Ribonucleotide reductase class I enzymes consist of two non-identical subunits, R1 and R2, the latter containing a diiron carboxylate center and a stable tyrosyl radical (Tyr), both essential for catalysis. Catalysis is known to involve highly conserved amino acid residues covering a range of ~35 Å and a concerted mechanism involving long range electron transfer, probably coupled to proton transfer in both the R1 and R2 proteins have been identified, but no direct model has been presented regarding the proton transfer side of the process. Arg265 is conserved in all known sequences of class Ia R2. In this study we have used site-directed mutagenesis to gain insight into the role of this residue, which lies close to the catalytically essential Asp266 and Trp103. Mutants to Arg265 included replacement by Ala, Glu, Gln, and Tyr. All mutants of Arg265 were found to have no or low catalytic activity with the exception of Arg265 to Glu, which shows ~40% of the activity of native R2. We also found that the Arg mutants were capable of stable tyrosyl radical generation, with similar kinetics of radical formation and R1 binding as native R2. Our results, supported by molecular modeling, strongly suggest that Arg265 is involved in the proton-coupled electron transfer pathway and may act as a proton mediator during catalysis.

The Involvement of Arg$^{265}$ of Mouse Ribonucleotide Reductase R2 Protein in Proton Transfer and Catalysis*

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Ribonucleotide reductase (RNR)$^2$ of the class I family catalyzes the reduction of nucleoside diphosphates to deoxyribonucleoside diphosphates and is essential for de novo synthesis of deoxyribonucleotides (dNTPs) required for DNA replication and repair (1–4). The RNR enzymes isolated so far have been grouped into three main classes (I, II, and III). The classification is based upon the different oxygen dependence and metal cofactors involved in the generation of the essential catalytically active stable free radicals as well as structural differences among them (4, 5). The class Ia is found in eukaryotes and some active stable free radicals as well as structural differences among the residues (15, 23, 25). However, so far no specific residue(s) directly involved in the transfer of the proton has been identified. In the amino acid sequence of protein R2 the arginine residue, Arg265, is conserved in all R2 sequences of E. coli and S. typhimurium R2F proteins (3, 26, 28). However, so far no data have been provided to support these hypotheses. Upon examination of several crystal
The position of Arg265, the fact that it is highly conserved and that it has been found to be coordinated to a water molecule residing in the vicinity of Asp266 and Trp103, suggest that it may play a role in the PCET transfer process. Taking into consideration the biochemical characteristics of arginine, this residue could most likely be involved in the proton transfer part of the PCET pathway rather than in electron transfer part. It has also been suggested to be involved in R1 binding (3, 23).

Arginine residues have been implicated in various systems as key members in proton-dependent transfer mechanisms. An arginine residue has been identified as the proton donor in the presence of a diiron site in the R2 protein. To obtain R2 apoprotein, the same procedure was employed except that 0.2 mM EDTA was added 20 min before induction, and all purification buffers contained 0.2 mM EDTA.

Preparation of the Anaerobic Fe^{2+} Solution—Buffer containing 50 mM Tris-HCl was thoroughly degassed in a septum-sealed 250-ml bulb for several hours by flushing oxygen-free argon and evacuation. A second bulb containing Mohr’s salt, (NH₄)₂Fe(SO₄)₂, was sealed and evacuated using the same procedure. A plastic syringe was made anaerobic by repeated washing with oxygen-free argon gas. Then the appropriate volume of degassed buffer was used to prepare the desired iron concentration by transferring the bulb containing Mohr’s salt.

Reconstitution Reaction of Apoprotein R2 with Fe^{2+} and Oxygen—Reconstitution of the iron site in the R2 proteins were carried out by mixing oxygen saturated apoprotein R2 with anaerobic Fe^{2+} solution. Rapid freeze quench was performed with a System 1000 apparatus from Update Instruments to obtain reaction times from 20 ms to 1 s. The EPR tubes were submerged in isopentane at a temperature of −110 °C. Anaerobic iron solution and apoprotein were rapidly mixed and then quenched by spraying them onto the EPR tube. The crystals were tightly packed into the tube using a packing rod made from Teflon. Isopentane from the EPR tube was completely removed by placing the tubes under vacuum for 15 min. For reaction times above 1 s, slow-freeze quench was achieved by hand mixing. A gas-tight Hamilton syringe washed several times with anaerobic Fe^{2+} was used to reconstitute the aerobic apoprotein previously placed in an EPR tube. Reactions were quenched by immersion of the EPR tubes into cold isopentane at −110 °C. To assess the reconstitution efficiency after EPR measurements, the protein was passed through a NAP™ 5 column to remove excess iron. The total iron content was deter-

ARG^{265} OF MOUSE RIBONUCLEOTIDE REDUCTASE R2

FIGURE 1. Structural view of the Tyr^{177}, diiron carboxylate site and residues in the PCET pathway in mouse R2 protein. Asp^{266} and Trp^{103} are known to participate in PCET events. The water molecule (sphere) can be seen between Asp^{266}, Trp^{103}, and Arg^{265}. The structure used is from Kauppi et al. (29).
minded using an iron/TIBC (ferrozine) reagent set from Eagle Diagnostics.

**Ribonucleotide Reductase Activity Assay**—The ability of native and mutant R2 proteins to catalyze the reduction of $[^3H]$CDP in the presence of R1 protein was determined as described earlier (42). One unit is defined as the amount of protein that, in excess of R1 protein, catalyzes the formation of 1 nmol of dCDP/min at 37 °C. The following reagents were incubated for 20 min at 37 °C in a total volume of 50 μl: 0.5 mM $[^3H]$CDP, 3 mM ATP, 10 mM dithiothreitol (DTT), 100 mM KCl, 6.4 mM MgCl$_2$, 0.02 mM FeCl$_3$, and 40 mM HEPES buffer, pH 7.5.

**Surface Plasmon Resonance Analysis of R1-R2 Complex Formation**—All surface plasmon resonance measurements were performed using Biacore 3000 equipped with research grade CM5 sensor chips. The R2 proteins were immobilized using the amino-coupling chemistry method as described earlier (11). After activation of the dextran layer, 30 μl of R2 proteins at concentrations of 40 μM were injected at a flow rate of 5 μl/min. The R2 proteins were allowed to interact with R1 protein at different concentrations previously equilibrated with HBS-EP buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) plus 2 mM DTT. Five different R1 concentrations were used to determine kinetics for the R1-R2 interactions. Increasing concentrations of pure R1 in the HBS-EP buffer containing 2 mM DTT and 0.125 mM dTTP were used to obtain kinetic data. A constant flow of 5 μl/min of the same running buffer was used as eluent during the subunit interaction. The surface was regenerated between each protein R1 with 10 μl of 0.5 M KCl in HBS-EP buffer. Data were collected and analyzed using the Bioeval 3.2 program to obtain relative unit values of bound R1 protein for each injection.

**Electron Paramagnetic Resonance Spectroscopy**—X-band EPR spectra were measured on a Bruker ESP 300 spectrometer as described earlier (43). All spectra were recorded at 20 K.

**Modeling of the Arg Site in Mouse R2**—To model the accessibility of the surface of the protein, the NACCESS program was used (44). Insight II was also used to measure distances and minimize the structures containing the different amino acid replacements.

**RESULTS**

**Activity of Apo and Reactivated R2 Proteins**—Because Arg$^{265}$ is a highly conserved residue, we were interested in studying the enzymatic activity as well as tyrosyl radical formation in several selected point mutants. The amino acids chosen to replace Arg$^{265}$ were chosen either to be small and hydrophobic (Ala), negatively charged (Glu), polar and non-hydrogen bonding (Gln), or aromatic (Tyr). The catalytic activities of the R2 point mutants were studied and compared with native R2 protein.

The specific activities found for the native and mutant R2 proteins in the mouse RNR enzyme reaction at pH 7.5 are summarized in Table 1. All proteins were assayed directly after preparation and as described in Mann et al. (41). Proteins R2 or R1 alone were used as controls. Each assay was repeated at least three times, and the reported values are the average with an approximate uncertainty of ±5% as can be observed in Table 1. The R265Q mutant showed insignificant activity, similar to those reported for R2 protein alone in the absence of R1, which were employed as controls. Therefore, we considered it to be inactive. A similar case was observed with the R265Y mutant, for which an activity of about 4% was detected. For the R265A mutant, an activity of ~10% when compared with the native protein was observed.

A different result was obtained for the R265E mutant protein. R265E was found to have ~40% of the activity compared with the native R2. This is a significant activity, which indicates that this mutant is capable of catalysis.

**Formation of the Stable Tyrosyl Radical in Native and Mutant R2 Proteins**—The tyrosyl radical is formed in vitro in a reaction between apoprotein R2, ferrous iron, and molecular oxygen. The formation of a tyrosyl radical was studied by EPR spectroscopy. The extent of tyrosyl formation was determined by EPR spectroscopy. The spectra were recorded at non-saturating conditions at 20 K. a.u., absorbance units.

| Protein | Specific activity (nmol/mg/min)a |
|---------|---------------------------------|
| Native R2 | 165 ± 5 |
| R265A | 17 ± 2 |
| R265E | 66 ± 8 |
| R265Q | <1.7 |
| R265Y | 7 ± 2 |

a Specific activity is the average of at least three assays.

**FIGURE 2.** X-band EPR spectra of the stable tyrosyl radical of the R265E, R265Q, and native R2 proteins. The spectra were recorded at non-saturating conditions at 20 K. a.u., absorbance units.
had substantially less enzymatic activity than the native enzyme, with the exception of the R265E mutant. These differences could be due to mechanistic or kinetic differences in the formation of the stable Tyr'. To assess the rate of formation of the Tyr', we recorded the time courses of the reconstitution reaction at room temperature with ratios [Fe(II)]/[polypeptide of R2] of 3 using apo forms of either native mouse R2 or mutant R265E proteins. The apo forms of protein R2 were obtained by adding 0.2 mM EDTA before induction and purifying them in the presence of 0.2 mM EDTA.

Before reconstitution, native apoR2 had an average iron content of 1–2% and less than 5% of Tyr (Table 2). After reconstitution, native R265E had substantially less enzymatic activity than the native protein regarding Tyr' formation. 68% of Tyr' formation was observed with a $k_{form}$ of 2.15 s$^{-1}$. Both quantitatively and kinetically these values are well in accordance with previous studies (45, 46). Formation of stable Tyr' was also assessed for the R265E mutant in the same manner as above. The $k_{form}$ of the R265E mutant R2 was 3.11 s$^{-1}$ (Table 2). The amount of Tyr' per polypeptide after reconstitution was 63%. These results show that R265E and native R2 have virtually the same quantitative and kinetic characteristics regarding Tyr' formation. It should be noted that attempts to reconstitute and increase radical concentrations in iron containing R2 proteins grown in LB media also resulted in increased radical concentrations. However, the extent of radical formation was less in those proteins, and the kinetics were slower.

### R1-R2 Interaction Studies by Surface Plasmon Resonance

The role of Arg$^{265}$ in the interaction between R2 and R1 was studied by immobilizing the native and mutant R2 proteins and allowing them to interact with the increasing concentrations of R1 protein containing DTT and the allosteric effector dTTP. Previous studies have shown that DTT increases the affinity between the R1 and R2 proteins (47). Sensorgrams showed an increase in bound R1 with increasing concentrations of R1 protein consistent with previous studies (data not shown). The kinetic parameters for R1-R2 binding are summarized in Table 3. As can be observed, no significant differences were observed between the native R2 protein and the mutants R265E and R265Q. The $K_D$ values varied from 0.43 μM$^{-1}$ in native R2 to 1.09 μM$^{-1}$ in the R265Q mutant. We conclude that the point mutations keep their overall structure integrity and have only minor effects on R1 binding.

### Modeling of the Arg$^{265}$ Site

A thorough comparison of the 1.45 Å resolution crystal structure of R2 from E. coli by Högberg et al. (28) and the mouse structure from Strand et al. at 2.2 Å (30) shows that both give practically the same picture (Fig. 3). The guanidino group from Arg$^{265}$ lies ~5.32 Å away from the carboxylic group of Asp$^{266}$ and at an equivalent distance to Trp$^{103}$. This distance is bridged by a highly conserved water molecule that is located ~3 Å from the guanidino group of Arg$^{265}$ and ~2.6 Å from the carboxylic group of Asp$^{266}$. The presence of this water molecule located between the Asp$^{266}$, Trp$^{103}$, and Arg$^{265}$ residues shortens the distance between the three residues. The positive charge of Arg$^{265}$ can, therefore, serve as a modulator to the negative charge of the Asp$^{266}$ upon catalysis. The water molecule appears to be highly conserved and is present in all known crystal structures of mouse R2. It is interesting to see that in oxidized- and cobalt-substituted crystal structures a slight movement of

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**TABLE 2**

| Protein  | iron/polypeptide before reconstitution | Tyr/polypeptide before reconstitution | Rate ($k_{form}$) |
|----------|----------------------------------------|--------------------------------------|-------------------|
| Native R2 | 0.03                                   | <0.05                                | 2.15 s$^{-1}$     |
| R265E    | 0.03                                   | <0.04                                | 3.11 s$^{-1}$     |
| R265Q    | 0.03                                   | <0.04                                | 3.11 s$^{-1}$     |

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**TABLE 3**

Surface plasmon resonance R1-R2 interaction parameters in the presence of 0.125 mM dTTP effector

| Protein  | $k_{assoc.}$ | $k_{dissoc.}$ | $K_D$ |
|----------|--------------|---------------|-------|
| Native   | 1.20 × 10$^{-3}$ | 0.52 × 10$^{-1}$ | 0.43 ± 0.20 |
| R265E    | 1.71 × 10$^{-1}$ | 1.15 × 10$^{-2}$ | 0.67   |
| R265Q    | 2.07 × 10$^{-1}$ | 2.27 × 10$^{-1}$ | 1.09   |

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**FIGURE 3.** Structural view of the environment of the Arg$^{265}$ residue in R2 proteins. A, the E. coli structure (E. coli numbering) shows Arg$^{257}$ guanidino group located 5.32 Å from Asp$^{266}$ carboxylate and 5.30 Å from Trp$^{103}$. A water molecule (star) is located between the guanidino group of Arg$^{257}$ and the carboxylate group Asp$^{266}$ in each structure, with distances 3.06 and 2.63 Å, respectively. B, the mouse R2 structure shows the water molecule located between the guanidino group of Arg$^{257}$ and the carboxylate group Asp$^{266}$ at distances, 3.05 and 2.64 Å, respectively. C, an overlay of the crystal structures of R2 from E. coli (black, PDB code 1MXR (28)), mouse (dark gray, PDB code 1XSM (29)), cobalt-substituted mouse (light gray, PDB 1H0O (30), and reduced mouse R2 at pH 6.0 (gray, PDB code 1W69 (31)). Stars represent water molecules.
Arg\textsuperscript{265} of Mouse Ribonucleotide Reductase R2

The present study shows the importance of Arg\textsuperscript{265} during catalysis and explores the role of this residue as a proton mediator in the PCET pathway. This highly conserved residue is critical for catalysis and in conjunction with other residues may regulate the proton transfer process in the R2 subunit of RNR.

**DISCUSSION**

PCET reactions have been extensively studied in various systems including respiration and photosynthesis (for review, see Refs. 20 and 22). In RNR a key feature of the enzyme involves an unusual long PCET chain that connects the essential tyrosyl radical in the R2 subunit to the putative thiol radical at the catalytic site in the R1 subunit. This dedicated transfer has been established by a combination of three-dimensional structure determinations, site-directed mutagenesis, and phylogenetic sequence comparisons. Extensive site-directed mutagenesis data support the hypothesis that the PCET process occurs via a series of hydrogen-bonded amino acid residues in the R1 and R2 proteins (for review, see Refs. 3 and 25). Mutant proteins designed to alter the PCET pathway in this enzyme have resulted in catalytically inert or substantially diminished proteins (18, 43, 45).

**TABLE 4**
Solvent-accessible surface calculations using the NACCESS program

| Residue numbering | Residue in E. coli | Mouse |
|-------------------|-------------------|-------|
|                   | Å\textsuperscript{2} | Å\textsuperscript{2} |
| His\textsuperscript{115} or His\textsuperscript{173} | 0 | 0 |
| Asp\textsuperscript{236} or Asp\textsuperscript{266} | 1.9 | 6.4 |
| Trp\textsuperscript{48} or Trp\textsuperscript{103} | 21.1 | 3.8 |
| Arg\textsuperscript{265} or Arg\textsuperscript{265*} | 95.20 | 95.8 |
| Arg\textsuperscript{67} (control)* | – | 231.3 |

* The calculated areas per residue depend on the size and structure of the residue; hence, Arg\textsuperscript{67} is used as a control. The crystal structures and PDB coordinates used for these calculations are from the Högbom et al. (28) E. coli crystal structure and Kauppi et al. (29) for the mouse.

The backbone is observed, resulting in a further 0.9 Å separation between the carboxylate of Asp\textsuperscript{266} and the guanidino group of Arg\textsuperscript{265}. Yet the water molecule retains its position between both residues (Fig. 3C).

An analysis of the accessible area of the protein surface of the E. coli and the mouse R2 proteins reveals that the Arg\textsuperscript{265} is much more exposed to the surface than the Asp\textsuperscript{266} and Trp\textsuperscript{103} (Table 4 and Fig. 4). The program calculates the solvent-accessible surface of a protein defined as a locus of the center of a probe sphere (representing the solvent molecule) as it rolls over the van der Waals surface of the protein (44). A larger surface area indicates a residue more exposed to the exterior. Hence, a residue such as the His\textsuperscript{173}, which is one of the iron ligands, is found to be completely buried and with a calculated surface area of zero, whereas Arg\textsuperscript{29} in a loop region fully exposed to water has an estimated area of 231.3 Å\textsuperscript{2} (Table 4). From this it is evident that Arg\textsuperscript{265} with a surface area of 95.8 Å\textsuperscript{2} is fairly close to the surface compared with Trp\textsuperscript{103} and Asp\textsuperscript{266} with surface areas around 5 Å\textsuperscript{2}. Arg\textsuperscript{265} is, therefore, a viable candidate to act as a proton mediator from the solvent or even to be hydrogen-bonded to and mediate proton transfer from the catalytically essential Tyr\textsuperscript{370}. The Tyr\textsuperscript{370} residue is not seen in the crystal structures since it is located in the flexible C terminus of R2, but it has been postulated to bridge the PCET pathway between the R2 and R1 proteins (17, 19, 24, 27, 48). Crystal structures of the R265E and R265Q mutants are under way to elucidate more on this question.

The present study shows the importance of Arg\textsuperscript{265} during catalysis and explores the role of this residue as a proton mediator in the PCET pathway. This highly conserved residue is critical for catalysis and in conjunction with other residues may regulate the proton transfer process in the R2 subunit of RNR.

**FIGURE 4.** Solvent-accessible surface-colored mouse R2 structure (PDB code 1XSM (29)). Dark sections show buried and solvent-inaccessible residues, whereas lighter sections show solvent-exposed residues. Arg\textsuperscript{265} and Asp\textsuperscript{266} are also shown (see Table 4).

Upon substitution of Arg\textsuperscript{265} by alanine, the specific activity decreased considerably when compared with native R2 (Table 1). The substitution by alanine should confer the inability to perform direct proton transfer due to the non-polar characteristics of alanine. The low but significant activity observed in this mutant may indicate that some proton transfer during catalysis is still possible. One explanation may be that replacement with alanine, which is a much smaller residue than arginine, leaves the possibility for the water molecule to take up its required space. The low but significant activity of the R265Y mutant may be that, despite the bulky tyrosine side chain which may prevent the binding of an adjacent water molecule, the OH group of tyrosine itself may participate to a small extent in the proton transfer.
Surprisingly, when Arg$^{265}$ was substituted by Glu, the mutant had a significant activity, about 40% that of the native R2. At pH 7.5 it may be expected that Glu may be deprotonated given the intrinsically low pK$\text{a}$ of ~4 in solution. It can be argued that the overall reduced activity may result from the presence of two negatively charged residues, Asp$^{266}$ and Glu$^{265}$, in close proximity. These electrostatic alterations may result in a change of the pK$\text{a}$ most likely of the Asp$^{266}$, obstructing the proton transfer process and resulting in the observed activity.

Substitution of arginine with glutamine results in total loss of activity. The loss of activity may be due to the fact that glutamine is a non-proton donor/acceptor and, hence, unable to participate in the proton transfer process. Glutamine is also devoid of a charge, and its size may prevent interaction of the water molecule and Asp$^{266}$.

Previous studies have suggested Arg$^{265}$ involvement in R1 binding (3, 23). Our results showed no evidence that Arg$^{265}$ participates in R1 binding. Hence, we propose that the crucial role of Arg$^{265}$ in catalysis is due to its participation in PCET.

Kinetics of Tyrosyl Radical Formation—All studied Arg$^{265}$ mutants were capable of forming tyrosyl radicals to the same extent and with similar kinetics as in native R2 protein. This indicates that Arg$^{265}$ is not directly involved in the tyrosyl radical generation. This in contrast to earlier observations on mutants to Asp$^{266}$ and Trp-103, residues that are proposed participants in the long range radical transfer chain. For these mutants dramatic differences in the kinetics of the radical formation were observed (43, 45).

The Arg$^{265}$ Environment—Examination of the crystal structures showed a highly conserved water molecule that appears to bridge the Asp$^{266}$ and Arg$^{265}$. This water molecule may play a role during deprotonation of Asp$^{266}$ in the PCET pathway. Although Asp$^{266}$ may be the major proton acceptor/donor in the PCET pathway, the Arg$^{265}$ residue is crucial for enzymatic activity. Similar observations have been reported in the bacteriorhodopsin photocycle in which an arginine residue interacts with an aspartate regulating proton release. The aspartate is directly involved in deprotonation processes, whereas the positively charged arginine is thought to modulate the aspartate pK$\text{a}$ (36, 39).

Our results suggest that Arg$^{265}$ is a good candidate to play a similar role. The proximity of Arg$^{265}$ to the known catalytically active Trp-103 and Asp$^{266}$ may also suggest another role for the Arg$^{265}$ residue in which it stabilizes the structure of the pair, most likely and, particularly, the Asp$^{266}$ residue. Further analysis is on its way including structures of these R2 mutants and other double mutants to fully assess the function of the Arg$^{265}$ and gain more insight into proton-coupled electron transfer processes in the R2 subunit of RNR.

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