Restoring Proper Radical Generation by Azide Binding to the Iron Site of the E238A Mutant R2 Protein of Ribonucleotide Reductase from Escherichia coli

The enzyme activity of Escherichia coli ribonucleotide reductase requires the presence of a stable tyrosyl free radical and dimer center in its smaller R2 component. The iron/radical site is formed in a reconstitution reaction between ferrous iron and molecular oxygen in the protein. The reaction is known to proceed via a paramagnetic intermediate X, formally a FeIII-FeIV state. We have used 9.6 GHz and 285 GHz EPR to investigate intermediates in the reconstitution reaction in the iron ligand mutant R2 E238A with or without azide, formate, or acetate present. Paramagnetic intermediates, i.e. a long-living X-like intermediate and a transient tyrosyl radical, were observed only with azide and under none of the other conditions. A crystal structure of the mutant protein R2 E238A/Y122F with a diferrous iron site complexed with azide was determined. Azide was found to be a bridging ligand and the absent Glu-238 ligand was compensated for by azide and an extra coordination from Glu-204. A general scheme for the reconstitution reaction is presented based on EPR and structure results. This indicates that tyrosyl radical generation requires a specific ligand coordination with 4-coordinate Fe1 and 6-coordinate Fe2 after oxygen binding to the diferrous site.

The enzyme ribonucleotide reductase (RNR) catalyzes the direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides. Because deoxyribonucleotides are building blocks of DNA, RNR is essential for DNA synthesis in living cells. A number of enzymes from different species has been described and classified into three major classes (1). Common for the RNRs known so far is that they all make use of a free radical mechanism to reduce ribonucleotides. RNR of class Ia, found in mammalian, plants, DNA viruses, and some procaryotes like Escherichia coli, consists of two non-identical homodimeric components, proteins R1 and R2 (2, 3).

The crystal structures of both components of E. coli RNR have been solved separately (4–6). The specific substrate reaction takes place in the larger R1 component, whereas the free radical on a tyrosyl side chain required for enzyme activity is generated in the smaller R2 component on Tyr-122 (1–3). It has been proposed that the substrate binding site in R1 is connected to Tyr-122 in the R2 component by an array of conserved hydrogen-bonded amino acids (4–6), considered to take part in a coupled electron/proton (H+) transfer between the two proteins during the enzymatic reaction (2, 7–10). The tyrosyl radical is formed together with a μ-oxo-bridged diferric center in a reconstitution reaction involving ferrous iron and molecular oxygen. The present study concerns the role in the radical generation reaction of one of the iron ligands, Glu-238, a conserved residue in protein R2. The E238A mutant R2 protein lacks a stable tyrosyl radical and enzymatic activity (11).

Despite many studies of the iron reconstitution reaction, a conclusive mechanism for the oxygen activation and radical formation is still missing. In the reduced protein, the iron ions are bridged by the carboxylate ligands Glu-115 and Glu-238 (for numbering of residues, see Scheme 1a). Fe1 has the monodentate ligands Asp-84 and His-118 and Fe2 has the monodentate ligands Glu-204 and His-241. At this point both Fe1 and Fe2 are 4-coordinate (12). Reduction of oxygen is proposed to result in a peroxy complex, and the ferrous ions are oxidized to ferric. The next step is proposed to be heterolytic cleavage of the peroxy complex, resulting in a high valent iron-oxo species. By proton/electron transfer from an external source via the radical transfer pathway, the so-called intermediate X is formed. This is the only relatively well characterized paramagnetic intermediate in the R2 wild-type system. It is considered to be the precursor to the tyrosyl radical on Tyr-122. Formally it is considered to be a FeIII-FeIV center with an iron-iron distance of about 2.5 Å (14–18). An OH (or aqua) ligand on Fe1 has been shown to originate from the molecular oxygen (19).

Finally Tyr-122 oxidation to the radical state, which involves abstraction of a H, and equilibration with solvent, result in the active radical-containing R2 protein with Glu-238 as a monodentate ligand and an additional H2O ligand to Fe2. In the active form of the enzyme the ferric ions are both 6-coordinate, antiferromagnetically coupled, and bridged by a μ-oxo bridge, also originating from the molecular oxygen (17). In this last...
step both Asp-84 and Glu-238 undergo carboxylate shifts, leaving Glu-238 a monodentate ligand to Fe2 and Asp-84 a bidentate ligand to Fe1.

The iron ligand Glu-238 has been shown to be able to adopt several different conformations depending on oxidation state and ligand binding mode of the metal site (4, 12, 13). This suggests that it is a key residue for controlling the oxygen activation, as was also indicated in structural studies of the azide complex of the reduced R2 F208A/Y122F mutant (13) and in the oxidized and reduced forms of the R2 E238A/Y122F mutant in the absence of azide (20).

In the present work the iron-oxygen reconstitution reaction in the E. coli R2 mutant E238A was studied by 9.6 GHz and 285 GHz EPR spectroscopy. The reconstitution reaction was investigated in the presence of a number of potential small molecule iron ligands. The objective was to determine whether the small molecule ligand could assume the role of Glu-238 and keep the iron center in a structure that would allow it to react properly with oxygen and give rise to a tyrosyl radical. This was observed for an azide complex. A crystal structure of the diferrous mutant R2 protein E238A/Y122F complexed with azide was obtained. Based on the combined EPR and structure data a plausible scheme for radical regeneration in the E238A mutant is proposed (Scheme 1b), in analogy with the proposed reconstitution reaction in wild-type protein R2 (13).

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of R2 Proteins**—Construction of plasmids for overexpression of wild-type and the two mutant R2 proteins has been described previously (11, 21). Overexpression of protein was performed either in MC1000/pGP1–2 (wild-type and E238A/Y122F) or K38/pGP1–2 (E238A). The wild-type protein was grown in low iron medium containing 45.4 mM phosphate buffer, pH 7.3, 7.57 mM (NH₄)₂SO₄, 0.4% glucose, 610 μM leucine, 406 μM MgSO₄, 50 μM EDTA, 5 μM CaCl₂, 0.6 μM ZnCl₂, 0.6 μM CuSO₄, 0.6 μM MnCl₂, 0.75 μM CoCl₂, 5.9 μM thiamine dichloride, 50 μg/ml carbenicillin, and 50 μg/ml kanamycin (22). The phosphate buffer, ammonium sulfate, glucose, and leucine solutions were all filtered through Chelex chelating ion exchange membranes (Bio-Rex ion exchange membrane, Bio-Rad) prior to the medium preparation. All glassware was washed with 0.1 M sulfuric acid to remove traces of iron. The mutant protein was grown in Luria-Bertani medium containing 50 μg/ml carbenicillin and 50 μg/ml kanamycin (22).

In the medium listed above the cells grew at 30 °C up to an optical density of \( A_{600} = 1.0 \). Then the temperature was raised to 42 °C to induce overexpression. Approximately 3 h after induction, the cultures were chilled and harvested by centrifugation at 6000 rpm for 12 min at

![Scheme 1](image-url)

**Scheme 1.** The proposed reaction paths for the radical generation reactions of the wild type (a) and the E238A+azide R2 (b) proteins. Hypothetical intermediates for the initial oxygen-bound forms and high-valent Fe IV forms are indicated within brackets. The iron ligand Glu-238 has been shown to be able to adopt several different conformations depending on oxidation state and ligand binding mode of the metal site (4, 12, 13). This suggests that it is a key residue for controlling the oxygen activation, as was also indicated in structural studies of the azide complex of the reduced R2 F208A/Y122F mutant (13) and in the oxidized and reduced forms of the R2 E238A/Y122F mutant in the absence of azide (20).
The frozen pellets were pressed 4–6 times in an X-press (BIOX) and stored at −80 °C. The pellets were pressed 4–6 times in an X-press (BIOX) and extracted with 50 mM Tris, pH 7.6, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride in a blender. The viscous solution was cleared by centrifugation at 19,000 rpm for 90 min at 5 °C (Sorvall centrifuge, SS-34 rotor). Nucleic acids were removed by precipitation with streptomyacin sulfate to a final concentration of 10%, and the solution was cleared by centrifugation at 15,000 rpm for 20 min at 5 °C (Sorvall centrifuge, SS-34 rotor). The proteins were then incubated at 5% ammonium sulfate and collected by centrifugation at 15,000 rpm for 20 min at 5 °C (Sorvall centrifuge, SS-34 rotor). The pellets were gently resuspended in milliliters of 50 mM Tris, pH 7.6 and desalted over a column containing Sephadex G-25 medium from Amersham Pharmacia Biotech, then applied to a column of DEAE-cellulose. Protein was eluted with a gradient from 0.15 to 0.3 M phosphate, pH 7.0. R2-containing fractions were pooled and concentrated to <20 μM by ultradialysis against a buffer containing 50 mM Tris, pH 7.6, and 10% glycerol, frozen in liquid nitrogen and stored at −80 °C. The R2 proteins were in the apo form (metal-free) after purification (21).

Protein purity was analyzed by SDS-polyacrylamide gel electrophoresis. The protein R2 concentrations were determined from the absorbance at 280 nm minus the absorbance at 310 nm (ε_{280-310} = 120 mM^{−1} cm^{−1}) using a Varian Cary-4 spectrophotometer.

**Preparation of the Anaerobic Fe^{2+} Solution**—Buffer containing 50 mM Tris, pH 7.5 was thoroughly degassed in a septum-sealed bulb for several hours by repeatedly flushing with oxygen-free argon and evacuation. A second bulb containing Mohr's salt crystals (NH_4)_2Fe(SO_4)_2 was sealed with a septum and degassed according to the same procedure. The bulbs were then left under a slight excess pressure of argon. A plastic syringe was made anaerobic by washing several times with oxygen-free argon gas. Then the appropriate volume of anaerobic buffer to prepare the desired iron concentration was transferred into the bulb containing Mohr's salt.

Reconstitution Reaction of Apoprotein R2 with Fe^{2+} and Oxygen—Reconstitution of the iron site in the R2 proteins was carried out by mixing equal volumes of apoprotein in oxygen-saturated 50 mM Tris, pH 7.6, with anaerobic Fe^{2+} solution. Where indicated 0.1 mM acetate, azide, or formate was added to the oxygen-saturated Tris buffer. Rapid freeze quenching (RFQ) was performed with a System 1000 apparatus from Update Instruments to obtain reaction times from 8 ms to 1 s. The reaction times were varied by changing the length of the reaction tube between mixer and spray nozzle. An EPR tube connected with a funnel fixed in a home-built holder was immersed completely into a n-pentane bath of −110 °C to 1 °C. Aerobic apo-protein and anaerobic Fe^{2+} solution were rapidly mixed and then quenched by spraying them into the EPR tube in the isopentane bath. The crystals were tightly packed into the EPR tube using a packing rod made from Teflon. The isopentane in the EPR tube was removed and then the EPR tubes were kept for at least 20 min under high vacuum to get rid of isopentane trapped between the crystals. During this time the tubes were stored in a separate n-pentane bath of −120 °C.

For reaction times longer than 2 s, slow freeze quench (SFQ) samples were obtained by hand-mixing. EPR tubes with the aerobic protein solution and a septum sealed bulb with the anaerobic Fe^{2+} solution were thermostated at the reaction temperature in a water bath. A gas-tight Hamilton syringe, equipped with a long needle, was washed several times with the anaerobic Fe^{2+} solution to remove oxygen. Then the anaerobic ferrous solution was mixed with the apoprotein, and the reaction was stopped by immersing the EPR tube into cold n-pentane (−120 °C).

**EPR Measurements**—9.6 GHz EPR spectra were recorded on a Bruker Elexsys E500 system using a rectangular dual mode Bruker EPR cavity (ER 4116DM). For measurements below 77 K, the spectrometer was equipped with an Oxford helium flow cryostat. Spin quantitation was performed using a Cu^{2+}/EDTA (1 mM/10 mM) primary standard or by a sample of E. coli wild-type R2 with a known tyrosyl radical content by comparing the double integrals under non-microwave saturating conditions. Spectra were evaluated using Xepr 2.0 software from Bruker.

26.8 GHz EPR spectra were recorded at 5 K in the Grenoble High Magnetic Field Laboratory (CNRS-MPI, Grenoble, France) as previously described (23). The frequency source used to generate a 285 GHz excitation is a Gunn-diode delivering a 95 GHz basic frequency, which is then tripled to obtain 285 GHz (system supplied by Radiometer Physics GmbH). The basic frequency can be changed ± 50 MHz. The exact frequency used was recorded automatically by the system.

**Crystallization and Crystal Soaking**—The E238A/Y122F mutant protein was crystallized using the hanging drop vapor-diffusion method as previously described for the wild-type R2 protein (24). The crystals attained a size of about 0.5 × 0.2 × 0.2 mm and belonged to spacegroup P2_1_2_1.

The E238A/Y122F mutant protein crystals were first soaked in mother liquor containing 80 mM FeCl_3 and 0.5% sodium dithionite for 90 min to reconstitute the reduced diiron site. The crystals were then moved to mother liquor containing 0.5% sodium dithionite and 500 mM sodium acetate for 30 min. The crystals were subsequently transferred to mother liquor with 20% glycerol as cryoprotectant, 0.5% dithionite, and 500 mM sodium azide, for about a minute before being flash frozen in liquid nitrogen.

**Data Collection and Structure Determination**—Data were collected at the Swiss-Norwegian beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The data were collected at a wavelength of 1.00 Å using a Mar Research imaging plate. The crystal was kept at 100 K using a cryosystem (Oxford cryosystems). The data were processed and scaled using the Denzo and Scalepack programs (25).

The structure was solved using molecular replacement with the refined structure of reduced wild-type R2 including water molecules (12) as the starting model. During the initial rounds of refinement the starting model was used as a rigid body. The resolution limits were gradually increased to their final values during the refinement. Rigid body and positional refinement was performed using the TNT refinement package (26). Electron density maps were calculated using TNT and programs from the CCP4 (27) suite. Interpretation of maps and model building was done using QUANTA (Molecular Simulations). After several cycles of refinement water molecules with B values exceeding 70 Å^2 were removed, new waters were added at peaks higher than 4σ in difference maps. The free R-factor (28) was calculated using 5% of the data and monitored throughout the refinement. Once the refinement had converged, a final round of refinement was performed with all the data included.

**RESULTS**

Reconstitution of the Iron Site Studied by 9.6 GHz EPR—Mutant apo-protein R2 E238A was reconstituted with Fe^{2+} (4 Fe^{2+/2} /R2) and molecular oxygen, either in the presence of acetate, azide, or formate, or without small potential iron ligand present. By combining RFQ and SFQ methods the reaction was followed from 8 ms to 10 min. EPR spectroscopy was used to detect any paramagnetic intermediates appearing in the reaction.

When the reconstitution reaction was performed in the absence of any exogenous ligand, no significant production of EPR observable species in the g = 2.00 region was observed. Increasing the iron concentration to 6 Fe^{2+/2} /R2 did not change this result (data not shown). Similarly, when the reconstitution (4 Fe^{2+/2} /R2) was performed in the presence of formate or acetate, no significant EPR observable species could be observed, as summarized in Table I. In contrast, when azide was present in the reaction mixture, the reconstitution reaction gave rise to EPR observable species.

Fig. 1 shows a series of EPR spectra obtained after reaction times at 5 °C spanning between 80 ms and 10 min in the presence of azide. The EPR signals can be explained as superposition of two components with different weights. After long...
reaction times the doublet signal is close, but not quite identical, to that of a wild-type Tyr-122 tyrosyl radical (Fig. 2). The composite spectra were quantitatively evaluated by subtracting a suitable amount of the spectra obtained after 90 s reaction (Fig. 1f), so that the corresponding resulting traces are EPR singlets with line widths (peak to through) of 2.3 mT after all reaction times (data not shown). When the reconstitution reaction was performed in the presence of $^{15}$N-labeled azide ($^{14}$N-$^{15}$N-$^{14}$N from Cambridge Isotope Laboratories) the EPR spectra were unchanged (data not shown). As will be shown later in the section on high-field EPR, the singlet spectrum dominating at early times in azide is virtually identical to that of intermediate X as it is observed in wild-type and Y122F protein R2, with a rate of formation of 8 s$^{-1}$ (including iron binding) and disappearance of 1 s$^{-1}$ (14), as illustrated in Scheme 1. The rate of formation of the doublet spectrum of the tyrosyl radical in R2 E238A is compatible with X$^{A}$ being its precursor.

The microwave relaxation behavior of the EPR spectrum dominated by X$^{A}$ (3-s reaction time, Fig. 1b) at different temperatures is presented in Fig. 3. Table III summarizes the EPR relaxation parameters of intermediate X$^{A}$ at different temperatures. The table gives the parameters of the saturation curves in terms of microwave power at half-saturation $P_{1/2}$ and $P_{b}$. The parameter $b$ describes the contribution of inhomogeneous broadening (32) to the saturation curve as shown in Equation 1,

$$I \propto 1/(1 + P/P_{1/2})^{b}$$  \hspace{1cm} (Eq. 1)$$

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

**High-Field EPR Studies of Intermediate X$^{A}$ and the Tyrosyl Radical in R2 E238A—EPR at 285 GHz corresponding to a magnetic field of about 10.1 T was used to further characterize the composite EPR spectra in a sample of reconstituted R2 E238A protein quenched after a 10-s reaction time (Fig. 4a). To calibrate the field-scale we used a sample of wild-type protein R2 from *E. coli*, known to contain no other radical than the pure tyrosyl radical, and with known g-values (Fig. 4b) (33). The spectra were recorded under identical conditions. Because the EPR signal from E238A was weak, we had to use a strong modulation amplitude, resulting in over-modulation (and loss of hyperfine resolution) of the wild-type EPR signal. The g-values were however not affected by the overmodulation. As
power saturation curves of the intermediate $X_A$ from components of intermediate $X_A$ were determined to be $g_2$ as an internal calibration standard, two of the $g$-value components, Table IV). Using the known $g$-values of the tyrosyl radical $4$, the signal is clearly composed of two $g$-value components, in agreement with the observations by $9.6$ GHz EPR (compare with Fig. 1c). The $g_1$ component of the tyrosyl radical was found to restore the proper course of the reconstitution reaction. In the O$_2$ activation of wild-type apo-protein R2, no other intermediate paramagnetic species than intermediate X has been detected (14, 15, 29–30). In the reconstitution reaction of mutant R2 protein, acetate or formate was found to restore the proper course of the reconstitution reaction. In the O$_2$ activation of wild-type apo-protein R2, no other intermediate paramagnetic species than intermediate X has been detected (14, 15, 29–30). In the reconstitution reaction of mutant R2 protein, acetate or formate was found to restore the proper course of the reconstitution reaction. The crystal structure of the azide-complexed diferrous E238A/Y122F mutant was determined. Extrapolating from previous experiences from structural studies of Y122F mutant R2 proteins, we expect the structure of the E238A/Y122F double mutant to correspond closely to the one of the single mutant E238A, which did not yield suitable crystals at the time of the study.

**DISCUSSION**

**Reactivity of the Protein R2 E238A Mutant**—In the present study we show that azide is required for the proper formation of a tyrosyl radical in the reconstitution reaction of mutant apo-protein R2 E238A (Table II). Neither acetate nor formate was found to restore the proper course of the reconstitution reaction. In the O$_2$ activation of wild-type apo-protein R2, no other intermediate paramagnetic species than intermediate X has been detected (14, 15, 29–30). In the reconstitution reaction of E238A with azide present, a paramagnetic species $X^A$ with a singlet EPR spectrum very similar to that of interme-

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**Table III**

| Temperature (K) | $P_{1/2}$ (mW) | $b$ |
|-----------------|---------------|-----|
| 10              | 0.07          | 0.83 |
| 25              | 0.30          | 0.74 |
| 35              | 3.07          | 0.76 |
| 40              | 19.33         | 0.92 |

$P_{1/2}$ and $b$ values were estimated from computer fitting of microwave saturation curves.

can be seen from Fig. 4a the signal is clearly composed of two components, in agreement with the observations by $9.6$ GHz EPR (compare with Fig. 1c). The signal can be explained as a superposition of a tyrosyl radical and intermediate $X^A$. The three $g$-value components of the tyrosyl radical are well resolved and almost identical to those of the radical found in wild-type E. coli R2 recorded under identical conditions (Fig. 4b, Table IV). Using the known $g$-values of the tyrosyl radical as an internal calibration standard, two of the $g$-value components of intermediate $X^A$ were determined to be $g_2 = 1.998$ and $g_3 = 1.993$. These values should be compared with what has been reported for the intermediate X from $36$ GHz studies of the E. coli R2 mutant Y122F (Table V) (15). The $g_2$ and $g_3$ components are close to identical in the two systems. The $g_1$ value of $2.007$ reported in Y122F may correspond to a weak feature at $2.006$ indicated in Fig. 4a. However, a $g_1$ component may also be hidden below the $g_2$ component of the tyrosyl radical.

**Structure of the Azide-complexed E238A/Y122F Mutant**—The crystal structure of the azide-complexed diferrous E238A/Y122F R2 mutant was determined. Extrapolating from previous experiences from structural studies of Y122F mutant R2 proteins, we expect the structure of the E238A/Y122F double mutant to correspond closely to the one of the single mutant E238A, which did not yield suitable crystals at the time of the study.

After crystallization, the crystals were reacted first with ferrous iron and then with $500$ mM sodium azide under reducing conditions, whereupon the crystals were rapidly frozen in liquid nitrogen. The X-ray data were collected at $100$ K. The structure was solved by molecular replacement, based on the refined structure of the reduced wild-type protein R2 (12). After the initial rigid body refinement, the Glu-238 and Tyr-122 of the starting model were converted to an alanine and a phenylalanine, respectively. A clear positive density appeared in the fo-fc maps at the iron site in the A subunit. This iron site of the R2 dimer has previously been shown to be more accessible for exogenous ligands than the iron site in the B subunit in this crystal form (13). An azide molecule was modeled into the density and included in the subsequent refinements. The bond lengths and topology of the azide molecule were taken from the literature and restrained accordingly during refinement. Table VI shows the data and refinement statistics. In the final model, differences were observed in the coordination of the Fe ligands Asp-84 and Glu-204 of the B subunit, as compared with the reduced E238A/Y122F structure without azide reported in (20). To confirm these differences omit maps were calculated for these residues and the azide molecule (Fig. 5a). Fig. 5b shows the coordination distances of the azide-complexed diferrous site determined here. Compared with the reduced E238A/Y122F structure without azide (20), Fig. 5c, the major differences are a more pronounced octahedral geometry around Fe2 with shorter metal-ligand distances and one free coordination position in the azide complex. Fe1 becomes 4-coordinate by a carboxylate shift of Asp-84 to a monodentate ligation mode. The azide replaces the coordination position of the bridging hydroxide in the reduced E238A/Y122F structure, albeit with shorter coordination distances. Hence the site has one 4-coordinate and one 5-coordinate Fe ion whereas both Fe ions are 5-coordinate in the reduced E238A/Y122F structure without azide reported in (20). The azide may also be hidden below the $g_2$ component of the tyrosyl radical.

**FIG. 3. EPR properties of intermediate $X^A$. a, EPR microwave power saturation curves of the intermediate $X^A$ from E. coli R2 E238A azide complex in the temperature range 10–40 K. The reconstitution reaction was quenched after 3 s at 5 °C. Continuous traces are computer fits as described in the text. 10 K (○); 25 K (●); 35 K (△); 40 K (▲). b, microwave power at half-saturation, $P_{1/2}$, as a function of temperature for intermediate $X^A$ from protein R2 E238A (●) and intermediate X from protein R2 Y122F (○) (10, 36).**

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2. P. Nordlund, unpublished observations.
Intermediate XA however behaves kinetically different from X, particularly in its slow conversion to the tyrosyl radical. After long reaction times (25–90 s) a transient doublet EPR signal dominates the EPR spectrum, which is similar to but not identical to the signal of the tyrosyl radical from wild-type protein R2 recorded under identical conditions (Fig. 2). The small spectral difference in the major splitting of the 9.6 GHz EPR spectrum of the radical from wild-type protein R2 and protein E238A could easily be explained by minor differences in the $\beta$ dihedral angles, caused by the different protein environments (34). The tyrosyl radical in protein R2 E238A is not as stable as the one in the wild-type protein, but has essentially decayed after 10 min. A possible explanation for the difference in stability is that Glu-238 is in close vicinity to the hydrophobic pocket, which has been shown to be important for the stability of the tyrosyl radical (4, 5, 12, 35). The structural disturbance caused by the mutation may make the pocket more accessible to the external solvent.

The microwave relaxation behavior of a sample dominated by intermediate XA yields the microwave power at half-saturation, $P_{1/2}$, at different temperatures (Table IV and Fig. 5b). Earlier microwave saturation studies of intermediate X from wild-type and mutant Y122F protein R2 gave $P_{1/2}$ = 0.02 milliwatts at 5 K, 0.2 milliwatts at 20 K, and 3.0 milliwatts at 29 K (10, 36), also shown in Fig. 3b. The observed $P_{1/2}$ values and their temperature dependence coincide closely for intermediates XA and X.

The 285-GHz EPR results were used to characterize the g-value tensors of the composite EPR spectra of XA and the tyrosyl radical obtained after a 10-s reaction time (Fig. 4a). The results support the assignment of the two paramagnetic species in R2 E238A to the corresponding wild-type species. What is remarkable however, is the fact that we were able to detect a signal at all from an X-like intermediate at this high field. Many earlier attempts to use 285 GHz EPR to investigate high-valent iron intermediates have failed3, most likely because of heterogeneities in the g-tensors (so called g-strain). This observation, taken together with the rather non-metal-like relaxation behavior and temperature dependence of the 9.6 GHz EPR signal, renews the question of the nature of intermediate X and XA (see the original proposal of a diferric radical intermediate by Stubbe et al. in Ref. 14).

In Scheme 1 there is an additional alternative description of X and XA with the unpaired spin of this $S = 1/2$ species residing on the hydroxyl ligand of Fe1 making it a hydroxyl radical. In fact, there may be a distribution of spin density over the whole

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3 A. Graslund, unpublished observations.
Table VI

Data collection and refinement statistics for azide-complexed E238A/Y122F

| Cell parameters | a (Å) | 73.9 |
|                | b (Å) | 84.9 |
|                | c (Å) | 114.0 |
| Space group | P2$_1$2$_1$2$_1$ |
| Resolution (Å) | 17–2.2 |
| No. of observations | 123580 |
| Unique reflns | 36078 |
| $R_{merge}^a$ | 0.123 |
| Completeness (%) | 97.3 |
| Refinement | Non-H atoms | 5925 |
| $R_p$ | 0.182 |
| Reflns used | 34260 |
| $R_{merge}^c$ | 0.256 |
| Reflns used | 1798 |
| $R_{cryst}$ (all data)$^b$ | 0.180 |
| Mean B factor (Å$^2$) | 35.4 |
| bond length (Å) | 0.019 |
| bond angles (deg) | 2.1 |

$^a$ $R_{merge} = \sum_h \sum_j |I_{hj} - I_{hj}^c|/\sum_h \sum_j I_{hj}$, where $I_{hj}$ is the jth observation of reflection h.

$^b$ $R_{cryst} = \sum_i |F_{obs,i} - F_{calc,i}|/\sum_i F_{obs,i}$, where $F_{obs}$ and $F_{calc}$ are the observed and calculated structure factor amplitudes, respectively.

$^c$ $R_{merge}$ is equivalent to $R_{cryst}$ for a 5% subset of reflections not used in the refinement.

diron site with the two “extremes” being full localization on either OH or Fe2 as indicated in the schemes. A dominant part of the unpaired spins residing on OH$^+$ might explain its probable role as a reactive group that accepts the H$^+$ from tyrosine when the radical is formed. In mouse protein R2, where the H bond to the radical remains after radical formation, the corresponding water ligand to Fe1 has been assigned as the H bonding partner to the tyrosyl radical (37) in agreement with this proposal.

Structure of the Azide-complexed E238A/Y122F Mutant—
The induction of a productive reaction path for tyrosyl radical generation when azide is added to the E238A protein, but not when other small potential ligands are added, shows a distinct role for azide in promoting the reaction. The structural study of R2 E238A/Y122F suggests that this effect is caused by a direct coordination of azide to the iron site. It has previously been shown that azide binding to the “wild-type-like” diferrous center of the F208A/Y122F protein can induce a conformational change of Glu-238 to a bridging-chelating mode (13). In this structure Fe1 is 4-coordinate and Fe2 octahedrally 6-coordinate with one of the coordination positions occupied by the azide molecule. The F208A/Y122F mutant was used in that azide binding study because in contrast to the wild-type protein it yielded a sufficiently high azide occupancy to allow crystallographic characterization. It was proposed that the coordination of azide in the F208A/Y122F structure mimics O$_2$ binding in the wild-type enzyme, which served as the basis for outlining a novel reaction mechanism (13).

In the present study we have solved the crystal structure of the reduced form of the double mutant E238A/Y122F soaked in azide. As previously explained, we expect the structure of the E238A mutant to be very similar to that of the E238A/Y122F mutant. The structure of the E238A/Y122F mutant in the absence of azide has previously been determined both in its reduced and oxidized states (20). In the reduced form without azide, the Fe ions are bridged by a hydroxide ion taking the position of the missing Glu-238 (see Fig. 5c). The E238A/Y122F mutant is still capable of O$_2$ activation where one possible outcome of the reaction is a self-hydroxylation of the nearby Phe-208 residue, indicating the importance of Glu-238 for controlling the O$_2$ activation process (20). In the azide-complexed form of the E238A/Y122F mutant determined here the azide molecule replaces the bridging hydroxide and binds at the same position but with shorter Fe coordination distances. Azide binding also induces a carboxylate shift of Asp-84, which becomes monodentate and leaves Fe1 4-coordinate (Figs. 5, b and c; 6).

Geometry of Radical Generation in Azide-complexed E238A—
Azide binding to the reduced iron site of the E238A mutant confers the ability of controlling oxygen activation, so that it results in a high-valent diron complex followed by tyrosyl radical formation. A possible explanation for this observation is that the azide molecule and the extra coordinating oxygen provided by Glu-204 mimic the bridging-chelating coordination of Glu-238 previously proposed to be required for a productive .
O₂ reaction in the wild-type protein (Scheme 1a). This asymmetric conformation of Glu-238 was proposed to be retained throughout the reaction and control the O₂ activation process in the wild-type enzyme (13). The shift induced by azide binding in the conformation of Asp-84, making it monodentate, makes Fe1 4-coordinate with two free coordination positions. These two positions are the same positions proposed to accommodate the terminal hydroxo ligand and the oxo-bridge in the wild-type mechanism. Taken together, these effects of azide binding induce a conformation of the metal center that leaves three coordination positions open, which are the correct ones to be used in O₂ activation and radical generation.

Alternative mechanisms for diiron-based O₂ activation have been proposed (reviewed in Ref. 39). One of the mechanisms assumes the formation of a symmetric bis-µ-oxo “diamond core” structure as an essential intermediate in the reaction. The bridging coordination of an azide, as observed in the E238A/Y122F structure, however, does not appear to be favorable for promoting such a bis-µ-oxo “diamond core” structure, because it limits the number of potential bridging positions. We therefore favor the reaction mechanism based on an asymmetric coordination geometry of the iron site as previously proposed (13).

Scheme 1, a and b shows the proposed analogous reaction pathways for tyrosyl radical formation in the wild-type protein and in the azide complex of the E238A mutant, respectively. After oxygen binding to the open coordination site on Fe2, only two free coordination positions are available on Fe1 to accommodate the products of oxygen cleavage. The fact that no other free positions are available seems to be the key feature for the controlled activation of oxygen. The close correspondence between observed structures and reaction intermediates in the wild-type and E238A/azide R2 proteins supports the proposed reaction geometry.

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Restoring Proper Radical Generation by Azide Binding to the Iron Site of the E238A Mutant R2 Protein of Ribonucleotide Reductase from *Escherichia coli*

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