Spectroscopic Studies of Co(II)-Reconstituted Ribonucleotide Reductase R2 from *Escherichia coli*

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A Co(II)-substituted form of the R2 protein of ribonucleotide reductase from *Escherichia coli* has been prepared by anaerobic addition of 4 equiv of Co(II) to the $R2_{apo}$ protein in order to explore structural changes that may occur when divalent metal ions are coordinated in the metal binding sites. The visible absorption and circular dichroism spectra of the Co(II)-substituted R2 protein contain multiple absorption features in the 500–600-nm region with two apparent maxima at 515 and 550 nm (ϵ_{550} = 115 M^{-1} cm^{-1}/Co(II)) indicative of Co(II) in a five-coordinate environment consisting of nitrogen or oxygen ligands. The intensity of the g = 5.44 signal in the EPR spectrum of the Co(II)–R2 complex, typical of $S = {}^3/_2$ high-spin mononuclear Co(II), indicates that the Co(II) moieties are uncoupled when bound to the R2 protein. The efficient electronic relaxation properties of Co(II) in this coordination environment have enabled us to obtain remarkably high-resolution paramagnetically shifted ^1H NMR spectra, relative to the diferrous and diferric protein, despite the 87 kDa size of R2. Preliminary assignments of several of these resonances are made. The spectroscopic properties described here remain unchanged when the protein is eluted from an air-saturated gel filtration column suggesting that, unlike the very air-sensitive diferrous R2 protein, the Co(II)–R2 complex does not readily bind or activate dioxygen.

Ribonucleotide reductase (RNR) catalyzes the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides, an essential step in the synthesis of DNA in all living cells.[1-3] The *Escherichia coli* enzyme contains two proteins, designated R1 (α_2) and R2 (β_2). The R1 protein possesses the substrate and effector binding sites as well as several redox-active thiols.[4-6] The active form of the R2 protein ($R2_{ox}$) contains a stable tyrosine radical at residue 122, essential for enzymatic activity,[7] and two nonheme (μ-oxo)diferric clusters.[8] The radical free diferric form ($R2_{met}$) has been crystallized and the X-ray structure determined.[9] The coordination environment of the diiron clusters is shown in Figure 1.

It is the diferrous form of the protein ($R2_{red}$) which reacts with dioxygen to generate the tyrosine radical.[10] The strong antiferromagnetic coupling of the diferric clusters[10] is significantly decreased upon reduction[8,11] most likely due to the elimination or modification of the oxo bridge that mediates the magnetic coupling in the diferric form. The nature of the changes in the ligand environment upon reduction is not yet fully understood. Near-IR circular dichroism studies of diferrous R2 suggest that neither of the ferrous ions retains the six-coordinate geometry of the diferric form; one iron is five-coordinate, and the other may be four- or five-coordinate.[12]

Substitution of active site metal ions has provided significant structural information for a variety of metalloproteins.[13] The iron can be readily removed from R2 to form $R2_{apo}$ by dialysis against the lithium salt of 8-hydroxyquinoline-5-sulfonic acid in a slightly denaturing 1.0 M imidazole buffer.[7] Reconstitution of $R2_{apo}$ with Fe(II) in the presence of ascorbate yields active protein.[14] Mn(II) reconstitution of $R2_{apo}$ was recently reported,[15] and its crystallographic characterization has provided insight into the structural changes that may occur when divalent metal ions are bound in the metal binding sites. The coordination environment of the dimanganese site shown in Figure 1 retains the same amino acid ligands as the diiron site, but with significant changes in the mode of carboxylate binding. Glu238, which is monodentate in the diferric structure, bridges the two metal ions in Mn–R2. Furthermore, the asymmetrically bidentate mode of

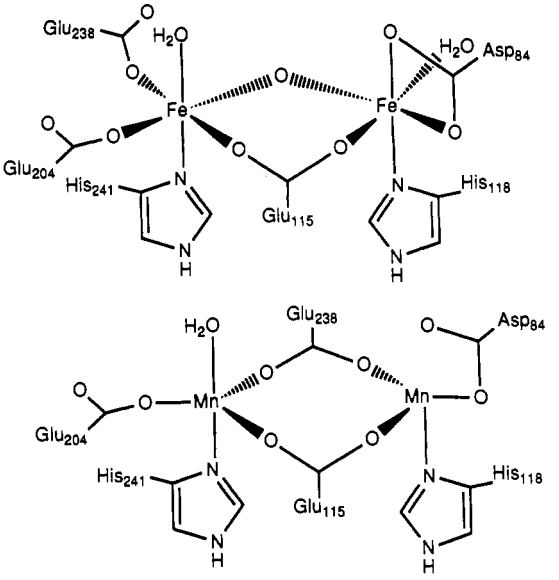

Figure 1. Metal cluster coordination environment of the native diferric[9] and manganese(II)-reconstituted[15] ribonucleotide reductase R2 protein.

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Asp84 in the diferric structure has changed to monodentate in the Mn(II) form. The dimanganese cluster does not appear to possess a single atom bridge (O^{2-}, HO^-, H_2O), but rather is bridged by two carboxylates (Glu115 and Glu238) resulting in a Mn–Mn distance of 3.6 Å (0.3 Å longer than the Fe(III)–Fe(III) distance). Both manganese ions show distorted trigonal bipyramidal coordination, in contrast to the octahedral coordination of the irons in the diferric form. The retention of the endogenous ligands following Mn(II) reconstitution and the observation that the protein structures are nearly identical for the $R2_{apo}$ and $R2_{met}$[15] demonstrates that the metal ion binding sites in R2 are well-defined. We have sought to further characterize these sites by reconstitution with Co(II). The coordination flexibility and rich spectroscopic properties of Co(II) make it ideal for reconstitution studies of $R2_{apo}$. We report here results which demonstrate Co(II) binding to R2 and the spectroscopic properties of this Co–R2 derivative.

Materials and Methods

R2 was isolated from *E. coli* strain N6405/pSPS2,[16] a heat-inducible overproducer, as previously described,[17] and its concentration was determined by absorbance at 280 nm (ϵ_{280} = 141 mM^{-1} cm^{-1}). R2 isolated by this method consistently displays a specific activity of 4000 ± 700 determined spectrophotometrically as previously described.[8] Iron removal was accomplished by dialysis against the lithium salt of 8-hydroxyquinoline-5-sulfonic acid in 1.0 M imidazole as previously described.[7] The concentration of $R2_{apo}$ was determined by absorbance at 280 nm (ϵ_{280} = 120 mM^{-1} cm^{-1}). Anaerobic addition of a Co(II) solution ($CoCl_2 \cdot 6H_2O$ (Johnson Matthey) or $Co(NO_3)_2$ (Aldrich)) to $R2_{apo}$ was accomplished using standard vacuum line techniques. The Co–R2 sample was passed down a Sephadex G-25 column to remove any unbound metal ions.

Inductively coupled plasma emission (University of Minnesota facility) was employed to quantify the amount of cobalt bound to the protein. This data was subsequently used to establish molar extinction coefficients for the visible ligand field bands. Optical absorption spectra were collected on a Hewlett-Packard 8541A diode array spectrophotometer. Circular dichroism experiments were performed on a JASCO 500 CD spectrophotometer. A 1.0-cm quartz cell was used for optical experiments. EPR spectra were collected on a Varian E109 spectrometer equipped with an Oxford ESR10 cryostat for low temperature studies. A $CoCl_2$ standard was used to quantify the $S = {}^3/_2$ EPR signal. Proton NMR spectra were obtained on IBM NR/300 and Bruker AMX360 spectrometers at 30 °C using a modified DEFT pulse sequence (D1–90°–τ–180°–τ–90°–AQ) to suppress the water and other resonances due to the protein backbone in the diamagnetic region.[18] The longitudinal relaxation times (T_1) of the paramagnetically shifted features at 360 MHz were estimated by the inversion–recovery method with a three-parameter least-squares fitting routine.

Results and Discussion

The anaerobic addition of 4 equiv of Co(II) to $R2_{apo}$ yields a pink cobalt R2 derivative within seconds. The visible absorption spectrum of this form of the protein (Figure 2A) contains a broad feature between 500 and 600 nm with two apparent maxima at 515 and 550 nm and is unaffected by passing the protein down a G-25 column. The circular dichroism spectrum of this sample in the visible region contains a dominant negative feature at ca. 550 nm (Figure 2C) and further demonstrates the presence of multiple absorption bands. These results indicate that the visible features in the absorption and CD spectra arise from cobalt bound to the R2 protein.

The visible absorption spectrum of the cobalt-reconstituted R2 is clearly red shifted and more intense than the spectrum of aqueous Co(II) in the buffer solution (Figure 2B). The visible difference spectrum resulting from the anaerobic addition of 2 equiv of Co(II) to $R2_{met}$, which has four Fe(III) coordinated in the metal ion binding sites, is similar in energy and intensity to that of aqueous Co(II) (Figure 2B), indicating that the features

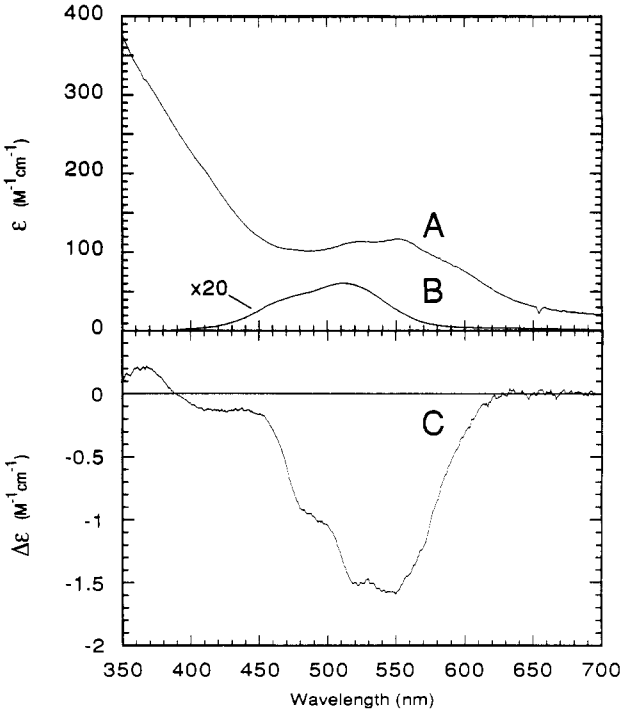

Figure 2. Visible absorption spectra of (A) anaerobic addition of 4 equiv of Co(II) to $R2_{apo}$ (0.33 mM, pH 7.6 in 25 mM HEPES) and (B) anaerobic addition of Co(II) to buffer (35.8 mM Co(II)) and (C) circular dichroism spectrum of the Co(II)–R2 complex (conditions same as A). Molar absorptivity is given per Co(II).

in the Co–R2 spectrum result primarily from Co(II) binding to the native metal binding sites as in the Mn(II) reconstitution study.[15] Furthermore, total protein and metal analyses for different preparations allow us to establish a binding stoichiometry of 4 Co/R2, a ratio consistent with full occupation of the two native dinuclear iron sites. This result is consistent with the Mn(II) reconstitution results for which it was shown that no more than 4 equiv of Mn(II) can bind to the protein, contrary to iron which can bind adventitiously.[10]

We assign the predominant feature of the optical spectrum at ca. 550 nm (ϵ_{550} = 115 M^{-1} cm^{-1}/Co(II)) to the highest energy ligand field transition, ${}^4T_{1g}$ (F) → ${}^4T_{1g}$ (P), of Co(II) in O_h symmetry. The intensity of this optical feature is greater than that of a similar transition observed in Co(II)-substituted hemerythrin (519 nm, ϵ = 34 M^{-1} cm^{-1}/Co(II))[19] whose ϵ/Co is within the range 5–40 M^{-1} cm^{-1} expected for high-spin six-coordinate Co(II).[20,21] The intensity of the 550-nm transition observed for Co–R2 is similar in intensity to the transitions observed for the acetate adduct of Co(II)-substituted bovine carbonic anhydrase II (571 nm, ϵ = 120 M^{-1} cm^{-1}/Co(II))[21] and consistent with high-spin Co(II) in a five-coordinate ligand environment consisting of nitrogen and oxygen ligands.[20,21] The reduction in coordination number compared to the diferric cluster is consistent with that reported for other divalent metal ions, Fe(II) and Mn(II), bound to the metal ion binding sites of R2.[12,15] The multiplicity of this feature may arise from several factors. The degeneracies of an O_h ligand field are removed upon lowering the symmetry resulting in multiple transitions. Spin forbidden transitions to the ${}^2G$ states may also contribute to the broadness of this feature as has been observed in several other high-spin Co(II) complexes.[20] Finally, there are likely to be two distinct Co(II) binding sites as found in the crystal structures of the native diiron[9] and manganese-reconstituted[15] R2 proteins.

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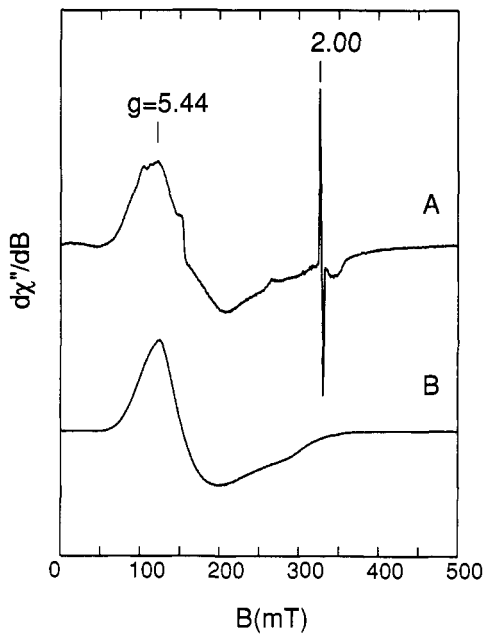

Figure 3. X-band EPR spectra of (A) anaerobic addition of Co(II) to $R2_{apo}$ and (B) anaerobic addition of Co(II) to buffer. Protein concentrations and conditions are identical to those in Figure 2. Instrument parameters: microwaves, 0.2 mW at 9.229 GHz; modulation, 20 G at 100 kHz; temperature, 2.5 K; gain, 2500; dB/dt, 2.1 mT/s.

The EPR spectrum of the Co(II)–R2 sample at 4 K contains a broad signal at ca. $g = 5.44$ (Figure 3), typical of $S = {}^3/_2$ high-spin mononuclear Co(II) species. The EPR signal for the Co(II)–R2 complex is of comparable intensity to that of an aqueous sample of $CoCl_2$ of identical concentration. This indicates that the majority of the Co(II)s bound to R2 do not interact with each other to form a magnetically coupled dinuclear site. These EPR results do not, however, rule out a bridged dicobalt cluster in Co–R2 as a recently synthesized complex containing a (μ-hydroxo)bis(μ-carboxylato)dicobalt(II) core[22] and the dicarboxylate-bridged Mn(II) reconstituted R2 protein[15] have both been shown to possess magnetically uncoupled metal centers.

The ^{1}H NMR spectrum of Co(II)–R2 in H_2O is shown in Figure 4. Despite its 87 000-Da size, several paramagnetically shifted resonances are observed. The number of features observed is significantly larger than that previously reported for the diferrous protein,[11] due to the more efficient electronic relaxation properties of the coordinated Co(II) ions. For presumably the same reason, the Co(II)–R2 spectrum is also better resolved than those of other Co(II)-substituted metalloproteins of comparable size, such as alcohol dehydrogenase[23] (80 kDa) and *E. coli* alkaline phosphatase[24] (94 kDa).

Two solvent exchangeable proton resonances at 64 and 42 ppm disappear following equilibration with D_2O (Figure 4). This allows us to assign these resonances as those arising from the solvent-exchangeable $N_\epsilon H$ protons of the two histidine residues (His241 and His118) N_δ-bound to the metal centers as found in the X-ray crystal structures of the diferric[9] and dimanganese[15] proteins. The signals at 44 and 40 ppm with respective T_1 values of 12.9 and 10.0 ms are likely due to the $C_\delta H$ protons of the coordinated histidines. Similar T_1 values and chemical shifts for the $C_\delta H$ protons have also been observed in Co(II)-substituted zinc proteins with distorted five-coordinate geometries, such as Co(II)–carbonic anhydrase and Co(II)–carboxypeptidase A.[25] Two very broad non-solvent-exchangeable signals are observed

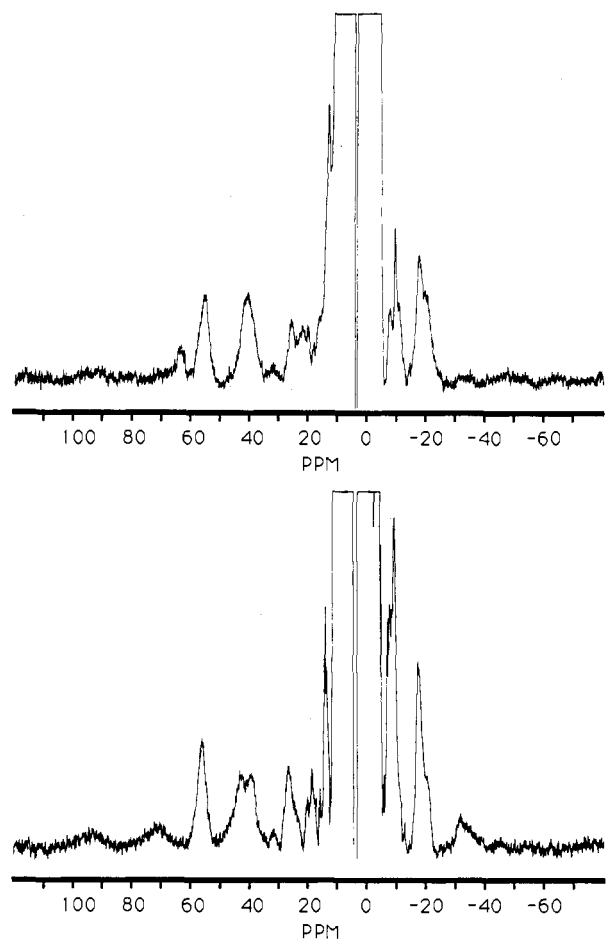

Figure 4. Proton NMR spectrum of the Co(II)–R2 complex (*303* K, *300* MHz) in 25 mM HEPES buffer (pH 7.6) in H_2O (upper) and D_2O (lower). Longitudinal relaxation values (T_1) for peaks a–d are 5.2, 12.9, 10.0, and 20.0 ms, respectively. The large diamagnetic envelope of the 87-kDa protein and the water resonance have been suppressed by the use of a modified-DEFT pulse sequence.

at 71 and 94 ppm. Their approximately 3–4-fold larger line width relative to those of the $C_\delta H$ and $N_\epsilon H$ protons suggests that the former may arise from the $C_\epsilon H$ protons of the coordinated histidine residues; this ratio is consistent with the expected r^{-6} dependence for relaxation times. A similar 3–4:1 ratio in line width has been estimated for the corresponding histidine protons in the spectra of cobalt(II) carbonic anhydrase and cobalt(II) carboxypeptidase A.[26]

Preliminary assignments of the resonances in Figure 4 may be made on the basis of the $1/r^6$ distance dependence of the relaxation time (T_1). Correlation of T_1 values to distances of ligands coordinated to the metal center, such as the histidine and the carboxylate-containing amino acid residues shown in the crystal structure of the diferric protein, is possible by this method. The remaining non-solvent-exchangeable resonances in the region of 20–60 ppm may well arise from the carboxylate-containing amino acids (i.e. Asp and Glu), which are also known to be endogenous ligands of native R2. Due to the broadness of these resonances, NOE experiments employing selective saturation to establish relative proton proximities have been unsuccessful, thus hampering a more definitive assignment of the other paramagnetically shifted

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resonances. On the assumption that histidine $C_\delta H$ protons have an average M–H distance of 5.1 Å, it is thus reasonable to assign the overlapped signals at 56 ppm (5.2 ms) and at 27 ppm (20 ms) to residues with relative M–H distances of ~4.5 and ~5.6 Å, respectively. These may be respectively associated with the $C_\beta H_2$ and $C_\alpha H$ protons of Asp84 or the $C_\gamma H_2$ and $C_\beta H_2$ protons of coordinated glutamate residues. The signals in the region of <20 ppm with relaxation times of >50 ms may be due to the protons one more bond removed from the metal center with M–H distances of >6.5 Å. Because the resolution of the Co(II)–R2 ^1H NMR spectrum is superior to that of the diferrous or diferric form of the protein[11] NMR comparisons may enable us to probe for active site structural similarities and differences between the *E. coli* R2 protein and those isolated from viral and mammalian sources. The success of these NMR experiments on Co–R2 warrants future NMR studies of Co(II)-substituted large metalloproteins (~100 kDa) possessing distorted five- or six-coordinate metal ion sites.

While the spectroscopic evidence provided here suggest that Co(II) is bound in the native metal binding sites of the protein, Co(II)–R2 does not exhibit the dioxygen sensitivity characteristic of the diferrous form of the protein. The visible absorption features shown in Figure 1A for Co–R2 remain unchanged following elution from an air-saturated gel filtration column, indicating that the Co(II) has not been oxidized by dioxygen. This apparent insensitivity to dioxygen is consistent with the recent report that Co(II)-reconstituted hemerythrin[19] does not appear to bind or activate dioxygen. However, following prolonged exposure of the Co(II)–R2 complex to dioxygen (several days), the spectral features in the 500–600-nm region decrease and a new feature appears around 350 nm, indicating partial oxidation to Co(III). This observation is similar to that observed for Co(II)-substituted hemocyanin which is oxidized slowly when exposed to air under similar conditions.[27] Coordinated in similar environments, Co(III) is expected to have a higher reduction potential than Fe(III) thereby explaining the reduction in dioxygen reactivity associated with Co–R2 relative to the diferrous form of the protein.

Conclusions

Substitution of Co(II) into the active site of R2 has allowed us to probe for structural changes associated with divalent metal ions bound to the active site. On the basis of our observed stoichiometry of 4 equiv of Co(II) bound per R2 protein, we conclude that the metal ion binding sites in R2 are structurally well-defined and serve to limit divalent metal ion binding to occupancy of the native cluster sites. Spectral and EPR data indicate that the Co(II) centers of Co–R2 are five-coordinate and magnetically uncoupled, properties similar to those observed for Mn(II) bound to the active site. Finally, the paramagnetically shifted ^1H NMR data for the Co(II)–R2 complexes demonstrates that this technique can provide relatively high resolution spectra which may ultimately provide a spectroscopic probe of structural differences and similarities between the metal ion sites of other R2 proteins such as those isolated from viral and mammalian sources.

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