Kinetic Evidence That a Radical Transfer Pathway in Protein R2 of Mouse Ribonucleotide Reductase Is Involved in Generation of the Tyrosyl Free Radical*

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Class I ribonucleotide reductases consist of two subunits, R1 and R2. The active site is located in R1; active R2 contains a diferric center and a tyrosyl free radical (Tyr'), both essential for enzymatic activity. The proposed mechanism for the enzymatic reaction includes the transport of a reducing equivalent, i.e. electron or hydrogen radical, across a 35 Å distance between Tyr' in R2 and the active site in R1, which are connected by a hydrogen-bonded chain of conserved, catalytically essential amino acid residues. Asp266 and Trp103 in mouse R2 are part of this radical transfer pathway. The diferric/Tyr' site in R2 is reconstituted spontaneously by mixing iron-free apoR2 with Fe(II) and O2. The reconstitution reaction requires the delivery of an external reducing equivalent to form the diferric/Tyr' site. Reconstitution kinetics were investigated in mouse apo-wild type R2 and the three mutants D266A, W103Y, and W103F by rapid freeze-quenched electron paramagnetic resonance with ≥4 Fe(II)/R2 at various reaction temperatures. The kinetics of Tyr' formation in D266A and W103Y is on average 20 times slower than in wild type R2. More strikingly, Tyr' formation is completely suppressed in W103F. No change in the reconstitution kinetics was found starting from Fe(II)-preloaded proteins, which shows that the mutations do not affect the rate of iron binding. Our results are consistent with a reaction mechanism using Asp266 and Trp103 for delivery of the external reducing equivalent. Further, the results with W103F suggest that an intact hydrogen-bonded chain is crucial for the reaction, indicating that the external reducing equivalent is a H'. Finally, the formation of Tyr' is not the slowest step of the reaction as it is in Escherichia coli R2, consistent with a stronger interaction between Tyr' and the iron center in mouse R2. A new electron paramagnetic resonance visible intermediate named mouse X, strikingly similar to species X found in E. coli R2, was detected only in small amounts under certain conditions. We propose that it may be an intermediate in a side reaction leading to a diferric center without forming the neighboring Tyr'.

The three established classes of ribonucleotide reductases catalyze the de novo synthesis of all four deoxyribonucleotides, the building blocks of DNA (1). The iron-containing class I is the one best characterized (2–4) and has been found e.g. in mammalian cells, some prokaryotes such as Escherichia coli and Salmonella typhimurium, and is coded for by DNA viruses of the herpes group. The common protein structure of class I ribonucleotide reductase is αβ2, i.e. it is composed of two homodimeric proteins, R1 and R2. Protein R2 contains a diferric iron center and a stable free radical on a tyrosine residue necessary for enzymatic activity, whereas protein R1 contains the substrate binding site. The sequence homology in both proteins is >90% between human, mouse, and hamster ribonucleotide reductases, but only about 25% between mouse and E. coli (3). Nevertheless, the general enzymatic properties are almost the same.

The three-dimensional structures of R1 and R2 from E. coli have been determined, and the holoenzyme has been model-built (5–7). In the model, the radical-forming residue Tyr-122 is situated in R2 in a hydrophobic pocket close to the μ-oxo-bridged diiron center, about 35 Å distant from the active site in R1. A long range electron transfer pathway consisting of conserved amino acids has been proposed to be essential for enzymatic activity of class I ribonucleotide reductases (2, 5–8). Recently, mouse R2 protein was crystallized, and its three-dimensional structure was determined (9). Despite the low sequence homology between mouse and E. coli R2 (3), the overall structure and the enzymatically important features are very similar (9). The ligands of the iron center as well as the R2 part of the proposed electron transfer pathway can be superimposed. Whereas the hydrogen bonds of the Fe1 ligand His173 involved in the proposed electron transfer pathway are the same as in E. coli R2, His270, a ligand of Fe2, has fewer hydrogen bonds and is, therefore, less constrained in mouse R2.

The electron transfer pathway was suggested to consist of the conserved amino acids (Fe1) His173-Asp266-Trp103-Tyr370 in R2 and Tyr738-Tyr737-Cys729 in R1 (mouse numbering) forming a hydrogen-bonded chain between the iron center in R2 and the active site in R1. Mutation of D266A and W103(Y/F) in mouse R2 leads to a complete loss of enzymatic activity (10), as is the case for the corresponding mutations in E. coli R2. The mutant mouse R2 proteins have been well characterized (10) and are identical to wild type R2 regarding secondary structure probed by CD spectroscopy (W103F) and the association/dissociation kinetics and binding constant for R1. Additionally, the unchanged Mn2+ binding constants, as a probe for Fe2+ binding, and the UV-visible spectra strongly indicate a stoichiometric iron binding in all mutant R2 proteins, including W103F (10).

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Reconstitution of active R2 can easily be achieved by mixing apoR2 with ferrous iron and oxygen (11–14). Four electrons reduce the molecular oxygen: one is delivered from the tyrosine residue, and two are supplied from the ferrous iron forming the diferric center. An external electron has to come from a third iron under our conditions. In wild type mouse and E. coli R2 (11–15) as well as in the mouse mutants D266A and W103Y (10), similar yields of about 1 tyrosyl free radical (Tyr$^\cdot$) per R2 ($\approx$60.5) were achieved by various similar reconstitution procedures. However, the low amount of about 0.01 Tyr$^\cdot$/R2 in apoW103F could not be increased by reconstitution with Fe$^{2+}$ (10).

The kinetics of the reconstitution reaction of E. coli R2 with Fe$^{2+}$/R2 has been studied intensively (13–15). Rapid freeze-quench (RFQ)-EPR and Mössbauer studies at 5 °C yielded an EPR- and Mössbauer-visible transient, denoted species X, that was kinetically competent to be a direct precursor of Tyr$^\cdot$ (14,15). Starting from aerobic apoR2, the formation rate of species X was 8 s$^{-1}$ (14), whereas preloading of the anaerobic R2 with ferrous iron yielded about 60 s$^{-1}$ (15), suggesting that iron binding to the aerobic R2 is the second slowest step in R2 reconstitution (Scheme 1). The slowest step in both procedures was found to be the formation of the stable Tyr$^\cdot$, with an unchanged rate of 1 s$^{-1}$. Mössbauer studies revealed no other iron center intermediates beside species X starting from apoR2 (14), whereas in the studies with preloaded R2, 10–20% of the iron showed unusual isomer shifts (15), possibly reflecting small amounts of accumulated iron center intermediates appearing before species X (e.g. peroxo or diferryl as proposed in Scheme 1). Our results on the reconstitution kinetics of mouse R2 proteins as well as the earlier findings in E. coli wild type R2 can be summarized in the reaction pathway sketched in Scheme 1.

Here, we have investigated the kinetics of the reconstitution reaction in wild type mouse R2 protein and in three mutants (D266A, W103Y, and W103F) on the R2 part of the proposed electron transfer pathway, thus probing its role in the reconstitution reaction. Our kinetic results show that the external electron is delivered via the proposed electron transfer pathway, and give strong evidence for the participation of an intact hydrogen-bonded chain in the reconstitution reaction. To explain our findings, we propose a novel reaction scheme consisting of the delivery of electron/proton pairs, i.e. hydrogen radicals, to the iron site. Theoretical calculations (16) have recently shown that movement of an uncharged H$^\cdot$ in a hydrogen-bonded system is energetically favorable compared with electron transfer, which involves charge separation. Thus we will refer to the electron transfer pathway as radical transfer pathway (RTP).

**EXPERIMENTAL PROCEDURES**

All experiments were done in 50 mM Tris/HCl buffer, pH 7.5, 100 mM KCl with a ratio of ferrous iron to protein R2 dimer of 4 or 6.

**Protein Purification**—The mouse proteins were overexpressed in logarithmically growing BL21(DE3) pLysS bacteria. They contained pETR2 plasmids encoding wild type mouse R2 protein (11) or the mutant mouse R2 proteins D266A, W103Y and W103F (10). The mut-
tant proteins were purified according to Rova et al. (10). Wild type protein was purified as in Mann et al. (11) with slight modifications. Grinding with aluminum oxide could be omitted due to the plgysS strain. Mutant and wild type mouse R2 were mainly obtained as apo protein. If necessary Tyr and the iron center in wild type mouse R2 were thoroughly degassed and subsequence through a Q-25 column. Fractionation yielded a protein pool with less than 10% N-terminal degraded protein monitored by SDS-polyacrylamide gel electrophoresis, whereas up to 50% N-terminal degraded protein were detected in a second protein pool. Reconstitution kinetics from both protein pools under identical conditions exhibited no significant difference (data not shown). E. coli apoproteins were prepared as described for wild type in (17). E. coli apoproteins were produced by growing cells in iron-depleted medium (18).

Preparation of the Anaerobic Fe²⁺ Solution—About 150 ml of buffer was thoroughly degassed in a septum-sealed 250-ml bulb for several h. A second bulb containing 5 to 15 mg of Mohr’s salt crystals (NH₄)₂Fe(SO₄)₂ was sealed with a septum and evacuated. The bulbs were repeatedly flushed with oxygen-free argon (L’air liquide, Alphagaz, Sorbal 500) and evacuated. The bulbs were left under a slight excess pressure of argon. A 60-ml plastic syringe was made anaerobic by washing twice with argon gas and twice partly filled with degassed buffer and argon gas. Then the appropriate volume of anaerobic buffer to prepare the desired iron concentration was transferred into the bulb containing the crystals.

RFQ—Rapid freeze–quench (19) was performed with a System 1000 apparatus from Update Instruments by mixing 1:1 aerobic protein solution (usually 100 μM) and anaerobic Fe²⁺ solution (400–600 μM). An EPR tube connected with a funnel fixed in a home-built holder was immersed completely into an isopentane bath. The stirred isopentane in the bath was cooled by a steady flow of gaseous N₂ which in turn was cooled by liquid N₂. For sample preparation stirring was switched off to avoid movement of the funnel and to allow its proper placement beneath the nozzle. Funnels were made from tips for 5-ml Gilmet pipetman by cutting off the small end and widening it.

One syringe of the System 1000 apparatus was washed twice with the anaerobic Fe²⁺ solution and then filled for experiments. The stability of both anaerobic Fe²⁺ solutions in the bulb and in the syringe was tested by transferring them to anaerobic cuvetts at different resting times and measuring their UV-visible spectra. Comparisons with a spectrum of freshly prepared Fe²⁺ and oxidized Fe³⁺ showed that in both solutions the oxidation of Fe²⁺ after 6 h was less than 10%.

The reaction mixtures were quenched by spraying into isopentane (–100 to –120 °C), and the crystals were packed to the bottom of an EPR tube with known inner diameter using a packing rod from Teflon. This comparison yielded the packing factor of about 0.5 in form of 50 l of oxygen-saturated water equilibrated at the reaction temperature. The results confirmed that this was done rapidly enough to avoid changes in the reaction temperature.

Reconstitution of Active R2 from Fe(II)-preloaded R2 Protein—To investigate if iron binding is the rate-limiting step in the reconstitution reaction or if it is affected by the mutation, both wild type and W103Y R2 proteins were preincubated with ferrous iron before oxygen addition. An EPR tube fitted with a stopcock was filled with 200 μl of protein solution (approximately 30 μM). Anaerobic protein solution was obtained by equilibration of the EPR tube with argon for 30 min on ice. Then a 3–4-fold molar excess of ferrous iron (a freshly prepared, argon-purged solution of Mohr’s salt) was added anaerobically to the protein. The mixture was then preincubated at 4 °C (wild type R2) or 16 °C (W103Y R2) for 5 min. Then a 3-fold molar excess of oxygen was added in form of 50 μl of oxygen-saturated water equilibrated at the reaction temperature. The reaction was stopped as described above (SFQ).

EPR Measurements—The EPR measurements were made at 9 GHz (X-band) on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments continuous flow cryostat for temperatures below 77 K or a cold finger Dewar for 77 K. Signals were measured at three different microwave powers to ensure that they were not saturated with microwave power. An average spin concentration was calculated by comparison with a 1 mM copper standard (12 mM H₃ClO₄, pH 1.8) or a mouse R2 standard with a well known radical content calibrated earlier against the copper standard. Composite spectra containing the signal of the stable tyrosyl radical (Tyr*) and the new EPR singlet (mouse X) were evaluated with subtracting fractions of the Tyr* spectrum of the corresponding 15-min sample using the ESP 300 software. The concentration of mouse X given by the resulting spectra was determined by comparison with the copper standard. The concentration of Tyr* in these composite spectra was calculated in two ways, from the multiplicity factor used and the Tyr* content of the 15-min samples and by the difference between total radical and mouse X concentration.

Evaluation of Kinetics—The Tyr* concentrations measured were corrected for the blank Tyr* content of the blank. The packing factor was estimated by comparing the Tyr* concentration in samples frozen directly and those that were produced by RFQ, both aged for long reaction times. The Tyr* concentrations were divided by the protein concentration corrected for the packing factor. Tyr/R2 versus time plots were first evaluated with a first order rate equation including a variable for the dead time, which varied between 13 and 30 ns. For presentation in the figures, the dead time was added to the reaction times calculated from the length of the reaction tube.

RESULTS

Formation of the Stable Tyr* in Wild Type Mouse R2—The reconstitution reaction of apo wild type mouse R2 at 5 °C with 4 or 6 Fe(II)/R2 was investigated at several temperatures between 5 and 32 °C by RFQ and SFQ methods. At 5 and 10 °C reaction temperatures none of the EPR spectra recorded between 4 and 77 K from RFQ or SFQ samples quenched from 8 ms reaction times up did reveal any EPR visible species beside Tyr*. As shown in the inset of Fig. 1, all spectra recorded have about the same shape as the final spectrum after 15 min, showing only the stable Tyr*.

Fig. 1 (filled symbols) shows the time course of the reconstitution reaction of apo wild type mouse R2 at 5 °C with 4 Fe(II)/R2. The curve obtained by RFQ samples could be fitted to the first order rate equation (1), yielding a formation rate k_form = 0.14 s⁻¹ (Table 1, Equation 1).

\[
\frac{[\text{Tyr}]}{[\text{R2}]} = \frac{[\text{Tyr}]}{[\text{R2}]}_0 \times (1 - e^{-k_{\text{form}} t})
\]  

(Eq. 1)

where \([\text{Tyr}]/[\text{R2}]_0\) are the concentration ratios at time t and at long times, respectively. Both \([\text{Tyr}]/[\text{R2}]\), and \(k_{\text{form}}\) (Table 1) are parameters determined by fitting Equation 1 to the experimental points \([\text{Tyr}]/[\text{R2}]\). With 4 Fe(II)/R2, the maximal yield was 0.8 Tyr/R2. With 6 Fe(II)/R2, the amount was increased to 1.1 Tyr/R2 without change of \(k_{\text{form}}\) in good agreement with previous results (11, 12). The SFQ samples gave the
same kinetic results as the RFQ samples, showing that both methods are compatible (Fig. 1, filled triangles).

Scheme 1 will yield a very complex kinetic pattern for formation and decay of its intermediates if the rates are such that all intermediates can accumulate to reasonable amounts (\( \leq 0.1 \) [Tyr]). If one or more intermediates do not accumulate, the mathematical description can be simplified.

In the case of mouse R2, the situation seems to be even simpler as we detect no intermediate but only the product Tyr. This is the classical pattern of a kinetic reaction including a rate-limiting step, where the time dependence of the product is described by Equation 1. Equation 1 derives from time dependence of C in a consecutive reaction (Reaction 1),

\[
A \rightarrow B \rightarrow C
\]

**REACTION 1**

shown in Equation 2 and the condition \( k_b \gg k_a \).

\[
C = A_0 \left(1 + \frac{k_a e^{-k_b t} - k_b e^{-k_a t}}{k_b - k_a}\right)
\]

(Eq. 2)

From \( k_b \gg k_a \), it follows that \( k_b \approx k_a \approx k_b \approx k_a \approx k_b \approx k_a \); inserting them in Equation 2 yields Equation 1. As we cannot follow the different states of the iron by EPR, we cannot exclude that early intermediates, e.g. R2 – [Fe2O2]4, might accumulate, but the effects of the mutations, as we will see below, show clearly that \( k_{\text{form}} \) is dominated by \( k_b \) in Scheme 1. Thus, we used Equation 1 for fitting the kinetics of Tyr in all our measurements.

Suppression of the Reconstitution Reaction upon Breakage of the Hydrogen-bonded Chain—The mutation W103F interrupts the hydrogen-bonded chain formed by conserved residues from the surface of the R2 protein to the iron site. Any change in the
reconstitution reaction of such a mutant protein may be regarded as a hint that this particular hydrogen bond is involved in the delivery of the external electron and may elucidate some aspects of the reaction mechanism. Up to now it was not clear whether W103F forms an unstable Tyr’ or does not at all form Tyr’ upon reconstitution, whereas its light absorption spectra indicates formation of a normal iron center (10). Therefore, we scanned the reconstitution kinetics of W103F completely down to the limit of time resolution by reaction times equally spaced on a logarithmic scale (inset of Fig. 2). Combined RFQ and SFQ experiments between 20 ms and 18 min at 20 °C 6Fe(II)/R2 show that W103P does not form any transient Tyr’ under our conditions. An unchanged background amount of about 0.01 Tyr’/R2 was measured in all samples (10).

Slow Down of the Reconstitution Reaction upon Alteration of the Hydrogen-bonded Chain—The mutant mouse R2 proteins D266A and W103Y have been shown to develop a stable Tyr’ up to a yield of 1 Tyr’/R2 with an anaerobic reconstitution procedure (10). We measured the kinetics of the reconstitution reaction of these mutant proteins with 6 Fe(II)/R2 at various temperatures. The W103Y and D266A R2 proteins developed the stable tyrosyl radical only to 0.3–0.5 Tyr’/R2 under our conditions. The time course of the generated stable Tyr’ is shown in Fig. 2 for D266A at 25 °C. All time courses for D266A and W103Y at different reaction temperatures exhibit the same kinetic pattern (data not shown) with different formation rates \( k_{\text{form}} \) (Table I). No EPR visible intermediates could be detected at any of the reaction temperatures investigated, neither in D266A nor in W103Y. The formation kinetics of the stable Tyr’ in these two mutant proteins are still first order without a detectable lag period but on average a factor of 20 slower than in wild type mouse R2 (Table I).

Reconstitution Kinetics of R2 Proteins Preloaded with Ferrous Iron—To elucidate if iron binding is a rate-limiting step in the reconstitution or if the mutations affect the kinetics of iron binding, we performed reconstitution experiments with Fe(II)-preloaded R2 proteins. SFQ measurements with wild type R2 at 4 °C yielded \( k_{\text{form}} \) of 0.10 s\(^{-1}\) (Fig. 1, open squares), which is not significantly different from \( k_{\text{form}} = 0.14 \) s\(^{-1}\) obtained without preloading of iron at 5 °C (Fig. 1, closed symbols). As in the nonpreloading measurements for wild type R2, no other EPR visible intermediate could be detected. Taken together these findings indicate that Fe(II) binding is not a limiting step in the reconstitution reaction of mouse wild type R2.

Analogous reconstitution experiments with the Fe(II)-preloaded mutant W103Y at 16 °C are shown in Fig. 3 (open squares) in comparison with the reconstitution data gained from apo R2 W103Y protein without Fe(II)-preloading at 15 °C (filled triangles). The nearly identical kinetics show clearly that the slower formation of Tyr’ in the mutants compared with wild type R2 is not due to a slower iron binding kinetics in the mutant proteins.

Temperature Dependence of the Reconstitution Reactions—The temperature dependence of the Tyr’ formation rates \( k_{\text{form}} \) of wild type and mutant mouse R2 proteins is shown in Fig. 4. At 5 °C the Tyr’ yield in the mutants D266A and W103Y was very low, and therefore the kinetics was not measurable. Above 30 °C the mutant apoproteins started to precipitate. However, between 10 and 25 °C the reconstitution kinetics of the mutant proteins gave reproducible results and could be evaluated according to the Arrhenius law (Equation 3).

\[
\ln k = \left( \frac{\Delta H_r}{R} \right) \times \frac{1}{T} + \ln A \tag{Eq. 3}
\]

The 1/T dependence of \( \ln(k_{\text{form}}) \) of wild type mouse R2 is linear between 5 and 32 °C and gave similar results for 6 (▲) Fe(II)/R2. The activation enthalpy \( \Delta H_r \) of about 140 ± 20 kJ/mol for wild type mouse R2 is only slightly affected by the mutations, leading to 110 ± 20 kJ/mol and 90 ± 20 kJ/mol for D266A and W103Y, respectively.

Appearance of a New EPR Singlet Species, Mouse X—Reconstitution of apo wild type mouse R2 with Fe\(^{57}\) resulted in the appearance of small amounts of a new EPR active intermediate at reaction temperatures between 18 and 32 °C (Fig. 5). Composite EPR spectra were obtained from RFQ samples up to 200 ms of reaction time, e.g. after 61 ms reaction time at 25 °C, the second EPR component is clearly detectable (Fig. 5A) compared with the final EPR signal of Tyr’ after 15 min of reaction time (Fig. 5B). After subtraction of a fraction of a pure Tyr’ spectrum, an EPR singlet-like spectrum denoted as mouse X became visible (Fig. 5C). The peak-to-trough line width of mouse X was determined to 18 ± 2 G and was found to be invariant between 4 and 77 K. The line width is apparently identical to the 18 ± 2 G of species X from E. coli R2 Y122F (Fig. 5D) at 8 K (13). Upon reconstitution with \(^{57}\)Fe and subtraction as above, broadening and hyperfine coupling to the nuclear spin of \(^{57}\)Fe is observed in both mouse X and species X of E. coli R2 Y122F (13 and data not shown).

The microwave power saturation behavior of mouse X was analyzed according to Hales (20), assuming Gaussian broadening and compared with that of species X from the E. coli R2
The $P_{50}$ values for mouse $X$ and $E. coli X$ were determined to 34 and 11 mW at 4 K, 3.2 and 3.0 mW at 29 K, and 190 and 160 mW at 49 K, respectively. The similar temperature dependence of their power saturation behavior taken together with the spectral similarities indicate that mouse $X$ is also located at the iron center and may have a similar structure as reported for species $X$, an Fe(III)-Fe(IV) intermediate (21).

**Kinetics of Mouse $X$**

The concentration of mouse $X$ after different reaction times was determined after spectra subtraction as described above. In Fig. 6 the time course for mouse $X$ after Fe(II)-preloaded W103Y mouse R2 compared with apo-W103Y mouse R2 at 15 °C ($\square$). For the experiments with Fe(II)-preloaded W103Y mouse R2, error bars were included to visualize the variance in the measurements. Points are averages of at least two experiments beside $\pm 1\sigma$ ($\square$). The time courses are identical in the error range of the measurements, indicating that the 20 times slower formation of Tyr$^+$ in the mutants is not due to a different iron uptake kinetics. The Tyr$^+$ formation in the wild type R2 at 15 °C is included for comparison ($\bigcirc$). Kinetic data (Tyr$^+$/R2), were normalized to the corresponding final yield (Tyr$^+$/[R2]), for a better comparison of the curves.

**Radical Transfer in Reconstitution of Mouse R2**

**Fig. 5.** Comparison of EPR spectra from mouse $X$ and $E. coli X$. The reconstitution of mouse R2 and $E. coli R2$ was performed at 25 °C with 6 Fe(II)/R2 and 4 Fe(II)/R2, respectively. The EPR spectra of mouse $X$ were obtained after subtraction of appropriate amounts of the stable tyrosyl radical spectrum (see text). A, 61 ms, mouse R2: 22 $\mu$m total radical; B, 15 min, mouse R2: 60 $\mu$m Tyr$^+$; C, 61 ms, mouse $X$ obtained by subtraction of a fraction of B from A, 4.2 $\mu$m; D, 200 ms, $E. coli R2 Y122F$ species $X$, 12 $\mu$m. The line shape and line width ($\pm 2$ G) of $E. coli R2 Y122F$ species $X$ and mouse $X$ are identical. Recording conditions: frequency 9.43 GHz, temperature 8 K, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 3 G, time constant 43 ms, scan time 84 s, and receiver gain $3.2 \times 10^4$.

**Fig. 4.** Arrhenius plots of $k_{form}$ from wild type ($\blacklozenge$) and $Fe(II)/R2$, D266A ($\square$), and W103Y ($\blacktriangle$) mouse R2 proteins. The reconstitution reaction of all three mouse proteins is strongly dependent on temperature and yields activation enthalpies of about 140, 110, and 90 kJ/mol, respectively (Equation 3).

(data not shown).
FIG. 6. Reconstitution kinetics of mouse R2 wild type with 6 Fe(II)/R2 at 25 °C. The formation of Tyr’ is consistent with a first order rate kinetics, $k_{\text{form}} = 7.1 \text{ s}^{-1}$. The inset shows a blow-up of the data obtained for mouse X. Here, the error bars show the deviations that result from judging the amount of Tyr’ spectra that was subtracted from the composite spectra. The curves represent mathematical fits assuming different models (see “Results, Kinetics of Mouse X”). The inset shows a blow-up of the intermediate formation. Inserting the parameters above in the time dependence of a consecutive reaction as shown in Equation 5 yielded a maximal difference of 0.08 at 35 ms. Such a small difference is not detectable with the usual error for spin concentration determination in EPR. Thus, a $k_6$ only 10 times faster than $k_5$ is already sufficient to not lead to any detectable lag period in the time course of Tyr’ formation. Inserting the parameters above in the time dependence of the intermediate B of a consecutive reaction as shown in Equation 4 allowed an accumulation of B to about 0.08. This is comparable with the amount of mouse X found in the reconstitution of wild type mouse R2 with 6 Fe(II)/R2 at 25 °C shown in Fig. 6.

$$\left(\frac{[X]}{[R2]}_t\right) = \left(\frac{[X]}{[R2]}_0\right) \times \left(\frac{k_5}{k_5 - k_6} e^{-k_5 t} - e^{-k_6 t}\right)$$  \hspace{1cm} \text{(Eq. 4)}$$

where $([X]/[R2])_0$, are the concentration ratios at time $t$ and at long times if no decay took place, respectively, and $k_{5,6}$ are rates from Scheme 1.

Is Mouse X a Kinetically Competent Precursor of Tyr’?—Assuming $k_5 > k_6$ and that mouse X is a precursor of Tyr’, then $([X]/[R2])_0$ in Eq. 4 have to be the same as $A_0$ and $k_5$ in Equation 2. As we know $([\text{Tyr’}]/[R2])_0 = 1.1$ and $k_{\text{form}} = 7 \text{ s}^{-1}$ from fitting (‘Tyr’)/[R2]), with Equation 1, which is a very good approximation for Equation 2, we can use these values as constant parameters in Equation 4. Determination of the variable $k_5$ by fitting Equation 4 to the mouse X data in Fig. 6 yielded $k_5 = 112 \pm 38 \text{ s}^{-1}$, which is much bigger than $k_5$, as expected, but the shape of the curve (solid line in the inset of Fig. 6) fits badly to the experimental points.

Assuming that mouse X is only the precursor of a part of Tyr’ allows one to set $([X]/[R2])_0$ as an additional variable and just keep $k_5$ fixed at 7.1 s$^{-1}$. The result of this fit is shown as the long dashed curve in Fig. 6 inset with $([X]/[R2])_0 = 0.37 \pm 0.33$ and $k_5 = 36 \pm 37 \text{ s}^{-1}$. We found that his assumption describes neither the measured data nor does it yield well defined variables. In another trial we fixed $([X]/[R2])_0$ to 1.1 and $k_5$ to 7.1 s$^{-1}$ assuming that Tyr’ formation is governed by X decay. The resulting best fitting curve (short dashes in Fig. 6) with $k_5 = 0.64 \pm 0.35 \text{ s}^{-1}$ does not give the expected $k_5 > k_6$ and lies way outside of the data. The other possible combinations of taking the parameters $([X]/[R2])_0$, $k_5$, and $k_6$ as variables and/or constants result in similar bad or worse fits. The data of mouse X found at other temperatures and 4 Fe(II)/R2 (Table 1) could similarly not be described by one of the models outlined above.

By including a lag period $\Delta t$ in Equation 4, yielding Equation 5, the measured data for mouse X could be described by manually inserting values for the four parameters $([X]/[R2])_0$, $k_5$, $k_6$, and $\Delta t$ into Equation 5 and fitting the resulting curve to the data by eye (dashed and dotted line in Fig. 6).

$$\left(\frac{[X]}{[R2]}_t\right) = \left(\frac{[X]}{[R2]}_0\right) \times \left(\frac{k_5}{k_5 - k_6} e^{-k_5 t + \Delta t} - e^{-k_6 t + \Delta t}\right)$$  \hspace{1cm} \text{(Eq. 5)}$$

Using $k_5 = 54 \text{ s}^{-1}$, $k_6 = 45 \text{ s}^{-1}$, $([X]/[R2])_0 = 0.22$, and $\Delta t = 35 \text{ ms}$ in Equation 5 yielded a good fitting of the data in Fig. 6 (dashed and dotted line). However, the rates as well as $([X]/[R2])_0$ are very different than $k_{\text{form}} = 7.1 \text{ s}^{-1}$, and $([\text{Tyr’}]/[R2])_0 = 1.1$ (25 °C, 6 Fe(II)/R2, see Table 1), indicating strongly that the amount of mouse X accumulating under this conditions is not competent to be an intermediate of the reaction sequence in Scheme 1 leading to Tyr’ formation. We propose that the visible mouse X is an intermediate in the side reaction sketched in Scheme 2, leading to a differ center without Tyr’. Further clarification is expected from ongoing Mossbauer RFQ experiments.

DISCUSSION

In respiratory and photosynthetic processes, unidirectional electron transport occurs between redox centers of different redox potentials (22–24). Two models for electron transport, one proposing a uniform traveling barrier for electrons unaffected by protein structure (23), the other including guidance of an electron through covalent and hydrogen bonds (24), are commonly discussed for distances up to 20 Å. For the enzymatic reaction in class I ribonucleotide reductases an electron has to be transferred over 35 Å, since it is believed that Ty” in R2 abstracts an electron from a cysteine at the active site of R1, and the radical transfer must be reversible (2, 7). These features are clearly different from those usually found in other redox proteins and raise the question what kind of transfer actually takes place during catalysis in ribonucleotide reductases. The presence of a conserved hydrogen-bonded chain between the two sites, Ty” in mouse R2 and Cys-429 in mouse R1, the fact that Tyr’ is a neutral radical lacking a H’, and that the first step of the enzymatic reaction is proposed to be H’ abstraction from the substrate suggests a transport of a proton concomitant with the transfer of an electron, i.e. H’ transfer. 

...
Therefore, the hydrogen-bonded chain might serve as a RTP. This is in good agreement with recent theoretical calculations leading to high activation energies for a pure electron transfer from one uncharged amino acid (R1-Cys429) to another (R2-Tyr177) due to the charge separation necessary (16).

Reactions at the Iron Center—For the following discussion we assume the reaction pathway for the reconstitution as outlined in Scheme 1 (13–15, 25). Binding of the two substrates FeII (+) (k1 and k3) and O2 (k2 and k4) is random, since both aerobic and anaerobic reconstitution procedures are possible. A probably very short lived, O2-R2-[FeII]14+ intermediate is proposed in analogy with the early O2-[FeII]14+ complex in the enzymatic cycle of soluble methane monoxygenase (26). The irons are oxidized via a peroxo intermediate (k7) to a diferryl compound (k8) in methane monoxygenase), which is converted to species X by one external reducing equivalent (k9). The source of the external reducing equivalent was shown to be either FeII (+), as under our conditions, or other reductants like ascorbic acid (12–14). The last step is the formation of TyrX by abstraction of an H‘ (k9) from the tyrosine residue adjacent to the iron center.

In the reconstitution of apo E. coli R2, two of the steps in Scheme 1 (k1 and k3) could be measured. The formation of TyrX is the rate-limiting step with k9 = 1 s–1 at 5 and 25 °C (data not shown). Species X accumulates during the reconstitution reaction of E. coli R2 and is the direct precursor of TyrX (27, 28), whereas no hydrogen bond has been found for E. coli R2 (29). Recent electron nuclear double resonance measurements confirm the presence of a D2O exchangeable, hydrogen-bonded proton in the vicinity of TyrX in mouse R2 (30). The results possibly reflect a shorter distance of the TyrX radical (14, 28), we likely detected mouse X instead of the tyrosine.

SCHEME 2. Proposed reaction mechanism for the formation of the diferric site without formation of TyrX, a side reaction of the reconstitution in vitro. For this reaction, evidenced by the finding of more diferric sites than TyrX radicals (14, 28), we likely detected mouse X as an intermediate. The difference from Scheme 1 is the source of the second H‘ (k9), which here might come from a second external [Fe(H2O)6]3+ instead of the tyrosine.

protein did not give rise to detectable amounts of the postulated intermediate mouse X, we conclude that iron binding is not rate-limiting for mouse X formation.

Nevertheless, we assume an identical pathway for the reconstitution of mouse as for E. coli R2 regarding the reaction steps and the intermediates formed (Scheme 1). The kinetic differences in reactions between the two proteins might arise from structural differences. In the mouse R2 protein, the channel leading from the iron binding site to the surface of the protein is more open to the solvent compared with the E. coli protein (6, 9). This might allow a faster transfer and binding of iron for the mouse protein. Minor amounts of mouse X observed in the present study were kinetically not competent for TyrX formation (Scheme 1) and are proposed to be part of a side reaction (Scheme 2). The differences in the ability to accumulate species X might be explained by different environments of TyrX. High-field EPR measurements suggested hydrogen bonding of TyrX in mouse R2 (27), whereas no hydrogen bond has been found for E. coli R2 (28, 29). Recent electron nuclear double resonance measurements confirm the presence of a D2O exchangeable, hydrogen-bonded proton in the vicinity of TyrX in mouse R2 (30). The results possibly reflect a shorter distance of the TyrX radical of the RTP is continued on the radical side of the iron center in mouse R2.

Nature of the External Reduction Equivalent—In contrast to W103Y, the mutant W103F does not even form a transient TyrX during reconstitution, although a diferric iron center seems to be formed (10). The only molecular difference between W103Y and W103F is the phenyl hydroxyl group, which is apparently crucial for the reconstitution. This observation cannot be explained by either of the two currently most discussed electron
transport models (22–24).

From structural data (6) it is known that Trp$^{103}$ is hydrogen-bonded to Asp$^{266}$ which in turn is hydrogen-bonded to a ligand of Fe1 (Fig. 7). A tyrosine at position 103 may still preserve a hydrogen bond to the δ-oxygen of Asp$^{266}$ by its phenyl hydroxyl group, but a phenylalanine should certainly interrupt it. From structure modeling it seems possible that a tightly bound water molecule in the position of the carboxylate group of Asp$^{266}$ compensates for the interruption of the hydrogen-bonded chain in the D266A mutation. The amount of Tyr$^\cdot$ formed in the mutant R2 proteins indicates that radical formation is not an all or none mechanism. Rather it seems that the yield of Tyr$^\cdot$ depends on the quality of the hydrogen-bonded chain: good in wild type, medium in D266A and W103Y, and very bad in W103F.

Based on these results we would like to put forward the model that the external reduction equivalent is not merely an electron but an electron accompanied by a proton, i.e. a hydrogen radical, H$^\cdot$, that is transported via the hydrogen-bonded chain of the RTP (Fig. 7). This process would require only small shifts of the hydrogen atoms forming the hydrogen-bonded chain sketched in Fig. 7, so that a former hydrogen bond becomes a covalent one and vice versa, in agreement with the recent theoretical model (16). From the slow kinetics of Tyr$^\cdot$ formation in D266A and W103Y, one might expect an accumulation of radical intermediates, e.g. Trp$^{103}$ and D266A and possibly Tyr$^{103}$ in W103Y. The formation of a transient radical on Trp$^{48}$ in E. coli corresponding to mouse Trp$^{103}$ has been proposed (31, 32). However, no such EPR visible transients have been detected in the present study.

Effect of Alteration of the Hydrogen-bonded Chain—Trp$^{103}$ and Asp$^{266}$ have been shown to participate in radical transfer during catalysis (10). Our present result shows that they are also involved in the reconstitution reaction, since the kinetics are on average slowed down by a factor of 20 compared with wild type mouse R2 (Table 1). Their activation enthalpies are very similar to that of wild type mouse R2 (Fig. 5). This suggests that the overall reaction mechanism is only little affected by these mutations.

Major structural changes are unlikely as shown earlier (10), although the mutations could possibly induce two kinds of minor structural changes, in the binding of Fe$^{2+}$ and O$_2$, or on the delivery pathway for the external reducing equivalent. We consider it very unlikely that the oxidation of ferrous iron by dioxygen (k$_{3,4}$ in Scheme 1), involving only the direct environment of the iron-oxygen center, is affected by the mutations. Therefore, they might affect three steps in Scheme 1, k$_{1,4}$, or k$_{5,6}$, or both. To rule out the first possibility we made two kinds of binding experiments under identical conditions, Fe$^{2+}$ binding of the aerobic protein (k$_{1,4}$) and O$_2$ binding of the Fe(II)-preloaded protein (k$_{5,6}$). Both experiments gave the same formation rates for Tyr$^\cdot$. The results indicate that only the delivery of the external reduction equivalent (k$_{5,6}$) can be affected by the mutations on the RTP. The striking feature of k$_{form}$ is that it is affected by mutations on the RTP. Thus, the most likely assignment is k$_{form}$ = k$_{D}$, i.e. the proposed transfer of an external H$^\cdot$ is the slowest step in the reconstitution of mouse R2 proteins.

Consequently, the calculated $\Delta$H$_D$ in Fig. 5 should be the activation enthalpy for the delivery of the proposed external H$^\cdot$. It is nearly unchanged in the D266A and W103Y mutants, suggesting that the activated complex is not formed on the hydrogen-bonded chain from residue 103 to the iron ligand His$^{173}$. Rather it seems to be formed at the iron site, probably when the proposed H$^\cdot$ is delivered from the histidine to one of the iron site oxygens. Energy might be necessary to adjust the otherwise flexible iron center of mouse R2 (9) into a rigid structure possibly required for the last step of the proposed hydrogen transfer.

From What Species Is the External Reducing Equivalent Delivered?—It is unlikely that a hydrogen radical is delivered from the (reduced) iron site of another R2 subunit without creation of an EPR visible signal, e.g. the mixed valent state (33, 34). It is more likely that some external iron binds to Trp$^{103}$ (Fig. 7) or maybe to Tyr$^{370}$ and delivers the electron/proton pair. An almost concomitant hydrogen transfer from a water ligand of an Fe$^{2+}$ bound to the surface of R2 as well as from the tyrosine to the proposed [Fe$_2$O$_2$]$^{4+}$ intermediate of the iron center is consistent with all findings in the mouse R2 reconstitution reaction so far (Fig. 7).

The proposed iron site intermediate [Fe$_2$O$_2$]$^{4+}$ (Fig. 7, left panel) is isoelectronic with a diferryl state analogous to compound Q in methane monoxygenase (35). Compound Q is EPR silent but observable by Mössbauer spectroscopy (26). Mössbauer spectroscopy suggests that the iron site in compound Q is significantly different from that of Fe$^{2+}$ complexed to the iron center of the RTP (Fig. 7). This suggests that the iron center is not complete in compound Q (36). The iron center in compound Q is probably stabilized by a protein environment and might also be a more labile iron complex than that of the iron center of the RTP. The latter process is not an all or none mechanism. Rather it seems to be formed at the iron site, probably when the proposed H$^\cdot$ is delivered from the histidine to one of the iron site oxygens. Energy might be necessary to adjust the otherwise flexible iron center of mouse R2 (9) into a rigid structure possibly required for the last step of the proposed hydrogen transfer.

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bauer investigations on E. coli R2 revealed only little of this intermediate (15), but it may be more favorable for observation in mouse R2 due to its different kinetics.

What Regulates the Functional State of the RTP?—The entire RTP seems to depend on intact hydrogen bonds, since it can be knocked out by mutations similar to W103F on residues in the E. coli R1 protein corresponding to mouse R1 residues Y737F and Y738F (36).

After the formation of Tyr (right-hand panel of Fig. 7), the hydrogen-bonded chain is disabled to deliver a second H
due to the presence of two N atoms in the His173 ligand that prevents the formation of the hydrogen-bonded chain. However, the Tyr in R2 may still be transported from the active site to Tyr in R2. This conformation (right-hand panel of Fig. 7) of the hydrogen-bonded chain has to flip back into the conformation postulated for the apoR2 (left-hand panel of Fig. 7) upon binding of either R1, effector, and/or substrate. This flip requires a real (reverse to Fig. 7) or virtual (rotation) movement of the hydrogen from one nitrogen to the other in His173, likely accompanied and facilitated by small structural changes of the iron center. To bridge the distance between the His173 ligand and the 

The present results are consistent with a new type of transfer process in proteins: radical transfer in the form of the shift between Tyr and the proposed highly oxidative [Fe2O2]+ intermediate over a distance of 10 Å. The difference in redox potentials between these two intermediates may still allow the proposed H+ transfer through an altered hydrogen-bonded chain, even if its structure is no longer perfectly adjusted to this reaction. On the other hand, in the enzymatic reaction, the difference in redox potentials between Tyr and the substrate in R1 is presumably much smaller, and the transfer distance is much longer. Thus, any misalignment of the hydrogen-bonded chain will suppress the proposed H+ transfer suggested to occur in the enzymatic reaction.

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