The iron-oxygen reconstitution reaction in protein R2-Tyr-177 mutants of mouse ribonucleotide reductase

EPR AND ELECTRON NUCLEAR DOUBLE RESONANCE STUDIES ON A NEW TRANSIENT TRYPTOPHAN RADICAL

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The ferrous iron/oxygen reconstitution reaction in protein R2 of mouse and Escherichia coli ribonucleotide reductase (RNR) leads to the formation of a stable protein-linked tyrosyl radical and a μ-oxo-bridged diferric iron center, both necessary for enzyme activity. We have studied the reconstitution reaction in three protein R2 mutants Y177W, Y177F, and Y177C of mouse RNR to investigate if other residues at the site of the radical forming Tyr-177 can harbor free radicals. In Y177W we observed for the first time the formation of a tryptophan radical in protein R2 of mouse RNR with a lifetime of several minutes at room temperature. We assign it to an oxidized neutral tryptophan radical on Trp-177, based on selective deuteration and EPR and electron nuclear double resonance spectroscopy in H2O and D2O solution. The reconstitution reaction at 22 °C in both Y177F and Y177C leads to the formation of a so-called intermediate X which has previously been assigned to an oxo (hydroxyl)-bridged Fe(III)/Fe(IV) cluster. Surprisingly, in both mutants that do not have successor radicals as Trp in Y177W, this cluster exists on a much longer time scale (several seconds) at room temperature than has been reported for X in E. coli Y122F or native mouse protein R2. All three mouse R2 mutants were enzymatically inactive, indicating that only a tyrosyl radical at position 177 has the capability to take part in the reduction of substrates.

The reduction of ribonucleotides to their corresponding deoxyribonucleotides is an essential reaction for all living cells and provides precursors for DNA synthesis. This reaction is catalyzed by the enzyme ribonucleotide reductase (RNR). A number of RNRs of different species have been discovered and classified into three major classes (1). A common feature of all RNRs within these three classes is that they use radical mechanisms to reduce ribonucleotides. RNR of class Ia, found in mammals, plants, DNA viruses, and some procaryotes like Escherichia coli, consists of two components, proteins R1 and R2 (2, 3).

The three-dimensional structures of proteins R2 of E. coli (4) and mouse (5) and of E. coli protein R1 (6) provide a basis for understanding the enzymatic reaction. A main step in ribonucleotide reduction, which takes place in the large subunit, protein R1, is the abstraction of the 3'-hydrogen from the ribose moiety of the substrate by a protein-linked radical (probably on Cys-439) (7, 8). Since Tyr-122 in the small subunit, protein R2 of E. coli (Tyr-177 in protein R2 of mouse RNR), is deeply buried inside the protein structure, it cannot take part directly in the substrate reduction in protein R1. A structure model of the enzyme (based on the known R1 and R2 structures) shows that Tyr-122 is connected to the substrate-binding site in protein R1 over a total length of about 35 Å via a network of conserved hydrogen-bonded amino acids, the so-called radical transfer pathway (2). It is believed that coupled electron/proton (H' radical) transfer takes place via this pathway during the enzymatic reaction (9). The enzyme loses activity when this network is disturbed, e.g. by mutations (10, 11).

The tyrosyl radical and its adjacent μ-oxo-bridged diferric iron center is formed in vitro in the so-called reconstitution reaction, when ferrous iron reacts with apoprotein R2 and molecular oxygen. This reaction, which results in the reduction of molecular oxygen to water, requires four reducing equivalents. Three are provided by the oxidation of the tyrosyl residue and the two ferrous iron. The fourth may come from an external ferrous iron ion via the radical transfer pathway. Schmidt et al. (12) showed recently that the radical transfer pathway in mouse protein R2 is involved in the generation of the tyrosyl radical/diferric iron site. In the E. coli protein R2 mutant Y122F, where the tyrosyl radical cannot be formed, other aromatic amino acids in the vicinity of the diferric iron center become oxidized, like tryptophans (13, 14) or a tyrosine (15). These radicals did not yield enzyme activity but show the existence of side pathways for the radical transfer in protein R2 when Tyr-122 is not available.

The unique high stability of the protein-linked tyrosyl free radical on Tyr-122 in protein R2 of E. coli RNR has been discussed since its discovery and spectroscopic assignment in 1972 (2, 16). As shown by site-directed mutagenesis, the tyrosyl radical might be protected by a cluster of hydrophobic amino acids, like in E. coli protein R2; changing only one hydrophobic amino acid for a hydrophilic one leads to a drop in the lifetime...
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EXPERIMENTAL PROCEDURES

Materials—Tryptophan (indole-2-carboxylic acid) and FeCl₃ (95.2%) were purchased from Merck, Darmstadt, Germany, and US Research Reagents, Inc., Detroit, MI, respectively. E. coli RNR, the radical carrying Tyr-177 (122 in mouse R2) was purified as described in the Methods section. Fe₂⁺ oxidation potentials, 0.94 and 1.05 V, respectively (18). We have used EPR and ENDOR to study the paramagnetic species formed in the Fe²⁺/oxygen reconstitution reaction of the mouse protein R2 mutants Y177W, Y177C, and Y177F. The results underline the uniqueness of the tyrosyl radical in the catalytic reaction of the native enzyme.

Expression and Purification of the Mutant R2 Proteins—The plasmid pETM2-Y177W (pETM2-Y177F or pETM2-Y177C) was transfected into E. coli strain BL21(DE3)pLysS that contains an IPTG-inducible chromosomal copy of the T7 RNA polymerase gene and a plasmid with the T7 lysozyme gene (20). LB medium (5 liters) containing carbenicillin 50 mg/ml; a plasmid with the T7 RNA polymerase gene and a plasmid with the T7 lysozyme gene (20). LB medium (5 liters) containing carbenicillin 50 mg/ml, was inoculated with the cells to an optical density at 600 nm of 0.5 and incubated at 37°C for 2 h. After a further 4 h at 37°C, the cultures were chilled, centrifuged at 2,500 × g for 20 min at 2°C, and gently resuspended in 100 ml of 50 mM Tris-HCl, pH 7.6. The suspensions were frozen in liquid nitrogen and stored at −70°C.

For preparation of protein R2 Y177W with indole-2-carboxylic acid and indole-2-carboxylic acid and indole-2-carboxylic acid, the liquid chromatographic mass spectrometry-coupled electrospray single quadrupole mass spectrometer (Applied Biosystems, Sciex, Perkin-Elmer Corp., Canada). For determining the mass by matrix-assisted laser desorption ionization-time-of-flight (TOF) mass spectrometry—The correct incorporation of isotope-labeled tryptophan into protein R2 was confirmed using a program for simulation and fitting of EPR spectra. Approximately 3 nmol of apoprotein R2 was dissolved in 1 ml of 50 mM Tris-HCl, 0.1 M KCl, pH 7.6, and gently resuspended in 10 ml of 50 mM Tris-HCl, 0.1 M KCl, pH 7.6. The reconstitution of the iron site in the mutant proteins R2 in H₂O or D₂O buffer at room temperature (22°C) was carried out as follows: apoprotein R2 in oxygen-saturated 50 mM Tris-HCl, 0.1 mM KCl, pH 7.5, was mixed with an equal volume of an anaerobic (N₂)ₕFeSO₄/₂H₂O solution (six times molar excess) dissolved in 50 mM Tris-HCl, 0.1 mM KCl, pH 7.5. A rapid freeze quench technique (System 1000 from Upstate Instruments) was used to obtain time points from 8 ms to 2 s. Both the aerobic apoprotein R2 and anaerobic Fe²⁺ solutions were rapidly mixed and sprayed into a cold isopentane bath (−120°C) to quench the reaction. The frozen spray was then tightly packed into an EPR tube (12). For reaction times >2 s, the anaerobic ferrous iron solution was mixed with the apoprotein R2 in an EPR tube using a gas-tight Hamilton syringe. The reaction was stopped by immersing the EPR tube into liquid nitrogen. The g values were determined by measuring the amount of free radical versus the reaction time of the sample warmed up to room temperature and refrozen again.

RESULTS

Mass Spectrometry—The correct incorporation of isotope-labeled tryptophan into protein R2 was confirmed using a program for simulation and fitting of EPR spectra. Approximately 3 nmol of apoprotein R2 was dissolved in 1 ml of 50 mM Tris-HCl, 0.1 M KCl, pH 7.6, and gently resuspended in 10 ml of 50 mM Tris-HCl, 0.1 M KCl, pH 7.6. The reconstitution of the iron site in the mutant proteins R2 in H₂O or D₂O buffer at room temperature (22°C) was carried out as follows: apoprotein R2 in oxygen-saturated 50 mM Tris-HCl, 0.1 mM KCl, pH 7.5, was mixed with an equal volume of an anaerobic (N₂)ₕFeSO₄/₂H₂O solution (six times molar excess) dissolved in 50 mM Tris-HCl, 0.1 mM KCl, pH 7.5. A rapid freeze quench technique (System 1000 from Upstate Instruments) was used to obtain time points from 8 ms to 2 s. Both the aerobic apoprotein R2 and anaerobic Fe²⁺ solutions were rapidly mixed and sprayed into a cold isopentane bath (−120°C) to quench the reaction. The frozen spray was then tightly packed into an EPR tube (12). For reaction times >2 s, the anaerobic ferrous iron solution was mixed with the apoprotein R2 in an EPR tube using a gas-tight Hamilton syringe. The reaction was stopped by immersing the EPR tube into liquid nitrogen. The g values were determined by measuring the amount of free radical versus the reaction time of the sample warmed up to room temperature and refrozen again.

The amount of iron bound protein in R2 was colorimetrically determined after removing unspecifically bound iron by running the protein R2 through a Sephadex G-25 column (23, 24). The apoproteins contained approximately 0.2 iron ion/protein R2 after purification. An acidic FeSO₄ stock solution was prepared as described in Sturgeon et al. (25). The reconstitution of the protein with FeCl₃ was done as described above except that the protein was dissolved in 200 mM HEPES, pH 7.5.

EPR and ENDOR Instrumentation—X band EPR spectra were recorded on a Bruker ESP 300E spectrometer using a standard rectangular Bruker EPR cavity (ER4102T) equipped with an Oxford helium flow cryostat (ER 4118-X-MD-5W2) equipped with an Oxford helium bath cryostat. Continuous wave (cw) ENDOR spectra were measured on a Bruker ESP 300E spectrometer using a self-built ENDOR accessory, which consists of a Rhode and Schwarz RF synthesizer (SMT201), an ENI A200L solid state RF amplifier, and a self-built high-Q TM110 ENDOR cavity (26). The cavity was adapted to an Oxford detection probes (ESR) probe which was measured, g value standard LiF/Li, with g = 2.00293 ± 0.000002 (27). Spin concentrations were determined by comparison of double integrals of EPR spectra with that of the tyrosyl radical in the mouse R2 wild type Tyr-177 which had been previously calibrated with a 1 mM Cu²⁺/EDTA standard. Evaluation of the microwave power saturation behavior of the radical was done by using the plot described in Ref. 28. The microwave saturation and the kinetic curves were fitted using the program Graft 3.01.

Mouse Protein R2 Mutants—All three mutated R2 proteins, Y177W, Y177C, and Y177F, behaved like the native protein R2.
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During the growth and purification procedure; the final yield was approximately 4–6 mg of pure protein R2/liter of bacterial culture. The light absorption spectra of all three mutants after 6Fe²⁺/R2 reconstitution are shown in Fig. 1. The absorbance of the mutants is significantly increased in the 300–400-nm region after the reconstitution reaction. However, the two bands at 320 and 370 nm, which are characteristic for a μ-oxo-bridged diferric iron center like in the active form of the native mouse protein R2 dimer (Fig. 1e), are not resolved in the mutant proteins. The Met form of native mouse protein R2 (19, 48) and other mouse R2 mutants (10) shows similar badly resolved light absorption spectra in the iron absorption region as seen for the mutants in Fig. 1, b–d. Upon reconstitution of Y177W, a broad transient light absorption band in the range 500–600 nm with a maximum at 550 nm and a half-width about 60 nm appeared initially (Fig. 1, inset). This band is completely gone after 2 min reaction time, which corresponds approximately with the decay of the transient EPR doublet signal in Y177W after 2 min reaction time, which corresponds approximately with the decay of the transient EPR doublet signal in Y177W after 2 min reaction time. Quantitation of this radical showed a maximum value of 0.34 unpaired spins/protein R2 dimer. The measured microwave power at half-saturation of the new radical at 32 °C was at least 1 order of magnitude lower than that of the tyrosyl radical at Tyr-177 in native protein R2 (P=0.030) at the same temperature, indicating a weaker magnetic interaction of the new radical with the diferric site in R2 Y177W.

Further characterization of the amount of bound iron in the protein R2 mutants by spectrophotometric analysis showed that all three mutants were able to bind approximately 3.5–4 iron ions/protein R2. In holoenzyme studies the interaction between native protein R1 and mutant protein R2 was analyzed by BLACore studies as described earlier (30). The binding efficiency of Y177W, Y177C, and Y177F to protein R1 was about the same as found for native protein R2 (data not shown). The enzyme activity of the mutated R2 proteins was measured by the reduction of [¹⁴C]CDP in the presence of an excess of pure recombinant mouse R1 protein and ATP as a positive effector (22). Neither of the mutated proteins showed any significant activity compared with native R2 protein (Table I). As demonstrated earlier and again clearly seen here for the native R2 protein, reactivation of apoR2 protein occurs continuously and with high efficiency during the assay in the presence of iron-dithiothreitol (31). Therefore, we should have been able to detect activity even from an R2 protein with a radical less stable than the normal tyrosyl radical.

**Table I**

| Protein R2 | Total protein | Formation of dCDP/30 min at 37 °C |
|------------|--------------|----------------------------------|
| Native     | 0            | 0.019                            |
| 0.5        | 3.7          |
| 1.0        | 8.0          |
| 2.0        | 9.35         |
| Y177W      | 0.5          | 0.034                            |
| 1.0        | 0.035        |
| 2.0        | 0.040        |
| Y177C      | 0            | 0.030                            |
| Y177W, R1 omitted | 2.0        | 0.020                            |
| Y177C      | 0            | 0.030                            |
| Y177F      | 0            | 0.030                            |
| 0.5        | 0.050        |

**Reconstitution of Protein R2 Y177W with 6Fe²⁺ and Oxygen**

**EPR Spectroscopy**—After addition of ferrous iron to aerobic apoY177W and a reaction time of 3 s at room temperature, an EPR spectrum was observed at 40 K with a giso value of 2.0029 (see Table II) and a dominating doublet hyperfine structure (hfs) (Fig. 2, top). The radical decays with a half-life time of 49 s at 22 °C (Fig. 3). Quantitation of this radical showed a maximum value of 0.07 mW than that of the tyrosyl radical at Tyr-177 in native protein R2 (P=0.2 mW) at the same temperature, indicating a weaker magnetic interaction of the new radical with the diferric site in R2 Y177W.

To identify the origin of the new radical in protein R2 Y177W we exchanged all tryptophans in the protein with selectively deuterated indole-δ-Trp. The incorporation of indole-δ-Trp into protein R2 Y177W was checked by mass spectroscopy (see “Experimental Procedures”). A comparison with the calculated molecular mass of Y177W R2 indole-δ-Trp shows that the isotopically labeled amino acid has been incorporated properly into the protein. A significant change of the hfs of the EPR spectrum was observed with the mutant R2 Y177W containing ring-deuterated tryptophans, indole-δ-Trp (Fig. 2, bottom). The small sub-splittings of each doublet component (Fig. 2, top) into three lines disappeared in the indole-δ-Trp labeled sample, proving that these lines resulted from a hyperfine interaction with the ring α-protons of Trp. Furthermore, well resolved lines became visible at the outer wings of the spectrum (Fig. 2, bottom). These lines are assigned to the A₁ component of an anisotropic hyperfine powder pattern from a ¹⁴N coupling. A similar line shape was observed also for tryptophan radical Trp-111 in mutant Y122F of E. coli R2 (14). The dominating doublet splitting of 2.25 mT (Fig. 2, top), which is not affected by indole-δ-Trp labeling is assigned to a large isotropic hfc of a β-proton. In the spectra simulations, the best fit of the spectrum in Fig. 2, bottom, was obtained by using a large isotropic proton hfc (A₁ = 2.25 mT) and an axially symmetric hyperfine tensor of ¹⁴N (A₂ = 0.07 mT, A₃ = 0.07 mT, and A₄ = 0.94 mT, see Table II). Due to the large A₁ tensor component of the ¹⁴N hyperfine tensor, we expect a significant spin density on the nitrogen of about 20%. An N-H proton...
Table II
 Experimental electronic g tensor (gi) and hyperfine tensor (Ai (mT)) principal components of the tryptophan radical Trp-177 in mouse R2 Y177W and Trp-111 in E. coli R2 Y122F

The tryptophan radical was obtained after a reaction time of 3 s. The reconstitution reaction was performed with 6 Fe²⁺/protein R2 at 22 °C. g and hyperfine tensor principal components were obtained from fits of the EPR powder spectra (Fig. 2) and from ENDOR spectra (Fig. 4) of the tryptophan radical in protein R2 Y177W indole-H₅-Trp and Y177W indole-d₅-Trp. Axes correspond for ¹⁴N, H(5), and H(7) to the molecular axes system (Fig. 5). Note that ¹⁴N and g tensors have axial symmetry in the given error margins.

| Protein R2 | Tensor element | A₁ (¹⁴N) | A₂ (H₅) | A₃ (H₇) | A₁ (H₅) | A₂ (H₇) | A₃ (H₇) |
|------------|----------------|---------|---------|---------|---------|---------|---------|
| Tyr-177 in mouse, Y177W | x | 2.0032 (1) | 2.25 (2) | 0.15 (2) | -0.65 (4) | -0.14 (4) | -0.12 (1) |
| | y | 2.0032 (1) | 2.25 (2) | 0.15 (2) | -0.14 (4) | -0.65 (4) | 0.18 (1) |
| | z | 2.0024 (1) | 0.94 (2) | 0.15 (2) | -0.49 (6) | -0.49 (6) | -0.06 (1) |
| This work | Iso | 2.0029 (1) | 0.36 | 2.25 | 0.15 | -0.42 | 0 |
| Trp-111 in E. coli, Y122F | x | 2.0031 (1) | 1.36 (2) | 2.83 (2) | -0.63 (2) | ≤0.15 | -0.118 (5) |
| | y | 2.0031 (1) | 1.36 (2) | 2.83 (2) | ≤0.15 | -0.56 (2) | 0.193 (5) |
| | z | 2.0023 (1) | 1.05 (2) | 1.36 (2) | 2.83 (2) | -0.52 (2) | -0.46 (2) | -0.079 (5) |
| Ref. 14 | Iso | 2.0028 (1) | 0.40 | 1.36 | 2.83 (2) | -0.40 | -0.36 | 0 |

| a Signs in analogy to Trp-111 in Y122F E. coli, which have been obtained from density functional and dipolar hyperfine tensor calculations (14).
| b Principal values of the hyperfine tensor assigned to a proton hydrogen-bonded to the indole-nitrogen (from ENDOR spectra; Fig. 4D). Principal axes deviate from the molecular axes.

Fig. 2. X band EPR spectra at T = 40 K of the tryptophan radical in mutant Y177W. Top, in protein R2 with indole-H₅-Trp (0.15 mM) in H₂O buffer; bottom, in protein R2 with indole-d₅-Trp (0.17 mM) in H₂O buffer, both frozen after 3 s reaction time with Fe²⁺ and O₂ at 22 °C. Solid line, experiment; dotted line, best simulation (fit). The obtained g and hyperfine parameters are given in Table II. The parameters for g, ¹⁴N, and the large β-proton coupling were obtained from R2 with indole-d₅-Trp. For the simulation of Trp-indole-H₅, these parameters were kept constant, and in addition the parameters for H-5 and H-7 from proton- and deuterium-ENDOR were used (see text). A single component Gaussian line width of 0.19 and 0.23 mT was used for indole-H₅-Trp and indole-d₅-Trp, respectively. Experimental conditions are as follows: microwave power, 10 μW; modulation frequency, 12.5 kHz; modulation amplitude, 0.15 mT.

Fig. 3. Decay of the tryptophan radical in protein R2 Y177W at 22 °C after 6Fe²⁺/R2 reconstitution. The data points were fitted with a single exponential decay equation (kₑ = 0.015 s⁻¹).

coupled to the indole nitrogen, as would be expected for a tryptophan cation radical, would exhibit a hyperfine tensor component of about 0.5 mT (32). Such a large coupling is, however, not observed in the EPR spectrum of Y177W indole-d₅-Trp in H₂O buffer (Fig. 2, bottom). Therefore, we conclude that there is no NH proton involved in the hfs. The observed tryptophan radical must therefore be neutral with the nitrogen atom deprotonated.

ENDOR Spectroscopy—cw-ENDOR experiments were performed at X band on Y177W with indole-H₅-Trp or Y177W indole-d₅-Trp in H₂O or in D₂O buffer. The ENDOR resonance condition is given to first order by Equation 1.

\[ \nu_v(\text{ENDOR}) = |\nu_v ± A/2|, \] (Eq. 1)

where \( \nu_v \) are the high and low frequency ENDOR transitions; \( \nu_v \) is the respective nuclear Larmor frequency (13.9 MHz for protons, 2.1 MHz for deuterons, and 1.0 MHz for ¹⁴N at a magnetic field of 326 mT); and A is the hfs constant of the respective nucleus. For each hfc A, two ENDOR lines are expected that are spaced symmetrically around \( \nu_v \), and separated by A in case of \( |A/2| < \nu_v \) (Equation 1). In frozen disordered solution, ENDOR resonances are expected over the full range of anisotropic hyperfine values. Prominent features are often observed in the first derivative cw-ENDOR spectra from...
which the principal values \( A_i \) of the hyperfine tensor of each nucleus can be obtained (33).

The large hfcs of the \( \beta \)-proton and the \( A_z \) component of the nitrogen were already determined by EPR. We attempted to observe ENDOR lines corresponding to the large \( \beta \)-proton coupling, which are expected at 17.6 and 45.4 MHz, but with no success. For such large \( \beta \)-proton couplings large ENDOR line widths are expected, in particular for methylene protons for which a variation of the side chain geometry leads to a distribution of the observed hyperfine couplings (see Equation 2). This effect and the low radical yield of 0.34 in Y177W reduce signal amplitudes for these couplings beyond detection.

The smaller hfcs, which are not resolved in EPR, were obtained from the ENDOR spectra. Fig. 4A shows the X band cw-ENDOR spectrum of Y177W with indole-\( h_5 \)-Trp in \( H_2 O \) buffer measured at 8 K. The spectrum is characterized by a strong matrix signal at the proton Larmor frequency \( v_H \) (13.9 MHz) and several pairs of lines that are symmetrically placed around 13.9 MHz and discussed below. Another pair of rather broad lines, at 4.8 MHz and at 23 MHz, yielding a proton hfc corresponding to 0.65 mT, is absent in the ENDOR spectrum of Y177W with indole-\( d_5 \)-Trp in \( H_2 O \) buffer (Fig. 4B). We conclude that this coupling represents one hyperfine tensor principal value of one (or two) indole ring \( \alpha \)-protons. Proton ENDOR signals corresponding to the other hyperfine principal values were not observed. This is due to the expected large hyperfine anisotropy of \( \alpha \)-protons, which broadens the ENDOR signals in frozen solution beyond detection.

Corresponding hyperfine components are, however, observed for deuterons at the ring \( \alpha \)-positions in Y177W with indole-\( d_5 \)-Trp. Fig. 4B shows additional lines between 2 and 4 MHz. These signals belong to \( \alpha \)-deuterons of the indole ring of ring-deuterated tryptophan. Their positions are shifted by a factor of 6.5 to lower frequencies, and their ENDOR spectrum is compressed by the same factor in comparison with the \( \alpha \)-protons in normal tryptophan due to the smaller magnetic moment of \( D \) as compared with \( H \). The amplitudes of the ENDOR signals are correspondingly increased. The lines are symmetrically spaced around the deuterium Zeeman frequency, \( v_D = 2.1 \) MHz. Only the high frequency part of the deuteron-ENDOR spectrum appears at useful ENDOR frequencies (>1.5 MHz, Fig. 4B, see inset 2.1–5 MHz with expanded frequency scale). Three couplings (\( A_1 = 0.6 \) MHz, \( A_2 = 2.1 \) MHz, and \( A_3 = 2.8 \) MHz) have been obtained. The second component is seen mostly as an inflection on the third component (Fig. 4, B and C). The couplings were assigned to the three principal values of one hyperfine tensor, since for \( \alpha \)-deuterons, as for \( \alpha \)-protons, a rhombic hyperfine tensor is expected. The values for the corresponding \( \alpha \)-proton are 3.9, 13.7, and 18.2 MHz or 0.14, 0.49, and 0.65 mT. The largest value agrees well with that obtained from the proton-ENDOR spectrum of the protein with indole-\( h_7 \)-Trp (see above, and Fig. 4A). This interpretation is supported by ENDOR simulations (dotted traces in Fig. 4A, 15–31 MHz, and Fig. 4C, 2.1–5 MHz) using solely the tensor principal values of the two \( \alpha \)-protons and assuming an inequivalence for the two smaller components of both tensors within the error margins given in Table II. The simulations show that for this case the largest component gives rise to the most intense peak as is observed in the proton- and deuterium-ENDOR spectra.

The three \( \alpha \)-deuteron and derived proton hyperfine tensor principal values agree remarkably well with those obtained earlier for the tryptophan neutral radical Trp-111 \(^*\) in E. coli protein R2 Y122F (14), see Table II. In this earlier work the tensor principal values were corroborated by EPR simulations and by McConnel-Strathdee based dipolar tensor calculations accounting for all carbon and nitrogen spin densities in the

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**Fig. 4.** cw-ENDOR spectra (X band) at \( T = 8 \) K of the tryptophan radical after 3 s reaction time at 22 °C. Spectra were recorded with a field setting at the maximum of the EPR low field absorption line, corresponding to 344.85 mT in Fig. 2. Since the ENDOR cavity exhibits a different resonance frequency than the EPR cavity used for recording the spectra in Fig. 2, the actual ENDOR spectra were recorded at 325.4 mT. The ENDOR splittings and the nuclear Zeeman frequencies for protons and deuterons (\( v_H \) and \( v_D \)) are indicated by dotted traces. A, protein R2 Y177W indole-\( h_5 \)-Trp in \( H_2 O \) buffer (0.15 mM). The dotted trace 15–31 MHz shows a proton ENDOR simulation using solely the tensor principal values of two \( \alpha \)-protons given in Table II and assuming an inequivalence of the two smaller tensor values for both protons within the given error margins (±0.06 and ±0.04 mT). The simulation indicates that the most intense peak results from the largest tensor component which is assumed to be equivalent for both protons. The same behavior is observed in the deuteron ENDOR spectrum, trace C. The ENDOR simulation program is described in Ref. 47. B, protein R2 Y177W indole-\( d_5 \)-Trp in \( H_2 O \) buffer (0.17 mM). Dotted trace, 2.1–5 MHz, expanded scale, is an ENDOR simulation with two \( \alpha \)-deuterons using the same values as for protons in trace A except for a scaling factor 1/6.5 accounting for the smaller magnetic moment of the deuteron. D, difference spectrum: indole-\( d_5 \)-Trp in \( H_2 O \) buffer minus indole-\( d_5 \)-Trp in \( D_2 O \) buffer. Splittings of a proton hydrogen bonded to N-1 are indicated (see text). Experimental conditions are as follows: microwave power, 5 mW; radiofrequency power, 100–150 W; modulation frequency 12.5 kHz; modulation amplitude, ±140 kHz. Total accumulation time 8–10 h each trace.
radical (14). The EPR simulations performed for Trp-177 (Fig. 2, top) showed, as in the earlier work (14), that two α-protons have to be assigned to the observed three hyperfine tensor values and that their hyperfine tensor axes of the largest value (0.65 mT) are rotated by 90° relative to each other in order to obtain the observed pattern in the experimental spectrum (see Table II). Based on earlier density functional calculations, these two proton hyperfine tensors were assigned to ring protons H-5 and H-7 (14) (see Fig. 5 for numbering of tryptophan ring). Since the largest hyperfine tensor component of an α-proton is expected for the magnetic field oriented perpendicular to the C–H bond and in the tryptophan ring plane, the largest component (0.65 mT) is assigned to $A_x$ (H-5) and $A_y$ (H-7) and the smallest component (0.14 mT) to $A_z$ (H-5) and $A_z$ (H-7), whereas $A_x$ is assumed to be similar (0.49(6) mT) for both α-protons (compare Fig. 4 and Table II).

The ENDOR spectra of Fig. 4, B and C, show two lines at 11.8 and 16.0 MHz corresponding to a 4.2-MHz coupling which is not affected by indole-D$_2$-Trp nor by D$_2$O buffer. Since a proton weakly coupled to the nitrogen would exchange in D$_2$O buffer, we assign this small coupling of 0.15 mT to the second methylene β-proton. This coupling is not resolved in the EPR spectrum in Fig. 2, bottom.

Exchangeable proton hfcs were not observed in the EPR spectra. However, from the ENDOR difference spectrum Y177W indole-d$_5$-Trp in H$_2$O minus Y177W indole-d$_5$-Trp in D$_2$O buffer (Fig. 4D), three small hyperfine tensor values of −0.12, +0.18, and −0.06 mT were obtained and assigned to one exchangeable proton. Previous ENDOR experiments on the neutral tryptophan radical Trp-111 in E. coli Y122F yielded very similar tensor components of an exchangeable proton of −0.12, +0.19, and −0.08 mT. Based on spin density and dipolar hyperfine tensor calculations, they were assigned to a hydrogen-bonded proton at the indole nitrogen (14). Since the tryptophan radical in Y177W exhibits a very similar $^{14}$N hyperfine tensor as Trp-111 in Y122F, we likewise assign the three values −0.12, +0.18, and −0.06 mT to the three hyperfine tensor principal values of a proton hydrogen-bonded to the nitrogen in the tryptophan indole ring (for signs, see Table II, legend and footnotes).

**Fig. 5. Molecular structure, molecular axis system, numbering scheme, and spin densities derived from the hyperfine coupling constants (see text) for the tryptophan radical in mouse R2 mutant Y177W.**

Reconstitution of Y177F and Y177C R2 with 6Fe$^{2+}$ and Oxygen

**EPR Spectroscopy**—When aerobic apoprotein R2 Y177F was mixed with 6Fe$^{2+}$ at 22 °C, an EPR singlet signal at $g = 2.00$ with a peak-to-peak line width of 1.8 mT was observed at 20 K. Fig. 6A shows this signal when the reaction was quenched after 3 s. A very similar EPR singlet signal, with regard to $g$ value, line width, and microwave saturation behavior, appeared also after reconstitution reaction in protein R2 Y177C under the same conditions (Table III). The EPR singlet in Y177F appeared with a rate constant of about 0.5 s$^{-1}$ and decays with a rate of 0.25 s$^{-1}$. The EPR singlet in Y177C had similar kinetics and appeared with a rate of 0.48 s$^{-1}$ and decayed with a rate of 0.23 s$^{-1}$. Both EPR singlets had completely disappeared after 20 s reaction time (Fig. 7). Upon reconstitution with apoprotein Y177F and $^{57}$Fe, we observed a doublet splitting ($\Delta = 2.6$ mT) of the EPR signal due to the nuclear spin of 1/2 for $^{57}$Fe (Fig. 6C). A similar transient EPR signal has also been observed in the reconstitution of wild-type and Y122F protein R2 of E. coli (34–36) and native mouse R2 (12) and has been assigned to an oxo (hydroxo)-bridged Fe(III)/Fe(IV) cluster, intermediate X (25). For comparison, Fig. 6, B and D, shows the corresponding spectra of E. coli R2 Y122F after a freeze-quenched reaction with $^{56}$Fe and $^{57}$Fe, respectively. The dotted trace is a simulation. Microwave saturation studies of the singlet EPR spectrum in mouse Y177F R2 and of intermediate X in E. coli Y122F R2 gave coinciding values of $P_{ss}$ 0.02 mW at 5 K and 0.2 mW at 20 K (data not shown). From the almost identical $g$ values, line shapes, microwave saturation behavior, and $^{57}$Fe hfcs, we assign also the transient EPR signals in Y177F and Y177C to such a bridged Fe(III)/Fe(IV) cluster, similar to intermediate X observed earlier in E. coli protein R2.

**DISCUSSION**

Among free radical enzymes (3), RNR from E. coli was the first enzyme in which a protein-linked tyrosyl free radical was identified. In a kinetic study of the formation of the tyrosyl radical, Bollinger et al. (36) have shown that an intermediate X, an Fe(III)/Fe(IV) cluster with spin delocalization, serves as precursor to the tyrosyl radical in the E. coli protein R2. When the tyrosyl radical cannot be formed due to a mutation like in E. coli protein R2 Y122F, intermediate X becomes longer lived and can oxidize other neighboring aromatic residues like tryptophans (13, 37). Two tryptophan radicals (located at Trp-111 and Trp-107) have previously been studied in detail and assigned to oxidized neutral radicals (13, 14).

An Oxidized Neutral Tryptophan Radical on Trp-177—We have observed a transient tryptophan radical in mouse protein R2 upon the reconstitution of Y177W with ferrous iron and oxygen. Its light absorption centered around 550 nm agrees with reported properties of photochemically produced tryptophan radicals (38, 49). Its dominant EPR doublet signal arises from the hyperfine splitting of one β-methylene proton, whereas two α-protons at the indole ring cause the small hfcs as seen in Fig. 2, top. In addition to the proton hfcs, the full $^{14}$N hyperfine tensor was obtained with the help of EPR simulations (see Fig. 2 and Table II). Finally, ENDOR difference spectra of Y177W with indole-d$_5$-Trp in H$_2$O and D$_2$O revealed the hyperfine tensor of an exchangeable proton assigned to a hydrogen-bonded proton at the indole-nitrogen.

The tryptophan radical in Y177W can now be compared with the previously reported oxidized neutral tryptophan radical at Trp-111 in E. coli R2 Y122F (13, 14). The tryptophan radical in Y177W exhibits only one large β-proton hfc. The hfc of the second β-proton is very small and only resolved in the ENDOR spectrum. In the tryptophan radical Trp-111$'$ of Y122F, there...
are two large $^3$-proton hfs. This indicates a difference in the
dihedral angles of the $^3$-methylene protons for the two trypto-
phan residues, whereas the spin densities in the indole rings
are very similar.

The spin density on C-3 in the tryptophan radical in Y177W
can be estimated from the observed hfc's of the $^3$-protons using
the empirical relationship (Equation 2)

$$A_{^3H} = \rho_p^B (B' + B'' \cos^2 \theta)$$

(Eq. 2)

where $\rho_p^B$ is the $\rho$ spin density of the neighboring carbon, $B'$
and $B''$ are empirical constants, and $\theta$ is the dihedral angle
between the axis of the $p_z$ orbital and the projected $C_pH_p$ bond
(39). Since the hfc of one $^3$-proton in Y177W is close to 0, its
dihedral angle $\theta$ must be close to 90°. For a tetragonal geometry
this implies $\theta = -30°$ for the second $^3$-proton ($\theta_1 - \theta_2 = 120°$).

Using $A_{^3H} = 2.25$ mT, $B' = 0$, $B'' = 5.8$ mT (14), and $\theta = -30°$,
a spin density $\rho_p^B$ of 0.52 is obtained for C-3 (see Fig. 5). The
same spin density was obtained for C-3 in Trp-111
$^{z}$ of Y122F
from

E. coli

Based on empirical constants between hyper-
fine tensor values and spin densities and assignments from
density functional calculations, for positions N-1, C-5, and C-7
(Fig. 5) in Trp-111 $^{z}$ of E. coli R2 Y122F spin densities of 0.20,
0.17, and 0.15 were determined, respectively (14). By using the
same assignment for the two ring protons (H-5 and H-7) as in
Ref. 14 and a $Q$ factor of $-2.48$ mT for $\alpha$-protons (40), a spin
density of 0.17 is obtained for each C-5 and C-7 in Y177W based
on the hyperfine tensor values given in Table II. For N-1 a spin
density of 0.18 is obtained for the tryptophan radical in Y177W
using the hyperfine tensor values of Table II and $B = 1.706$ mT
(41) for $^{14}$N ($\rho^B(N) = 1$; $B = (A_p - A_{^3H})/2$). This shows that the
spin densities in the indole rings of these two tryptophan rad-

The spin density on C-3 in the tryptophan radical in Y177W
can be estimated from the observed hfc's of the $\beta$-protons using
the empirical relationship (Equation 2)

$$A_{\beta H} = \rho_p^B (B' + B'' \cos^2 \theta)$$

(Eq. 2)

where $\rho_p^B$ is the $\rho$ spin density of the neighboring carbon, $B'$
and $B''$ are empirical constants, and $\theta$ is the dihedral angle
between the axis of the $p_z$ orbital and the projected $C_pH_p$ bond
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FIG. 6. X band EPR spectra at $T = 20$ K of intermediate X formed in the reconstitution reaction of mouse protein R2 Y177F (0.1 mM)
(A and C) and E. coli protein R2 Y122F (1 mM) (B and D). Apoprotein R2 in 200 mM HEPES buffer, pH 7.5, was 1:1 mixed with 0.6 mM $^{56}$Fe
(A), 4 mM $^{56}$Fe (B), and 0.6 mM $^{55}$Fe in 2.5 mM H$_2$SO$_4$ (C), and 4 mM $^{53}$Fe in 2.5 mM H$_2$SO$_4$ (D, dotted trace simulation, see text). The reaction was
stopped after 3 s at 22 °C (A), 300 ms at 22 °C (B), and 2 s at 5 °C (C and D), see text. Experimental conditions are as follows: microwave power,
0.03 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT.

FIG. 7. Time course of formation and decay of intermediate X
in mouse protein R2 Y177C and Y177F upon addition of 6
Fe$^{57}$/R2 at 22 °C as measured by EPR. The data points were fitted
with a double exponential equation assuming a consecutive reaction.
Rates for formation and decay of X are presented in Table III.

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dihedral angles of the $^3$-methylene protons for the two trypto-
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phan residues, whereas the spin densities in the indole rings
are very similar.
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cals are indeed very similar. Furthermore, in both radicals the expected large coupling from an N—H proton is missing, and the observed hyperfine tensors from H-bonded exchangeable protons clearly indicate that the radicals are in their neutral state, deprotonated but H-bonded at N-1.

Another common feature of both tryptophan radicals in mouse R2 Y177W and E. coli R2 Y122F is that they exhibit a rather small g anisotropy (g_u - g_g = 8 × 10^{-4}, see Table II). This is typical for radicals with high spin densities only on carbon and nitrogen atoms, like tryptophan. For comparison, tyrosyl radicals with a high spin density on an oxygen atom (larger spin-orbit interaction than nitrogen or carbon) exhibit a larger g anisotropy (for native mouse R2 g_u - g_g = 54 × 10^{-4} (42)). It should also be pointed out that in these two mutant R2 proteins we have established that protein-linked neutral tryptophan radicals exhibit a light absorption band centered around 550 nm (Fig. 1, inset, and see Ref. 13).

Which is the site of the observed oxidized neutral tryptophan radical in protein R2 Y177W? Including Trp-177, there are 7 tryptophans per polypeptide chain in mouse R2 Y177W. We expect the transient radical to be located relatively close to the iron site (cf. Ref. 13), and we expect the geometry of the β-protons with respect to the indole plane (dihedral angles θ) as derived from the crystal structure (5) to agree with the experimental observations. Tryptophans 103, 214, 210, 92, 246, and 117 have distances to their indole-nitrogen from Fe2 (the iron ion most distant from Tyr-177) of 11, 15, 17.4, 21, 24.7, and 25.5 Å, respectively. Only tryptophans 92 and 246 have dihedral angles of the β-methylene protons θ1 = −27°, θ2 = 93°, and θ1 = −37°, θ2 = 83° (5), which agree within ±10° with those obtained for the tryptophan radical in Y177W (−30 and 90°). Trp-92 and Trp-246 are, however, 21 and 24.7 Å from the Fe2 atom, far away from the iron site. Tryptophans 111 and 107 in E. coli, which carry radicals in the mutant Y122F (13, 14), are much closer to the iron site (5 and 7 Å). The closest tryptophan to the iron site in native mouse R2 (Trp-103) is 11 Å from Fe2, but its dihedral angles (θ1 = −43° and θ2 = 77°) do not agree well with the experimental data. Therefore, we assign the observed neutral tryptophan radical to residue Trp-177, which should be the tryptophan closest to the iron site. Reconstitution with 57Fe did not change the EPR spectrum of the radical (data not shown), indicating only weak interaction with the iron site like in native enzymes with tryrosyl radicals (43) or the Trp-111' radical in E. coli Y122F (13). The Trp-177 radical is not enzymatically active (Table I).

The Long-lived Intermediate X in Protein R2 Mutants Y177F and Y177C—The reconstitution of apoY177F and Y177C R2 with an excess of Fe_{6}^{2+} showed the formation of an S = 1/2 paramagnetic species. The X band EPR spectrum of 57Fe-labeled R2-Y177F protein could be successfully simulated using the g tensor and the two hyperfine tensors of 57Fe from the Q band studies of intermediate X (25) (Fig. 6D). We therefore assigned the intermediates in mouse R2 Y177F and Y177C to Fe(III)/Fe(IV) clusters, like intermediate X in E. coli R2, with similar structures as reported for intermediate X in E. coli R2 Y122F (25). However, the intermediates X in the two mouse mutants differ kinetically from that in E. coli Y122F in the rates of formation and decay, i.e. in their time window of appearance. Intermediate X in E. coli R2 Y122F became reduced after 2 s at 25 °C, whereas in the two mouse mutants intermediates X are still detectable after 10 s reaction time at 22 °C (Fig. 7).

In E. coli R2 Y122F intermediate X becomes reduced by the oxidation of the non-conserved tryptophans 111 and 107 (13). E. coli Trp-111 and Trp-107 correspond to Gin-167 and Phe-163, respectively, in mouse protein R2, which cannot be easily oxidized and may explain the missing successor radicals in Y177F. Besides Tyr-177 in native mouse protein R2, there are no other aromatic amino acids in the close vicinity of the iron site, which can reduce intermediate X. This may be the reason for its long lifetime.

Whereas phenylalanine in Y177F is not expected to be oxidized, cysteine in Y177C was expected to form a cysteine radical upon reconstitution. The crystal structure of the analogous mutant E. coli R2 Y122C shows a much larger distance of the −SH group to Fe1, the iron ion closest to Cys-122, (8.9 Å) than the OH group of tyrosine 122 in E. coli wild type R2 (5.3 Å) and a hole at the site of the missing phenyl ring. In neither of the R2 mutants E. coli Y122C and mouse Y177C did we detect any EPR signal at 77 K in the time window of 8 ms to 20 s (comitant with the decay of X) up to 20 min reaction time at room temperature after the reconstitution. We have to conclude that either due to the larger distance between Cys-177 and Fe1 and no cysteine radical has been formed or, if it was formed, it is short lived, or it has a low signal intensity caused by its large g anisotropy (44). Functionally related cysteine-based radicals have been detected in a class II RNR with strong magnetic interaction with a covalent complex (45) and very recently in an E. coli protein R1 mutant with a lifetime of several seconds (46).

Concluding Remarks—The present study shows that the iron/oxygen reconstitution reaction in protein R2 is able to create protein-linked free radicals in the close vicinity of the dfferic iron center in mouse protein R2. When the tyrosine 177 is mutated for another redox-active amino acid with a suitable side chain and an appropriate redox potential, like tryptophan, this residue can be oxidized. Despite the formation of a transient tryptophan radical, the loss of the enzymatic activity in all three mutants, Y177W, Y177F, and Y177C, suggests strongly that the tyrosyl radical 177 cannot be replaced by other amino acids. The long lifetime and high relative yield of intermediate X in mouse Y177F and Y177C mutants with no observable successor radicals may facilitate further studies of the properties of this important intermediate in iron-oxygen chemistry of diiron-oxygen proteins.

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