A New Tyrosyl Radical on Phe^{208} as Ligand to the Diiron Center in *Escherichia coli* Ribonucleotide Reductase, Mutant R2-Y122H

**COMBINED X-RAY DIFFRACTION AND EPR/ENDOR STUDIES***

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The R2 protein subunit of class I ribonucleotide reductase (RNR) belongs to a structurally related family of oxygen bridged diiron proteins. In wild-type R2 of *Escherichia coli*, reductive cleavage of molecular oxygen by the diferric diiron center generates a radical on a nearby tyrosine residue (Tyr^{122}), which is essential for the enzymatic activity of RNR, converting ribonucleotides into deoxyribonucleotides. In this work, we characterize the mutant E. coli protein R2-Y122H, where the radical site is substituted with a histidine residue. The x-ray structure verifies the mutation. R2-Y122H contains a novel stable paramagnetic center which we name H, and which we have previously proposed to be a diferric iron center with a strongly coupled radical, Fe^{II}Fe^{II}H. Here we report a detailed characterization of center H, using ^1H/^2H-^14N/^15N- and ^57Fe-ENDOR in comparison with the Fe^{II}Fe^{IV} intermediate X observed in the iron reconstitution reaction of R2. Specific deuterium labeling of phenylalanine residues reveals that the radical results from a phenylalanine. As Phe^{208} is the only phenylalanine in the ligand sphere of the iron site, and generation of a phenyl radical requires a very high oxidation potential, we propose that in Y122H residue Phe^{208} is hydroxylated, as observed earlier in another mutant (R2-Y122F/E238A), and further oxidized to a phenoxy radical, which is coordinated to Fe1. This work demonstrates that small structural changes can redirect the reactivity of the diiron site, leading to oxygenation of a hydrocarbon, as observed in the structurally similar methane monoxygenase, and beyond, to formation of a stable iron-coordinated radical.

The R2 protein is a homodimer which in its active form contains two μ-oxo bridged diferric iron centers and a stoichiometric amount of a tyrosyl radical. The active form can be generated *in vitro* by the so-called reconstitution reaction by adding a 6-fold molar excess of Fe^{2+} and molecular oxygen to the iron-free apoR2 protein (13). The tyrosyl radical is stabilized by a surrounding cluster of hydrophobic side chains as well as by the diiron center, and survives for a couple of days at room temperature. During the reconstitution reaction, several intermediate states of the diiron center have been proposed (7, 14–17), some of which have been observed by spectroscopy, such as a high valence Fe^{III}Fe^{IV} state, known as intermediate X. Intermediate X has a net electron spin of $S = \frac{1}{2}$, and exhibits a 1.8 mT broad singlet EPR spectrum at X-band frequency and cryogenic temperatures (14–17).

To investigate the redox chemistry of R2 and the functional abilities of diiron centers in general, it is of great interest to
modify the proteins by site-directed mutagenesis and to trap and characterize paramagnetic intermediates during the reconstitution reaction of the apoprotein with ferrous iron and molecular oxygen. The substitution of the radical site at Tyr122 in E. coli R2 with other amino acids has been performed for several reasons: (i) A protein-linked radical generated on another aromatic amino acid, e.g. tryptophan, is expected to be similarly well shielded and stabilized by the hydrophobic environment as is the tyrosyl radical. Such radicals and their interactions with the protein may be studied as models for functional amino acid radicals in other enzymes, where radicals might be more difficult to detect. (ii) A radical from another amino acid residue at this site may or may not assume the function of Tyr122 as a temporary electron and proton acceptor for the active site in the R1 protein in the catalytic cycle. (iii) By altering the normal target for oxidation by the diiron center, the latter may perform a different chemistry, which could help in understanding the different reactions of other structurally related diiron proteins, such as MMO.

Results for several Tyr122 mutations have been reported previously. In R2-Y122F the precursor intermediate X was observed to have an increased lifetime compared with the wild type, and it was succeeded by transient tryptophan radicals at 18–20 °C. The mutant X found in R2-Y122F has identical spectroscopic properties to the wild-type X found in R2-Y122H. In protein R2 of mouse RNR the paramagnetic center (95.2%) of the protein, and information on the structure of the paramagnetic center can be obtained only from EPR and ENDOR spectroscopy. Detailed EPR and ENDOR studies of R2-Y122F were grown either in Luria-Bertani (LB) medium, or in minimal medium (27). Both media contained carbenicillin 50 mg/ml and kanamycin 50 mg/ml. R2-Y122H was purified using the standard methods as described previously (28). Protein purified by these methods was normally 90% pure. For crystallization the mutant was further purified on FPLC Mono Q 16/10 and Superdex 200 columns. The bacterial iron-chelating agent (29). The cells expressing R2-Y122F were only grown in the minimal medium to produce the iron-free apo form.

Isotope Labeling of R2-Y122H—An acidic 57Fe stock solution was prepared as described by writing (16). The iron content in the Fe solution was determined using the bathophenanthroline assay (29) and was found to be 1.6 ± 0.2 mg/ml. Protein purification of R2-Y122H was grown in minimal medium as described by writing (29) except that the trace metal solution was omitted, and substituted with 1 ml of 0.5% 57FeCl3×6H2O for the unlabeled control, or a corresponding amount of the 57Fe stock solution for the labeled sample. This modified minimal medium was also used for global 13N labeling and for 13C and 15N labeling of amino acid residues, where the medium was supplemented with sterile filtered solution of the isotope labeled L-amin acids to a final concentration of 160 μM. The cells were harvested, and the protein was purified as described above.

Reconstitution of Apoprotein R2 with Ferrous Iron and Oxygen—The reconstitution of the iron site in R2 carried out at room temperature as follows: a solution of 2 mM apoR2 (Y122H or Y122F) in air-saturated 50 mM Tris-Cl, pH 7.6 was mixed in an EPR tube. The reaction was stopped by quenching the reaction at time points from 8 °C to 120 °C) to quench the reaction. The reaction mixture was then incubated at 4 °C. After 30 min of incubation time the reaction was quenched by running R2 through a Sephadex G-25 column. Hydroxyurea did not react with center H.

EXPERIMENTAL PROCEDURES

Materials—57Fe (95.2%) foil was purchased from Chemotrade, Düsseldorf, Germany; D2O (99.97%), [1-13C]Asp, [δ-15N]Glu, and D2O were purchased from Isotec (Sigma-Aldrich). 2-[13C]Glucose, 8-hydroxyquinoline-5-sulfonate, 8-hydroxyquinoline, and ethylenediaminetetraacetic acid, sodium salt, disodium salt, and sodium salt were purchased from Sigma. Diammoniumiron(II)sulfate hexahydrate, hydrogen peroxide, sodium dithionite, and dithionite were purchased from Merck KGaA, Darmstadt, Germany. Tryptsin Gold (Mass Spectrometry Grade) was purchased from Promega (Madison, WI), diethanolamine from Biomed, Hamburg, Germany, and sodium palmitate from BALBI (Rome, Italy). The oligonucleotide used for generating the mutant Y122H d(5′-CTTACTTAGCTCCCATCTACTATC-3′) was synthesized and purified by Scandinaviam Gene Synthesis AB, Köping, Sweden. A new construction, plasmid pTB2-Y122H, was verified with dyeoxyribonucleotide sequencing.

Bacterial Strains and Plasmids—E. coli MV 190 (Δlac-proAB), thi, supE, dars-recA306; ΦN10/Fd36, proAB, lacZAM15) obtained from Bio-Rad was used for mutagenesis, cloning, and plasmid preparation. MC1061 [pACYC184, gale, gale, ara-λ-leu7697, araD139, recA, rcl+::Tn10] obtained from Amersham Biosciences was used for expression. Plasmid pTB2 containing the nrdB gene coding for protein R2 was used in combination with a second plasmid, pGP1-2, for overexpression of R2-Y122H using heat induction of the T7 RNA polymerase (26).

Expression and Purification—The cells carrying the R2-Y122H mutation were grown either in Luria-Bertani (LB) medium, or in minimal medium (27). Both media contained carbenicillin 50 mg/ml and kanamycin 50 mg/ml. R2-Y122H was purified using the standard methods as described previously (28). Protein purified by these methods was normally 90% pure. For crystallization the mutant was further purified on FPLC Mono Q 16/10 and Superdex 200 columns. The iron absorption index (ε1%·1 cm−1), and 13C and 15N labeling of the unlabeled control, or a corresponding amount of the 57Fe stock solution for the labeled sample. This modified minimal medium was also used for global 13N labeling and for 13C, and 15N labeling of amino acid residues, where the medium was supplemented with sterile filtered solution of the isotope labeled L-amin acids to a final concentration of 160 μM. The cells were harvested, and the protein was purified as described above.

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Chemical Reduction—Reduction of R2-Y122H was performed in septum-sealed vials with alternating evacuation and argon flushing to minimize the presence of oxygen. To a solution of 0.2–1.0 mM protein in 50 mM Tris-HCl, pH 7.6, an equimolar concentration of phenazine methosulfate was added as redox mediator as well as a four times excess of either dithionite or ascorbate as reducing agent (30). Samples were extracted by an airtight Hamilton syringe and transferred to a 4-mm outer diameter EPR tube before freezing in liquid nitrogen after incubation at room temperature from 1 to 10 min.

Trypsin Digestion—The trypsin digestion was performed on wild-type R2 and R2-Y122H using the following protocol (31): 10 µl of the 0.35 mM protein R2 was added to 100 µl of 6 µm urea in 0.1 mM Tris-HCl, pH 7.8, to unfold the protein. Then 5 µl of 200 mM dihydrothreitol was added to reduce the cysteine residues, and the mixture was incubated for 60 min at room temperature. Then 20 µl of 200 mM iodoacetamide was added to block all cysteine thiol groups and thereby prevent formation of unwanted disulfide bridges. After 60 min of incubation at room temperature, 20 µl of 200 mM dihydrothreitol was added, which was allowed to react with any excess of iodoacetamide for another 60 min at room temperature. The solution was then diluted with 775 µl of 0.1 mM Tris-HCl, pH 7.8, to reduce the urea concentration to below 0.6 M where trypsin is active, and then 100 µl of 0.2 mg/µl trypsin was added. The reaction mixture was incubated at 37 °C overnight. The reaction was terminated by addition of 5 µl of 100% 200 mM Tris base, and the samples frozen for later analysis by mass spectrometry.

Continuous Wave (CW) EPR and ENDOR Instrumentation—X-band (9.5 GHz) EPR spectra were recorded on a Bruker ESP 300E spectrometer using a standard rectangular (TE_{x,y}) EPR cavity (Bruker ER4102ST) equipped with an helium flow cryostat (Oxford, ESR9). CW-ENDOR spectra were measured on a Bruker ESP 300E spectrometer using a self-built ENDOR accessory, which consists of a Rhode & Schwarz RF synthesizer (SMT02), an EII A200L solid state RF amplifier, and a self-built high-Q TM_{110} ENDOR cavity (32). The cavity was adapted to an Oxford helium flow cryostat (ESR 910). The measured g-values were calibrated using the known g-value standard Li_{2}LiF, with g = 2.002293 ± 0.0000002 (33). Spin concentrations were determined by comparison of the double integrals of the EPR spectra with that of a 20% water standard.

Pulse ENDOR—The pulse ENDOR spectra were recorded at X-band using a Bruker ESP 380E spectrometer equipped with an Oxford Instruments helium flow cryostat (CF935) and a Bruker ER 4118X-MDS-EN resonator. For all measurements the Davies pulse sequence (34) was used. The lengths of the microwave (MW) pulses (π and π/2), the radiofrequency pulse (RF), π, and the shot repetition rate are given in the figure captions for each experiment.

Simulation of EPR Powder Spectra—The EPR powder spectra have been analyzed using a program for simulation and fitting of EPR spectra with anisotropic g and hyperfine tensors based on the work of Rieger (35), which is described in Ref. 19 and references therein. Thereby, a residual activity of less than 10 units/mg (nmol of product formed) was verified by dideoxy DNA sequencing (not shown), mass spectrometry (see below). The specific activity of R2-Y122H during substrate turnover in R1 was determined using the 5′-[3H]CDP assay at room temperature in the presence of excess protein R1, as described by Thelander et al. (22); wild-type R2 was used as control. A residual activity of less than 10 units/mg (nmol of product formed) at 1.9-Å resolution was hence less than 0.5%, and probably arises from contamination of chromosomally encoded wild-type R2.

RESULTS

Expression of R2-Y122H—The mutant protein R2-Y122H of E. coli RNR behaves like the wild-type protein during the cell growth and purification procedures; we obtained ~6–7 mg of pure protein R2 per liter of bacterial culture grown in minimal medium and about 6 times more pure protein when cells were grown in LB medium. The mutation Tyr→His at position 122 was verified by dideoxy DNA sequencing (not shown), mass spectrometric analysis (see above) as well as x-ray crystallography (see below). The specific activity of R2-Y122H during substrate turnover in R1 was determined using the 5′-[3H]CDP assay at room temperature in the presence of excess protein R1, as described by Thelander et al. (22); wild-type R2 was used as control. A residual activity of less than 10 units/mg (nmol of product formed) at 1.9-Å resolution was hence less than 0.5%, and probably arises from contamination of chromosomally encoded wild-type R2.

X-ray Structure—The x-ray structure of R2-Y122H at 1.9-Å resolution clearly confirms the mutation Tyr→His at position 122 of the R2 structure (see Fig. 1). All ligands of the iron center are well defined by the electron density, and their conformations are comparable to the structure of the wild-type oxidized diferric R2 (metR2) (42, 43). There are, however, some noticeable differences. In the R2-Y122H dimer, the Fe–Fe distance is 3.5 Å in monomer A and 3.4 Å in monomer B, whereas in the wild-type metR2 dimer at 1.4-Å resolution, the distances are smaller and more equal, (3.39 and 3.35 Å, PDB ID 1MXR)

### Table I

| Data collection | Resolution range (Å) | Completeness (%) | Observed reflections | Unique reflections | R_{merge} (%) |
|-----------------|---------------------|-----------------|----------------------|-------------------|--------------|
|                 | 20–19              | 99.1 (98.4)     | 191503               | 57178             | 8.4 (23.3) |

**Statistics for the highest resolution shell are given in parentheses.**

R_{merge}(j, i) = \sum_k [I_{j,k} - \langle I_i \rangle]/\sum_{j,k} I_{j,k}, where I_{j,k} are the k individual observations of each reflection j and I(i), is the weighted mean.

R_{free}(%) = R_{merge}(F) = \sum_{j,k} [F_{j,k} - \langle F_i \rangle]/\sum_{j,k} F_{j,k}, where [F]_j and [F]_i are the observed and calculated structure factors for reflection i.

**R.m.s., root mean square.**

### Data collection and refinement statistics for E. coli R2 mutant Y122H

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A New Iron-Phenoxyl Radical Center in R2-Y122H

Fig. 1. Stereo view of the oxidized diferric iron centers of RNR R2 from E. coli mutant R2-Y122H chain A (blue) and chain B (green), (1.9-Å resolution), wild-type R2 (red, 1.4-Å resolution, PDB entry 1MXR (43)) and mutant F208Y (cyan, 2.5-Å resolution, PDB entry 1RRR (44), bottom). The two iron ions of the diiron center (Fe1 and Fe2), the μ-oxo bridge, and four water molecules (Wat) are indicated by spheres. In R2-F208Y, no water molecules or bridging oxygens were assigned near the diiron center; however, Tyr208 was observed to be hydroxylated to DOPA. The waters in position Wat3 and 4 are only observed in R2-Y122H. A new hydrogen bond is indicated between Asp 84 and His122. The Fe1-Fe2 distances are 3.52 Å (R2-Y122H-Chain A), 3.40 Å (R2-Y122H-Chain B), 3.38 Å (R2-wt-Chain A), and 3.63 Å (R2-F208Y-Chain A). Fe1-Wat1 distances are 2.23 Å (R2-Y122H-Chain A), 2.12 Å (R2-Y122H-Chain B), 2.30 Å (R2-wt-Chain A), and 2.01 Å (meta-OH-group of DOPA-208 in R2-F208Y-Chain A) Fe1-Wat3 distances are 2.68 Å (R2-Y122H-Chain A) 2.46 Å (R2-Y122H-Chain B), and 2.18 Å (R2-F208Y-Chain A). Fe1-Wat1 distances are 2.23 Å (R2-Y122H-Chain A), 2.12 Å (R2-Y122H-Chain B), 2.30 Å (R2-wt-Chain A), and 2.01 Å (meta-OH-group of DOPA-208 in R2-F208Y-Chain A) Fe2-Wat2 distances are 2.20 Å (Y122H-Chain A), 2.40 Å (Y122H-Chain B), and 2.30 Å (wt-Chain A). The figures were designed in DeepView (81).

In wild-type metR2, the carboxyl group of Asp84 is a monodentate ligand to Fe1 with the free carboxylate oxygen making a H-bond to the water coordinated to Fe2, the Fe1-bound oxygen making a second weak H-bond to Y122-OH (43). In R2-Y122H, D84 is rotated further away from the iron site, leaving one oxygen atom ligated to Fe1 and the free oxygen forming a new short hydrogen bond to Ne of His122 (2.7-Å O-N distance). The position of Asp84 in diferric R2-Y122H resembles the situation in the diferrous reduced form of wild-type R2 (39) and mutant R2-Y122H (44) (not shown). Another commonly flexible side chain in R2, Glu238, is found in the same conformation in both monomers of R2-Y122H as in wild-type metR2, i.e. with one carboxyl oxygen coordinated to Fe2 and the second oxygen hydrogen-bonding to a water molecule that is coordinated to Fe1 (Wat1 in Fig. 1). There is also an additional water molecule located in the enlarged pocket around His122 in R2-Y122H (Wat4 in Fig. 1) in both monomers.

The iron centers in the two crystallographically independent protein chains A and B of the R2-Y122H dimer are almost identical. Differences between chains A and B have been observed in other R2 mutants (39, 44) and were explained by a different accessibility for small molecules in the two halves due to crystal packing effects. However, the only differences near the diiron center of R2-Y122H are in the conformation of Phe208 (see Fig. 1), and in the coordination of Fe1. The phenyl ring of Phe208 is shifted between the two monomers because of a 16° torsion angle. The χ1 values are −115° in chain A and −131° in chain B, compared with −105° and −103° in chains A and B of wild-type metR2. Rotation about χ1 keeps the plane of Phe208 approximately at the same distance from Fe1. For instance, in wild-type metR2 the distance between the Phe208 C4 (Cα) carbon and Fe1 is 4.6 Å in both monomers and it is 4.4–4.5 Å in R2-Y122H chain A and B, despite the different χ1 torsions. Crystallographic B-factors and electron density maps indicate somewhat higher flexibility of Phe208 in chain B than in chain A. In subunit B of R2-Y122H, elongated electron density could allow the placement of two water molecules, one coordinating Fe2 (Wat2) and the other coordinating Fe1 (Wat3), in addition to Wat1 in wild-type metR2 (see Figs. 1 and 2). However, the B-factor for the Fe1 ligand is higher (27 versus 21 Å2), the coordinating distance longer (2.5 versus 2.4 Å), and the distance between the two water molecules is only 2.0 Å. This suggests that the water molecule Wat3 coordinating Fe1 is only partially occupied and probably not present at the same time as the water molecule Wat2 coordinating Fe2. The differences between these two water ligands are even more pronounced in subunit A (B-factor 33 versus 14 Å2, coordinating distance 2.7 versus 2.2 Å) indicating lower occupancy for Wat3 in subunit A. The high resolution structure of wild-type metR2 indicates 5-coordinated Fe1 (43). The presence of the extra water ligand to Fe1 may be made possible partly by the shift of Asp84 toward His122 described above.

Optical Spectra—The optical absorption spectrum of R2-Y122H obtained from cells grown in LB medium shows two broad bands at 325 and 370 nm, which are characteristic for a μ-oxo-bridged diferric iron center, and is identical to the spectrum of wild-type R2 after hydroxyurea treatment (i.e. metR2) (45) (compare middle spectra in Fig. 3). The intensity of these bands suggests that R2-Y122H has fully occupied iron sites, i.e. 4 iron per dimer. This was confirmed by quantification of iron from acid-denatured protein using the bathophenanthroline
assay (29), which yielded 3.6–4.2 irons per dimer in different preparations. The characteristic narrow peak at 410 nm resulting from the tyrosyl radical in the active wild-type R2 (upper trace in Fig. 3) is missing in the optical absorption spectra of R2-Y122H. Upon treatment with the strong iron chelator 8-hydroxyquinoline in the presence of imidazole as a mild denaturing agent, and subsequent gel filtration to isolate the protein, the optical bands at 325 and 370 nm disappear (Fig. 3, lower trace), as also observed for wild-type R2 (13). However, traces of up to 0.2 irons/dimer could still be detected in R2-Y122H using the more sensitive bathophenanthroline assay. When R2-Y122H was isolated from cells grown in iron-free minimal medium, we obtained a similar optical spectrum without the bands at 325 and 370 nm (not shown). In consistency with previous studies of wild-type R2 from iron-free growth medium (27); however, a residual amount of up to 0.2 irons/dimer could also be detected here using the bathophenanthroline assay.

EPR Spectra of the Paramagnetic Center in R2-Y122H—Purified R2-Y122H contains a small amount of a novel and stable paramagnetic species, called center H (24). Quantification of center H showed that it appears in ~3–5% of the R2-Y122H dimers at pH 7.6. In contrast to the tyrosyl radical Y122 of wild-type R2, which gives rise to a doublet EPR spectrum \((g_{iso} = 2.0047 \pm 0.0002)\) (46) (not shown), center H exhibits a Gaussian-shaped isotropic singlet EPR signal at X-band \((g_{iso} = 2.0029 \pm 0.0002, \text{linewidth } 2.2 \pm 0.1 \text{ mT})\) (Ref. 24, see also Fig. 4C). Center H exhibits a microwave power saturation behavior and temperature dependence that is unusual for a free radical in a protein. The EPR spectra of R2-Y122H recorded at increasing temperatures exhibit increasing EPR linewidths, and above 70 K the spectrum is no longer observable (24).

The overall lineshape of the EPR spectrum of center H at X-band (9.5 GHz) is similar to the EPR spectrum of intermediate X (Ref. 24), which is a short-lived \(\text{Fe}^{III}\text{Fe}^{IV}\) intermediate that appears in wild-

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**Fig. 3. Light absorption spectra of *E. coli* R2-Y122H and wild-type R2.** Upper trace, active wild-type R2 containing a tyrosyl radical. Middle traces, wild-type R2 treated with hydroxyurea (dark trace) and R2-Y122H as isolated (light trace). Lower trace, R2-Y122H after treatment with hydroxyquinoline and imidazole.

**Fig. 4. X-band EPR spectra of intermediate X in *E. coli* R2-Y122F and center H in *E. coli* R2-Y122H.** A, 1 mM \(^{56}\text{Fe}-\text{R2-Y122F}\). Experimental conditions: temperature 35 K, microwave power: 1 milliwatt, modulation frequency: 12.5 kHz, modulation amplitude: 0.2 mT, 3 sweeps. B, 1 mM \(^{57}\text{Fe}-\text{R2-Y122F}\). Experimental conditions: temperature 35 K, microwave power: 2 milliwatt, modulation frequency: 12.5 kHz, modulation amplitude: 0.2 mT, 3 sweeps. C, 1.0 mM \(^{56}\text{Fe}-\text{R2-Y122H}\). Experimental conditions: temperature 35 K, microwave power: 1 milliwatt, modulation frequency: 12.5 kHz, modulation amplitude: 0.2 mT. D, 0.9 mM \(^{57}\text{Fe}-\text{R2-Y122H}\). Experimental conditions: temperature 30 K, microwave power: 1 milliwatt, modulation frequency: 12.5 kHz, modulation amplitude: 0.7 mT.
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Table II

| g-Tensor components of different paramagnetic species in wild-type and mutant R2 | $g_1$ | $g_2$ | $g_3$ | Ref. |
|---|---|---|---|---|
| Center H in R2-Y122H | 2.0086(1)* | 2.0040(1) | 1.9955(2) | This work |
| Fe$^{3+}$Fe$^{2+}$ in R2-Y122H | 1.930 | 1.843 | 1.802 | This work |
| Fe$^{3+}$Fe$^{2+}$ in E. coli R2 | 1.936 | 1.936 | 1.818 | 85 |
| Intermediate X in R2-Y122F | 2.007 | 1.999 | 1.994 | 16 |
| Y122' in wild-type R2 | 2.00912 | 2.00457 | 2.00225 | 47 |
| Fe$^{3+}$Fe$^{2+}$ in mouse R2 | 1.92 | 1.73 | 1.60 | 30 |
| Fe$^{3+}$Fe$^{2+}$ in MMOH, M. capsulatus | 1.94 | 1.86 | 1.74 | 48 |

* Number in parenthesis denotes error in the last digit.

type R2 and mutant R2-Y122F during the oxygen activation reaction (14–17). However, the spectrum of center H is somewhat broader (2.2 mT) and rather stable. EPR signals were observed from Y122H protein that had been kept for 2 weeks at room temperature (not shown).

The g tensor principal components were obtained from 94 GHz (W-Band) high-field EPR spectra of frozen solutions of R2-Y122H, published in a previous report and are given in Table II (24). The $g_1$- and $g_2$-values are similar to the respective values reported for the wild-type tyrosyl radical in E. coli (20, 47); however, the smallest g-value, $g_3$, which is below 2.0, is untypical for an isolated organic radical and resembles the $g_3$-value of the Fe$^{III}$Fe$^{II}$ intermediate X of 1.994 (from Ref. 16) (see Table II), providing evidence for a metal nature of center H. As observed at X-band EPR, no hyperfine structure was resolved either in the W-band EPR spectra. Therefore ENDOR experiments were required, see below.

Intriguingly, center H was also visible in samples obtained from cells grown in minimal medium, when no iron was added. However, as mentioned above and also reported for the wild-type, these preparations still contain traces of up to 0.2 iron/mg protein; however, as mentioned above and also reported for the wild-type, these preparations still contain traces of up to 0.2 iron/mg protein. Attempts to generate center H by reconstitution of apoR2-Y122H with iron(II) and molecular oxygen did not lead to a significant increase of the yield of H.

Chemical Stability of Center H—The paramagnetic center H in R2-Y122H exhibits unusual stability. We have treated R2-Y122H with different chemical agents and compared the results with wild-type R2. The X-band EPR spectra recorded at low microwave power (not shown) are partly superimposed by a signal resulting from the tyrosyl radical Tyr122, confirming a small contamination of chromosomally encoded wild-type R2, as detected in the activity assay (see above). Therefore we used hydroxyurea, a well-known scavenger of the tyrosyl radical in wild-type R2 (45), to quench selectively the superimposed doublet signal from wild-type Tyr122. Hydroxyurea had no effect on the intensity or lineshape of the EPR spectrum of center H.

Treatment with hydroxyquinoline/imidazole and subsequent gel filtration removes 95% of the iron coordinated to the protein (total spin $S = 0$) by one electron reduction. In center H, as measured by EPR, was merely reduced to 40% of its original level after this treatment. Addition of 6 x molar excess of Fe$^{3+}$ and molecular oxygen to the iron-depleted R2-Y122H restored the optical absorption bands at 325 and 370 nm. However, the magnitude of the EPR signal of center H was unaffected by this reconstitution.

Chemical reduction of R2-Y122H using dithionite ($E'_o = -460$ mV) and phenazine methosulfate (PMS) as mediator ($E'_o = -270$ mV) under anaerobic conditions led to a decrease of the EPR signal of center H. After 5 min reaction time 80% of the original EPR spectrum of center H vanished, and a new EPR spectrum exhibiting large g-anisotropy, already resolved at X-Band, appeared. (Fig. 5). The g tensor values obtained from a simulation (Fig. 5, dotted trace) are listed in Table II. Based on these g tensor values we assign the new signal to a mixed valence Fe$^{III}$Fe$^{II}$ center as observed in the structurally similar enzyme MMOH and in mammalian R2 after chemical reduction (30, 8). After removing the PMS and dithionite by gel filtration we were able to regain the signal of center H through treatment with hydrogen peroxide, although with slightly lower spin concentration than before reduction. Treatment of R2-Y122H with sodium ascorbate/PMS, or dithionite alone, also led to the reduction of center H and simultaneous appearance of the mixed valence Fe$^{III}$Fe$^{II}$ center (not shown). As can be seen in Fig. 5, the integrated intensity of the broad mixed valence Fe$^{III}$Fe$^{II}$ EPR signal exceeds by far that assigned to center H. This clearly shows that the mixed valence signal is generated from the Fe$^{III}$Fe$^{II}$ state in the majority of the protein (total spin $S = 0$) by one electron reduction. In center H, dithionite reduction leads to an EPR silent species, e.g. a Fe$^{III}$Fe$^{II}$ state with a phenoxylate ligand, or a Fe$^{III}$Fe$^{II}$ state with a coordinated radical, coupled to an $S = 0$ ground state. Whatever the nature of this state is, it is at least in part reoxidized to the radical ligated Fe$^{III}$Fe$^{III}$ by hydrogen peroxide.

X-band EPR of Center H and X Labeled with $^{57}$Fe—In a previous study (24), we observed a significant broadening of the EPR spectra of center H labeled with $^{57}$Fe. Here, we compare...
and signals in the low frequency range (1–10 MHz) resulting from nitrogen nuclei ($\nu_{14N} = 1.02$ MHz at 330 mT) (see below). ENDOR lines from hydrogens occur as pairs in the spectra according to the first-order resonance condition in Equation 1,

$$\nu_{\text{ENDOR}} = |\nu_s \pm A/2|$$  \hspace{1cm} (Eq. 1)

where $\nu_s$ represents the Larmor frequency of a nucleus with spin $I > 0$ nucleus, and $A_j$ the electron-nuclear hyperfine coupling for this nucleus. In frozen solution the hyperfine coupling is anisotropic, defined by three tensor components, $A_1$, $A_2$, and $A_3$ (j = 1, 2, 3), which all give rise to spectral features in the ENDOR spectra (49).

$X$ in Y122F—The spectra of the samples labeled with $^{57}$Fe exhibit large couplings from $^{57}$Fe ($^{57}$Fe = 0.45 MHz at 330 mT). The ENDOR spectrum of the labeled intermediate $X$ ($^{57}$Fe$^{111}$ $^{57}$Fe$^{111}$) in R2-Y122F shows two groups of $^{57}$Fe-ENDOR lines, one group at ~35 MHz, and a second group at frequencies below 20 MHz partly overlapping with the proton ENDOR lines (Fig. 6B). The larger $^{57}$Fe tensor obtained from the group of lines at 35 MHz exhibits only small anisotropy, indicative of high spin Fe$^{111}$; whereas the group of lines below 20 MHz shows a larger $^{57}$Fe hyperfine anisotropy, indicative of Fe$^{111}$ in high spin Fe$^{111}$, for which the ENDOR assignments are consistent with the earlier reported Q-band ENDOR data (16). The $^{57}$Fe hf tensor components obtained from simulations (Fig. 6B, dotted trace) are given in Table III and are consistent with the $^{57}$Fe-splitting seen in the EPR spectra, Fig. 4B.

$H$ in Y122F—The appearance of two new groups of ENDOR lines around 25 and 35 MHz in the ENDOR spectra of R2-Y122F labeled with $^{57}$Fe (Fig. 6D) compared with the non-labeled protein (Fig. 6C) clearly shows that center $H$ is also a diiron center. The $^{57}$Fe hf tensor values obtained from ENDOR simulations (dotted line in Fig. 6D) are presented in Table III. As can be seen from comparing Figs. 6B and 6D, the large $^{57}$Fe tensor of center $H$ is comparable to the large $^{57}$Fe tensor of Fe$^{111}$ in intermediate $X$ in Y122F. However, the small $^{57}$Fe tensor of center $H$ is far more isotropic, is significantly larger than that of Fe$^{111}$ in intermediate $X$ and is also different from $^{57}$Fe tensors found for Fe$^{111}$ in Fe$^{111}$Fe$^{111}$ centers (48), indicating that the small $^{57}$Fe tensor of center $H$ is neither Fe$^{111}$ nor Fe$^{111}$ (16, 24). The similarly small anisotropy of the two $^{57}$Fe tensors of center $H$ suggests that both tensors derive from Fe$^{111}$, and we therefore describe center $H$ as an antiferromagnetically coupled Fe$^{111}$Fe$^{111}$ center. However, this diferric center needs to be strongly coupled to a radical, $R^\bullet$, in order to obtain a total spin of $S = \frac{1}{2}$. Interestingly, such a model was originally suggested for intermediate $X$ (14, 50), but later revised (16).

$^{14}$N and $^{15}$N ENDOR—For nuclei with a nuclear spin $I > \frac{1}{2}$, such as $^{14}$N with $I = 1$, the observed ENDOR resonance frequencies ($\nu_{\text{ENDOR}}$, see Equation 1) are also influenced by the anisotropic quadrupolar splitting, which may result in an additional splitting, according to the first-order expression (51) in Equation 2,

$$\nu_{\text{ENDOR}} = |\nu_s \pm A/2 \pm 3P/2|$$  \hspace{1cm} (Eq. 2)

$P_j$ ($j = x, y, z$) is an element of the traceless quadrupole coupling tensor $P$, with $P = q^2Q/2h$, where $Q$ is the nuclear quadrupole moment, $q$ the electric field gradient tensor at the nucleus, $e$ the elementary charge, and $h$ is the Planck constant. The traceless tensor is usually described by the value $q^2Q/4h$, and the asymmetry parameter $\eta = (P_{x} - P_{y})/P_{z}$.

In the CW ENDOR spectra of R2-Y122F containing interme-
A New Iron-Phenoxyl Radical Center in R2-Y122H

### Table III

| 15N-hf tensor components and 14N-quadrupole parameter $e^qQ/h$ and $\eta$ of H in R2-Y122H and 57Fe-hf tensor components of X in R2-Y122H |
|-----------------|-----------------|-----------------|-----------------|
|                  | Fe(III)*        | Fe(IV)*         |                  |
| $A_1$ MHz        | 74.7 ± 0.1      | 73.4 ± 0.1      |                  |
| $A_2$ MHz        | 71.0 ± 0.1      | 38.0 ± 0.1      |                  |
| $A_3$ MHz        | 73.65 ± 0.1     | 36.0 ± 1        |                  |
|                  |                  | 33.8*           |                  |
|                  | 57Fe H122H       |                  |                  |
| $A_1$ MHz        | 66.6 ± 0.2      | 45.1 ± 0.2      | 48.3*           |
| $A_2$ MHz        | 69.7 ± 0.2      | 47.6 ± 0.2      |                  |
| $A_3$ MHz        | 72.5 ± 0.2      | 52.1 ± 0.2      |                  |
|                  |                  |                  |                  |
| $N_1^a$ MHz      | 15.4 ± 0.5      | 9.2 ± 0.4       | 9.9*            |
| $N_2^a$ MHz      | 16.5 ± 0.5      | 10.6 ± 0.4      |                  |
|                  | 18.7 ± 0.6      |                  |                  |
| $e^qQ/h$         | 1.6 ± 0.1       | 1.3 ± 0.1       |                  |
| $\eta$           | 0.9 ± 0.1       | 0.9 ± 0.1       |                  |

* This work was from simulations of the ENDOR spectra, Figs. 6 and 7.

* Fe(IV) values from an earlier Q-band ENDOR study (16): Fe(IV) A$_1$ = 27.5, A$_2$ = 36.8, A$_3$ = 36.8 MHz, Fe(III): A$_1$ = -74.2, A$_2$ = -72.2, A$_3$ = -73.76 MHz. We introduced a small rhombicity in the A$_3$ values from Fe(IV) for better agreement between simulated and experimental Fe(IV) ENDOR spectra (Fig. 6). Assignment of A$_{1,2,3}$ to g-values, g$_{1,2,3}$ (Table II), and to Fe(III) and Fe(IV) in analogy to Ref. 16.

* Isotropic value (A$_1$ + A$_2$ + A$_3$) and assignment to Fe1 and Fe2 could be exchanged. However, the larger 14N hf-tensor is assigned to the histidine coordinated to the iron with the larger hf-tensor.

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To unravel the hyperfine and quadrupolar interactions of center X (Fig. 6A), ENDOR lines from only one 14N nucleus are observed. Previous X-band (52) and Q-band (17) ENDOR studies of intermediate X have assigned these lines to the N$_2$ of the histidine ligand of the Fe(III). From the crystal structure of R$_2$, each of the two iron is known to be coordinated by a separate histidine residue (6) (see Fig. 1), however, the hyperfine interactions from the histidine ligated to Fe(III) were believed to be too small to be observed by ENDOR spectroscopy (16, 17, 52).

In the ENDOR spectrum of R2-Y122H (Fig. 6C), the 14N ENDOR line pattern is clearly different from those in intermediate X (Fig. 6A). In fact, four resonance positions can easily be observed, which were unambiguously identified as 14N lines by recording spectra at different microwave frequencies (9.55 and 9.15 GHz) and correspondingly different magnetic fields (data not shown). Due to the larger nuclear g-value of protons ($g_{1H} = 2.0023$) and hence larger nuclear Larmor frequency ($\omega_{1H} = 9.15$ GHz) and correspondingly different magnetic fields ($B_{1H} = 330$ mT) and the hyperfine interaction with the electron, and therefore, the 15N-ENDOR lines are shifted with respect to the respective 14N ENDOR lines. The $v_\text{ENDOR}$ transition (Equation 2) of N$_1$ is shifted from about 10 MHz for 14N to 14 MHz for 15N, and therefore buried under the proton matrix ENDOR region. However, the $v_\text{ENDOR}$ transition (Equation 2) of N$_1$, which is symmetric to $v_\text{ENDOR}$ is observed at 9–10 MHz (Fig. 7B). Both ENDOR lines, $v_\text{ENDOR}$ and $v_\text{ENDOR}$ are observed for the smaller 15N hyperfine tensor. The best simulation of the 14N ENDOR spectrum (Fig. 6B) is obtained using the 15N hyperfine tensor values of two nitrogens, N$_1$ and N$_2$, scaled by the smaller nuclear g-value of 14N and introducing for both 15N nuclei weak quadrupolar couplings. The deduced 14N hf tensor components and quadrupole parameters are presented in Table III.

Because of the weak intensities of the lines below 7 MHz in CW ENDOR spectra, we also recorded pulsed Davies 14N-ENDOR spectra (34, 53), see Fig. 7A. This spectrum confirms all four 14N-ENDOR lines. The dashed line represents a simulation of the nitrogen ENDOR lines in the pulsed ENDOR spectrum of center H using the parameters in Table III. It is important to note that the small anisotropy of the two 57Fe hf tensors and the detection of a second 14N hf tensor, which is missing in the Fe(III)Fe(IV) center X, both indicate that center H is made up of two Fe(III) ions.

### Experiments to Identify the Ligand Radical Site of Center H: Selective Isotope Labeling of Iron Site Ligands of R2-Y122H—Candidates for the location of a ligand radical in center H are the amino acid residues or small molecule ligands in close vicinity to the iron site, including histidine, aspartate, and glutamate residues. In a first attempt, we prepared R2-Y122H proteins labeled with [13C]aspartate and [13C]glutamate by adding labeled amino acids to the minimal medium used for overexpressing the E. coli cells. Specific 13C-labeling of histidine residues is more difficult in a minimal medium, since histidine will also be used for purine biosynthesis (54). The incorporation of labeled amino acids was checked by MALDI mass spectrometry determining the difference between theoretical and measured mass of the whole molecule (for data, see “Experimental Procedures”). None of these samples displayed any change in the EPR or ENDOR spectra of center H, expected from electron spin density at the magnetic isotope 13C (data not shown). This excludes aspartates and glutamates as the origin of the radical site.

We then replaced the phenylalanine residues in R2-Y122H...
with fully deuterated phenylalanine D₈-Phe, since hydroxylation of Phe²⁰⁸ by high valence diiron-oxygen intermediates has been observed in other mutants (44, 55). The incorporation of D₈-Phe was verified by MALDI-MS. In the pulse ENDOR spectrum from samples with deuterated Phe (Fig. 8B), two new features appeared compared with the non-labeled sample (Fig. 8A, see also expanded inset). These new features are centered around the nuclear frequency of deuterium, τ₀ = 2.16 MHz at 330 mT according to the resonance condition (Equation 1). The ENDOR difference spectrum (Fig. 7C, Phe H₈ - Phe D₈) reveals corresponding proton couplings that are missing in the spectrum from samples with deuterated Phe (Fig. 8B). The deuterium lines are split by 0.54 MHz, which corresponds to a splitting of 3.5 MHz for the respective proton ENDOR lines (Fig. 8C). The ratio of 6.5 between these values represents the difference in the magnetic moments of protons and deuterons. The deuterium quadrupole coupling is usually very small (53). Spectra were recorded at 10 K: radiofrequency pulse, 8 milliwatt microwave power, and 100 watt RF power; total accumulation time 40 min (¹⁴N), 9 h (¹⁵N).

FIG. 7. ¹⁴N/¹⁵N-ENDOR of center H. A, Davies Pulse ENDOR of 2 mM ¹⁴N-R2-Y122H. Experimental conditions: temperature: 10 K, center field 345.9 mT, microwave π-pulse width: 192 ns, τ = 488 ns, radiofrequency π-pulse width τ₀ = 8 µs, shot repetition time: 20.4 ms, accumulation time: 20 h. B, CW ENDOR spectra of 2 mM ¹⁴N-R2-Y122H and 1.3 mM ¹⁵N-R2-Y122H, respectively, with simulations (dotted lines) using the parameters given in Table III. Experimental conditions: T = 10 K, 8 milliwatt microwave power, and 100 watt RF power; total accumulation time 40 min (¹⁴N), 9 h (¹⁵N).

FIG. 8. X-band pulse ENDOR spectra of A: 2.4 mM non-labeled R2-Y122H (347 sweeps) and B: 2.5 mM R2-Y122H labeled with deuterated phenylalanine, Phe-D₈ (393 sweeps). Inset, expanded scale of the low frequency range of spectra A and B, as indicated by dashed lines, amplitude magnified 14 times, showing two resolved deuterium ENDOR lines for trace B, see text. C, difference spectrum (A - B) showing exclusively hf interactions of center H belonging to protons of phenylalanine. Simulation (dotted line) using the hf parameters listed in Table IV, which are derived from a tyrosyl radical using the proper spin projection factor (29, see text). The ENDOR lines flanking the proton Larmor frequency of the protons (14.8 MHz), which correspond to the small hf couplings, appear with larger intensity in the simulation than in the experiment because of the fact that Davies ENDOR intensities are inherently weak when the hf couplings are very small (53). Spectra were recorded at 10 K: radiofrequency pulse, 8 µs; microwave frequency, 9.7 GHz; magnetic field, 345.8 mT; microwave π-pulse length, 296 ns.

DISCUSSION
The Nature of Center H

EPR Spectra of Center H and Intermediate X—To characterize the novel center H, observed in R2-Y122H, we compared its spectroscopic parameters with those of the short-lived intermediate X, that has been observed both in wild-type R2 and in the mutant R2-Y122F (16, 56). Q-band ENDOR experiments of ¹⁷O- and ⁵⁷Fe-enriched R2 protein (16, 56) have shown that intermediate X is a spin-coupled Fe³⁺O-Fel²⁺ center with significant spin density on both iron ions and on oxygen ligands. The unusually fast spin relaxation for center H, deduced from the microwave saturation behavior and temperature dependence of the X-band EPR spectra (see “EPR Spectra of the Paramagnetic Center in R2-Y122H” in the “Results” section), as well as the small g₂ component obtained from high-field EPR at W-band (see Table II), provided strong evidence for center H being a metal-centered spin system, similar but not identical to intermediate X.

⁵⁷Fe-ENDOR of Center H and Intermediate X—The CW ENDOR data of the R2-Y122H protein labeled with ⁵⁷Fe revealed two large ⁵⁷Fe hf tensors, clearly showing the involvement of both iron ions. In contrast to intermediate X where the ⁵⁷Fe hf tensor of Fe⁴⁺ has large anisotropy, both ⁵⁷Fe hf tensors in center H exhibit relatively small anisotropy, typical for Fe⁴⁺, indicating an Feº⁴⁺Feº³⁺ center. In order to obtain an S = ½ ground state for center H, a third spin needs to be coupled to the diiron center, resulting in a three spin system of S₁(R₁) = ½, S₂(Fº²⁺) = ½, and a coupled radical S₂(R) = ½, which may be located on a ligand. The radical spin can couple to one Feº³⁺, either ferromagnetically, leading to an intermediate spin S₂ = 3, or antiferromagnetically, leading to intermediate spin S₂ = 2. In either case the respective intermediate spin would then couple antiferromagnetically to the remaining Feº³⁺ (S₁ = ½) to give a total S₁₂₃ = ½. Spin exchange interaction (57),
leads to different “spin projection factors” $c$ for the different spins within the three spin system. These spin projection factors are $c_1 = -5/3$, $c_2 = 20/9$, $c_3 = 4/9$ for ferromagnetic coupling, when $S_{z3} = 3$, and $c_1 = 7/3$, $c_2 = -14/9$, $c_3 = 29/9$ for antiferromagnetic coupling, when $S_{z3} = 2$ (24, 50). These factors scale all nuclear hyperfine couplings from the respective iron ions ($^{57}$Fe$^{III}$, and ligand $^{14}$N$-\text{ENDOR}$-hyperfine couplings) and those from the radical, compared with the respective uncoupled species. In particular, for the antiferromagnetic case, a rather small value, $c_2 = 29/9$, and hence small hyperfine couplings, are expected for the radical. When assuming the same intrinsic hyperfine couplings for both $^{57}$Fe$^{III}$ nuclei Fe1 and Fe2, the ratio of the isotropic parts of their $^{57}$Fe hf tensors should reflect their different spin projection factors. This hyperfine ratio (69.6 MHz/48.3 MHz, see Table III) is 1.44, which is closer to the expected ratio of the spin projection factors of 1.50 for the case of antiferromagnetic coupling compared with 1.33 for the case of ferromagnetic coupling.

$^{14}$N ENDOR of Center H—In view of the structure of the diiron site in R2-Y122H (Fig. 1), $^{14}$N-ENDOR lines may be expected from $N_s$ and $N_r$ of the histidines ligating the two iron ions, and perhaps also from nitrogenous of the protein backbone (58–63). For the case of a backbone N-H ligating a quinone, quadrupole couplings ($e^2qQ/h$) of 3.2 MHz, $\eta = 0.52$ ($Q_{\alpha}$ in *Rhodopseudomonas (R.) viridis*) and 3.05 MHz, $\eta = 0.54$ ($Q_{\beta}$ in *R. sphaeroides*) have been reported (58). These $e^2qQ/h$ values are much larger than the quadrupole couplings for the ligating nitrogens in center H (see Table III). A similarly high value of $e^2qQ/h = 3.96$ MHz for histidine N$_r$ is reported in reference (59) and similar values were reported for heme Fe$^{III}$ ligating imidazole $e^2qQ/h = 2.3 - 3.2$, $\eta = 0.1 - 0.3$ (60, 61). The magnitudes of the observed nitrogen couplings in center H are, however, in better agreement with those observed for histidine coordinated in N$_r$ position to heme Fe$^{III}$, and also histidine N$_r$ ligating a quinone (60, 62) which lie in a range of $e^2qQ/h = 1.44 - 1.65$ and $\eta = 0.69 - 0.91$. Values of $e^2qQ/h$ of about 1.4 MHz and $\eta = 0.9$ have been reported for the remote N$_r$ of histidine coordinated to copper in azurin (63). However, the $e^2qQ/h$ values for $^{14}$N of center H in R2-Y122H (1.6 and 1.3 MHz) are in remarkably good agreement with those reported for the histidine ligands in the structurally very similar diiron complexes of MMOH and semimethemerythrin sulfide (1.4 MHz), for which also comparable $^{14}$N hf tensors were observed (64). This indicates that the resonances in center H indeed derive from two $N_s$ of two histidines. It can also be excluded that the couplings arise from the non-coordinating remote nitrogen of the histidines, since these are known to exhibit only small hyperfine coupling of about 2 MHz (63, 65). The $^{14}$N hf tensors observed for center H have isotropic parts of 16.9 and 9.9 MHz, and a low hyperfine anisotropy, which is typical for the $^{14}$N nucleus of histidines directly coordinated to a metal. We therefore assign the nitrogen resonances to two coordinating $N_s$ of histidines His118 and His121. The position of the mutation, His122, is too remote, for the large and rather isotropic nitrogen hyperfine couplings observed in ENDOR.

The ratio of the isotropic part of the $^{14}$N hyperfine tensors (16.9 MHz/9.9 MHz) is 1.7, which is even larger than expected for the case of antiferromagnetic radical-Fe1 coupling (1.5) and is not in agreement with the ratio of 1.33 expected for ferromagnetic coupling (see above). For intermediate X (Fe$^{III}$ Fe$^{IV}$), where spin projection factors of 7/3 and $-4/3$ with a ratio of 1.75 are expected, only the histidine $N_s$ ligated to Fe$^{III}$, and not the one ligated to Fe$^{IV}$, could be observed in the ENDOR spectra (see Fig. 6, A and B, and Refs. 16, 17, and 50).

**MALDI-TOF Experiments, Hydroxylation of Phe*208*—Evidence for the nature of the ligand radical, R*, came from pulsed ENDOR data of R2-Y122H labeled with D$_{2}$-Phe, which clearly demonstrates significant spin density on a phenylalanine residue, as expected for a radical on this residue. From the x-ray structure of R2-Y122H, it is clear that Phe*208* is the only phenylalanine residue that could become a direct ligand of the diiron center. In view of the remarkable stability of center H, a phenyl radical at Phe*208* seems however highly unrealistic, since this type of radical is known to be highly reactive and unstable. Interestingly, for another mutant of R2 (Y122F/E238A), hydroxylation of Phe*208* has been observed (44). Therefore, we assume a similar hydroxylation reaction for R2-Y122H, which leads in ~5% of the protein to a further oxidation to a phenoxyl radical at residue 208. In order to prove this assumption, we have trypsin-digested the R2-Y122H protein and investigated the obtained fragments by MALDI-TOF mass spectrometry. The trypsin fragment with Phe*208* (208-FYVVSFACSFAFAER) has a theoretical mass of 1645 daltons. This value will be increased by 57 due to the acetamide group at the cysteine (R-S-CH2-CNH2, see “Experimental Procedures”) to 1702 daltons. Hydroxylation of Phe*208* in a fraction of the protein will increase the mass of this fragment by 16 to a value of 1718 daltons. In the recorded MALDI-TOF spectra of R2-Y122H fragments we found indeed a peak at 1702 daltons, and a weaker peak at 1718, which had only about 15% of the intensity. The corresponding spectra from R2 wild-type protein showed only the peak at 1702 dalton (Fig. 9). This strongly supports our assumption of hydroxylation of Phe*208* in a fraction of the R2-Y122H protein. The different yield of hydroxylation (~15%) found by MALDI-TOF compared with the yield for center H (~5%) from EPR might result from different sensitivity in the MALDI-TOF spectra of the corresponding two protein fragments, or it might indicate that not all of the hydroxylated Phe*208* becomes a radical.

**H/D ENDOR of Center H—** Individual proton hf tensor values could not be directly obtained from the proton ENDOR difference spectrum (Fig. 8C), due to overlapping of signals from several protons, and simulations of the spectra were required, based on hyperfine interactions found in similar systems. On the above assumption of a phenoxyl radical F208-O’, we used the proton hyperfine couplings from Y122’ (66) and scaled these down by the spin projection factor, $c_2 = 29/9$, which we found for the ligand radical from the $^{57}$Fe ENDOR data for an antiferromagnetic Fe$^{III}$-radical coupling. The fact that none of the observed proton couplings in Fig. 8 are larger than ~6 MHz excludes the alternative value, $c_2 = 4/9$, for the ferromagnetic coupling. This is in line with the observed ratios of $^{57}$Fe hyperfine couplings (Fe/
the crystal structure of R2-Y122H are 85° and 

and coordination to Fe2 rather than Fe1 have dihedral angles of the side chain β protons of residue 208 outside the range estimated from the ENDOR experiments (Fig. 8A), excluding these structures as models for center H: in subunit A of R2-Y122F/E238A (44) the angles are 105° and −15° and in both subunits of R2-D84E/W48F the angles are 132° and 12°.

The simulation of the ENDOR spectrum in Fig. 8C based on a para-phenoxyl radical fits reasonably well with the experiment. For a meta-phenoxyl radical large spin densities are expected for C4, C5, and C6. In this case, there would be only a very small hf coupling from the β-methylene protons of the side chain due to the low spin density on C4. However, there would be an additional large anisotropic hf tensor expected from the α proton on C6 with estimated values of −9 MHz, −7 MHz, and −2.6 MHz. In the ENDOR difference spectrum (Fig. 8) we found no evidence for such a large hyperfine splitting of −9 MHz. However, the largest hf tensor value of such an α proton would appear in the ENDOR spectrum as a broad and weak wing of an anisotropic pattern, and we cannot rigorously rule out that such a large anisotropic component might be broadened beyond detection. Nevertheless, both the ENDOR spectra and the overlaid structures of R2-Y122H and R2-F208Y (Fig. 1) suggest oxidation of Phe208 at the para position. A model of this center is presented in Fig. 11.

Remarkably, it is almost impossible to remove the iron ions of center H with chelating agents, in contrast to the majority of the R2-Y122H protein, which does not contain center H and for which the diiron center can be reversibly removed (Fig. 3). The x-ray structure (Fig. 1) of the diferric form, which represents the majority species, shows two terminal water molecules for Fe1 and one for Fe2. We propose that in those protein molecules that form center H there is no second terminal ligand at Fe1. Instead, Phe208 is oxygenated and oxidized to a phenoxyl radical, which then occupies the place of the second water ligand to Fe1. The additional coordination to Fe208-O2 might explain the unusually strong iron binding of center H. Relatively stable phenoxyl ligand radicals have been reported for FeIII model complexes (68). The terminal water ligands of center H are expected to give rise to large anisotropic hyperfine couplings from their protons, which should be exchangeable against deuterium. In an extensive study by Willems et al. (69) on intermediate X in R2 of E. coli, it was shown that from a terminal water ligand on FeIII a dipolar proton hyperfine tensor with approximate components of −10 MHz, −10 MHz, and +20 MHz is expected. Thus, similar large dipolar hf tensors are expected from the protons of the two terminal water molecules in center H. However, such a large anisotropic hf tensor leads to a very broad, weak pattern in the ENDOR spectra, extending over a range of 15 MHz, with signal amplitudes lowered by at least a factor of 10 compared with the other proton ENDOR lines in Fig. 8. This, together with the low yield of center H in R2-Y122H (typically 3–5%) explains why we were not able to detect these proton hyperfine couplings in our ENDOR spectra. However, simulations of the EPR spectrum of H (Fig. 4C) show, that such large additional hyperfine couplings are required to explain the observed EPR linewidth of center H (2.2 mT). Upon H2O/D2O exchange (data not shown) a narrowing of the EPR spectrum was observed. Hence, the observed width of the EPR

Fe1. The dihedral angles of the side chain δ protons of residue Tyr208 in the structure of R2-F208Y, where residue Tyr208 is further oxygenated to DOPA and coordinated to Fe1 (48° and −72° for subunit A, and 42° and −78° for subunit B), agree better with the range of values estimated from the experiments. We thus propose a very similar orientation for the oxidized coordinated phenoxyl radical of center H in R2-Y122H, except for the second oxygen at the meta position of residue 208 in R2-F208Y, which is absent in center H. Furthermore, the oxygen at C4 (para position) of Tyr208 in R2-F208Y is very close to the extra water Wat3 in R2-Y122H (Fig. 1). This suggests that Phe208 is hydroxylated at the para position in H, as also suggested by the spectroscopic results (see below). Two other mutants showing hydroxylation of Phe208 at the meta position and coordination to Fe2 rather than Fe1 have dihedral angles of the side chain β protons of residue 208 outside the range estimated from the ENDOR experiments (Fig. 8A), excluding these structures as models for center H: in subunit A of R2-Y122F/E238A (44) the angles are 105° and −15° and in both subunits of R2-D84E/W48F the angles are 132° and 12°.

The simulation of the ENDOR spectrum in Fig. 8C based on a para-phenoxyl radical fits reasonably well with the experiment. For a meta-phenoxyl radical large spin densities are expected for C4, C5, and C6. In this case, there would be only a very small hf coupling from the β-methylene protons of the side chain due to the low spin density on C4. However, there would be an additional large anisotropic hf tensor expected from the α proton on C6 with estimated values of −9 MHz, −7 MHz, and −2.6 MHz. In the ENDOR difference spectrum (Fig. 8) we found no evidence for such a large hyperfine splitting of −9 MHz. However, the largest hf tensor value of such an α proton would appear in the ENDOR spectrum as a broad and weak wing of an anisotropic pattern, and we cannot rigorously rule out that such a large anisotropic component might be broadened beyond detection. Nevertheless, both the ENDOR spectra and the overlaid structures of R2-Y122H and R2-F208Y (Fig. 1) suggest oxidation of Phe208 at the para position. A model of this center is presented in Fig. 11.

Remarkably, it is almost impossible to remove the iron ions of center H with chelating agents, in contrast to the majority of the R2-Y122H protein, which does not contain center H and for which the diiron center can be reversibly removed (Fig. 3). The x-ray structure (Fig. 1) of the diferric form, which represents the majority species, shows two terminal water molecules for Fe1 and one for Fe2. We propose that in those protein molecules that form center H there is no second terminal ligand at Fe1. Instead, Phe208 is oxygenated and oxidized to a phenoxyl radical, which then occupies the place of the second water ligand to Fe1. The additional coordination to Fe208-O2 might explain the unusually strong iron binding of center H. Relatively stable phenoxyl ligand radicals have been reported for FeIII model complexes (68). The terminal water ligands of center H are expected to give rise to large anisotropic hyperfine couplings from their protons, which should be exchangeable against deuterium. In an extensive study by Willems et al. (69) on intermediate X in R2 of E. coli, it was shown that from a terminal water ligand on FeIII a dipolar proton hyperfine tensor with approximate components of −10 MHz, −10 MHz, and +20 MHz is expected. Thus, similar large dipolar hf tensors are expected from the protons of the two terminal water molecules in center H. However, such a large anisotropic hf tensor leads to a very broad, weak pattern in the ENDOR spectra, extending over a range of 15 MHz, with signal amplitudes lowered by at least a factor of 10 compared with the other proton ENDOR lines in Fig. 8. This, together with the low yield of center H in R2-Y122H (typically 3–5%) explains why we were not able to detect these proton hyperfine couplings in our ENDOR spectra. However, simulations of the EPR spectrum of H (Fig. 4C) show, that such large additional hyperfine couplings are required to explain the observed EPR linewidth of center H (2.2 mT). Upon H2O/D2O exchange (data not shown) a narrowing of the EPR spectrum was observed. Hence, the observed width of the EPR
spectrum of center \(H\) is consistent with the presence of large anisotropic proton hyperfine couplings from terminal water ligands, not detected in the ENDOR spectra.

Hydroxylation of Phe\(^{208}\) in Other R2 Mutants—Hydroxylation of Phe\(^{208}\) has also been observed in several other R2 mutants. In the double mutant R2-Y122F/E238A residue Phe\(^{208}\) is hydroxylated at the meta position and becomes a phenolate ligand to Fe2, replacing the normal ligand E238 in wild-type R2 (44). A recent density functional study comparing the centers in R2 and MMOH with focus on the shifting carboxylate E238 (E234 in MMOH) concluded that the carboxylate shifts have very low energy barriers in both cases and may be essential for the oxygen activation (70). In mutant R2-F208Y, residue Tyr\(^{208}\) is converted to a DOPA (27), where, the para-OH group of DOPA is ligated to both iron Fe1 and Fe2, and the meta-OH group is only ligated to Fe1 (44). Interestingly, in R2-F208Y a stable paramagnetic species called center \(Z\), with an EPR spectrum similar to that of center \(H\), has been generated in an alternative route to the formation of DOPA when high concentrations of ascorbate were present during the reconstitution reaction (71). A \(^{1}H\)-\(^{2}H\)-d\(^{2}\)-Tyr labeled preparation of this protein showed a narrowing of the X-band EPR signal of center \(Z\) (71, 72). This indicates that centers \(Z\) in R2-F208Y and \(H\) in R2-Y122H may be very similar, both most probably involving a tyrosyl radical on residue 208, which, however, has to be formed by hydroxylation of Phe\(^{208}\) in the case of R2-Y122H.

Hydroxylation of Phe\(^{208}\) at the meta position has also been observed in R2 mutant D84E/W48F (55), where the D84E mutation was introduced to correct the only difference of the iron ligands between R2 and the structurally related protein MMOH, and the W48F mutation served to block the radical transfer chain, which is absent in MMOH. In these mutants hydroxylation was observed at the meta position of Phe\(^{208}\). Our data indicate hydroxylation of the para position of Phe\(^{208}\), which corresponds to the tyrosyl radical (66)). For the para-phenoxyl radical, large hf tensor values of approximately \(-9\) MHz, \(-7\) MHz, and \(-2.6\) MHz (see text) are obtained for the ring proton at position 6, when assuming a large spin density of 0.38 at 6 (same as for position 1 in the para-phenoxyl radical), and using a spin projection factor of 2/9 and proper scaling of the hyperfine tensor values for the \(\alpha\) protons at positions 3 and 5 (spin density 0.25) from the tyrosyl radical Tyr\(^{177}\) (43). The \(^{1}H\) and D-ENDOR data are in better agreement with oxidation of Phe\(^{208}\) at the para position (left, see text).
Y122H. This is most probably due to the significantly higher redox potential of histidine (1.4 V) in comparison with tryptophan (0.9 V) (73). However, using strongly oxidizing OH$^\cdot$ radicals, generated in a Fenton reaction, transient histidine OH$^\cdot$ adduct cation (74) and neutral radicals (75) have been generated and investigated in a liquid aqueous solution of histidine. Recently, histidine radicals were also observed in a reaction of superoxide dismutase with hydroperoxide (23). In R2 mutant Y122H, either the oxidation power of the intermediate X ($Fe^{III}Fe^{IV}$) is not sufficient for generating a histidine radical, or the hydroxylation of Phe$^{208}$ and subsequent formation of a coordinated phenoxyl radical as a rather stable product, is energetically more favorable.

In mutant R2-Y122F of *E. coli*, where the active site residue Phe$^{122}$ is also very difficult to oxidize, transient tryptophan radicals were observed at residues Trp$^{107}$ and Trp$^{111}$ (19–21), and not hydroxylation of Phe$^{208}$ as in case of R2-Y122H. This difference in the reaction pathways may rely on structural differences in the iron coordination. Indeed, the histidine His$^{122}$ in R2-Y122H forms a hydrogen bond to aspartate Asp$^{84}$ making Asp$^{84}$ a monodentate iron ligand, whereas in R2-Y122F, Asp$^{84}$ is bidentately ligated to the iron (44). Interestingly, the corresponding aspartate in the met-R2 form of mouse RNR is monodentate (76), similar to the reduced diferrous form of *E. coli* RNR, indicating that this residue is rather flexible. In MMOH from *Methylosinus trichosporium* OB3b the complementary Glu$^{114}$ forms a monodentate bond to Fe1 (3). This residue is less flexible due to the extended length of the side chain, which probably ensures that the substrate oxidation reaction in MMOH always happens directly at the iron site, and not at a more distantly located amino acid residue, as in R2. Furthermore, in R2-Y122H, as well as in MMOH, there is an extra water ligand to Fe1 at the place that is normally occupied by one of the Asp$^{84}$ carboxyl oxygens. This structural difference, may also explain why the rhombic g tensor of the mixed valence species, $Fe^{III}Fe^{III}$, of the R2-Y122H protein shows greater similarities with the g tensor of the $Fe^{III}Fe^{II}$ center in MMOH than with the g tensor of $Fe^{II}Fe^{III}$ in wild-type R2 (Table II). It could be further speculated that in the diferrous state R2-Y122H has also one terminal water ligand at Fe1 as observed for MMOH (77).

In MMOH, the hydroxylation of the substrate is performed by the $Fe^{IV}Fe^{IV}$ intermediate Q (5, 78), which has so far not been observed in R2. In the wild-type R2, intermediate X performs the one-electron oxidation of Y122 (15). This intermediate was originally described as a $Fe^{III}Fe^{III}$ species with a strongly coupled hydroxyl radical ligand bound to Fe1 (50). Later, this model was changed to a $Fe^{II}Fe^{IV}$ species, based on ENDOR and Mössbauer data with a substantial portion of the spin delocalized to the oxygen ligands (16). However, the actual electronic structure of X could be a $Fe^{II}Fe^{IV}$ state with a hydroxyl ligand with some admixture of a $Fe^{III}Fe^{III}$-hydroxyl radical character. In the case of R2-Y122H this equilibrium might be shifted to the hydroxyl radical form, which could react with residue Phe$^{208}$ forming a stable phenoxyl radical.

Investigation of the mechanism of hydroxylation of Phe$^{208}$ and generation of the paramagnetic center H in R2-Y122H using the iron reconstitution reaction was not possible, since center H was already present in all preparations of R2-Y122H, and its yield could not be further increased by this reaction. However, it seems reasonable that it is generated in an alternative route of the oxygen activation reaction leading to oxidation of a hydrophobic amino acid residue in the close vicinity of the iron. Thereby different possible reaction pathways may be considered. Center H could be generated in a reaction with one molecule of oxygen. In order to explain the experimental data, a branching has to be assumed. The main branch leads for the majority to the diamagnetic $mu$-oxo-bridged $Fe^{III}Fe^{III}$ center and to formation of an additional terminal water ligand at Fe1, which is seen in the x-ray structure. This reaction requires two extra-electrons (in addition to those from the two irons) and two protons, to form the water molecule. A second, much less frequent branch leads also to formation of a $mu$-oxo-bridged $Fe^{III}Fe^{III}$ center, but to oxygenation and oxidation of Phe$^{208}$ to a phenoxyl radical, which is coordinated to Fe1. The formation of the differic $mu$-oxo bridged iron center requires two electrons from the iron, whereas for the oxygenation and generation of the phenoxyl radical one electron and one proton has to be removed from Phe$^{208}$ and replaced by the second oxygen from the split $O_2$ molecule.

Alternatively, two rounds of reactions with molecular oxygen could be involved in generation of center H. The first round leads for the main branch as above to the $mu$-oxo-bridged $Fe^{III}Fe^{III}$ center and an additional water ligand to Fe1. A second less frequent branch leads to formation of the $mu$-oxo-bridged $Fe^{IV}Fe^{III}$ center and to hydroxylation of Phe$^{208}$, as observed in other self-hydroxylating mutants. When in this subensemble of R2-Y122H protein the diiron center is thereafter reduced to the diferrous form, a second round of reaction with molecular oxygen could generate the $mu$-oxo-bridged differmic state and the radical on the previously hydroxylated residue Phe$^{208}$, which then coordinates to Fe1. This latter scheme is attractive. It could explain, why center H is not generated in the normal iron reconstitution reaction, since after hydroxylation of Phe$^{208}$ a reduction to the diferrous form is required before the second round can start. Such a mechanism would represent a very interesting combination of the function of MMOH, hydroxylation, in the first round with the function of RNR, radical generation, in the second round. However, in contrast to wild-type R2, in R2-Y122H the phenoxyl (or tyrosyl) radical is generated, not at residue 122, but at the hydroxylated residue Phe$^{208}$, where it is much more stable, and less reactive compared with the radical at Tyr$^{122}$, because of its coordination to Fe1.

From an enzymology point of view it is interesting to note that the mutant R2-Y122H does not exhibit any measurable enzymatic activity except that of remaining chromosomally encoded wild-type R2 in the sample. A complete loss of enzymatic activity was also found in mouse R2-Y177W even when a tryptophan free radical was formed (W177') (21). These observations support the unique role of the tyrosyl radical Y122' in protein R2 of *E. coli* RNR, and Y177' in mouse RNR, respectively, for the catalytic activity. Interestingly, in the intracellular bacterial parasite *Chlamydia trachomatis*, the RNR has been classified as class Ic since there is no tyrosyl residue at the expected position for the tyrosyl radical (79, 80); however, the RNR reaction is sensitive to hydroxyurea and an alternative radical mechanism involving the intermediate X has been suggested (80). The present combined EPR/ENDOR and x-ray diffraction study underlines the diversity of redox reactions possible in mutants of the diiron carboxylate protein R2 of *E. coli*, which is important for the understanding of the functional intermediate states in other native diiron carboxylate enzymes.

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A New Tyrosyl Radical on Phe208 as Ligand to the Diiron Center in Escherichia coli Ribonucleotide Reductase, Mutant R2-Y122H: COMBINED X-RAY DIFFRACTION AND EPR/ENDOR STUDIES

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