Crystal Structural Studies of Changes in the Native Dinuclear Iron Center of Ribonucleotide Reductase Protein R2 from Mouse*

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Class I ribonucleotide reductase (RNR) catalyzes the de novo synthesis of deoxyribonucleotides in mammals and many other organisms. The RNR subunit R2 contains a dinuclear iron center, which in its diferrous form spontaneously reacts with O2, forming a μ-oxo-bridged diferferic cluster and a stable tyrosyl radical. Here, we present the first crystal structures of R2 from mouse with its native dinuclear iron center, both under reducing and oxidizing conditions. In one structure obtained under reducing conditions, the iron-bridging ligand Glu-267 adopts the μ-(η1,η2)-coordination mode, which has previously been related to O2 activation, and an acetate ion from the soaking solution is observed where O2 has been proposed to bind the iron. The structure of mouse R2 under oxidizing conditions resembles the non-radical diferferic R2 from Escherichia coli, with the exception of the coordination of water and Asp-139 to Fe1. There are also additional water molecules near the tyrosyl radical site, as suggested by previous spectroscopic studies. Since no crystal structure of the active radical form has been reported, we propose models for the movement of waters and/or tyrosyl radical site when diferferic R2 is oxidized to the radical form, in agreement with our previous ENDOR study. Compared with E. coli R2, two conserved phenylalanine residues in the hydrophobic environment around the diiron center have opposing rotameric conformations, and the carboxylate ligands of the diiron center in mouse R2 appear more flexible. Together, this might contribute to the lower affinity and cooperative binding of iron in mouse R2.

The enzyme ribonucleotide reductase (RNR)1 catalyzes the reduction of the four ribonucleotides to the corresponding deoxyribonucleotides, which is essential for DNA synthesis and repair in all living cells (1, 2). The RNR enzymes isolated so far have been grouped into three main classes based upon the different oxygen dependence and metal cofactors involved in the generation of the catalytically essential free radicals as well as structural differences (3, 4). Class I can further be subdivided into Ia and Ib based on differences in amino acid sequence (4). Class Ia is found in eukaryotes and some bacteria and viruses, whereas class Ib is not found in eukaryotes. Class I RNRs are composed of two homodimeric protein subunits, termed R1 and R2 (2–6); however, the R1 subunit of mouse RNR has been suggested to also occur in an active hexameric form (7). In mammals, three genes for class Ia RNR have been identified, one gene for the large subunit, R1, and two isoforms of the small subunit, R2 and p53R2. Whereas the “normal” R2 is only present during replication of DNA in the S phase (8), p53R2 can be induced throughout the cell cycle by the signaling protein p53 in response to DNA damage (9) and probably also a p53-independent pathway (10). In this work, we focus on the normal S phase-specific isoform of R2. The normal mammalian R2 has recently also been suggested to be an oncprotein under hypoxia (10).

Each R2 subunit contains a dinuclear iron center and a tyrosyl radical site (Tyr-177 in mouse R2) which are essential for initiation of the nucleotide reduction process taking place at the active site in R1. The diiron center of R2 can exist in different oxidation states. If molecular oxygen is allowed to react with the diferferic R2 (Fe(II)Fe(II)), it will spontaneously oxidize the diiron center through a series of intermediate states, leading to a μ-oxo-bridged diferferic iron cluster (Fe(III)Fe(III)) and a stable tyrosyl radical. The dinuclear iron center is located within a four-helix bundle (11) and is coordinated by carboxylate (Glu/Asp) and histidine side chains from the four helices (12, 13). Very similar centers are found in many other proteins (e.g. the hydroxylase component of methane monooxygenase (MMOH) and Δ9-stearyl-acyl carrier protein desaturase (Δ9-D)). All of these proteins have dinuclear iron centers that bind dioxygen, and together they form a family called diiron-oxygen proteins (14), but their biological roles are very different (11, 14–17); MMOH oxidizes methane to methanol in methanotrophic bacteria, Δ9-D introduces a double bond into saturated fatty acids, and the R2 subunit of RNR generates, as mentioned above, a tyrosyl radical essential for the reduction of ribonucleotides to deoxyribonucleotides in eukaryotes and many bacteria and viruses. It is the first and second coordination sphere of the diiron center in each protein that determines the outcome of the reaction with oxygen. Therefore, it is of great interest to

The atomic coordinates and structure factors (codes 1WW6 and 1WW9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).§ These two authors contributed equally to this work.¶ Present address: Dept. of Chemistry, University of Tromsø, NO-9037 Tromsø, Norway.** To whom correspondence should be addressed. Tel.: 47-22856625; Fax: 47-22854449; E-mail: k.k.andersson@biojemi.uio.no.

1 The abbreviations used are: RNR, ribonucleotide reductase; R2, the small protein subunit of class Ia RNR; p53R2, the p53-Inducible isomerase of R2; R2F, the small protein subunit of class Ib RNR; ENDOR, electron nuclear double resonance spectroscopy; MMOH, the hydroxylase component of methane monoxygenase; Δ9-D, Δ9-stearyl-acyl carrier protein desaturase.
obtain detailed knowledge of the environment of the diiron center and compare it with iron-clusters from different proteins derived from various species.

Although R2 from both mouse and *Escherichia coli* belongs to the same class of RNR (class Ia), the sequence identity is only ~20\% (18), and the iron-oxygen cluster in mouse R2 is much more labile compared with *E. coli* R2 (19). There are also significant differences in the spectroscopic properties (14, 20–27). Variable temperature saturation recovery EPR studies have shown that the antiferromagnetic coupling between the ferric iron ions in mouse R2 is weaker than in *E. coli* R2; resonance Raman studies have shown weaker symmetric Fe(III)-O-Fe(III) stretch modes for mouse R2 compared with *E. coli* R2; and a deuterium shift of ~5 cm\(^{-1}\) is observed in mouse R2 that is not observed in *E. coli* (21, 26). Furthermore, high field EPR have shown different \(g_1\) values of the tyrosyl radical from mouse R2 and *E. coli* R2, which indicates a hydrogen bond to the phenoxy oxygen in mouse that is absent in *E. coli* (22–25, 27), and the dihedral angle of the \(\beta\)-hydrogens with respect to the phenoxy ring is different (14, 24, 25). Also in class Ib R2F, there is no hydrogen bond to the tyrosyl radical (28, 29); the dihedral angles are largely different compared with both mouse and *E. coli* R2 (20, 28, 29).

The crystal structure of *E. coli* R2 has been solved for several different conditions (e.g. the reduced differferous form (30, 31) and the dipherfer met form lacking the radical (12, 13, 32) in addition to several mutant forms (see, for example, Ref. 2)). The x-ray structure of the active radical containing R2 protein, however, is not known. In a recent study combining single crystal EPR and x-ray crystallography on the same crystal, it was suggested that the tyrosine ring could be shifted by about 1 Å away from the diiron center in the radical state compared with the nonradical dipherferate state (32).

The high resolution structure (1.4 Å) of the dipherfer R2 pre-

presented in that work showed that the two irons are five- and six-coordinated, respectively, whereas previous structures suggested two six-coordinated irons (12, 13). Also in the reduced form of R2, different coordination numbers for the irons have been proposed based on x-ray structures; the original structure suggested that both ferrous iron ions were four-coordinated (30), but a more recent structure, which was obtained after iron reconstitution into apo-R2 crystals at neutral pH, suggested that the two irons here were best described as four- and five-coordinated, respectively (31), which is more consistent with, for example, CD and magnetic CD spectroscopic data (33).

The first x-ray structure of the R2 protein from mouse was solved in 1996; however, under the crystallization conditions used (pH 4.7), only one of the two metal binding sites was partially occupied (site 2, Fe2; the site furthest away from the diiron core) (34). Recently, we published the first crystal structures of mouse R2 with fully occupied metal sites by soaking crystals in solutions of cobalt(II) at different pH values (35). In agreement with the earlier results, only one metal ion was bound to the protein when the protein crystal was equilibrated at pH 4.7, but when the protein was soaked at pH 6.0, crystals with two fully occupied metal ion sites were obtained.

In this work, we describe the first crystal structures of mouse R2 with intact native dinuclear iron center, both under reducing and oxidizing conditions. We observe interesting carboxylate shifts of the carboxylate ligands, showing a very flexible structure around the dinuclear iron center. Under reducing conditions, the bridging ligand Glu-267 of mouse R2 adopts the \(\mu-(\eta^1-\eta^2)\) coordination mode, which is postulated to be very important for proper oxygen activation (36). Based on these new structural data, we discuss the observed differences in the spectroscopic data for the class I RNR enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**

All commercial reagents were used as obtained: sodium acetate (Sigma), ammonium iron(II) sulfate ((NH\(_4\))\(_2\)Fe(II)(SO\(_4\))\(_2\) \(\cdot\)6H\(_2\)O) (Merck), methylviologen (1,1′-dimethyl-4,4′-bipyrindinium dichloride hydrate) (Sigma), sodium dithionate (Aldrich), 96% glycerol (Prolabo), pheno-

saffrane (Aldrich), sodium l-ascorbate (Sigma), and dipotassium hy-

drogen phosphate/potassium dihydrogen phosphate (Merck).

**Crystallization**

Cloned mouse apo-R2 protein was prepared and purified as reported earlier (37). The protein was crystallized using the hanging drop vapor diffusion method at pH 4.7 according to the previously published protocol (38). The crystals were shaped like thin sheets with a size of up to 0.05 × 0.10 × 0.50 mm and belong to space group C222\(_1\).

**Crystal Soaking**

**Reducing Conditions**—For Structure 1, a 10-μl solution of (NH\(_4\))\(_2\)Fe(II)(SO\(_4\))\(_2\) was added to crystals of apo-R2 protein in 10 μl of mother liquor (pH 4.7) (final iron concentration 5 mM) and incubated for about 3 h. The (NH\(_4\))\(_2\)Fe(II)(SO\(_4\))\(_2\) solution consisted of 0.1 M sodium acetate buffer (pH 6.5) to which the redox mediator methylviologen and phosphate-buffered (pH 6.0) redox agent sodium dithionate was added, giving a final concentration of 2 mM methylviologen and 0.5% dithionite in the crystal soaking solution. The methylviologen works also as a redox indicator, since its blue color would disappear in the presence of oxygen. The final pH in the soaking solution was measured with pH paper to pH 6.0. One crystal was then moved to a cryosolution (mother liquor containing 25% glycerol and 0.5% sodium dithionite solution, pH 6.0) for 30 s, mounted in a rayon loop, and flash-frozen in liquid nitrogen.

Two other batches of reduced crystals were obtained where the iron(II) solution was modified to contain either 2 mM sodium ascorbate as reducing agents and 2 mM pheno-saffrane as redox indicator (Structure 3) or unbuffered 0.5% sodium dithionite (Structure 4) as reducing agent and no redox indicator.

**Oxidizing Conditions**—For Structure 2, a 10-μl solution of (NH\(_4\))\(_2\)Fe(II)(SO\(_4\))\(_2\) was added to crystals of apo-R2 protein in 10 μl of mother liquor (pH 4.7) (final iron concentration 5 mM) and incubated for about 3 h. The (NH\(_4\))\(_2\)Fe(II)(SO\(_4\))\(_2\) solution consisted of 0.1 M sodium acetate buffer (pH 6.5), sodium ascorbate (final concentration 2 mM), and pheno-saffrane (final concentration 2 mM) as redox indicator, since an excess of oxygen changed the color of the solution from yellow to red. One crystal was then moved to an aerobic cryosolution (mother liquor containing 25% glycerol) for 30 s, mounted in a rayon loop, and flash-

frozen in liquid nitrogen. The final pH in the soaking solution was measured to pH 6.0.

**Data Collection and Refinement**

Several diffraction data sets were collected at MAXII-I711 in Lund, the Swiss-Norwegian beam line (BM1) at the European Synchrotron Radiation Facility in Grenoble, and at Deutsches Elektronen-Synchro-

tron in Hamburg (see Table I). All data sets were processed and scaled using Denzo and Scalepack (39), and a mouse apo-R2 protein structure (34) (water and iron excluded) was used as a starting model in the refinements of the structures. The CNS program suite (40) was used for initial rigid body refinements, simulated annealing, positional refinements, and map calculations. Interpretation of maps and model building were carried out in the program O (41). The electron density maps and omit maps clearly showed electron density for iron ions in all of the structures in the previously described metal binding site 1 and 2 in R2. The iron ions were included in the coordinate files, and water molecules were added to the model in positions with well defined electron density and reasonable hydrogen bond geometries and refined in CNS. The free \(R\)-value was calculated from 5% of the data and monitored throughout the refinement. Data and refinement statistics for all structures can be found in Table I.

**EPR Measurements**

The EPR spectra were recorded at X-band on a Bruker ESP 300E, fitted with a Bruker ER4111DM dual mode cavity and an Oxford ESR 900 Helium Flow Cryostat.
RESULTS AND DISCUSSION

Quality of the Models—In this paper, we describe the x-ray crystal structures of the dinuclear Fe-R2 center formed under reducing (Structure 1 in Table I) and oxidizing (Structure 2 in Table I) conditions. Data from two additional structures obtained under different reducing conditions are also presented in Table I (Structures 3 and 4). The Ramachandran plots for Structures 1 and 2 reveal that between 91.9 and 94.2% of the amino acids are part of a loop region that forms the dimer interface of R2 (Arg-79 to Pro-85), and the improved orientation of this loop provides a better contact region between the two protomers. We now observe that Arg-80 in one protomer forms an interaction with Glu-144 in the other protomer and possibly also Arg-79 with Glu-192. Interestingly, we obtain a better fit to the electron density map for the amino acids Phe-84 and Pro-85 when Pro-85 is in the cis configuration.

As mentioned above, the C-terminal end (residues 349–390) is poorly resolved or not visible at all in the electron density maps due to large flexibility. In the oxidized structure, we observe additional electron density within the previously proposed "open" channel reaching from the protein surface to the diiron site (34). This density could not be fitted with water molecules and might originate from amino acids in the flexible C-terminal tail of the enzyme. The C-terminal tail might fold back and into the channel as part of the mechanism that controls the access to the dinuclear cluster from the protein surface.

The Dinuclear Cluster of Mouse R2 RNR Made under Reducing Conditions—The structure under reducing conditions was obtained from crystals of apo-R2 soaked in mother liquor to which an Fe(II)-solution was added. A buffered dithionite solution (final pH = 6.0) was used as reducing agent. The crystallographic data are listed in Table I (Structure 1). Fig. 1, A and B, shows an omit map and stick model of the dinuclear iron center. Analogous to other diiron-oxygen proteins (11, 14), each Fe(II) in mouse R2 is coordinated by histidines (His-173 and His-270, respectively), and Glu-170 bridges the two Fe(II) in a $\mu$-($\eta^1$,$\eta^1$) fashion with each iron coordinated by a separate carboxyl oxygen, also called a $\mu$-($\eta^1$,$\eta^1$) bridge. Asp-139 and Glu-233 are monodentate terminal ligands to Fe1 and Fe2, respectively.

The electron density map of reduced mouse R2 reveals density near the dinuclear cluster that cannot be accounted for by iron, water, or protein side chains, and it is best fitted with an acetate ion. One carboxyl oxygen of the acetate is coordinated...
to Fe2, with a bond length of 2.1 Å, whereas the second carboxyl oxygen is 2.7 Å away from Fe1, which is slightly too long for an iron ligand. This acetate ion is most likely a result of the presence of sodium acetate in the soaking solution, but its position may have implications for the binding of small ligands to the iron site during the iron-oxygen reconstitution reaction. The selective binding to Fe2 resembles the binding of azide in the azide-soaked double mutant R2-F208A/Y177F from E. coli (36); both azide and one carboxylate oxygen of acetate bind to Fe2 on the side facing away from the histidine ligand (His-241 and His-270 in E. coli and mouse R2s, respectively). Acetate has also been observed near the diiron center in x-ray structures of other diiron-oxygen proteins, such as in an oxidized form of MMOH at 4 °C (42) and in a reduced form of /H9004 9-D (43).

However, in these cases, the acetate has been interpreted to be coordinated in a bridging mode binding both Fe1 and Fe2. There is also one example of a structure of MMOH solved at cryogenic conditions (160 K), where the acetate is not directly coordinated to any iron (44).

The Fe-Fe distance in the reduced mouse R2 (Structure 1) is rather short (3.4 Å) and seems to be correlated to the coordination mode of Glu-267, which is \( \mu-(\eta^1,\eta^2) \) with one carboxyl oxygen bridging Fe1 and Fe2 and Fe2 being chelated by both carboxyl oxygens (see Fig. 1B). This is very similar to the situation observed in the diferrous MMOH (42, 44, 45), but in the originally published x-ray structures of diferrous R2 from E. coli, which were obtained from chemically or photolytically reduced crystals of diferrie R2, the corresponding Glu-238 was found in a \( \mu-(\eta^1,\eta^2) \) mode, and the Fe-Fe distance was significantly longer, 3.9 Å (30). Recently, however, a new x-ray structure of diferrous E. coli R2 was published where the diferrous form was generated by soaking crystals of apo-R2 in a solution of Fe(II), and then Glu-238 adopted a \( \mu-(\eta^1,\eta^2) \) coordination with a Fe-Fe distance of 3.6 Å, as measured in the released Protein Data Bank file (1PIY) (31). The authors argued that the coordination pattern was a result of the soaking conditions, although they observed the reverse pattern in the mutant R2-D84E, where Glu-238 was found in the \( \mu-(\eta^1,\eta^2) \) mode in the chemically reduced protein (30), and the \( \mu-(\eta^1,\eta^2) \) mode was found in the Fe(II)-soaked apo-R2-D84E (31). The mutant R2-D84E is a mimic of the diiron site of MMOH with all carboxylate ligands being glutamate. The wild-type R2F protein of class Ib RNR from Corynebacterium ammoniagenes is also described with the corresponding Glu-202 as a \( \mu-(\eta^1,\eta^2) \) bridge after soaking of apoprotein with Fe(II) (46).

A \( \mu-(\eta^1,\eta^2) \) bridge and a corresponding short Fe-Fe distance...
of 3.4 Å has also been observed in the azide-soaked double mutant R2-F208A/V122F from E. coli (36). In that paper, it was argued that the $\mu$-(η^1,η^1)-coordination is induced by binding of a small ligand to Fe2, such as azide or dioxygen, and that the transition from $\mu$-(η^1,η^2) to $\mu$-(η^1,η^2) of Glu-238 (i.e. Glu-267 in mouse R2) represents the first step in the oxygen activation reaction of R2. The oxygen molecule was proposed to bind down to Fe1 and form a peroxide complex (36). It is possible that the presence of acetate in our reduced structure of R2 has induced this transition, which results in the observed short Fe-Fe distance. However, Glu-267 is also observed in the $\mu$-(η^1,η^2) orientation in the other structures of mouse R2 obtained under different reducing conditions where no acetate was observed and with corresponding short Fe-Fe distances (3.4–3.6 Å) (see Fig. 1C; see below). Furthermore, a distorted orientation of Glu-267 was observed in the dicobalt-substituted mouse R2 structure (35), with a Co(II)-Co(II) distance of 3.4 Å. 

The observed pattern for the reduced diiron center in mouse R2 is four- and six-coordination for Fe1 and Fe2, respectively (Fig. 1B). However, since the acetate would not be expected to be present in solutions of R2, the diiron center could be four- and five-coordinated or even four- and four-coordinated if the bidentate coordination of Glu-267 to Fe2 was induced by the presence of acetate. This is supported by a combined CD and variable temperature variable field magnetic CD spectroscopic study of mouse R2 in a D2O solution (pD 7.5 and >50% glycerol), where a four- and five-coordinated diferrous center was observed with zero field splitting D values of $-15 < D_1 < -10$ and $+7 < D_2 < +10$, respectively (47). A very weak exchange coupling was found between the two iron ions, supporting the presence of carboxylate bridges. The magnetic CD data are in agreement with the interpretation of the observed integer spin EPR signal in mouse R2 (47, 48).

**Novel Orientation of Phe-237 and Phe-241 Makes the Diiron Site More Open**—Compared with the previously published mono-Fe structure of mouse R2 (34), we observe a totally different orientation of the side chains of Phe-237 and Phe-241 in the reduced structure as well as in the oxidized structure (see below). The torsion angles over the C$_\alpha$-C$_\beta$ bond differ by more than 100°, and thus the phenyl rings are pointing away from the diiron center in our mouse R2, whereas they are pointing toward the diiron center in the mono-Fe mouse R2. In E. coli R2 and mouse R2, there is a channel reaching from the surface to the proposed oxygen reaction site close to Tyr-177 (34). A striking difference between the E. coli R2 and mouse R2 is that this channel is blocked by a tyrosine residue in E. coli R2, whereas in mammalian R2, it is replaced by a smaller serine residue. In mouse R2, the channel is leading all the way to the conserved amino acids Phe-237 and Phe-241 that are part of the hydrophobic environment around the dinuclear cluster. In the oxidized E. coli R2 structure, Phe-208 is located 4.5 Å away from Fe1 (32), whereas the counterpart Phe-237 in mouse R2 is located 6.4 Å away from Fe1. The orientation of the phenylalanines in E. coli R2 gives a less open environment around the dinuclear center compared with the dinuclear center of mouse R2 and might for that reason provide a more stable environment for iron binding. This could explain why mouse R2 is much more sensitive toward bulkier, hydrophobic radical scavengers, which indicates a more open structure around the iron/radical in the mouse protein compared with E. coli R2 (49).

**Other Structures Obtained under Reducing Conditions**—We obtained also an x-ray structure from crystals of apo-R2 soaked in mother liquor containing Fe(II) and with ascorbate instead of dithionite as reducing agent. This structure is nearly identical to the one obtained with dithionite (Fig. 1C, red; detailed result not presented) (Structure 3 in Table I). In the ascorbate-reduced structure, the electron density that was associated with the acetate ligand above is significantly smaller and corresponds instead to a water molecule. In contrast to the acetate seen in the dithionite-reduced structure, this water molecule is too far away from Fe2 to be a direct ligand (2.7 Å). Both Glu-267 and Asp-139 have high B-factors and might be very flexible. In this structure, Glu-267 might equally well be fitted as a monodentate ligand to Fe2 or with one oxygen atom bridging the two iron ions and both oxygen atoms coordinated to Fe2.

We have additional x-ray data sets from mouse R2 obtained from crystals of apo-R2 that were soaked in mother liquor containing Fe(II), as above, but an unbuffered solution of dithionite (pH < 6.0) was used as reducing agent (Fig. 1C, blue; detailed result not presented) (Structure 4 in Table I). The most striking difference resulting from lowering the pH in the soaking solution is that the relative electron density on Fe1 is low, indicating that this iron is loosely bound. The lower occupancy of site 1 compared with site 2 at pH < 6.0 is in agreement with the pH 4.7 mono-Fe R2 mouse structure (34) and the structures of cobalt-substituted R2 from mouse (35).

This collection of three structures presented of the same protein provides important information about the possible rotation and motion of amino acid side chains during oxygen activation at the active site. In all the structures obtained under reducing conditions, Asp-139 and Glu-267 have relative high B-factors compared with the other amino acids coordinating the dinuclear iron site. When the structures made under reducing conditions are superposed (Fig. 1C), Asp-139 is particularly flexible. The variations observed for the different structures of the same protein show that the ligands are very sensitive to the pH in the soaking solution and make relatively large carboxylate shifts in the crystal, which also influence the coordination number of the iron ions. There are variable amounts of water molecules close to the diiron center in all three structures that are not present in the structure of different E. coli R2 (30, 31).

**The Dinuclear Cluster of Mouse R2 RNR Made under Oxydizing Conditions**—The electron density map and the ball-and-stick representation for R2 made under oxidizing conditions is obtained from crystals of apo-R2 (final pH 6.0) that were soaked in mother liquor containing Fe(II) and then oxidized in air (Fig. 2A) (Structure 2 in Table I). The electron density map reveals a peak between the two iron ions that is best fitted with a $\mu$-oxo bridge, which is located 1.8 Å away from both Fe1 and Fe2 (Fig. 2A). The most significant change occurring at the diiron center of mouse R2 upon oxidation to the dfferferic state, in addition to the introduction of the $\mu$-oxo bridge, is a large carboxylate shift of the flexible amino acid Glu-267. A result of this shift is that the coordination to Fe1 is broken, and Glu-267 is now only a terminal monodentate ligand to Fe2. Small shifts in the orientations of Asp-139 and Glu-233 are also observed, but they do not change the coordination mode to Fe1 or Fe2. The rigid framework of the binding site is formed by the histidines (His-173 and His-270) and Glu-170, which do not move significantly upon oxidation.

**More Flexible Carboxylate Ligands Compared with the Differferic Form of E. coli R2—Fig. 2C** compares the two differferic (met) structures of mouse R2 (red) and E. coli R2 (blue, Protein Data Bank code 1MXR) (32). Most ligands have a very similar arrangement in the two R2 proteins, but there are some differences. The $\mu$-oxo group of mouse R2 is located 1.8 Å from both Fe1 and Fe2, which is slightly shorter than the 1.94- and 1.93-Å distances measured for E. coli R2 (protomer A). This difference is also reflected in the Fe1-Fe2 distance of 3.3 Å and the angle defined by Fe1-O-Fe2 of 131° in mouse R2, which in E. coli are 3.38 Å and 122°, respectively.

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**The Diiron Center of R2 Ribonucleotide Reductase from Mouse**

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Both *E. coli* and mouse R2 have two water molecules close to the irons. In *E. coli*, one is coordinated to Fe1 and one to Fe2, but the former coordination is lost in mouse R2 (water-Fe1 distance 3.8 Å). This makes the metal cluster of the diferric form in *E. coli* R2 four- and six-coordinated, as opposed to five- and six-coordinated in *E. coli* R2. The most apparent difference between the two structures in Fig. 2C is the carboxylate shift of Asp-139 in mouse R2 and Asp-84 in *E. coli* R2. In *E. coli* R2, this residue was previously described as a bidentate ligand to Fe1 but has in the more recent high resolution structure (1.4 Å) been established as a monodentate ligand with both carboxylate oxygens still facing Fe1 (32). In mouse R2, however, one carboxyl oxygen is pointing away from Fe1, and Asp-139 is clearly monodentate as in reduced *E. coli* R2 (30). Glu-267 in *E. coli* R2, however, one carboxyl oxygen is pointing away from Fe1, and Asp-139 is clearly monodentate as in reduced *E. coli* R2 (30). Glu-267 in mouse and Glu-238 in *E. coli* have similar terminal monodentate coordination to Fe2, although mouse Glu-267 shows great flexibility judged by the high B-factor.

**Relation to Spectroscopic Data**—The kinetic and spectroscopic data of the dinuclear center of R2 are typically acquired at pH 7.6, and for that reason it would be preferable to have a crystallographic model at this pH. However, we were not able to increase the pH above 6 for the crystals of mouse R2 without dissolving the crystals in the soaking solution. We have verified by X-band EPR that the “normal” tyrosyl radical can be regenerated from apomouse R2 at pH 6.0 by iron reconstitution in solution, and traces of tyrosyl radicals were also observed in the iron-soaked crystals of apo-R2 (data not shown). For crystals of *E. coli* R2, it has been demonstrated that moderate changes of the pH between 5 and 7.6 have little effect on the coordination geometry of the diiron center (32, 50). Thus, in the following, we will use the present structural model of the oxidized diiron center of mouse R2 as a basis for discussion of the unique spectroscopic properties of mouse R2 compared with *E. coli* R2.

**Hydrogen Bond to the μ-Oxo Group**—Variable temperature saturation recovery EPR studies have shown that the antiferromagnetic coupling between the ferric irons in mouse R2 (J = −77 cm⁻¹ in H₂O and −70 cm⁻¹ in D₂O) is weaker than in *E. coli* (J = −92 cm⁻¹ in H₂O), suggesting a hydrogen bond to the μ-oxo bridge in the mouse R2 protein (20). Resonance Raman studies show weaker symmetric stretch Fe(III)-O-Fe(III) modes for mouse (486 cm⁻¹) R2 compared with *E. coli* R2 (493 cm⁻¹), and a deuterium shift of −5 cm⁻¹ in mouse R2 showed the presence of the postulated hydrogen bond to the μ-oxo group (21). Such a hydrogen bond could also explain why the oxygen in the μ-oxo bridge is very hard to exchange in mouse R2 (21), where one must perform the whole reconstitution reaction in presence of [¹⁸O]water to get an isotope effect on the resonance Raman spectra of the μ-oxo bridge, whereas in *E. coli* R2, the μ-oxo bridge can be exchanged very rapidly by soaking the active protein in H²¹⁸O (51). A clear donor candidate for this hydrogen bond is not obvious from the crystal structure, which might reflect the pH difference of pH 6 in the crystals and pH 7.5 in the spectroscopic measurements. The change in pH might move a water into hydrogen bond position. One other speculative possibility is Glu-267, which is located 2.6 Å away from the μ-oxo bridge and could be a hydrogen bond donor if protonated. However, a protonation of a carboxylate ligand to Fe(III) is unusual, since the iron induces a decrease of the pKᵣ value by 2 units (52).

**Hydrogen Bond to the Radical**—Several spectroscopic tech-
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Since the H–O bond distance in water is \( \sim 1.0 \, \text{Å} \), this water oxygen must have moved 1.2 Å to a position about 2.9 Å away from Tyr-177 in order to become hydrogen-bonded to the Tyr-177 radical. Another possibility is that the tyrosyl radical has moved, shown in Fig. 3B, where the cyan tyrosine represents the new position of Tyr-177 that will give a distance of 2.9 Å from the phenoxyl oxygen to the water molecule. A similar rotation of the tyrosyl radical was suggested for \( E. \ coli \) R2 by comparing the orientation of the \( g \)-tensor of the tyrosyl radical in the active form and the orientation of the tyrosyl ring in the crystal structure of the nonradical diferric form (32). Interestingly, the dihedral angle between the \( C^-C^- \) bond and the phenoxyl ring is in the 45–55° range (see Fig. 2C) in the x-ray structures of the nonradical met-R2 forms of both \( E. \ coli \) and mouse, whereas the magnitudes of the hyperfine interactions from the \( \beta \)-protons on the tyrosyl radical measured by ENDOR spectroscopy indicate that this angle should be 10–30° in the radical state of mouse R2 (22, 54). This confirms that a rotation of the tyrosyl side chain must take place in mouse R2 and that this rotation is different from the one proposed for \( E. \ coli \) (32).

In the active form of mouse R2, probably both the water and the tyrosyl radical have moved, accompanied by a movement of the \( \alpha \)-helix.

The water on the opposite side of Tyr-177 might also be responsible for the H/D exchangeable proton bonded to the tyrosyl radical (see Fig. 2A), but this water is too far away from Fe1 to account for a very weak exchange coupling (see below) that is observed between the diiron center and the Tyr-177 radical in mouse R2 (20, 53).

**A Weak Exchange Coupling between the Tyrosyl Radical and the Diiron Center**—A weak exchange coupling has been observed between the tyrosyl radical and the diiron center in R2 from mouse and some other species, e.g. \( Salmonella \ typhimurium \) R2F (20). In the x-ray structure of mouse R2, the distance between the tyrosyl radical oxygen and the closest iron is 5.9 Å, and in the class Ib R2F from \( S. \ typhimurium \), it is even 7 Å (55), which is too long, since exchange couplings need overlapping electron orbitals. Eriksson \ et al. \ (55) have therefore suggested that the water molecule close to the radical could mediate the very weak exchange coupling in \( S. \ typhimurium \), consistent with our finding of a water molecule between Tyr-177 and Fe1 in mouse R2.

Even when a water molecule is included, however, the tyrosyl radical and the diiron center need to move a little bit closer to be able to facilitate the radical transfer that is needed in the R1-R2 holoenzyme complex. As mentioned above, it has been proposed that a shifting of the tyrosine ring away from the diiron center occurs in isolated \( E. \ coli \) R2 when it is converted from nonradical to radical form (32). In the R1-R2 complex, this shifting might well be inverted to allow for electron/proton transfer between the tyrosyl radical in R2 to the active site in R1, where a cysteine radical has been proposed to initiate the substrate turnover (2, 5, 6). An interesting idea is that there might exist a triggering mechanism for radical transfer in R2 proteins involving movement of the \( \alpha \)-helix with the tyrosyl radical, which could possibly be facilitated by the binding of R1. Such movements might be in the range 0.2–1 Å but are probably not as large as observed in the classical movement of \( \alpha \)-helix F of hemoglobin upon binding of oxygen (1 Å). At this point, we cannot exclude backbone movements in the R2 proteins as an explanation for the radical shifts. This is in agreement with recent findings that the presence of the R1 protein changes the redox properties of the diiron center in R2 from \( E. \ coli \) (56).

**Biological Implications**—The x-ray structures of mouse R2 have shown that the dinuclear iron cluster is surrounded by

**Fig. 3. Two alternative models of the active tyrosyl radical form of R2 from mice based on ENDOR and the crystallographic structure.** The x-ray structure of oxidized mouse R2 has been modified to take into account the hydrogen bond to Tyr-177 that was detected by Q-band Mims ENDOR measurements (22), where the distance between a water oxygen (hydrogen bond donor) and the tyrosyl radical oxygen is 2.9 Å (see “Results and Discussion”). The images were drawn in Bobscript (59), a modified version of Molscript (60). The cyan sphere (59), a modified version of Molscript (60). The cyan sphere represents the new position of the water molecule.
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very dynamic carboxylate ligands and well defined water molecules. In addition, we confirmed that the diiron center is connected with the surface via a relatively open hydrophobic channel. The well defined positions of the water molecules near the diiron center might indicate that they have specific biological roles, but they could also just be a result of the greater accessibility of the diiron center. The relatively open diiron center, which loses its iron easily, might be a convenient way to switch on and off the activity of mammalian R2 (34, 35).

The dynamic protein structure of mouse R2 with the observed carboxylate shifts and proposed radical and water movements might be of great importance for controlling the reactivity of mammalian R2. We have recently shown that both binding of Co(II) and Fe(II) to mouse R2 is highly cooperative (35, 47). The CD and magnetic CD measurements showed that the two iron binding sites in mouse R2 have similar and low binding affinities at pH 7.5 (47), whereas in E. coli R2 the affinity for Fe(II) is much higher and different between the two sites (33, 57). The lower kinetic and thermodynamic affinity for Fe(II) and the unique cooperative binding of metal ions in mouse R2 compared with E. coli R2 can possibly be explained by the greater flexibility of the carboxylate ligands that we observe in the various x-ray structures of mouse R2 presented here.

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