Reconstitution of the tyrosyl radical in ribonucleotide reductase protein R2 requires oxidation of a dipherous site by oxygen. The reaction involves one externally supplied electron in addition to the three electrons provided by oxidation of the Tyr-122 side chain and formation of the μ-oxo-bridged dipherous site. Reconstitution of R2 protein Y122F, lacking the internal pathway involving Tyr-122, earlier identified two radical intermediates at Trp-107 and Trp-111 in the vicinity of the di-iron site, suggesting a novel internal transfer pathway (Sahlin, M., Lassmann, G., Pötsch, S., Sjöberg, B.-M., and Gräsland, A. (1995) J. Biol. Chem. 270, 12361–12372). Here, we report the construction of the double mutant W107Y/Y122F and its three-dimensional structure and demonstrate that the tyrosine Tyr-107 can harbor a transient, neutral radical (Tyr-107). The Tyr-107 signal exhibits the hyperfine structure of a quintet with coupling constants of 1.3 mT for one β-methylene proton and 0.75 mT for each of the 3 and 5 hydrogens of the phenyl ring. Rapid freeze quench kinetics of EPR-visible radicals in Y122F show a hyperfine structure at very short lived and is no longer detectable because of the formation kinetics of the stable tyrosyl radical in wild type R2 suggests that these protein-linked radicals are substitutes for the missing Tyr-122. However, in contrast to Tyr-122 these radicals lack a direct connection to the radical transfer pathway utilized during catalysis.

Three classes of ribonucleotide reductases can be distinguished on the basis of their composition and cofactor requirements, but they have in common the generation of protein-linked radicals during reduction of ribonucleotides to the corresponding deoxyribonucleotides (1, 2). The class I ribonucleotide reductase of Escherichia coli consists of two homodimeric proteins denoted R1 and R2. The larger R1 protein contains the catalytic site with redox-active cysteines, whereas the function of the smaller R2 is to store a stable tyrosyl radical (Tyr-122) in E. coli that is essential for catalysis (3). Tyr-122 is formed by oxidation of an adjacent iron site and stabilized by the resulting μ-oxo-bridged dipherous center. Tyr-122 is located 10 Å from the surface and 5.3 Å from the closest iron (4).

Reduction of substrate by ribonucleotide reductase is proposed to happen via a radical mechanism involving the formation of a thylial radical at cysteine 439 (E. coli numbering) at the substrate binding site in R1. This raises the question how a radical is transferred from Tyr-122 in R2 to Cys-439 in R1 and vice versa. By means of separately solved crystal structures of R1 and R2 and site-directed mutagenesis experiments, an electron transfer pathway, of about 35 Å in length, has been suggested, leading from the assembled cofactor in R2 to the active site in R1 (5). We prefer to call it a radical transfer pathway (RTP), i.e. transfer of a hydrogen atom (radical), as the pathway is hydrogen-bonded, and all radicals hitherto observed in protein R2 are of the oxidized, neutral form (6–9). The participating amino acids are conserved among all class I ribonucleotide reductase species described so far. The radical transfer in R2 is presumed to occur via Trp-48, situated on the R2 protein surface, the hydrogen-bonded Asp-237 and His-118, a ligand of Fe-1 (see Fig. 1). In the mobile carboxyl-terminal part of R2, which interacts with R1, the invariant residues Tyr-356 and possibly Glu-350 might connect Trp-48 in R2 with Tyr-731 in R1, which is linked via Tyr-730 to Cys-439 in the active site (3, 10).

ApoR2, lacking both iron center and tyrosyl radical, can be obtained either by reducing the radical and chelating the iron or by growing the overproducing bacteria in iron-depleted medium (11, 12). The iron center and the tyrosyl radical assemble spontaneously in a reconstitution process involving apoprotein R2, ferrous iron, and molecular oxygen (Equation 1).
apoR2-Tyr-122 + 2Fe^{2+} + H^{+} + e^{-} + O_{2} \rightarrow Fe^{3+}O^{2-} - Fe^{3+} - R2-Tyr-122^{+} + H_{2}O \quad (Eq. 1)

Radical Transfer in Protein R2 W107Y/Y122F

The reduction of oxygen to water requires four electrons. Two of them are provided by the two Fe^{2+} forming the dipheric center and one by Tyr-122 forming the tyrosyl radical, and, at least in vitro, the fourth electron can be supplied by external reductants such as Fe^{2+} or ascorbate (13, 14). The reconstitution reaction thus provides a possible trap to trap and characterize radical intermediates, and one Fe^{3+}-Fe^{2+} intermediate and several other intermediates have been observed previously, generation of the oxidized di-iron site in wild type and mutant protein (9, 14–18). In addition, recent kinetic investigations on wild type and mutant mouse R2 support the use of the RTP for bringing an external electron/H^{+} pair.

Mutation of residue Tyr-122 to the non-oxidizable residue phenylalanine causes a deficit of one electron in the reconstitution reaction which consequently must be supplied from elsewhere. Although it is possible that one more electron could be delivered via the catalytically essential RTP described above, it has been found that it may come at least to some percentage from other oxidizable amino acids in the vicinity of the iron center that are part of the hydrogen-bonded network of the iron ligands. Transient radicals have been assigned to the non-conserved tryptophans 107 and 111 based on their EPR and electron nuclear double resonance characteristics and molecular geometry (9, 15, 20). The conserved Trp-111 is at 4 Å distance from Fe-2 and hydrogen-bonded via a water molecule to the backbone of Fe-2 ligand His-241 (Fig. 1). The transient neutral Trp-107 was observed as a room temperature doublet EPR signal indicating relatively weak interaction with the iron center (9, 20). Trp-111 is at 4 Å distance from Fe-2 and hydrogen-bonded via N-1 to Fe-2 ligand Glu-204 (Fig. 1). The transient Trp-111 was observed as a low temperature (77 K) EPR quartet with a weakly coupled proton and relatively strong interaction with the iron center (9, 20).

In this report we have determined the three-dimensional structure of the double mutant W107Y/Y122F and compared transient radical kinetics in this double mutant with the single mutant Y122F during the reconstitution reaction. From the results we propose a pathway through which an electron/proton pair is delivered by the oxidizable amino acids surrounding the iron site.

EXPERIMENTAL PROCEDURES

[HFeβ]-Tyrosine was prepared as described elsewhere (23, 24).

MutaGene of W107Y/Y122F—The oligonucleotide used for mutagenesis (mismatches are underlined) W107Y d(5'-CTGTTTACCAGAAAACC-TACGTCCGAAC-3') was synthesized and purified by Scandinavian Gene Synthesis AB. The start plasmid was the wild type R2 protein without the N-terminal Met-Arp.

The mutant proteins Y122F and W107Y/Y122F were purified as described earlier (29). Apo forms of Y122F and W107Y/Y122F were obtained by growing E. coli in iron-depleted medium as described in Ref. 12 but modified slightly by omitting the iron chelation procedure. Protein preparations were highly purified by using Cm and Hg ions in the final step. The purity of the preparation was determined by polyacrylamide gel electrophoresis.

Protein Purification—The mutant R2 proteins Y122F and W107Y/Y122F were purified as described earlier (29). Apo forms of Y122F and W107Y/Y122F were obtained by growing E. coli in iron-depleted medium as described in Ref. 12 but modified slightly by omitting the iron chelation procedure. Protein preparations were highly purified by using Cm and Hg ions in the final step. The purity of the preparation was determined by polyacrylamide gel electrophoresis.

Cryocrystallization of W107Y/Y122F Protein and Data Collection—Crystals of R2 mutant Y122F/W107Y were grown as described previously (31). A crystal measuring approximately 1.0 × 0.3 × 0.3 mm was transferred from crystallization mother liquor containing 20% PEG 4000, 0.2 mM NaCl, 1 mM ethyl mercurichloride, and 0.1 mM MES buffer, pH 6.0, to an almost identical solution containing 24% PEG 4000 and an additional 20% glycerol as cryoprotectant. After less than 1 min in this solution, the crystal was flash frozen in a stream of liquid nitrogen at ~170 °C and maintained at ~160 °C for the duration of the data collection.

The crystal diffracted to 1.9 Å on a rotating anode x-ray source. Diffraction data were collected at two points, exploiting the length of the crystal. The data set collected was 91% complete to 1.9 Å with Rmerge(I) = 0.092. In the highest resolution shell (1.98–1.95 Å) the data were 77% complete, with Rmerge(I) = 0.054. The average I(1) was 13.2, in the highest resolution shell 2.4. Reflection intensities were integrated using Denzo (32) and reduced using Scalepack (32).

Refinement—The starting model for refinement was the structure of reduced R2 determined at 100 K (33). The occupancies of the side chains of residues Tyr-122 and Trp-107 were set to zero before beginning refinement. The program TNT was used for all refinement (34). A cross-validated R factor (35) was calculated on 5% of the data (2418 reflections). The initial model had R_{model} = 0.215 and R_{data} = 0.254. The final model contained 2576 protein atoms, 267 water molecules, 4 Fe atoms, and 14 partially occupied Hg atoms. The model was refined to R_{model} = 0.207 and R_{data} = 0.275.

Molecular Simulations—Molecular simulations (Molecular Simulations Inc., Burlington, MA) were used for this and other model building operations.

After 15 cycles, clear difference and 2F_{o} - F_{c} density was seen for a phenylalanine at position 122 and a tyrosine at position 107 of both monomers. These were refined as such from that point. The refined B factors of all four iron atoms were higher than usual with respect to their surroundings, but more normal values could be obtained by setting their occupancy to 0.8. After 31 cycles, the reflections used for cross-validation were returned to the refinement and a further 7 cycles gave R_{model} = 0.208. The model contains 5576 protein atoms, 267 water molecules, 4 Fe atoms, and 14 partially occupied Hg atoms and has good geometry: r.m.s. deviations from the ideal bond lengths and angles of C back and H atoms (36) are 0.01 Å for bonds, 1.0° for angles, and 16.9° for torsion angles. All residues are in allowed regions of the Ramachandran plot.

Kinetics of Reactivation of ApoR2 with Fe^{2+} Ions and O_{2}—All kinetic reactions were performed at 25 °C by rapidly mixing equal volumes of an aerobic solution of apoR2 in 50 mM Tris-HCl, pH 7.6, with an anaerobic solution of (NH_{4})_{2}Fe(SO_{4})_{2} dissolved in 50 mM Tris-HCl, pH 7.6, to keep the protein in an aerated state. The substrate was used 80–100 μM apoR2 protein and 320–400 μM Fe^{2+}. The protein concentration was determined by absorbance at 280 nm (ε_{280} = 120 mM^{-1} cm^{-1}) using a Perkin-Elmer Lambda 2 spectrophotometer. The iron content after reconstitution was measured colorimetrically (30) after removing the unspecifically bound iron by desalting the R2 protein on a G-25 column.

Rapid freeze quenching (System 1000, Update Instrument) was used.

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to obtain time points from 700 ms to 2 s. The solutions were mixed rapidly and sprayed into a −120 °C isopentane bath to quench the reaction. The resulting crystals were packed into an EPR tube. Longer reaction times (5 s to minutes) were achieved by mixing the solutions directly in the EPR tube and freezing them in isopentane at −120 °C.

For kinetics at room temperature (20 °C, not thermostated) the EPR spectrometer was coupled to a stopped flow accessory as described previously (37). The transient species was detected by starting a rapid scan after stopping the flow. The field was adjusted at the maximum of the first derivative line of a transient to measure the kinetics. The dead time is about 10 ms and a baseline recorded under exactly the same conditions was subtracted.

EPR spectra at 9 GHz were recorded on a Bruker ESP 300E spectrometer at 8 K equipped with an Oxford Instrument liquid helium cryostat or at 77 K using a cold finger Dewar. Spin quantification was performed with GraFit 3.01® including an intermediate of a consecutive reaction using GraFit 3.01® including a "lag time."

\[
F(t) = \frac{[Trp]}{[R2]} \left( e^{-k_1t} - (e^{-k_2t} - e^{-k_1t}) \right) \quad \text{(Eq. 2)}
\]

\( F(t) \) is the time-dependent ratio of radical to R2 dimer. \( [\text{Trp}]/[\text{R2}] \) is the maximal yield of the intermediate; \( k_1 \) is rate of formation; \( k_2 \) is the rate of decay, and \( dt \) the lag time.

RESULTS

Crystal Structure—The structure of W107Y/Y122F is generally well resolved at high resolution (1.95 Å) with good geometry (Fig. 1B). The electron density around residues 122 and 107 of both monomers (Fig. 2) establishes unambiguously that the mutations to phenylalanine and tyrosine have occurred. Tyrosine 107 has almost the same geometrical arrangement as tryptophan 107 in the wild type protein. The dihedral angles, \( \theta_{111} \), of the \( \beta \)-methylene protons as defined by the orientation to the \( p_z \) axis of tyrosine 107 (perpendicular to the ring plane) are 40 and 80°. Two \( \mu \)-oxo-bridged di-iron sites are observed. However, the iron atoms do not appear to be fully occupied, and a rough estimate of their occupancy based on crystallographic B factors is 0.8. Perhaps for this reason the density is poor for two of the coordinating side chains in both monomers, namely Glu-238 and Glu-204. However, Glu-204 clearly has monodentate coordination, and since analysis of the radical transfer pathway presented herein does not depend on the coordination mode of Glu-238, we leave further analysis of the iron center for a future study.

Formation of the \( \mu \)-Oxo-bridged Diferric Center in W107Y/Y122F—Formation of the di-iron site in W107Y/Y122F was studied using light absorption. The features apparent at 325...
Radical Transfer in Protein R2 W107Y/Y122F

Fig. 3. UV/Vis spectra of the E. coli apoR2 and the reconstituted R2 protein of the mutants W107Y/Y122F and Y122F. Apo wild type R2 shows the same spectrum as the apo mutant protein (data not shown). The protein concentration was 10 μM. The apoR2 protein was reconstituted with 4 Fe/R2 in 50 mM Tris-HCl, pH 7.5, at 25 °C, and afterward the excess iron was removed with a G-25 column. Inset, stopped flow kinetic trace following the formation of the dieric center at 370 nm in W107Y/Y122F and Y122F. Inset, stopped flow kinetic trace following the formation of the dieric center at 370 nm in W107Y/Y122F and Y122F. Inset, stopped flow kinetic trace following the formation of the dieric center at 370 nm in W107Y/Y122F and Y122F. Inset, stopped flow kinetic trace following the formation of the dieric center at 370 nm in W107Y/Y122F and Y122F. Inset, stopped flow kinetic trace following the formation of the dieric center at 370 nm in W107Y/Y122F and Y122F.

and 370 nm indicate that the μ-oxo-bridged dieric center in W107Y/Y122F is properly formed (Fig. 3), and the extinction coefficients are similar to those of Y122F (25). The stability of the iron center is not affected by the additional mutation. The iron content was of the same order of magnitude (~3 Fe/R2) for W107Y/Y122F, for the single mutant Y122F, and for the wild type protein implying at most 75% di-iron sites. In both mutants the iron center is assembled spontaneously within 5 s. The observed kinetics at 370 nm are biphasic. From the inset of Fig. 3 we calculated for W107Y/Y122F a rate constant of 2.1 ± 0.2 s⁻¹ for the first phase, and 8.7 ± 0.2 s⁻¹ was determined for Y122F.

Stopped Flow and Freeze Quench EPR Studies of Transient Radicals—In Y122F we earlier assigned a transient room temperature doublet EPR signal, visible in the time regime 10 s to minutes, to Trp-107 (9). In the double mutant W107Y/Y122F this room temperature doublet is absent, confirming that this transient is indeed likely to be generated at Trp-107 in Y122F. Instead a new radical species in the time window 500 ms to 20 s is formed and decays (Fig. 4, Table I). The hyperfine splitting of this room temperature signal in W107Y/Y122F can be resolved in low temperature freeze quench studies that show a quintet spectrum with estimated hyperfine couplings from one proton of 1.3 ± 0.1 mT and from two additional protons of 0.75 ± 0.1 mT each (Fig. 5A). In Y122F a quartet signal appears in this time range which was assigned to Trp-111 (9). In Table II the characteristics of the different EPR signals found in W107Y/Y122F and Y122F are listed. The microwave power at half-saturation (P₁₀) of the quintet in W107Y/Y122F is almost 2 orders of magnitude lower than that determined for the quartet in Y122F, indicating a weaker magnetic interaction (38, 40) of the new radical species with the iron center. Evaluation of the intensity of the EPR signal showed that it is linearly dependent on the temperature (8–190 K) as expected from the Curie-Weiss law. The total spectral width defined by the spacing of the outermost peak to outermost trough was not broadened by increasing temperature. However, after annealing for 15 min at 260 K (~13 °C) followed by cooling to 8 K, the yield of the transient radical was only recovered to 60%, suggesting that this radical is not stable at higher temperatures.

In both mutants the axial EPR signal (Fig. 5C; component I in Ref. 9) tentatively assigned to a Trp-48-derived radical is present, even though it is considerably delayed in W107Y/Y122F and accumulates to only 10% of the yield found in Y122F. This radical species is long lived (~20 min). The weak magnetic interaction with the iron center would be consistent with the large distance of 8 Å between Trp-48 and the di-iron site. A similar axial line shape was reported for a tryptophan radical species weakly coupled to the heme iron in cytochrome c peroxidase (21, 22) and for a peroxyl radical observed in myoglobin treated with hydrogen peroxide (41). This supports the hypothesis that the axial signal in W107Y/Y122F or W107Y/Y122F is a tryptophan-derived radical, probably an oxygen adduct of Trp-48 or perhaps other surface-located tryptophans.

Assignment of W107Y Transient Radical by Labeling with [2H]β, β-Tyrosine—To assign the EPR quintet to a tyrosyl radical the reconstitution reaction was performed with R2 protein from cells grown in media containing [2H]β, β-tyrosine. Replacing a proton involved in EPR hyperfine coupling with deuterium causes the number of hyperfine lines to change from two to three and their spacing to decrease about 6-fold (42). In practice this is usually observed as a collapse of the line width of the EPR signal. The EPR spectrum obtained with specifically deuterated R2 W107Y/Y122F is shown in Fig. 5B. The presence of deuterated tyrosine in the mutant resulted in a significant narrowing of the hyperfine splitting and yielded a singlet alike EPR spectrum.

We performed a computer simulation of the EPR spectrum of the new tyrosyl radical (Fig. 5A). After iterations leading to a best fit to the experimental spectrum, we obtained the following parameters (hyperfine coupling constants in mT): -1.28, -0.1, -0.89 for H3, -0.87, -0.37, -1.02 for H5, and 1.69, 1.23, and 0.94 for one of the β-methylene protons, whereas the hyperfine coupling of the other one is not resolved outside the line width. The isotropic hyperfine couplings are therefore 1.28, 1.02, and 1.69 mT for H3 and H5, respectively, in reasonable agreement with the values estimated directly from the EPR spectrum. The g value was fixed to 2.0020 and the two other components of the g tensor were obtained to g = 2.0075 and 2.0050. These EPR parameters are compatible with a tyrosyl radical of the oxidized, neutral form. According to the theoretical calculations of Himo et al.,3 the simulated and experimental coupling constant with an

3 Himo, F., Gräslund, A., and Eriksson, L. A. (1997) Biophys. J. 72, in press.
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The reconstitution reaction was performed with 4 Fe/R2, in 50 mM Tris-HCl, pH 7.5, at 25 °C in mutants W107Y/Y122F, Y122F, and wild type R2 protein. ND, not determined.

TABLE I
Rate constants (k) and lag times (dt) of transient radicals formed in R2 mutant proteins

| Protein       | Type of EPR signal | Assignment | Lag time (dt in s) | Formation (k_1 in s⁻¹) | Decay (k_2 in s⁻¹) | Yield radical/R2 |
|---------------|--------------------|------------|-------------------|------------------------|-------------------|-----------------|
| W107Y/Y122F  | Quintet⁺⁺         | Tyr-107    | 0.7               | 1.4 ± 0.4              | 0.026 ± 0.004     | 0.08            |
|               | Axial              | Trpaxial   | 20 ± 4            | 0.3 ± 0.02             | 0.029 ± 0.002     |                 |
| Y122F         | Quartet⁺⁺         | Trp-111    | 0.500             | 1.4 ± 0.9              | 0.05 ± 0.01       | 0.07            |
|               | Doublet⁺⁺         | Trp-111    | 10                | 0.027                  | ~0.001            | ~0.1            |
|               | Axial              | Trpaxial   | ~2                | ND                     | ~0.001            | ~0.1            |
| Wild type     | Doublet⁺⁺         | Tyr-122    | 1.7 ± 0.3         | Stable                 | 1.2               |                 |

⁺⁺ Observation temperatures of the EPR signals are 77 K.
⁺ Confirmation at low temperature (see annealing experiment) is consistent with a relatively weak metal interaction and the observed distance of 8 Å between Tyr-107 and the iron center. These considerations strongly indicate that the new tyrosine at position 107 harbors the transient radical.

Kinetics—The transient Tyr-107⁺ observed at low temperature in rapid freeze quench samples of W107Y/Y122F showed rate constants of formation (k_1) and decay (k_2) of 1.4 and 0.03 s⁻¹, respectively, and a lag time of 0.7 s. These rate constants of Tyr-107⁺ are confirmed by stopped flow EPR at room temperature (Table I). The difference in k_1 obtained by the two methods, although still in the same range, may be caused by systematic errors. In the low temperature measurements an EPR signal of an early EPR singlet intermediate, possibly equivalent to species X (9, 14), is superimposed on the Tyr-107⁺ quintet. The amount of Tyr-107⁺ was estimated by subtraction of the quintet spectrum and inspection of the form of the residual, which should show a perfect singlet. Small amounts of the quintet are difficult to determine by this method and therefore the earliest values of Tyr-107⁺ may be judged too small. Thus, the lag time (dt) and the k_1 of Equation 2 will be increased and the values obtained may be regarded as upper limits. The amount of deuterated Tyr-107⁺ was easier to calculate by this method and gave the same results. The kinetic trace measured with EPR stopped flow at room temperature showed different start and end values. Thus the lag time of Tyr-107⁺ could not be calculated accurately, so that this formation rate can be regarded as a lower limit. Nevertheless, the decay constants, for which these problems are not significant, are the same by both methods. Taken together, the kinetic data and the results of the annealing experiment make it most likely that the transient radical species observed at 293 and 8 K are the same. Because the kinetics of Trp-111⁺ in Y122F and formation of the stable Tyr-122⁺ in wild type R2 were measured by rapid freeze quenching, we considered only the kinetic data obtained by this method.

The decay rate of 0.03 s⁻¹ for Tyr-107⁺ is faster than that for Trp-107⁺ (0.001 s⁻¹), suggesting that a tyrosyl radical is less stable than a tryptophan radical in this environment. Interestingly, the observed formation and decay rate constants for the Tyr-107⁺ transient in the double mutant are almost identical to those found for the putative Trp-111⁺ in Y122F (Fig. 6, A and B, Table I). We therefore assume a consecutive reaction forming first Trp-111⁺ and then Trp-(Tyr)107⁺ double mutant formation of Trp-111⁺ is the rate-limiting step and formation of Tyr-107⁺ is fast, whereas in Y122F the formation of Trp-111⁺ is 50-fold faster than that for Tyr-107⁺. The overall yield (given in Table I) of Tyr-107⁺ and Trp-111⁺ is about the same, with 0.07 – 0.08 unpaired spins/R2 obtained by fitting the kinetics to Equation 2.

DISCUSSION

The reconstitution reaction of R2 apoprotein with ferrous iron/O₂ yields the μ-oxo-bridged diferric center and a stable tyrosyl radical at Tyr-122. Certain aspects of this reaction may serve as a model for delivery of hydrogen radicals to the iron site in R2 that may occur during catalytic reaction. The fact...
that transient protein-linked free radicals are trapped concomitant with and subsequent to the assembly of the diferric center in Y122F (9, 15–18) indicates that if Tyr-122 is missing other radical transfer in proteins takes place via hydrogen in the near vicinity of the diferric center (9, 20). If we assume that neutral radicals can be generated at tryptophan residues EPR and electron nuclear double resonance studies revealed side chains can be oxidized to contribute an electron. Earlier work on Y122F (9, 15–18) indicates that if Tyr-122 is missing other transient protein-linked free radicals are trapped concomitant with and subsequent to the assembly of the diferric center, consistent with the distance of 8 Å from Tyr-107 to the diferric site. The Trp-107 transient in Y122F observed as an EPR doublet at room temperature disappeared as expected in the double mutant W107Y/Y122F. Together, these observations demonstrate that Tyr-107 harbors the new transient radical and thereby conclusively identify the earlier assignments of the transient tryptophan radicals in Y122F R2 protein (9, 15, 20).

The Tyr-107 EPR spectrum has a striking similarity with those of the Tyr107 of photosystem II and the tyrosyl radical of R2 from Salmonella typhimurium (7, 44). These three EPR signals have in common that only one β-methylene proton shows a detectable hyperfine splitting. However, the isotropic hyperfine splitting of 1.29 mT in Tyr-107 is somewhat larger compared with 0.83 mT in Tyr107 of photosystem II and 0.91 mT Tyr107 in R2 from S. typhimurium. The anisotropy of the hyperfine structure of these signals is caused by coupling with the 3 and 5 hydrogens of the phenyl ring and has similar coupling constants.

Our kinetic studies on the reconstitution of the Y122F and W107Y/Y122F mutant R2 proteins suggest that a hydrogen radical transfer occurs along a hydrogen-bonded pathway, combined with an electron transfer, utilizing the π-interaction of aromatic rings. This last step is most likely proton-coupled since again the observed transient radical is neutral. Fig. 1 shows the crystal structure of W107Y/Y122F in comparison with the structure of wild type R2. Trp-107 is hydrogen-bonded via a water molecule to the backbone of the Fe-2 ligand His-241, whereas Trp-111 is directly connected via a hydrogen bond to the Fe-2 ligand Glu-204. Thus, the residues Trp-107 and Trp-111 in wild type R2 and in Y122F are connected to Fe-2 in a similar fashion as are Trp-48, Asp-237, and His-118 to Fe-1 (Fig. 1) and may therefore be regarded as short distance “model RTP” of the catalytically important RTP in wild type R2.

If we first consider Y122F, two possibilities exist for the delivery of the “missing” electron from tryptophans Trp-107 and Trp-111 (Fig. 6A). The electron might be delivered in parallel from Trp-111 and from Trp-107 using their specific hydrogen bonding described above. It then follows that the kinetics and yields of both intermediates, Trp-111” and Trp-107”, may be quite different. Another possibility is that Trp-111 is a direct precursor of Trp-107”, because Trp-111 has the most direct connection to the iron center considering the number of the covalent and hydrogen bonds involved (Fig. 6A). The two tryptophan planes are stacking in a “head to tail” manner with respect to their indole rings, i.e. the pyrrole ring from one indole stacks on the phenyl ring of the other and vice versa. The distance of ~4–5 Å suggests electrostatic interactions between
them. We observed that generation of Trp-111$^\cdot$ is followed by formation of Trp-107$^\cdot$. The determined decay constant of 0.05 s$^{-1}$ for Trp-111$^\cdot$ and formation constant of 0.027 s$^{-1}$ for Trp-107$^\cdot$ and the accumulated yield of each of about 0.08 radical/R2 strongly suggest that Trp-111$^\cdot$ is the precursor of Trp-107$^\cdot$. We suggest that the radical is transferred utilizing the “stacking complex” configuration of their aromatic rings as illustrated by $k_s$ in Fig. 6A and that the route over the backbone of the ligand His-241 to the dipheric center is less efficient.

With the mutation Trp$\rightarrow$Tyr at position 107, a switch takes place from the appearance of Trp-111$^\cdot$ followed by Trp-107$^\cdot$ to the immediate observation of Tyr-107$^\cdot$. Surprisingly, the transient Trp-111$^\cdot$ signal is absent in the double mutant W107Y/Y122F, and the formation and decay constants of Tyr-107$^\cdot$ are identical to those of Trp-111$^\cdot$ measured in Y122F (Fig. 6B). The structure of the double mutant shows that Tyr-107 is still bound to His-241 via a water molecule and that the aromatic rings of the Tyr-107 and Trp-111 now form a stacking complex with the phenyl rings on the top of each other, the phenyl ring of Tyr-107 is slightly tilted (Fig. 1). Assuming that this pathway (as was also proposed for Y122F above) is valid for W107Y/Y122F, the transient Trp-111$^\cdot$ will no longer be detectable if Tyr-107$^\cdot$ is formed much faster than Trp-107$^\cdot$ in Y122F. In the double mutant the formation of Trp-111$^\cdot$ becomes the rate-determining step, and we measure the unchanged rate $k_{111}$ for the formation of Trp-111$^\cdot$ as the overall rate constant of the proposed consecutive reaction Trp-111$^\cdot$ $\rightarrow$ Trp-107. The alteration of the kinetics in W107Y/Y122F also underlines that the RTP Fe-2-E204-W111-W107 is the preferred one in the Y122F single mutant (Fig. 6A).

Our results indicate that the Fe-2-Glu-204-Trp-111-Trp-107 pathway utilizes a radical transfer, combined with a proton-coupled electron transfer via the $\pi$-interaction of the indole rings. Why is the Tyr-107 radical in the double mutant protein more rapidly formed than the corresponding tryptophan radical in Y122F? According to earlier studies on electron transfer proteins, the presence of aromatic residues facilitates electron transfer in general (45). The kinetics of the Tyr-107$^\cdot$ formation in W107Y/Y122F may reflect that phenyl rings arranged on top of each other allow a more efficient electron transfer than does the head-to-tail configuration of the tryptophans in Y122F, where the phenyl rings do not overlap. An additional explanation might be found in slightly different redox potentials of tyrosine and tryptophan. According to experiments with single amino acids in aqueous (H_2O) and hydrophobic (CH_2CN) solutions, tyrosine is easier to oxidize in a hydrophilic environment than tryptophan, whereas tryptophan is easier to oxidize in a hydrophobic environment (46). Probably the environment of the tryptophans 111 and 107 is predominantly hydrophilic because these residues are not shielded in a hydrophobic pocket like Tyr-122. Therefore Tyr-107 becomes a better candidate for oxidation than Trp-111. The more rapid decay of the tyrosyl radical is not explained by this argument. Based on the redox potentials an even slower decay of a tyrosyl radical would be expected. The rapid decay is therefore most likely due to structural differences, e.g. increased accessibility to solvent.

The radical transients formed during reconstitution in the R2 mutants Y122F and W107Y/Y122F are substitutes for the missing stable tyrosyl radical of wild type R2, and the rate constants for formation of Tyr-107$^\cdot$ and Trp-111$^\cdot$ in the mutants (1.7 s$^{-1}$ and Tyr-122$^\cdot$ in wild type R2 (1.4 s$^{-1}$) are remarkably similar under our conditions. This raises the question as to whether a tyrosine at position 107 would compete with the Tyr-122 for radical formation. A situation, where competition occurs, has been reported earlier with the mutant R2 protein F208Y. Here the introduction of a tyrosine in the near vicinity of the iron center resulted in the hydroxylation of the Tyr-208 phenyl ring instead of reconstitution instead of formation of Tyr-122$^\cdot$, and the formed catechol subsequently becomes an iron ligand (47). Our results suggest that competition might be possible in a Tyr-122-containing protein if Trp-107 or -111 is replaced by tryptophane. Note that Trp-111 is H-bonded via a carbonylate to Fe-2 in almost the same fashion as tyrosine 122 is linked to Fe-1. Analyzing the sequence homologies among the class I R2 proteins with respect to positions 107 and 111 (E. coli numbering), one finds combinations like tryptophan-tryptophan (e.g. E. coli), tyrosine-107-glutamine 111 (e.g. herpes simplex), or phenylalanine 107-glutamine 111 (e.g. mouse). Accordingly, in the Y177F mutant R2 protein from mouse (corresponding to Y122F) no transient tryptophan radicals can be observed. The combination tryptophan (tyrosine)-tyrosine is not found among any class I R2 protein so far sequenced. One might conclude that nature avoids these combinations to have no competition for the stable radical formation at the catalytically essential tyrosine site.

In class I ribonucleotide reductase a hydrogen-bonded chain of conserved amino acids is believed to transfer a radical from Tyr-122$^\cdot$ in R2 to the active site in R1 during catalysis. Site-directed mutagenesis studies have revealed the importance of intact hydrogen bonding, but kinetic evidence is still lacking for a RTP during catalysis. The geometric arrangement of Trp-111 and Tyr-107 in R2 is reminiscent of the stacking configuration of the tyrosine residues 730 and 731 in the proposed RTP in protein R1. Both these tyrosine side chains in R1 are essential for catalysis: exchange of either of them to phenylalanine (Y730F or Y731F) results in an inactive enzyme, even though the positioning of the phenyl rings is unchanged (48). The present results in the mutant R2 protein, yet catalytically inactive, might model the RTP processes occurring between the R1 and R2 proteins during catalysis. Our kinetic and EPR results suggest that during reconstitution of Y122F and W107Y/Y122F a hydrogen radical is abstracted from Tyr(Trp)-107 via Trp-111 and Glu-204 by the iron site. This consecutive reaction of radical abstraction might resemble a consecutive radical transport from the active site in R1 to Tyr-122$^\cdot$ in R2. In addition it presents the first kinetic evidence for the transfer of a radical (electron/proton) to the iron site.

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Kinetics of Transient Radicals in *Escherichia coli* Ribonucleotide Reductase: FORMATION OF A NEW TYROSYL RADICAL IN MUTANT PROTEIN R2

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