obtained at 278 K and model compounds at 298 K. nd = not determined. [b] For data obtained at 90 K. [c] Exact frequencies uncertain due to occurrence of protein tryptophan vibration at 758 cm^{-1}. [d] Data from Shiemke et al. (1984, 1986). [e] Data for cis form, which has Fe–OH hydrogen bonded to oxo bridge. [f] Extremity of upward shift due to coupling of ν_s(Fe–O–Fe) with δ(Fe–OD). [g] $Fe_2O(Ac)_2(HBpz_3)_2$, where $HBpz_3$ is tris(1-pyrazolyl)borate; data from Armstrong et al. (1984). [h] $[Fe_2O(Ac)_2(tacn)_2]^{2+}$, where tacn is triazacyclononane; data from Spool et al. (1985).

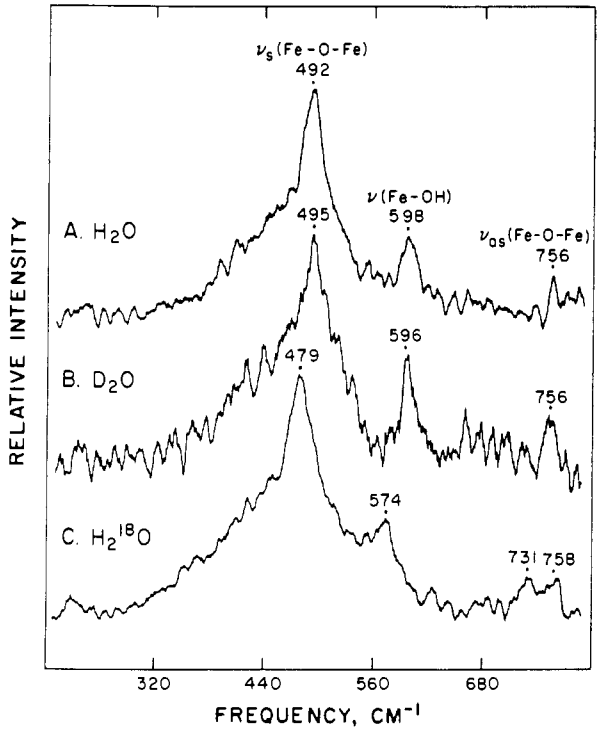

FIGURE 1: Resonance Raman spectra of radical-free protein B2 of *E. coli* ribonucleotide reductase in solvents of different isotopic composition: (A) protein in 25 mM phosphate (pH 8.1); (B) protein exposed to D_2O for 6 h at 277 K in 25 mM phosphate (pD 8.7); (C) protein exposed to $H_2{}^{18}O$ for 6 h at 277 K in 25 mM phosphate (pH 7.9). Spectra were obtained at 278 K with 406.7-nm excitation (13 mW) and are accumulations of 15, 6, and 10 scans, respectively; scan rate was 2 cm^{-1} s^{-1}; slit width was 5 cm^{-1}. Spectra have been subjected to background subtraction and a 25-point third-order polynomial smoothing function (Loehr et al., 1979). The broad underlying band centered at ~450 cm^{-1} is scattering from glass.

To further substantiate these assignments, we tested the exchangeability of these oxy ligands with solvent.

Examination of $H_2{}^{18}O$-equilibrated protein B2 at 278 K (Figure 1C, Table I) reveals a 13-cm^{-1} decrease in ν_s(Fe–O–Fe), similar in magnitude to that observed previously (Sjöberg et al., 1982). In the region characteristic of the Fe–O–Fe asymmetric stretch, there is clearly a new peak at 731 cm^{-1} in $H_2{}^{18}O$ as well as a residual feature at 758 cm^{-1} (Figure 1C). The latter is most likely due to a protein moiety such as tryptophan (Lord & Yu, 1970) and, thereby, complicates the assignment of ν_s(Fe–O–Fe) in $H_2{}^{16}O$.

In various forms of methemerythrin, the asymmetric Fe–O–Fe vibration occurs at 750–780 cm^{-1} and decreases 30–42 cm^{-1} in $H_2{}^{18}O$ (Shiemke et al., 1984). These frequencies agree well with those expected for an Fe–O–Fe angle of 135°, which is the known value in the azidomet form (Stenkamp et al., 1984). A similar set of ν_s and ν_{as} peaks is observed for the tribridged Fe–O–Fe model compounds (Table I) in which the two Fe(III)'s are also bridged by two carboxylates as in methemerythrin and the resulting Fe–O–Fe angle is <130° (Armstrong et al., 1984; Wieghardt et al., 1983; Spool et al., 1985). The close match of the ν_s(Fe–O–Fe) frequency and ^{18}O shift in ribonucleotide reductase implies an analogously small angle for the Fe–O–Fe bridge. Application of the secular equation for ν_s(Fe–O–Fe) (Wing & Callahan, 1969) leads to a calculated Fe–O–Fe angle of 138° for radical-free protein B2. The 25-cm^{-1} shift from 756 to 731 cm^{-1} is somewhat small for an asymmetric stretch according to normal coordinate analysis calculations (W. D. Wheeler, A. K. Shiemke, B. A. Averill, T. M. Loehr, and J. Sanders-Loehr, unpublished results). However, the cyano and cyanato forms of methemerythrin have similarly small oxygen isotope shifts of 28 and 26 cm^{-1}, respectively, for ν_{as} (Fe–O–Fe) (Shiemke, 1986). Thus, 756 cm^{-1} appears to be a possible frequency for the asymmetric vibration in ribonucleotide reductase, but its exact location is obscured by the presence of a protein peak in this region.

The 598-cm^{-1} vibration of radical-free B2 shifts 24 cm^{-1} to lower energy in $H_2{}^{18}O$. The 4.0% decrease demonstrates that this vibration is due to a solvent-exchangeable oxygen atom. The theoretical decrease predicted for a 598-cm^{-1} Fe–OH vibration on the basis of the increased mass of the oxygen is 4.1% or 25 cm^{-1}. Similarly, in hydroxomethemerythrin, the Fe–OH vibration at 565 cm^{-1} undergoes a shift of 27 cm^{-1} or 4.8% in $H_2{}^{18}O$ (Shiemke et al., 1986). The nature and magnitude of the shift is, thus, highly suggestive of the presence of a hydroxide ligand in ribonucleotide reductase. Further evidence for the ν(Fe–OH) assignment has been obtained from the pH dependence and deuterium isotope sensitivity of this vibrational mode, as described below.

Effect of pH on Fe–OH Vibration. In hydroxomethemerythrin, protonation of the hydroxide group ($pK_a \sim 8$) results in the disappearance of the Fe–OH mode at 565 cm^{-1} (Shiemke et al., 1986). Crystallographic studies indicate that the low-pH form known as methemerythrin has actually lost its hydroxo group and that the remaining iron is pentacoordinate (Stenkamp et al., 1984). The amplitude of the 598-cm^{-1} peak of ribonucleotide reductase is also quite sensitive to pH. By comparison with the 492-cm^{-1} ν_s(Fe–O–Fe) peak (whose intensity appears constant, but somewhat obscured by the underlying glass band), it can be seen that the 598-cm^{-1}

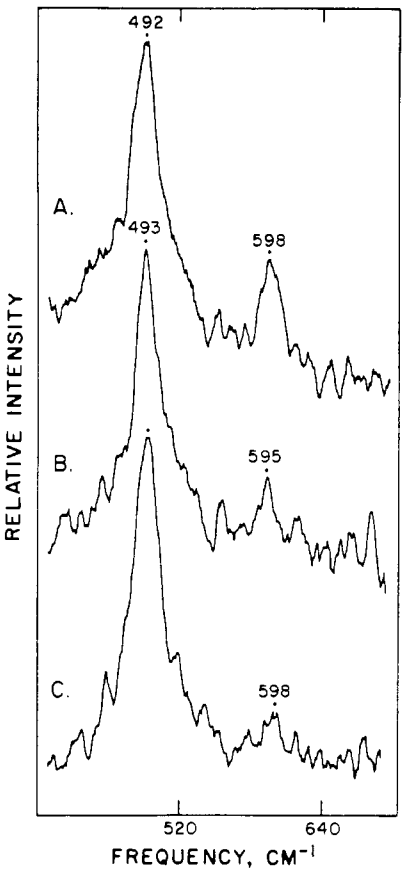

FIGURE 2: Resonance Raman spectra of native and radical-free protein B2 at different pH values: (A) radical-free protein at pH 8.1; (B) native protein at pH 8.0; (C) radical-free protein at pH 6.7. Spectra were obtained as described in Figure 1 and are accumulations of 15, 19, and 15 scans, respectively.

peak in radical-free B2 undergoes a 2.6-fold decrease in intensity between pH 8.1 (Figure 2A) and pH 6.7 (Figure 2C). For the protein in D_2O solvent (data not shown), lowering the pD from 8.7 to 6.9 results in only a 1.8-fold decrease in the intensity of ν_s(Fe–OH). These data are consistent with the protonation of an iron-coordinated hydroxo group. They indicate that the pK_a in H_2O is close to 7 and slightly lower than the pK_a in D_2O.

Analysis of the Raman spectrum of radical-free B2 at pH 6.7 gives no indication of a new vibrational mode between 200 and 600 cm^{-1}, which might be assigned to an Fe–OH_2 stretch. This could be due either to a loss of the water molecule, as in methemerythrin, or to a lack of resonance enhancement as, for example, if the ligated water molecule were no longer part of the optical chromophore. In the case of the conversion of the hexacoordinate iron in hydroxomethemerythrin to the pentacoordinate iron in methemerythrin, there is a marked change in the optical spectrum around 350 nm (Garbett et al., 1969). In ribonucleotide reductase we have not been able to observe any such change in the optical spectrum of native B2 between pH 6.5 and pH 9.5 (data not shown). Thus, the water molecule may still be associated with the iron atom in ribonucleotide reductase.

Since the B2 protein is composed of two identical polypeptide chains, the binuclear iron site must lie on a subunit interface and exhibit 2-fold symmetry with respect to the protein ligands of the two iron atoms. In keeping with this symmetry, we would expect each of the iron atoms in radical-free B2 to have a coordinated hydroxide ligand. In native B2, this symmetry would be partially destroyed since one of the two subunits has its Tyr-122 converted to a free radical and since this free radical is located in close proximity to the binuclear iron center (Larsson & Sjöberg, 1986; Reichard & Ehrenberg, 1983; Sahlin et al., 1987). The Raman spectrum of the native form of protein B2 at pH 8.0 (Figure 2B) shows a peak corresponding to an Fe–OH mode at 595 cm^{-1}. However, the intensity of this peak relative to the Fe–O–Fe peak at 493 cm^{-1} appears to be lower than that observed for radical-free B2 at a similar pH value (Figure 2A). The lowered intensity of the 595-cm^{-1} peak in native B2 suggests the possibility that the hydroxide ligand associated with the radical side of B2 may have become protonated.

The tyrosyl radical in native B2 has been previously identified by its characteristic optical and EPR spectral properties (Reichard & Ehrenberg, 1983). Another property that is typical of phenoxy radicals is the appearance of a strongly enhanced resonance Raman mode near 1500 cm^{-1} (C–O stretch) upon excitation within the narrow ~400-nm absorption band (Tripathi & Schuler, 1984). Raman spectra of native B2 obtained at 90 K with either 406.7- or 413.1-nm excitation [using conditions described in Sjöberg et al. (1982)] do exhibit a resonance-enhanced feature at 1500 cm^{-1} that is not seen with longer excitation wavelengths or with the apoprotein. Since a vibrational mode at this frequency is generally associated with a deprotonated phenoxy radical (Tripathi & Schuler, 1984), it is likely that the tyrosyl radical in native B2 is also deprotonated. This tyrosyl moiety could be responsible for the specific removal and replacement of the C-3 hydrogen of the ribosyl substrate during the catalytic cycle (Reichard & Ehrenberg, 1983; Stubbe et al., 1983).

Effect of Deuterium on Fe–O–Fe and Fe–OH Vibrations. Earlier observations on the ν_s(Fe–O–Fe) vibration of ribonucleotide reductase showed a 4–5-cm^{-1} shift to higher energy in D_2O for both the native and radical-free forms of B2 (Sjöberg et al., 1982). Subsequent studies of the oxy and hydroxomet forms of hemerythrin indicated similar increases in their respective symmetric Fe–O–Fe stretching frequencies upon deuteriation (Table I) and were attributed to a hydrogen bond between the exogenous ligand (hydroperoxide or hydroxide, respectively) and the μ-oxo bridge (Shiemke et al., 1986). Furthermore, the strength of this intramolecular hydrogen bond could be judged from the temperature dependence of ν_s(Fe–O–Fe). While both oxy and hydroxomet species exhibited a single vibrational mode at 90 K, raising the temperature to 278 K resulted in the appearance of a second ν_s(Fe–O–Fe) peak from a non-hydrogen-bonded form of hydroxomethemerythrin. However, no new peak was observed in oxyhemerythrin.

We have performed a similar set of experiments on ribonucleotide reductase between 90 K and 278 K and failed to observe any temperature effect on either the intensity or the band contour of the Fe–O–Fe symmetric stretch. Although the frequency of the vibration gradually decreases as the temperature increases, the shape of the peak does not change, and no shoulders appear at higher temperatures. This behavior is analogous to that of oxyhemerythrin, and it clearly shows that whatever hydrogen bonds are involved in the μ-oxo bridge of ribonucleotide reductase, they must be relatively strong. This observation would tend to rule out the hydroxide ligand as the direct hydrogen bond donor since the strain in that four-membered ring results in a rather weak hydrogen bond. Nevertheless, the involvement of the oxo bridge as a hydrogen-bond acceptor is likely since polar groups tend to make as many hydrogen bonds as possible when buried inside a protein (Baker & Hubbard, 1984). For example, each of the bridging sulfido groups of the binuclear iron–sulfur cluster in

Spirulina ferredoxin is involved in a hydrogen bond to the protein backbone (Tsukihara et al., 1981).

The Fe–OH mode of radical-free B2 is also deuterium sensitive, shifting from 598 cm^{-1} in H_2O to 596 cm^{-1} in D_2O (Figure 1B). This 2-cm^{-1} decrease is considerably smaller than the 13-cm^{-1} decrease expected on the basis of the change in reduced mass, but it is analogous to the behavior of the Fe–OH mode of hydroxomethemerythrin, which shifts only 5 cm^{-1} in D_2O (Table I). The unusually small deuterium isotope effect in both cases may be due to the involvement of the OH group as a hydrogen-bond donor. Alternatively, the smaller-than-expected frequency shift with deuterium could signal coupled Fe–O–Fe and Fe–OH vibrational modes. For example, in the oxo-bridged complex $[Fe_2O(4\text{-chloro-2,6-pyridinedicarboxylato})_2(H_2O)_4]\cdot 4H_2O$, the protons of the aquo ligands are 2.65–2.78 Å away from the oxo group (Ou et al., 1978) and, hence, beyond a reasonable hydrogen-bonding distance (Baker & Hubbard, 1984). In this case, resonance-enhanced vibrations at 431 and 457 cm^{-1} attributed to $Fe–OH_2$ stretching (W. D. Wheeler, A. K. Shiemke, B. A. Averill, T. M. Loehr, and J. Sanders-Loehr, unpublished results) undergo anomalously small deuterium isotope shifts of −5 and −7 cm^{-1}, respectively, compared to the expected value of −26 cm^{-1} from the change in reduced mass. In the absence of hydrogen bonding, the possible coupling of these vibrations to the 381-cm^{-1} ν_s(Fe–O–Fe) may account for the weak deuterium isotope dependence.

Model for Binuclear Iron Center in Ribonucleotide Reductase. Binuclear Fe(III) complexes with a single bridging oxo group exhibit Fe–O–Fe angles of 180–139° (Thich et al., 1981). Oxo-bridged iron centers readily add two bridging carboxylates to form structures such as $[Fe_3O(alanine)_6(H_2O)_3]$ (Thich et al., 1981) or $[Fe_2O(Ac)_2(L_2)]$, where L is a tridentate ligand, notably tris(1-pyrazolyl)borate (Armstrong et al., 1984), triazacyclononane (Wieghardt et al., 1983), or half of a tetrakis(picolyl) diamine (Toftlund et al., 1986). The Fe–O–Fe angles for the tribridged model complexes range from 124 to 119°. The tribridged centers in methemerythrins have Fe–O–Fe angles ranging from 143 to 130°, on the basis of X-ray crystallography (Stenkamp et al., 1984) and ν(Fe–O–Fe) oxygen isotope effects (W. D. Wheeler, A. K. Shiemke, B. A. Averill, T. M. Loehr, and J. Sanders-Loehr, unpublished results). For ribonucleotide reductase, an Fe–O–Fe angle of 138° can be calculated from the Raman spectroscopic data for ν_s(Fe–O–Fe). A similar value has been derived from EXAFS analyses on the basis of an iron–iron distance of 3.22–3.48 Å and an Fe–O distance of 1.78 Å (Bunker et al., 1987; Scarrow et al., 1986). Thus, it is likely that the binuclear iron site of ribonucleotide reductase, like hemerythrin, contains a tribridged core.

In hemerythrin the bridging groups are one μ-oxo and two protein carboxylates, Glu-58 and Asp-106. In ribonucleotide reductase one bridging group is the μ-oxygen. We expect to find the other two bridging groups among the six evolutionarily conserved carboxylate residues. Residues like Glu-115, Asp-237, and Glu-238 are likely candidates, since they occur in areas of high general homology and include the only two conserved histidine residues (Sjöberg et al., 1985). In view of the 2-fold symmetry of the B2 protein, it is likely that each of the subunits contributes the same carboxylate residue and that only one of the conserved carboxylates occurs in a bridging position. This proposed structure is depicted in Figure 3.

The proposed structural model for the iron cluster in ribonucleotide reductase also shows at least one histidine ligand per iron atom. There is considerable evidence for this. First,

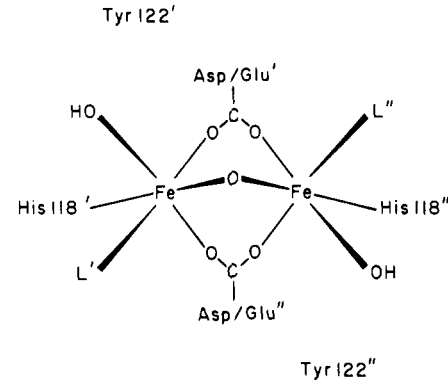

FIGURE 3: Model for the binuclear iron center of *E. coli* ribonucleotide reductase in the radical-free state. Possible protein ligands have been indicated; L = unassigned ligand. Each iron is presumed to be associated primarily with one of the two identical polypeptide chains. The ligands arising from each subunit are denoted by the superscripts (′) and (″).

a paramagnetically shifted, deuterium-exchangeable resonance was observed by NMR spectroscopy at 24 ppm (Sahlin et al., 1986) and assigned to a histidine ligand by its analogy with histidine resonances in hemerythrin (Maroney et al., 1984). The histidine resonance in B2 was ~3 times less intense than another unassigned paramagnetically shifted resonance at 19 ppm. This is clearly different than in the case of several hemerythrin NMR samples, where the histidine resonances are the most prominent features in the spectrum. Second, the amino acid sequence of protein B2 includes two evolutionarily conserved histidine residues, His-118 and His-241 (Sjöberg et al., 1985). Third, EXAFS data indicate a mixture of N and O ligands in the first shell and one to two histidines per iron in the third shell (Bunker et al., 1987; Scarrow et al., 1986). Fourth, the resonance Raman enhancement factor for ν_s(Fe–O–Fe) is ~300 relative to $\nu_1(SO_4^{2-})$; scattering factors of this magnitude have only been observed in binuclear iron sites with unsaturated ligands such as imidazole and pyrazole (W. D. Wheeler, A. K. Shiemke, B. A. Averill, T. M. Loehr, and J. Sanders-Loehr, unpublished results). The ligand referred to as L in Figure 3 is most likely a protein residue. The NMR and EXAFS data favor a carboxylate rather than a histidine.

The remaining coordination site on each iron atom is assigned to a hydroxide ion, with tyrosine-122 in close proximity. It is likely that only one of the two hydroxide ligand sites is utilized for dioxygen binding during activation of the tyrosyl radical. This process is known to require a reduced form of the protein reacting with O_2 (Petersson et al., 1980; Barlow et al., 1983; Eliasson et al., 1986). By analogy to deoxyhemerythrin (Reem & Solomon, 1984; Stenkamp et al., 1985) and a ferrous model compound (Chaudhuri et al., 1985), the Fe(II) form of ribonucleotide reductase would be expected to have a similar structure to the Fe(III) form but with a hydroxo bridge instead of the μ-oxo bridge. In the ferrous form of the protein the two hydroxide ligands would be less strongly coordinated or even absent, as appears to be the case in deoxyhemerythrin (Reem & Solomon, 1984), thus creating a readily exchangeable or available site for O_2 binding. It is assumed that only one O_2 binds since a single molecule of dioxygen should be capable of oxidizing both of the iron atoms and the tyrosine ring. This activation reaction might actually bear some resemblance to the oxygenation of deoxyhemerythrin with an initial step being the reduction of dioxygen to peroxide. The random binding of O_2 to one of the two iron atoms in ribonucleotide reductase provides a structural ex-

planation for the stoichiometry of one tyrosyl radical per dimeric iron center (Petersson et al., 1980) and the half-of-sites reactivity of the enzyme (Sjöberg et al., 1987).

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