Cobalt Substitution of Mouse R2 Ribonucleotide Reductase as a Model for the Reactive Diferrous State

SPECTROSCOPIC AND STRUCTURAL EVIDENCE FOR A FERROMAGNETICALLY COUPLED DINUCLEAR COBALT CLUSTER

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The R2 dimer of mouse ribonucleotide reductase contains a dinuclear iron-oxygen cluster and tyrosyl radical subunit. The dinuclear diferrous form reacts with dioxygen to generate the tyrosyl radical essential for the catalytic reaction that occurs at the R1 dimer. It is important to understand how the reactivity toward oxygen is related to the crystal structure of the dinuclear cluster. For the mouse R2 protein, no structure has been available with a fully occupied dinuclear metal ion site. A cobalt substitution of mouse R2 was performed to produce a good model for the very air-sensitive diferrous form of the enzyme. X-band EPR and light absorption studies revealed a strong cooperative binding of cobalt to the dinuclear site. In perpendicular mode EPR, the axial signal from mouse R2 incubated with Co(II) showed a typical $S = 3/2$ signal, and its low intensity indicated that the majority of the Co(II) bound to R2 is magnetically coupled. In parallel mode EPR, a typical integer spin signal ($M_s = \pm 3$) with $g \sim 12$ is observed at 3.6 K and 10 K, showing that the two Co(II) ions ($S = 3/2$) in the dinuclear site are ferromagnetically coupled. We have solved the 2.4 Å crystal structure of the Co(II)-substituted R2 with a fully occupied dinuclear cluster. The bridging Co(II) carboxylate ligand Glu-267 adopts an altered orientation compared with its counterpart Glu-238 in Escherichia coli R2. This might be important for proper $O_2$ activation of the more exposed native diferrous site in mouse R2 compared with E. coli R2.

The enzyme ribonucleotide reductase (RNR) catalyzes the conversion of ribonucleotides to deoxyribonucleotides, providing the cell with the essential building blocks for DNA synthesis and repair (1, 2). A common feature for all RNRs isolated so far is a similar reaction mechanism involving free radicals and redox-active cysteines. The RNR enzymes can be grouped into three major classes based on the mechanism used for radical generation and on structural differences (3–6). RNR from mouse belongs to class Ia. RNRs in this class are composed of $\alpha_2\beta_2$ tetramers. The $\alpha_2$ dimers are called R1, and each subunit hosts an active site and binding sites for allosteric effectors. Each subunit of the $\beta_2$ dimers (called R2) contains a $\mu$-oxo-bridged diferrous center (iron-oxo-cluster) (7) and a free tyrosyl radical in its active form. Upon binding of the substrate to the R1 protein, the enzymatic reaction is initiated. The radical originating from the tyrosyl radical of the R2 protein is transferred to the active site cysteines in the R1 protein (5, 7–11). This radical or hydrogen atom is proposed to be propagated via a conserved chain of hydrogen-bonded amino acid residues within the R2 and the R1 proteins. Mutations of the amino acids in this chain lead either to a decreased or a total loss of enzyme activity, which supports this radical transport pathway (12–14).

RNR isolated from Escherichia coli is so far the best characterized enzyme and prototype of class Ia. For mammalian RNs, the mouse enzyme may serve as a better model because there are a number of significant differences between the E. coli enzyme and RNR in higher organisms (7, 15). The sequence identity between mouse R2 and E. coli R2 is only about 25% (15), although the identity is higher among the eukaryotic R2 proteins (60–82%) (16). The newly characterized human p53R2, which is possibly important in DNA repair, also shows large sequence similarity to the mouse R2 protein (17, 18).

The three-dimensional structure of mouse RNR R2 has been determined at 2.3 Å resolution (16). The x-ray structure of Kauppi et al. (16) showed that the iron center of mouse R2 had a more water-exposed and less hydrogen-bonding environment around the metal site than in E. coli R2. So far it has only been possible to crystallize the R2 mouse protein at pH 4.7, and at this pH only one of the metal binding sites is occupied by iron. Structures of the biologically relevant diferrous and diferrous forms of the E. coli R2 enzyme have been solved. Both structures showed fully occupied di-iron sites (8, 19). No crystal structure has been published on the active form harboring a tyrosyl radical. In this paper, we present a model for the diferrous form of mouse R2, the form that reacts with dioxygen and forms the essential tyrosyl radical and the $\mu$-oxo-diferrous cluster (20–22). The diferrous form of R2 is difficult to work with because it is very $O_2$-sensitive. Substitution studies with other divalent metals that are less air-sensitive can provide useful information about this biological relevant form of the enzyme. In this study, Co(II) was chosen because cobalt ions exhibit...
characteristic spectra depending on ligand environments and thus provide the electronic structure for the metal ion site of mouse R2 (23, 24). Analogous studies where the metal ion cluster has been replaced has provided significant structural information for many metalloproteins, among which are manganese (25–27) and cobalt- (28) substituted R2 RNRS. The x-ray manganese structures have served as good models for the reduced form of the E. coli R2 enzyme.

Among the isolated and characterized non-corrin-cobalt-containing enzymes, the most relevant for comparison with R2 is the methionine aminopeptidases isolated from E. coli. The x-ray structure revealed a dinuclear cobalt site with carboxylate and histidine ligands (29). This rich carboxylate coordination environment is very similar to the carboxylate-bridged dinuclear iron centers we find in the iron-oxogyn proteins such as class I R2 RNR, methane monooxygenase hydroxylase (MMOH), and acyl-carrier-protein Δ-desaturase, which are all capable of activating dioxygen for subsequent oxidation chemistry. However, this is not the case for the cobalt-containing methionine aminopeptidases, which from E. coli requires two Co(II) atoms for hydrolytic activity to cleave the N terminus from newly translated polypeptide chains.

In this study we report the first crystal structure of a fully occupied dinuclear site of mouse R2 RNR at pH 6. A structure at this pH is more physiologically relevant than the previous structure at pH 4.7. It also gives new insights into the structure and spectroscopic properties of the reduced diferrous center of mouse R2. Our EPR studies show that the cobalt atoms in the dinuclear site are ferromagnetically coupled in contrast to cobalt-substituted E. coli R2 (28). Several other non-corrin-cobalt enzymes other than methionine aminopeptidases have been isolated and characterized (prolidase, nitrile hydratase, glucose isomerase, methylymalonyl-CoA carboxyltransferase, aldehyde decarboxylase, lysine 2,3-aminomutase, and bromoperoxidase) (30), and the ferromagnetic coupled dinuclear Co(II) cluster in mouse R2 can serve as a model for active dinuclear cobalt-containing proteins.

**EXPERIMENTAL PROCEDURES**

**Protein**—The cloned mouse R2 apoprotein was prepared as reported by Mann et al. (21). The apoR2 protein obtained after purification was pretreated with hydroxyurea and EDTA to remove all iron before incubation with Co(II). Aqueous solutions of Co(II) (PDB ID 1XSM) was added to the solution to prevent oxidation of the Co(II) ions. One crystal was transferred to a cryosolution of 25% glycerol, 5 mM Co(II), and 2 mM sodium ascorbate and flash-frozen in liquid nitrogen.

A 2.4 Å dataset was collected on a MAR CCD detector at beamline ID14-3 at ESRF in Grenoble. The 120 frames were measured, processed in DENZO, and merged in Scalepack (33) and gave 99.3% completeness (15,274 unique reflections) between 30.0 and 2.4 Å resolution, a Rmerge of 5.7% and Rfactor value of 9.6.

**Refinement and Model Building**—Although our cobalt-substituted R2 and apoR2 proteins were crystallized using the same conditions as for the iron-reconstituted mouse R2 protein reported by Kauppi and co-workers (16, 32), the cell dimensions (space group C2221, one monomer in the asymmetric unit) were shorter. Therefore, the molecular replacement technique had to be used for the structure determination. The R2 protein (PDB ID 1XSM) was used as search model, and all water molecules and the single iron ion were deleted. The rotation and translation searches were carried out with the CNS program package (34).

After the rigid body refinement, simulated annealing and positional refinement in CNS electron density maps (Fo-Fc and 2Fo-Fc) showed well defined electron density at an 8-Å level for the cobalt ions in the proposed metal center in the protein. The program O (35) was used for map inspection and model building. The cobalt ions were included in the coordinate file, and 113 water molecules were added in several iterations to the model and refined in CNS. Only water molecules with well defined density and reasonable hydrogen bond geometry were chosen. The refinement brought R and Rfree, based on 5% of the data down to 21.8 and 29.7%, respectively.

The 65–352-residue model of the Co(II)-substituted R2 protein refined to 2.4 Å showed good stereochemistry with deviations from ideal geometry of 0.024 Å for bond lengths, 2.1° for bond angles, and 21.8° for dihedral angles. In the CNS package (34), the mean coordinate error was estimated to be 0.31 Å for the entire structure. The program PROCHECK (36) estimated that 89.0% of the nonglycine and proline residues were in the most favored regions, whereas 9.5 and 1.5% were found either in other allowed or in generously allowed regions, respectively. Four amino acids (Arg-79, Phe-81, Arg-149, and Glu-307) in the latter mentioned region are all in flexible loops at the surface of the protein and have high temperature factors. The coordinates for the structures of mono-Co(II)-R2 and di-Co(II)-R2 are deposited in the Protein Data Bank and have been assigned PDB ID 1h0o.pdb and 1h0n.pdb, respectively.

**RESULTS AND DISCUSSION**

**EPR Evidence for Formation of a Magnetically Coupled Dinuclear Co(II) Cluster**—In this study, we have substituted the natural iron cluster with cobalt. We chose Co(II) because it would make a good model for the native dinuclear ferrous cluster of the enzyme. The mouse apoR2 protein after purification was pretreated with hydroxyurea and EDTA to remove all iron before incubation with Co(II). Aqueous solutions of Co(II) are readily oxidized to Co(III). The air-sensitive Co(II) buffer solutions were made fresh just before use. Unlike the very air-sensitive diferrous R2 protein, the cobalt-containing form of mouse R2 (Co(II)-R2) shows no difference in oxidation state whether the reconstitution was performed aerobically or anaerobically (data not shown). Unless otherwise indicated, all of the work represented in this paper is done aerobically. This apparent insensitivity to dioxygen is consistent with reports of other cobalt-substituted proteins such as the anaerobic prepared cobalt-substituted R2 protein from E. coli (28). This protein did not appear to bind or activate dioxygen.

**Incubation of R2 with a Large Excess of Cobalt**—The reconstitution was first made at pH 7.6 by the aerobic addition of 10 molar excess of Co(II)/apopR2 dimer (typical [R2] was 0.2–0.5 mM). This yielded a pink cobalt R2 derivative within seconds. The sample was passed down a small Sephadex G-25 column at 4 °C to remove any unbound cobalt from the protein. The EPR spectrum at 4 K of the gel-filtered Co(II)-R2 sample had a broad signal from 50 to 370 mT, typical of Co(II) high spin S = 3/2 (Fig. 1, dotted line).

Systems with S > 1/2 can be described by the spin Hamiltonian (23, 24, 28, 37–40)

\[ H = \beta \cdot S \cdot g \cdot B + D(S_z^2 - S(S+1)/3) + E(D(S^2 - S_z^2)) \]  

(Eq. 1)

in which \( \beta \) is the Bohr magneton, \( B \) is the applied magnetic
field, $g$ is the Landé matrix, $D$ is the axial zero-field splitting parameter, and $E$ is the rhombic zero field splitting parameter. $|E/D|$ ranges between 0 and $1/3$. $|E/D| = 0$ corresponds to the fully axial case, and $|E/D| = 1/3$ is the fully rhombic case.

The observed broad axial X-band EPR signal from Co(II)-R2 is typical for the lowest Kramers doublet ($M_g = \pm 1/2$) from $S = 3/2$ high spin mononuclear Co(II) species. The signal has a $g_{\text{eff}}$ at $-170$ mT ($g \sim 4.1$) with a $g_{\text{max}}$ value at $-5.4$ and a signal at $g = 2.02$ over a temperature range from 3.6 to 30 K. The $g$ values of the Co(II)-R2 sample are close to the theoretical axial $S = 3/2$ $g$ values at 4.0, 4.0, and 2.0. Different traces of Co(II) low spin ($S = 1/2$) impurities are observed above $g = 2$. No tyrosyl radical was generated during the aerobic reaction of apoR2 with Co(II) (Fig. 1, dotted line).

A full occupancy of the native $\mu$-oxo-diferric binding site will give a binding stoichiometry of 4 metal ions/R2 dimer. Quantitation of the observed broad axial $g_{\text{max}} \sim 5.4$ EPR signal from the gel-filtered Co(II)-R2 sample against Co(II) in buffer showed that about 15–20% of the expected metal binding sites were occupied with EPR-active Co(II). To rule out the presence of EPR-silent Co(II), a reduction of the protein with dithionite was performed. This gave no change in the observed EPR signal (data not shown). The Co(II)-R2 sample was acid denatured (1.4 M HCl, 12 h at room temperature). The EPR spectrum of the acid-denatured Co(II)-R2 sample (Fig. 1, solid line) showed an increase in the intensity of the Co(II) $S = 3/2$ EPR signal compared with the intensity from the Co(II)-R2, pH 7.6, sample (Fig. 1, dotted line). Assuming that the intensity of the Co(II) signal after acid denaturation represents all Co(II) bound to R2, the results suggest that 80% of the metal binding sites in the Co(II)-R2 sample at pH 7.6 were occupied with EPR-silent Co(II). The EPR spectra of Co(II) in buffer did not change in shape or intensity when HCl was added. These results imply that the majority of the Co(II) atoms bound to R2 at pH 7.6 interact with each other to form a magnetically coupled dinuclear site that cannot be detected by perpendicular mode X-band EPR. The binding ratio of cobalt to R2 indicates that most of the available metal sites are occupied with magnetically coupled cobalt atoms in the gel-filtered Co(II)-R2 sample.

**The Formation of the Dinuclear Co(II) Cluster**—The formation of the perpendicular mode X-band EPR-silent metal site at pH 7.6 can be followed by preparing EPR samples with an increasing ratio of Co(II) to apoR2 without gel filtration. The EPR spectra from the titration of R2 with 1–8 equivalents Co(II) are identical in shape to the spectrum observed from the gel-filtered Co(II)-R2 sample, although with different intensities (Fig. 2, B and C). When the ratio of the concentration of added Co(II) to apoR2 reached the level at which formation of magnetically coupled dinuclear cobalt sites becomes possible, the relative intensity of the $g_{\text{max}} = 5.4$ Co(II) $S = 3/2$ EPR signal from the cobalt-substituted R2 will no longer be comparable in intensity with that of a buffer sample of CoCl$_2$ of identical concentration (Fig. 2, A and B). Thus, a quantitation of the Co(II) EPR signal of a sample containing 4 equivalents of Co(II)/R2 dimer (6 Co(II)-R2) in the same buffer as A shows (C). At 127 mW, 3.8 K, the X-band EPR spectra of 1.8 mM Co(II) in 0.1 M MES, 1.2 M NaCl, pH 6.0 (* 0.2) (D) and 6 equivalents of Co(II)/R2 dimer (6 Co(II)-R2) in the same buffer as D are shown (E). [R$_2$] = 0.3 mM in all samples. Instrumental conditions: microwave frequency, 9.6 GHz; modulation, 1.0 mT.
Tris-HCl, pH 7.6, 20% glycerol). Table I summarizes the EPR relaxation parameters of the $g_{\text{max}}$ signal from the different samples at different temperatures. The parameters of the saturation curves in terms of microwave power at half-saturation $P_{1/2}$ and $b$ are summarized in Table I. The parameter $b$ describes the contribution of nonhomogeneous broadening (41) to the saturation curve as shown in Equation 2,

$$I \alpha 1/(1 + P/P_{1/2})^{b}$$

(Eq. 2)

where $I$ is the EPR amplitude and $P$ is the microwave power.

The $P_{1/2}$ values were estimated to 0.2–0.3 mW at 3.6 K and 8–16 mW at 5.8 K with $b = 0.7$ or $b = 1$ for both Co(II)-R2

samples. The $P_{1/2}$ for the Co(II) in buffer was estimated to 0.3 mW at 3.6 K. The saturation behaviors of the three samples are similar (Table I). In the Co(II)-R2 samples, there might be a small fraction of mononuclear Co(II) bound to R2. Integration of the EPR signal under non-saturating conditions demonstrates a 20–30% decrease at 5.8 K compared with 3.6 K. A negative $D$ value would have increased the EPR-active Co(II) signal when the temperature was increased. Taken together with the microwave saturation behavior this shows a positive $D$ value for the EPR-active species in Co(II)-R2 (37, 38). The acid-treated Co(II)-R2 also has EPR properties identical to the high spin EPR-active Co(II) in Co(II)-R2 and in buffer (data not shown). The observed cobalt signals in all three of these samples have similar positive and large $D$ values. This validates the quantitation of the EPR-active Co(II) in Co(II)-R2. It also shows that the majority of the EPR-active Co(II) present in mouse Co(II)-R2 is very similar to Co(II) in buffer.

Fig. 4 presents the amount of EPR-active Co(II) in the Co(II)-R2 samples (dotted line) when increasing amounts of Co(II) are added to apoR2. At 100%, all Co(II) added is EPR-active, whereas lower values show the presence of EPR-silent cobalt. With 1 equivalent of Co(II)/apoR2 dimer, ~90% of the cobalt is EPR-active. Addition of more equivalents of Co(II) decreases the intensity of the signal compared with the same amount of cobalt in buffer. This demonstrates the formation of the dinuclear cobalt site that is magnetically coupled and may therefore be EPR-silent in perpendicular mode. The lowest relative amount of EPR-active Co(II) and a nearly saturated cluster is at ~6 equivalents of Co(II) added per R2 dimer. Adding 8 equivalents of Co(II)/apoR2 dimer gave an EPR signal with ~60% intensity compared with the same amount of Co(II) in buffer. If R2 is saturated when 8 equivalents of Co(II) is added per R2 dimer this should give an intensity of the observed $S = 3/2$ Co(II) EPR signal corresponding to ~50% of the signal observed for the same amount of Co(II) in buffer. The result indicates nearly saturated dinuclear sites with ~4 equivalents of EPR-silent dinuclear Co(II)/R2 dimer when 8 equivalents are added. The titration illustrates that the affinity for Co(II) is low, and more than 4 Co(II)/R2 dimer is needed to occupy fully the four well defined metal binding sites in the mouse R2 dimer.

**Light Absorption Spectra of Co(II)-R2**—The visible spectrum of the fully occupied Co(II)-R2 (Fig. 5A, solid line) is clearly red shifted and more intense than the spectrum of Co(II) in buffer solution (Fig. 5A, dotted line). The induction of this red shift was followed spectrophotometrically by adding increasing amounts of cobalt to the apoR2 protein (Figs. 4 and 5B). The appearance of the 550 nm absorption maximum was not significant until the addition of 2–3 equivalents of cobalt/R2 dimer and saturated by ~7 equivalents of Co(II)/R2 dimer. The formation of the dinuclear cluster observed by EPR also confirmed this low affinity for cobalt. The affinity toward cobalt is lower for mouse R2 compared with that reported for cobalt-substituted *E. coli* R2 where there is saturation with 4 equivalents of Co(II)/R2 dimer (28).

**TABLE I**

| Temperature | $P_{1/2}$ | $b$ | $P_{1/2}$ | $b$ | $P_{1/2}$ | $b$ |
|-------------|---------|-----|---------|-----|---------|-----|
| 3.6 K       | 0.2     | 0.65| 0.2     | 0.76| 0.3     | 1.18|
| 5.8 K       | 8.0     | 0.62| 8.0     | 0.66|         |     |

**FIG. 3.** Microwave power saturation of the $g_{\text{max}}$ 5.4 component of the Co(II) signals. Continuous traces are computer fits (Equation 2). [R2] = 0.5 mM in all samples. EPR microwave saturation curves of A are 4 equivalents of CoII/apoR2 at 3.6 K (□) and 5.8 K (○). B, 6 equivalents of CoII/apoR2 at 3.6 K (▲) and 5.8 K (●) and 3 mM Co(II) in buffer (▼) at 3.6 K (50 mM Tris-HCl, pH 7.6, 20% glycerol).

**TABLE I**

| Temperature | $P_{1/2}$ | $b$ | $P_{1/2}$ | $b$ | $P_{1/2}$ | $b$ |
|-------------|---------|-----|---------|-----|---------|-----|
| 3.6 K       | 0.2     | 0.65| 0.2     | 0.76| 0.3     | 1.18|
| 5.8 K       | 8.0     | 0.62| 8.0     | 0.66|         |     |
The formation of the 550 nm chromophore when cobalt binds to apoR2 can be fitted to a sigmoidal binding model (Fig. 4, solid line). This indicates a strong cooperative interaction between the protein and the dinuclear Co(II) center. The dinuclear site is formed concomitantly with a strong 55 nm absorption. Possibly both Co(II) ions contribute to the observed pink color because both binding sites of Co(II) have almost the same geometry (see below, Fig. 7B). The affinity for the dinuclear Co(II) cluster is low because 2 mM Co(II) is needed for half-saturation of 0.5 mM R2. Co(II) binding to R2 followed by light absorption at pH 7.6 corresponds well with the EPR behavior because both demonstrate the presence of a fully occupied dinuclear site at 7–8 equivalents of Co(II)/R2 dimer.

Comparison with the Cobalt-substituted R2 from E. coli—

The anaerobic cobalt-substituted R2 from E. coli showed a pink color and had a very similar absorption spectra regarding intensity and $\lambda_{max}$ to what we have observed for the Co(II)-substituted R2 from mouse (28). The EPR spectrum of Co(II)-R2 from E. coli is typical for $S = 3/2$ high spin mononuclear Co(II) species, and the intensity of the signal is consistent with the Co(II) atoms not being magnetically coupled when bound to the R2 protein. More hyperfine structure and the presence of a rhombic component are seen for the EPR-active Co(II) in the cobalt-substituted E. coli R2 than we observe for the cobalt-substituted mouse R2 at pH 7.6. This is probably due to the fact that the EPR-active Co(II) observed for mouse R2 is mostly Co(II) in buffer and not mononuclear cobalt bound to R2 as in E. coli R2. For our cobalt-substituted mouse R2 protein, most of the cobalt was silent in perpendicular mode EPR.

Evidence for a Ferromagnetically Coupled Dinuclear Cobalt Cluster in R2—

The EPR and light absorption studies of Co(II)-R2 were performed at pH 7.6. In this paper, we present the first crystal structure of a fully occupied metal site in mouse R2 at pH 6 (see below, Fig. 7). At pH above that, the crystals dissolve in the buffer. To be able to compare the EPR and light absorption results with the crystal structure, we prepared a Co(II)-R2 EPR sample as close to crystal conditions as possible. The EPR spectrum of the Co(II)-R2 prepared in 0.1 M MES, pH 6.0, 1.2 M NaCl, 20% glycerol, did show an EPR signal of high spin Co(II) $S = 3/2$, similar to what we observed when the sample was prepared at pH 7.6, although with a more pronounced shoulder at ~100 mT and possible hyperfine structure (Figs. 2E and 6A). At pH 6.0, the intensity of the $S = 3/2$ signal is also low compared with that of identical concentrations of cobalt in buffer (Fig. 2, D and E). This shows that if the reconstitution is carried out at pH 7.6 or 6.0, most of the cobalt bound to R2 will form magnetically coupled dinuclear sites. In addition, the pH 6.0 sample contains a low field perpendicular mode EPR signal at $g = 9.2$, both at 127 $\mu$W (Fig. 2E) and more clearly visible at 10 mW (Fig. 6A), where Co(II) in buffer is partially saturated. The $g = 9.2$ signal is not clearly visible even at a microwave power of 10 mW for the sample prepared at pH 7.6 (data not shown).

In parallel mode EPR, the only signal observed for the Co(II)-R2 samples prepared at both pH 7.6 and 6.0 at 3.5 K is a low field signal at $g = 12$ (Fig. 6, B and D). The parallel mode EPR signal can be described as a broad negative peak, expanding from 20 to 120 mT at X-band. The Co(II) $S = 3/2$ signal is no longer EPR-active as expected. The g = 12 signal is strong enough to be observed below 0.1 mW. The shape of the parallel mode signal as well as the shift in g value compared with the signal observed in perpendicular mode EPR is typical for integer spin signals (42, 43). The integer spin signal observed for the dimeric form of the mouse R2 protein showed a similar behavior (44).

The intensity of the $g = 12$ integer spin signal at pH 6.0 decreased when the temperature rose to 10 K (Fig. 6C) and was hardly observed at higher temperatures, indicating that the signal arises from a ground-state transition. The observed g
value at 12 is the expected theoretical $g_{\text{eff}}$ value for an $M_s = \pm 3$ transition. We assign the signal to arise from the $M_s = \pm 3$ ground state spin system that would arise from a ferromagnetic coupling of the two high spin $S = 3/2$ Co(II) ions in the dinuclear cobalt site. Both ferromagnetic and antiferromagnetic coupling are observed in other dinuclear cobalt-substituted proteins. A ferromagnetically coupled dinuclear cobalt center was observed in a Co(II)-substituted hemocyanin (45) and a Co(II)-substituted aminopeptidase from *Aeromonas proteolytica* (46). The cobalt-substituted dinuclear form of aminopeptidase from *A. proteolytica* showed that most of the Co(II) added to the protein has substituted dinuclear form of aminopeptidase from *Aeromonas proteolytica* is more pronounced at pH 6.0 than 7.6 is probably because of $hv_X$, the perpendicular mode EPR signal being higher at pH 7.6 and therefore difficult to detect with another zero field splitting parameter in integer spin EPR. We assign the signal to arise from the $S = 3/2$ transition. We assign the signal to arise from the $S = 3/2$ Co(II) ions in the active site of the aminopeptidase (46). Bennett and Holz (46) assign this signal to a similar to what we observe for the cobalt-substituted mouse R2. In the first experiments, the mouse R2 crystals were co-crystallized with 3.8 equivalents of Co(II)/R2 dimer at pH 4.7. These conditions only led to crystals with partially mononuclear occupancy of the metal binding sites (the site furthest away from the radical Tyr-177, see below). Both EPR and light absorption data had revealed that the affinity for Co(II) is weak. This might be because of the open structure around the metal site leading to loss of bound cobalt into the solution as for iron or because the cobalt is not the natural element for R2. To get fully occupied metal centers, the 4 equivalents of Co(II)/R2 dimer had to be increased. To find the proper conditions for a fully occupied metal site, the crystals were studied under a microscope to determine the incubation time and the right concentration of Co(II) to use in the soaking solution.

In this work we managed to raise the pH in the Co(II)-R2 crystals to 6. The mono-iron mouse R2 structure was solved at pH 4.7 (16). In both structures, only residues 65–352 of the 390 amino acids are seen in the electron density maps, indicating that the N- and C-terminal ends are flexible. Two peaks in the proposed metal binding sites are clearly seen in the difference-Fourier maps $2F_o-F_c$, showing that the cobalt ions were bound to the protein with full occupancies after soaking the crystals in a 5 m Mol Co(II) solution for 3 h at room temperature (Fig. 7A). Average temperature factors for dicobalt R2 mouse protein main chain and side chains are 36.8 and 38.2 Å$^2$, respectively; for the site 1 cobalt ion, 37.4 Å$^2$; and for the site 2 cobalt ion, 29.4 Å$^2$. This shows that site 2 has a higher occupation of cobalt ions and less flexibility than site 1 at pH 6.

Fig. 7A shows the electron density, and Fig. 7B shows a model of the metal ligands, the cobalt ions, and the stable free radical site Tyr-177. All residues that make interactions with the cobalt ions are well defined in the density map. The glutamic acids at 170 and 267 have a bidentate bridging binding mode to the cobalt ions. The binding of Asp-139 and His-173 to the Co(II) in site 1 (Co1) and Glu-233 and His-270 to the Co(II) in site 2 (Co2) make both metal ions 4-coordinated. The distance between the cobalt ions is 3.40 Å (Table II).

Mononuclear and Dinuclear Cobalt R2 Mouse Structures—The protein crystallizes only at pH 4.7, and at this pH when co-crystallized with cobalt only one of the metal sites is occupied with mononuclear cobalt (site 2). The occupancy of only one of the metal binding sites is the same as was observed in the first x-ray structure of mouse R2 by Kauppi et al. (16). The monooiron mouse R2 crystals were soaked in a solution of ferrous salt at pH 4.7. This increased the occupancy on the already occupied site without any detectable binding at the second site. We managed to increase the pH in our crystals by simultaneously soaking the crystals in a buffer solution containing Co(II). This treatment also led to occupancy of site 1. A comparison of the
monocobalt-substituted structure at pH 4.7 and the dicobalt-substituted structure at pH 6 of mouse R2 is presented in Fig. 8A. In the monocobalt structure, only site 2 is occupied by cobalt ions. The cobalt atom is 4-coordinated, and the ligands are Glu-170, His-270, Glu-233, and Glu-267. Greatest flexibility is shown by the carboxylate ligands upon binding of the second cobalt ion. The occupation of site 1 moves Asp-139 closer to the metal site, and a change in the orientation is observed. The orientation of Glu-233 is also altered. In the dinuclear structure, Glu-267 and Glu-170 make carboxylate bridges between the cobalt ions. The histidines (at 173 and 270) do not change significantly. They form the rigid framework of the binding site. Upon increasing the pH to 6, we do not observe any changes in the position of the stable radical Tyr-177 or the hydrogen bonding pattern and position of the amino acids involved in the radical transport pathway. Neither do we observe any changes in the acceptability and the hydrophobicity of the environment around the metal site compared with the structure at pH 4.7 solved by Kauppi et al. (16).

Comparisons of the Mononuclear Co(II) and Iron Mouse R2 Structures (Both at pH 4.7)—The monocobalt structure is overall similar to the previously reported mono-iron structure (16) (Fig. 8B). The iron ion (probably Fe(III)) is located closer to Glu-267 and has a bidentate binding mode. In the monocobalt structure, the cobalt ion makes only one interaction with this residue. His-270 and His-173 are also altered. In the dinuclear structure, Glu-267 and Glu-170 make carboxylate bridges between the cobalt ions. The histidines (at 173 and 270) do not change significantly. They form the rigid framework of the binding site. Upon increasing the pH to 6, we do not observe any changes in the position of the stable radical Tyr-177 or the hydrogen bonding pattern and position of the amino acids involved in the radical transport pathway. Neither do we observe any changes in the acceptability and the hydrophobicity of the environment around the metal site compared with the structure at pH 4.7 solved by Kauppi et al. (16).

**Table II**

| Atom | Atom | Å     | Atom | Atom | Å     | Atom | Atom | Å     |
|------|------|-------|------|------|-------|------|------|-------|
| Co1  | Co2  | 3.40  | Fe1  | ND1 H173 | 2.11 | Fe1  | ND1 H118 | 2.00 |
| Co1  | OD1 D139 | 1.76 | Fe1  | OD1 D84  | 1.65 | Fe1  | ND1 E114 | 2.13 |
| Co1  | OE1 E170 | 2.18 | Fe1  | OE1 E115 | 1.99 | Fe1  | ND1 E144 | 2.23 |
| Co1  | OE2 E267 | 2.54 | Fe1  | OE2 E238 | 1.67 | Fe1  | O2 H2102 | 2.49 |
| Co2  | ND1 H270 | 2.23 | Fe2  | ND1 H241 | 2.02 | Fe2  | ND1 H246 | 2.17 |
| Co2  | OE1 E267 | 1.83 | Fe2  | OE1 E238 | 1.77 | Fe2  | O2 E243  | 2.44 |
| Co2  | OE2 E170 | 2.04 | Fe2  | OE2 E115 | 1.95 | Fe2  | O2 E243  | 2.45 |
| Co2  | OE2 E233 | 1.73 | Fe2  | OE2 E204 | 2.16 | Fe2  | O2 E209  | 2.00 |
| Co2  | ND1 H270 | 2.23 | Fe2  | ND1 H241 | 2.02 | Fe2  | ND1 H246 | 2.17 |
| Co2  | OE1 E267 | 1.83 | Fe2  | OE1 E238 | 1.77 | Fe2  | O2 E243  | 2.44 |
| Co2  | OE2 E170 | 2.04 | Fe2  | OE2 E115 | 1.95 | Fe2  | O2 E243  | 2.45 |
| Co2  | OE2 E233 | 1.73 | Fe2  | OE2 E204 | 2.16 | Fe2  | O2 E209  | 2.00 |
| Co2  | ND1 H270 | 2.23 | Fe2  | ND1 H241 | 2.02 | Fe2  | ND1 H246 | 2.17 |
| Co2  | OE1 E267 | 1.83 | Fe2  | OE1 E238 | 1.77 | Fe2  | O2 E243  | 2.44 |
| Co2  | OE2 E170 | 2.04 | Fe2  | OE2 E115 | 1.95 | Fe2  | O2 E243  | 2.45 |
| Co2  | OE2 E233 | 1.73 | Fe2  | OE2 E204 | 2.16 | Fe2  | O2 E209  | 2.00 |
| Co2  | ND1 H270 | 2.23 | Fe2  | ND1 H241 | 2.02 | Fe2  | ND1 H246 | 2.17 |
| Co2  | OE1 E267 | 1.83 | Fe2  | OE1 E238 | 1.77 | Fe2  | O2 E243  | 2.44 |
| Co2  | OE2 E170 | 2.04 | Fe2  | OE2 E115 | 1.95 | Fe2  | O2 E243  | 2.45 |
| Co2  | OE2 E233 | 1.73 | Fe2  | OE2 E204 | 2.16 | Fe2  | O2 E209  | 2.00 |
| Co2  | ND1 H270 | 2.23 | Fe2  | ND1 H241 | 2.02 | Fe2  | ND1 H246 | 2.17 |
| Co2  | OE1 E267 | 1.83 | Fe2  | OE1 E238 | 1.77 | Fe2  | O2 E243  | 2.44 |
| Co2  | OE2 E170 | 2.04 | Fe2  | OE2 E115 | 1.95 | Fe2  | O2 E243  | 2.45 |
| Co2  | OE2 E233 | 1.73 | Fe2  | OE2 E204 | 2.16 | Fe2  | O2 E209  | 2.00 |
The essential carboxylate ligands are shown to be able to adopt several conformations that can also vary with the oxidation state of the metals (5, 19, 25–27, 47, 48). Carboxylate shifts are believed to be important to accommodate different redox states during redox cycling of these kinds of centers.

The x-ray structures of the dinuclear clusters of the cobalt-substituted mouse R2 and the diferrous R2 E. coli are similar (Fig. 9, pink and black structures, respectively). In both complexes, the metal ions are 4-coordinated and have the same binding mode to their ligands. The histidines His-173(His-118) and His-270(His-241) show very little variation in their orientation in the different x-ray structures (E. coli numbering in parentheses). Significant variations in the position of all of the carboxylate ligands Glu-267/Glu-233/Asp-139/Glu-170 are observed. Compared with E. coli, both Glu-233(Glu-204) and Asp-139(Asp-84) move closer to the metal binding sites forcing the metal atoms closer together, and therefore the metal distance between Co1 and Co2 becomes 0.5 Å shorter that the corresponding metal to metal distance in the diferrous wild type E. coli (3.9 Å) (19) (Table II). This agrees well with the EPR studies showing that the cobalt ions in mouse R2 have a strong magnetic coupling; whereas this is not the case for the diferrous ions in E. coli R2, which are weakly antiferromagnetically coupled (49). The most interesting change that we observe in the cobalt-substituted mouse R2 structure compared with that of the reduced wild type E. coli structure is the carboxylate shift seen for the bridging carboxylate ligand Glu-267 compared with the E. coli counterpart Glu-238.

Even though mouse and E. coli R2 proteins belong to the same RNR class (class Ia), and their metal ions ligands are conserved, several differences in the behavior of these two clusters are observed. The redox properties of the R2 protein from mouse are significantly different from those of the R2 from E. coli. For example, the reduced mouse R2 in glycerol exhibits an integer EPR signal with a g value observed around 14 (44) which is not shown for the E. coli enzyme. The mixed valence form (Fe(II)Fe(III)) of mouse R2 can be induced by a mild chemical reduction with a 30% yield (44, 50). In the E. coli R2 protein, this redox state is only produced in small amounts. Even using drastic conditions as high pH only increases the yield to ~5%. The coupling between the iron atoms in the oxidized state is weaker in mouse than in E. coli (51, 52). The redox properties of the mouse iron-oxygen center resemble those of MMOH. In MMOH, both the integer spin signal in the reduced form and the mixed valence state is observed (7, 53, 54). The x-ray structure of the dinuclear cobalt cluster in R2 from mouse reveals that it is similar to the chemically reduced di-iron form of MMOH from Methylococcus capsulatus (48, 55) (Fig. 9, MMOH represented in green). The reduced structure of MMOH contains two iron centers bridged by one μ-1,3- and one μ-1,1-carboxylate ligand. One histidine and one monodentate glutamate are coordinated to each iron, and additional water is bound to one of the iron. Both iron atoms in the metal center are 5-coordinated. The Glu-144 in the MMOH structure and the Glu-170 in the cobalt-substituted mouse R2 structure are in similar orientations as are the histidines (Fig. 9). MMOH has a water molecule that hydrogen bonds to Glu-114. This water is absent in both the cobalt-substituted mouse R2 and the diferrous E. coli R2 structure. The orientation of the carboxylate ligands in the CoII-R2 mouse structure resembles the MMOH counterparts more than the E. coli counterparts do. This might contribute to the observed 3.4 Å Co1-Co2 distance in CoII-R2 being closer to the 3.3 Å Fe1-Fe2 distance in MMOH than the 3.9 Å Fe1-Fe2 distance in E. coli R2 (Table II).

In the reduced form of MMOH, Glu-243 has a bidentate chelating mode to Fe2 along with monodentate bridging between the iron atoms. This μ-(η1,η1) coordination is suggested by Eklund et al. (5), Andersson et al. (47), and Merkx et al. (56) to be a well suited conformation for binding of oxygen without any large structural changes in the di-iron-oxygen cluster. Such an orientation is also found in the reduced MMOH mimic mutant D84E of E. coli R2 (57). The dioxygen bound state has not been trapped in R2 because the iron-oxygen species is highly reactive. In the crystal structure of the azide-soaked R2 E. coli mutant F208A/Y122F, azide binds to the iron farthest away from tryrosine 122 (site 2), inducing a carboxylate shift with Glu-238 (47). In analogy with how azide binds, oxygen is...
intermediates are formed for mouse R2. In the study by Bollinger and co-workers (64) that the formation of the reactive Fe(II)-R2 diferrous form of mouse R2 observed either with perpendicular or parallel mode EPR has been analyzed further by using saturation magnetization (44). The two ferrous ions were as-signed the same sign and magnitude for the D values, indicating no or weak magnetic coupling in this study. The ferromagnetic coupling of the cobalt ions in Co(II)-R2 could support another possible interpretation of the saturation magnetization data assuming different signs of the D values and a weak magnetic coupling between the ferrous ions. This is consistent with magnetic circular dichroism analysis of both the diferrous form of R2 from E. coli (49) and mouse (58).2

The stability of the iron/radical center is the most striking difference between the mouse and the E. coli RNR R2 proteins (5, 7). The metal site of the mouse enzyme is labile at physiological conditions, and a continuous supply of ferrous iron and oxygen is needed to keep the enzyme fully active (21, 59, 60). The expression of the mouse R2 protein is normally regulated by the cell cycle (61, 62). A novel study indicated that during low oxygen (hypoxic) conditions the human R2 protein might be by the cell cycle (61, 62). A novel study indicated that during low oxygen (hypoxic) conditions the human R2 protein might

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**Cobalt Substitution of Mouse R2 Ribonucleotide Reductase as a Model for the Reactive Differrous State: SPECTROSCOPIC AND STRUCTURAL EVIDENCE FOR A FERROMAGNETICALLY COUPLED DINUCLEAR COBALT CLUSTER**

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