Evidence by Mutagenesis that Tyr\textsuperscript{370} of the Mouse Ribonucleotide Reductase R2 Protein Is the Connecting Link in the Intersubunit Radical Transfer Pathway*

Ulrika Roß\textdagger, Annie Adrait\textsection, Stephan Pötsch\textsection, Astrid Gräslund\textsection, and Lars Theander‡‡

From the ‡Department of Medical Biosciences, Medical Biochemistry, Umeå University, SE-901 87 Umeå, Sweden and the §Department of Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden

Ribonucleotide reductase catalyzes all \textit{de novo} synthesis of deoxyribonucleotides. The mammalian enzyme consists of two non-identical subunits, the R1 and R2 proteins, each inactive alone. The R1 subunit contains the active site, whereas the R2 protein harbors a binuclear iron center and a tyrosyl free radical essential for catalysis. It has been proposed that the radical properties of the R2 subunit are transferred \textit{via} long-range electron transfer between the subunits.

All ribonucleotide reductases characterized up to now use free radical chemistry to catalyze the \textit{de novo} synthesis of deoxyribonucleotides from ribonucleotides (1, 2). Ribonucleotide reductases in mammalian cells, DNA viruses, plants, and some prokaryotes (\textit{i.e.} \textit{Escherichia coli}) belong to the same class and are composed of two non-identical subunits, the R1 and R2 proteins. Each subunit is inactive alone, but together they form an active enzyme complex of the type $\alpha_2\beta_2$.

The large subunit, the R1 protein, can be considered the business end of the enzyme as it contains the binding site for the ribonucleoside diphosphate substrates (3). The small subunit, the R2 protein, does not bind substrate, and its function consists of two non-identical subunits, the R1 and R2 proteins. Interruption of the hydrogen-bonded chain in Y370F inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.

Alteration of the same chain in Y370W inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.

To identify the linking amino acid residue in the radical transfer pathway, the R2 protein with tryptophan or phenylalanine. This residue is located close to the flexible C terminus, known to be essential for binding to the R1 protein. Our results strongly indicate that Tyr\textsuperscript{370} links the RTP between the R1 and R2 proteins. INTERRUPTION OF THE HYDROGEN-BONDED CHAIN IN Y370F INACTIVATES THE ENZYME COMPLEX. ALTERATION OF THE SAME CHAIN IN Y370W SLOWS DOWN THE RTP, RESULTING IN A 58 TIMES LOWER SPECIFIC ACTIVITY COMPARED WITH THE NATIVE R2 PROTEIN AND A LOSS OF THE FREE RADICAL DURING CATALYSIS.

All ribonucleotide reductases characterized up to now use free radical chemistry to catalyze the \textit{de novo} synthesis of deoxyribonucleotides from ribonucleotides (1, 2). Ribonucleotide reductases in mammalian cells, DNA viruses, plants, and some prokaryotes (\textit{i.e.} \textit{Escherichia coli}) belong to the same class and are composed of two non-identical subunits, the R1 and R2 proteins. Each subunit is inactive alone, but together they form an active enzyme complex of the type $\alpha_2\beta_2$.

The large subunit, the R1 protein, can be considered the business end of the enzyme as it contains the binding site for the ribonucleoside diphosphate substrates (3). The small subunit, the R2 protein, does not bind substrate, and its function consists of two non-identical subunits, the R1 and R2 proteins. Interruption of the hydrogen-bonded chain in Y370F inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.

Alteration of the same chain in Y370W inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.

All ribonucleotide reductases characterized up to now use free radical chemistry to catalyze the \textit{de novo} synthesis of deoxyribonucleotides from ribonucleotides (1, 2). Ribonucleotide reductases in mammalian cells, DNA viruses, plants, and some prokaryotes (\textit{i.e.} \textit{Escherichia coli}) belong to the same class and are composed of two non-identical subunits, the R1 and R2 proteins. Each subunit is inactive alone, but together they form an active enzyme complex of the type $\alpha_2\beta_2$.

The large subunit, the R1 protein, can be considered the business end of the enzyme as it contains the binding site for the ribonucleoside diphosphate substrates (3). The small subunit, the R2 protein, does not bind substrate, and its function consists of two non-identical subunits, the R1 and R2 proteins. Interruption of the hydrogen-bonded chain in Y370F inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.

All ribonucleotide reductases characterized up to now use free radical chemistry to catalyze the \textit{de novo} synthesis of deoxyribonucleotides from ribonucleotides (1, 2). Ribonucleotide reductases in mammalian cells, DNA viruses, plants, and some prokaryotes (\textit{i.e.} \textit{Escherichia coli}) belong to the same class and are composed of two non-identical subunits, the R1 and R2 proteins. Each subunit is inactive alone, but together they form an active enzyme complex of the type $\alpha_2\beta_2$.

The large subunit, the R1 protein, can be considered the business end of the enzyme as it contains the binding site for the ribonucleoside diphosphate substrates (3). The small subunit, the R2 protein, does not bind substrate, and its function consists of two non-identical subunits, the R1 and R2 proteins. Interruption of the hydrogen-bonded chain in Y370F inactivates the enzyme complex. Alteration of the same chain in Y370W slows down the reaction, but in a 58 times lower specific activity compared with the native R2 protein and a loss of the free radical during catalysis.
transfer pathway between the R1 and R2 subunits, we substituted the conserved Tyr<sup>370</sup> in the mouse R2 protein with tryptophan or phenylalanine. Tryptophan has the potential to form a hydrogen-bonded linkage, whereas phenylalanine cannot form such a linkage. We here report data strongly supporting Tyr<sup>370</sup> as the connecting link in the intersubunit RTP.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The T7 RNA polymerase-responsive vector pET3a (28, 29) containing the cDNA encoding the mouse R2 protein (7) was used as a source for site-directed mutagenesis. The Clal/EcoRI sites in the original pET3a vector were removed (12), and the resulting plasmid pETR2-Cla/R1, containing unique EcoRI and Clal sites.

**Oligonucleotide-directed Mutagenesis—**Tyr<sup>370</sup> was mutagenized to phenylalanine or tryptophan (Y370F and Y370W) by the polymerase chain reaction technique, using the primers indicated below and pETR2-Cla/R1 as a template. All oligonucleotides used were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer. The primers were 5′-CTGGCGAATTCGATACTGACCTCTCGACTGGAAACCTGTTACGGC (Y370F) and 5′-CTGTGCAATTCGATACGATCTCCCTTTAACCCTGGTCGATCCGCC (Y370W). Boldface letters denote the mutagenic codon, and the recognition site for EcoRI used in the subcloning is underlined. The same upstream primer was used for both of the mutants: 5′-GGAGCTCATATGGAGAACGCGTGCA-3′. The underlined nucleotides denote an MluI recognition site. A polymerase chain reaction mixture containing 1 ng of pETR2-Cla/R1, 330 ng of each primer, and 0.2 mM dNTPs (Amersham Pharmacia Biotech) in a buffer recommended for Pfu polymerase (Stratagene) was preheated for 5 min at 97 °C before the addition of Pfu polymerase. After 30 cycles (94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min), EDTA was added to a final concentration of 10 mM, and the two polymerase chain reaction products were purified by phenol/chloroform extraction. The mutated fragments were recovered by digestion with MluI and EcoRI, resulting in a fragment of 459 base pairs. This fragment was finally cloned into pETR2-Cla/R1 digested with the same enzymes.

To amplify the mutated constructs, they were transfected into E. coli strain MC1061 to permit large-scale plasmid preparations. To ensure that no unwanted mismatches had been incorporated during the polymerase chain reaction, both constructs were verified by double-stranded DNA sequencing of the area covering the subcloned fragments. Finally, the constructs pETR2Y370F and pETR2Y370W were transfected into E. coli strain BL21(DE3) pLysS (29), which enables expression of the native or mutated R2 protein to catalyze reduction of [3H]CDP in the presence of the R1 protein and CDP.

**Expression and Purification of the Mutated and Native R2 Proteins**—The native or mutated R2 proteins were overexpressed by induction of bacterial construct. The cells were then grown and induced with isopropyl-1-thio-D-galactopyranoside as described previously (31). Approximately 0.17 nmol of dimeric native R2 protein or mutated R2 protein was injected during the amino coupling procedure.

**RESULTS**

**Expression and Purification of the Mouse Y370F and Y370W R2 Proteins**—The construction of the pETR2Y370F and pETR2Y370W R2 expression vectors and the expression and purification of the mutated R2 proteins were described under “Experimental Procedures.” The procedures resulted in a yield of 10–15 mg of R2 apoprotein/liter of bacterial culture. SDS-polyacrylamide gel electrophoresis of the R2 proteins showed them to be as pure as the native R2 protein (Fig. 1). All preparations showed the presence of full-length R2 protein (45 kDa) as well as variable amounts of N-terminally degraded forms (43.5 and 41 kDa). However, as shown earlier (7), this truncation does not affect the catalytic activity of the R2 protein; indeed, deletion of up to 60 amino acid residues previously (31). Approximately 0.17 nmol of dimeric native R2 protein or mutated R2 protein was injected during the amino coupling procedure. During the immobilization, there was a constant flow (5 µl/min) of HBS running buffer (10 mM Hepes, 0.15 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.05% (v/v) Surfactant P20 (Biacore AB) (pH 7.4)).

**Subunit Interaction**—The interaction of the mutated R2 proteins with the R1 protein was studied by allowing the immobilized R2 protein to interact with two different concentrations of R1 protein (200 and 800 nm dimeric protein) in a solution of HBS running buffer supplemented with dithiothreitol and dTTMP (2 and 0.1 mM final concentrations, respectively). The same buffer was used as eluent during the subunit interaction. After the first R1 injection, the surface was regenerated with 10 mM Tris-HCl running buffer prior to the next injection. Increasing subunit interaction was monitored as an increase in surface plasmon resonance, detected as resonance units (RU).

**Reconstitution of the Iron/Radical Center—**Before use, the native and mutated R2 apoproteins were reactivated with iron. An anaerobic ferrous ammonium sulfate solution was freshly prepared as described previously (16). The aerobic R2 protein was incubated for 30 s at 25 °C with a 5-fold molar excess over the R2 polypeptide of the anaerobic ferrous ammonium sulfate. Excess iron was removed by equilibrating the protein solution with 50 mM Tris-HCl (pH 7.6) and 100 mM KCl on a Sephadex G-25 column.

**SEP Analyses—**EPR spectra at 9.3 GHz were measured on a Bruker ESP 300 spectrometer using a standard rectangular Bruker EPR cavity (ER4102T) equipped with an Oxford helium flow cryostat or a cold finger Dewar flask for measurements at 77 K. Spin concentrations were determined by comparison of double integrals of EPR spectra with that of the tyrosyl radical in the mouse wild-type R2 protein that had been calibrated with a 1 mM Cu<sup>2+</sup> standard. Rapid freeze-quench experiments were performed as described (16).

**Iron Assay and Light Absorption Spectra**—The amount of iron/R2 polypeptide was determined by a colorimetric method (32). Light absorption spectra of the reactivated native R2 protein (20 µM polypeptide) and mutated R2 proteins (44 µM polypeptide) were recorded between 280 and 500 nm using a Perkin-Elmer Lambda 40 UV-visible spectrometer.

**Ribonucleotide Reductase Activity**—The ability of the mutated R2 polypeptide to catalyze reduction of [3H]CDP in the presence of the R1 protein was determined as described previously (33), where 1 unit corresponds to the amount of enzyme that catalyzes the formation of 1 nmol of dCDP/min at 37 °C. In the assay, the following reagents were used in a final volume of 50 µl: 25 µM of [3H]CDP (specific activity, 20,000 cpm/nmol), 2 µM of 4-2-hydroxyethyl-1-piperazine-sulfonic acid buffer (pH 7.6), 0.15 µM of ATP, 0.32 µM of MgCl<sub>2</sub>, 5 mM dithiothreitol, 5 µM of KCl, 1 mM of FeCl<sub>3</sub>, and enzyme as indicated.

**Kinetics of the Tyrosyl Radical during CDP Reduction**—A mixture containing 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 5 mM ATP, 50 µM R1 protein, and 25 µM reactivated native or mutated R2 protein was freshly prepared. The reaction was started by the addition of CDP to a final concentration of 0.5 mM. The mixture was transferred into an EPR tube and incubated at 37 °C for a certain time and then quenched by immersing the EPR tube into liquid nitrogen (77 K). Formation of transient species and quantification of tyrosyl radical content were made by EPR spectroscopy.

**RESULTS**

**Expression and Purification of the Mouse Y370F and Y370W R2 Proteins**—The construction of the pETR2Y370F and pETR2Y370W R2 expression vectors and the expression and purification of the mutated R2 proteins were described under “Experimental Procedures.” The procedures resulted in a yield of 10–15 mg of R2 apoprotein/liter of bacterial culture. SDS-polyacrylamide gel electrophoresis of the R2 proteins showed them to be as pure as the native R2 protein (Fig. 1). All preparations showed the presence of full-length R2 protein (45 kDa) as well as variable amounts of N-terminally degraded forms (43.5 and 41 kDa). However, as shown earlier (7), this truncation does not affect the catalytic activity of the R2 protein; indeed, deletion of up to 60 amino acid residues...
from the N-terminal end does not affect the specific activity of the recombinant mouse R2 protein.2

Subunit Interaction—It is known that the C-terminal part of the R2 protein is essential for binding to the R1 protein. We therefore investigated if the Y370W and Y370F mutations affected R1 binding, using biosensor analyses as described previously (12, 31). The native and mutated R2 proteins were immobilized to the sensor chips, yielding 1500–2200 immobilized RU, where 1 RU corresponds to a surface concentration of 1 pg of protein/mm² of a 100-nm-thick dextran layer (35).

Fig. 2 shows the sensorgram for the interaction between the R1 protein and the native or mutated R2 protein. The association and dissociation of the R1-R2 complex can be followed as changes in surface plasmon resonance, which are detected as RU in the sensorgram. After injection of the R1 protein in the running buffer, the association phase in Fig. 2 was detected as an increase in RU. The association reached a steady-state equilibrium level, and this occurred faster with increasing concentrations of R1 protein. When the injection of the R1 protein was stopped and only running buffer with dTTP was used, the R1-R2 complex started to dissociate as seen by the decrease in RU. As shown in Fig. 2, there was no significant difference in shape between the response curves for the native and mutated R2 proteins. Control experiments in which the same concentrations of R1 protein were injected on sensor chips without the immobilized R2 proteins gave only an insignificant response of ~50 RU (data not shown). Therefore, we conclude that the R2 protein point mutations did not influence R1 protein binding in a significant way.

Properties of the Iron/Radical Center in the Y370F and Y370W R2 Proteins—The iron/radical center in the mutated proteins was reconstituted by the addition of a 3-fold molar excess of an anaerobic ferrous iron solution to an aerobic protein solution (see “Experimental Procedures”). EPR spectroscopy of the reactivated proteins, shown in Fig. 3 (traces B and C), displayed a normal EPR signal with the same hyperfine pattern of the tyrosyl radical as for the native R2 protein (Fig. 3, trace A). The comparison of the microwave saturation behavior of the tyrosyl free radical in the two R2 protein mutants with that of the wild-type R2 protein at 20 and 30 K did not show any significant difference in the $P_{50}$ value. Therefore, the mutation did not influence the magnetic interaction of the tyrosyl free radical with the metal site, indicating that the radical structure is not disturbed. Determination of radical and iron content showed that the Y370F and Y370W R2 proteins are similar to what was previously reported for the native R2 protein (7), with 0.6 and 0.8 nmol of tyrosyl radical/nmol of R2 polypeptide; trace C, Y370F protein R2 (0.6 nmol of tyrosyl radical/nmol of R2 polypeptide). mT, milliteslas.

3, trace A). The comparison of the microwave saturation behavior of the tyrosyl free radical in the two R2 protein mutants with that of the wild-type R2 protein at 20 and 30 K did not show any significant difference in the $P_{50}$ value. Therefore, the mutation did not influence the magnetic interaction of the tyrosyl free radical with the metal site, indicating that the radical structure is not disturbed. Determination of radical and iron content showed that the Y370F and Y370W R2 proteins are similar to what was previously reported for the native R2 protein (7), with 0.6 and 0.8 nmol of tyrosyl radical/nmol of R2 polypeptide; trace C, Y370F protein R2 (0.6 nmol of tyrosyl radical/nmol of R2 polypeptide). mT, milliteslas.

Fig. 3. X-band EPR spectra of R2 protein Tyr177 tyrosyl radicals recorded at 30 K. Trace A, native R2 protein (0.75 nmol of tyrosyl radical/nmol of R2 polypeptide); trace B, Y370W R2 protein (0.55 nmol of tyrosyl radical/nmol of R2 polypeptide); trace C, Y370F protein R2 (0.6 nmol of tyrosyl radical/nmol of R2 polypeptide). mT, milliteslas.

2 L. Thelander, unpublished results.
control experiment in which the highest amount of Y370W protein was assayed in the absence of added mouse R1 protein showed only column background values (0.04–0.05 nmol). We therefore conclude that the Y370W protein has ~1.7% of normal R2 protein activity, and this activity is not due to any contamination of *E. coli* ribonucleotide reductase.

**Substrate-dependent Inactivation of the Tyrosyl Radical of the Y370W R2 Protein during Catalysis**—One reason for the low specific activity of the Y370W R2 protein compared with the native R2 protein might be a slower rate of radical transfer. This might allow the detection of transient EPR reaction intermediates. We therefore used EPR spectroscopy to study the kinetics of the CDP reduction catalyzed by the native and mutated R2 proteins in the presence of a 2-fold molar excess of the R1 protein, but in the absence of added iron. Under these conditions, where the incubation mixture was incubated at 27 °C in an EPR tube, we were not able to detect any visible transient EPR intermediates. Instead, an unexpected time-dependent decay of the tyrosyl radical was observed with the Y370W R2 protein (Fig. 5A, ). Neither the native R2 protein nor the Y370F R2 mutant showed such a decay under similar incubation conditions (Fig. 5A, and , respectively). Furthermore, control samples with the native or mutated R2 proteins in the presence of the R1 protein, but without the addition of CDP, showed no significant decrease in radical content (data not shown). Therefore, the Y370W-specific tyrosyl radical decay is absolutely dependent on the presence of the CDP substrate.

The iron/radical center of the mouse R2 protein is labile under physiological conditions. After 30 min at 37 °C, the protein loses ~50% of its iron and radical content. Continuous loss of ferric iron in the presence of the reductant dithiothreitol in the assay mixture results in a simultaneous decay and reconstitution of the tyrosyl radical (34). This may explain the observed rapid initial decay of the radical, followed by a steadystate plateau at ~60% of the initial value (Fig. 5A). To support this hypothesis, we repeated the CDP reduction at 37 °C in the presence of the previously characterized iron chelator desferrioxamine (34). When the reactivated Y370W R2 protein was incubated with desferrioxamine in the absence of the R1 protein and CDP, the decay of the radical was much more pronounced (Fig. 5B, ). This further strengthens our hypothesis that the decay of the tyrosyl radical in the case of the Y370W protein is dependent on ongoing catalysis.

**Reconstitution Kinetics of the Native and Mutated R2 Proteins**—To investigate if Tyr370 is involved in the reconstitution of the diferric/tyrosyl free radical center of the R2 protein, we used rapid freeze-quench EPR with 3 nmol of Fe(II)/nmol of R2 polypeptide and a reconstitution temperature of 5 °C. With

| R2 protein | Total protein | Formation of dCDP/30 min at 37 °C |
|------------|---------------|-----------------------------------|
| Native     | 1.0           | 6.84                              |
| Y370F     | 2.0           | 0.04                              |
| Y370F     | 4.0           | 0.05                              |
| Y370F     | 6.0           | 0.05                              |
| Y370W     | 2.0           | 0.22                              |
| Y370W     | 4.0           | 0.56                              |
| Y370W     | 6.0           | 0.65                              |
| Native     | 1.0           | 6.84                              |

* The activity was measured in the presence of 10 μg of recombinant mouse R1 protein (33). The Dowex column background was ~0.04–0.05 nmol and has not been subtracted.

### Enzyme Activity

The enzyme activity of the reactivated Y370W and Y370F R2 proteins was measured in the presence of the R1 protein using [3H]CDP as a substrate and ATP as a positive effector. In Table I, the results are compared with the activity of the reactivated native R2 protein. No activity was detected for the Y370F R2 protein. In contrast, the Y370W R2 protein showed a low but still significant activity, which increased in a linear way with increasing amounts of added mutated protein. The reaction was linear during the 30-min incubation (data not shown). The reactivated native R2 protein assayed in parallel had a specific activity of 228 units/mg. The specific activity of the Y370W R2 protein was ~3.9 units/mg. A
Radical Transfer in Mouse Ribonucleotide Reductase

FIG. 5. Time-dependent decay of the Y370W tyrosyl radical during CDP reduction. A, the content of the tyrosyl radical in the R2 proteins incubated at 27 °C with the R1 protein and CDP under assay conditions, but in the absence of added iron, was quantified by EPR spectroscopy. ■, Y370W R2 protein; ●, native R2 protein; □, Y370F R2 protein. B, time-dependent decay of the Y370W tyrosyl radical during CDP reduction in the presence of 100 μM desferrioxamine at 37 °C. ■, Y370W R2 protein incubated with the R1 protein and CDP; ▲, Y370W R2 protein incubated in the absence of the R1 protein and CDP.

native R2 apoprotein, we could confirm the earlier reported 
$k_{\text{form}}$ for the tyrosyl radical of 0.14 s$^{-1}$ (16). The values for the Y370W and Y370F R2 proteins (0.19 and 0.16 s$^{-1}$, respectively) did not differ significantly from the value of the native protein. Furthermore, the final yield of the tyrosyl free radical was the same for all three proteins (0.5–0.6 nmol of radical/nmol of polypeptide chain).

The key to understanding the mechanism of the reaction catalyzed by the enzyme ribonucleotide reductase is a better knowledge of how the radical properties of the R2 protein are transferred to the active site of the R1 protein. After the three-dimensional structures of the R1 and R2 proteins were reported (8, 10), many studies have been made to address this question.

Biological electron transfer has been extensively studied for redox reactions involved in the respiratory and photosynthetic chains. The two commonly discussed models for electron transfer over distances of up to 20 Å argue whether or not the intervening protein structure is an important factor for the efficiency of the electron transfer (36, 37). In contrast to these models, the electron transfer in ribonucleotide reductase has to propagate quite a long distance of ~35 Å between the oxidized tyrosine in the R2 protein and a neutral cysteine at the active site of the R1 protein. It is unlikely that the current electron transfer theories can be applied to this system since too much energy would be required to overcome the charge separation arising from a pure electron transfer. Instead, a more recent theoretical model explains this transfer as a coupled electron/proton transfer, i.e., a hydrogen radical transfer (18). The advantage of this model is that it minimizes charge separation as an uncharged hydrogen radical is moved along a conserved hydrogen-bonded chain, i.e., an RTP. This model also accounts for the reaction that initially takes place during ribonucleotide reduction, i.e., the abstraction of an H from the 3′-position of the substrate.

We have previously tried to obtain experimental evidence for the proposed RTP of the mouse R2 protein during the catalytic reaction as well as during the reconstitution of the iron/tyrosyl radical center (12, 16). Our results strongly support the RTP model and stress the importance of a proper hydrogen-bonded chain for the formation of such a pathway, both during catalysis and the generation of the iron/radical center. Similar results were obtained for the E. coli R1 and R2 proteins (14, 15).

The connection between the R1 protein RTP with the R2 protein RTP is unknown since no co-crystallization of these proteins has been obtained. In the E. coli R2 protein, the conserved tyrosine 356 has been suggested as the connecting link since substitution of this residue with alanine and phenylalanine inhibits enzyme activity (19, 38). Tyr$^{356}$ is part of the E. coli R2 protein C-terminal tail, which is required for binding to the R1 protein (26). In the mouse R2 protein, this residue corresponds to tyrosine 370, which is not involved in the 7-amino acid-long C-terminal region required for R1-R2 complex formation (22). By substituting the mouse R2 protein Tyr$^{370}$ with either of 2 aromatic residues, tryptophan or phenylalanine, with completely different hydrogen bond-forming properties, we aimed to probe the importance of an intact hydrogen-bonded chain for the RTP.

Using the biosensor technique, we could not detect any major difference between the native and mutated R2 proteins regarding their ability to bind to the R1 protein. This was expected since Tyr$^{370}$ does not belong to the C-terminal amino acids involved in R1 binding. Furthermore, the results argue against any major collapse of the R2 protein structure. The ability to form a normal iron/tyrosyl free radical center in the mutated R2 proteins also indicates that the overall protein conformation was retained.

However, the Y370F R2 protein was completely inactive, whereas the Y370W R2 protein had a low but significant activity of ~1.7% compared with the native R2 protein. Since the R1-R2 complex formation and the iron/tyrosyl radical center are unaffected in these mutated R2 proteins, the results strongly indicate the conserved Tyr$^{370}$ to be involved in the RTP. According to the model of Siegbahn et al. (18), the radical transfer is dependent on a proper hydrogen-bonded chain. Our results are in good agreement with this prediction since interruption of the RTP totally inhibits the catalytic reaction (Y370F), whereas an alternation allows partial catalysis (Y370W). In contrast to the catalytic reaction, Tyr$^{370}$ appears not to be involved in the reconstitution of the iron/tyrosyl free radical center in the isolated R2 protein. This is different from the conserved Trp$^{103}$ and Asp$^{266}$ (16) and may be a consequence of its position in the flexible C-terminal tail of the protein.

The importance of proper hydrogen bonds as linkages in the
Radical Transfer in Mouse Ribonucleotide Reductase

RTP was also addressed in studies in which the conserved aspartic acid 237 in the E. coli R2 protein, proposed to be involved in the radical transfer, was substituted with asparagine or glutamate (15). The D237N R2 protein was catalytically inactive, whereas the D237E R2 protein retained activity of the native R2 protein. The three-dimensional structure of the D237E protein showed that this residue could indeed form the same hydrogen bonds to Trp48 and His118 as in the native R2 protein. However, the reduced activity might be explained by the slightly longer distance of the bond to Trp48.

One unexpected observation in our study is the time-dependent decay of the tyrosyl free radical when the Y370W R2 protein was incubated with an excess of the R1 protein and CDP under assay conditions, but in the absence of added iron. EPR measurements showed a significant time-dependent loss of the tyrosyl radical. As this loss was detected neither in the native R2 protein nor in the Y370W R2 protein. Instead, our results show that the entire catalytic cycle is slowed down in the Y370W mutant, and this slow rate of radical transfer may explain the loss of radical probably as a consequence of structural changes.

Together with our earlier results (12, 16), our observations strongly support a radical transfer mechanism along the previously suggested array of conserved residues, where an intact hydrogen-bonded chain is a prerequisite for an efficient transfer. Even if the C-terminal 37 amino acids of the mouse R2 protein are too flexible to be mapped in the crystal structure, they contain at least one catalytically essential residue, Tyr370. Upon R1-R2 complex formation, this residue may be placed in a well defined structural position, bridging the RTP of the R1 protein with the RTP of the R2 protein.

REFERENCES

1. Reichard, P. (1997) Trends Biochem. Sci. 22, 81–85
2. Sjo¨berg, B.-M. (1997) Struct. Bonding 88, 139–173
3. von Do¨beln, U., and Reichard P. (1976) J. Biol. Chem. 251, 3616–3622
4. Stubb, J. (1990) Adv. Enzymol. Relat. Areas Mol. Biol. 63, 349–419
5. Ehrenberg, A., and Reichard, P. (1972) J. Biol. Chem. 247, 3485–3488
6. Petersson, L., Gra¨slund, A., Ehrenberg, A., Sjo¨berg, B.-M., and Reichard, P. (1980) J. Biol. Chem. 255, 6706–6712
7. Mann, G., Gra¨slund, A., Ochiai, E.-I., Ingemarson, R., and Thelander, L. (1991) Biochemistry 30, 1939–1947
8. Nordlund, P., Sjo¨berg, B.-M., and Eklund, H. (1990) Nature 345, 595–598
9. Kauppi, B., Nielsen, B. B., Ramaswamy, S., Larsen, I. K., Thelander, M., and Eklund, H. (1996) J. Mol. Biol. 262, 706–720
10. Uhlin, U., and Eklund, H. (1993) J. Mol. Biol. 232, 123–164
11. Nordlund, P., and Eklund, H. (1995) Biochim. Biophys. Acta 1264, 323–329
12. Rova, U., Goodto¨va, K., Ingemarson, R., Behravan, G., Gra¨slund, A., and Thelander, L. (1995) Biochimie 77, 1825–1832
13. Behravan, G., Srikanta, S., Rova, U., Thelander, L. Eckstein, F., and Gra¨slund, A. (1995) Biochim. Biophys. Acta 1264, 323–329
14. Ekberg, M., Sahlb, M., Eriksson, M., and Sjo¨berg, B.-M. (1996) J. Biol. Chem. 271, 20655–20659
15. Ekberg, M., Po¨tsch, S., Sandin, E., Thunnissen, M., Nordlund, P., Sahlb, M., and Sjo¨berg, B.-M. (1998) J. Biol. Chem. 273, 21463–21472
16. Schmidt, P. F., Rova, U., Katterle, B., Thelander, L., and Grasland, A. (1998) J. Biol. Chem. 273, 15758–15761
17. Siegbahn, P. E. M., Blomberg, M. R. A., and Crabtree, R. H. (1997) Theor. Chem. Acc. 97, 289–300
18. Climent, I., Sjo¨berg, B.-M., and Huang, C. H. (1992) Biochemistry 31, 4801–4807
19. Lyckessel, P.-O., Ingemarson, R., Davis, R., Grasland, A., and Thelander, L. (1994) Biochemistry 33, 2838–2842
20. Sjo¨berg, B.-M., Karlsson, M., and Jornvall, H. (1987) J. Biol. Chem. 262, 9736–9743
21. Yang, F. S., Spanevello, R. A., Celiker, I., Hirschmann, R., Rubin, H., and Cooperman, B. S. (1990) FEBS Lett. 272, 61–64
22. Filatov, D., Ingemarson, R., Gra¨slund, A., and Thelander, L. (1992) J. Biol. Chem. 267, 15816–15822
23. Cohen, A. A., Gaudreau, P., and Langelier, Y. (1986) Nature 321, 441–443
24. Duan, E. M., Frame, M. C., SukhNetarpale, J. H., Clark, W. N., and Marsden, H. S. (1986) Nature 321, 439–441
25. Climent, I., Sjo¨berg, B.-M., and Huang, C. H. (1991) Biochemistry 30, 5164–5171
26. Cosentino, G., Lavalleé, P., Rakhit, S., Pante, R., Gaudette, Y., Lawetz, C., Whitehead, P. W., Duceppe, J.-S., Lépine-Frenette, C., Dansereau, N., Guilbault, C., Langelier, Y., Gaudreau, P., Thelander, L., and Guindon, Y. (1991) Biochim. Cell Biol. 69, 79–83
27. Roseanneg, A. H., Lade, B. N., Chai, D.-S., Lin, S.-W., Dunn, J. J., and Studier, F. W. (1987) Gene (Amst.) 56, 125–135
28. Studyer, F. W., Rosenbag, A. H., Dunn, J. J., and Dubendorff, J. W. (1996) Methods Enzymol. 265, 60–89
29. Davis, R. W., Thelander, M., Mann, G. J., Behravan, G., Souchy, P. B., Lavalleé, P., Grasland, A., and Thelander, L. (1994) J. Biol. Chem. 269, 23171–23176
30. Ingemarson, R., and Thelander, L. (1996) Biochemistry 35, 8603–8609
31. Atkin, C. L., Thelander, L., Reichard, P., and Lang, G. (1973) J. Biol. Chem. 248, 7464–7472
32. Engstrom, Y., Eriksson, S., Thelander, L., and Åkerman, M. (1979) Biochim. Biophys. Acta 18, 2941–2952
33. Nyholm, S., Mann, G. J., Johansson, A. G., Bergeron, R. J., Grasland, A., and Thelander, L. (1993) J. Biol. Chem. 268, 26200–26205
34. Jonsson, U., Fatgerstam, L., Ivarsson, B., Jonsson, B., Karlsson, R., Lundh, K., Lof, S., Persson, B., Roos, H., Rönner, I., Sjölander, S., Stenben, E., Stihlberg, R., Urbaniczky, C., Ostlin, H., and Malmqvist, M. (1991) BioTechniques 11, 620–627
35. Moser, C. K., Keske, J. M., Warnecke, K., Farid, R. S., and Dutton, P. L. (1992) Nature 355, 796–802
36. Beratan, D. N., Orucuz, J. N., Winkler, J. R., and Gray, H. B. (1992) Science 258, 1740–1741
37. Tong, W., Burdi, D., Riggs-Gelasco, P., Edmondson, D., Huynh, B. H., Stubbe, J., Han, S., Arvai, A., and Tainer, J. (1998) Biochemistry 37, 5840–5848