The mechanistic consequences of disabled electron transfer in the D266A variant of the R2 protein of mouse Ribonucleotide Reductase

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Disabled electron transfer in variant of Mouse R2

To fully understand the mechanism of tyrosyl radical formation in the subunit of ribonucleotide reductase from mouse, it is necessary to examine the kinetics of electron transfer in variants of the protein. A change in mouse aspartate 266 (D266), a conserved residue along the electron transfer pathway, is expected to block electron transfer into the diiron center of R2. A mutation in D266 was introduced by PCR, and the resulting variant gene was transformed into DH5α cells. Consequently, disabled electron transfer in mouse R2 variants will allow for the evaluation and understanding of the mechanism of cofactor formation. The assembly of the R2 cofactor is extraordinary from a chemical perspective and studies of homologous R2 proteins from various organisms will provide chemical and evolutionary insight.

Ribonucleotide reductase (RNR) is an enzyme that converts ribonucleotides to deoxyribonucleotides, which is the rate determining reaction in DNA biosynthesis (1-3). It is an enzyme that is essential to living cells and vital in DNA biosynthesis and repair. RNR is composed of two homodimeric subunits, the large (90 kD) R1 and the small (45 kD) R2. The R1 subunit contains the substrate and allosteric effector binding sites, while the small R2 subunit harbors a diiron center and the catalytically essential tyrosyl radical cofactor (2, 4, 5). Tyrosyl radical formation in mouse R2 is governed by the rate-determining step of iron acquisition. In vitro, the cofactor assembles spontaneously when metal-free R2 is mixed with Fe (II) and molecular oxygen. The reaction involves initial acquisition of Fe (II) by the protein followed by the reductive activation of O₂ by the diiron center to effect a one electron oxidation of an internal tyrosine residue (Y177). Studies with Escherichia coli R2 show that electron transfer in this reaction is rapid.

Most knowledge about R2 has been obtained from studies on the E. coli protein. These studies suggest that the main function of this cofactor is to generate a reactive thyl radical in the R1 subunit, which in turn is responsible for the initiation of the first step in the catalytic mechanism of RNR (the hemolytic scission of the 3’ C-H bond of the nucleoside substrate). For this thyl radical to be produced, an electron must be transferred from the cysteine residue (Cys439 in E. Coli) in R1 to the tyrosyl radical (Tyr122 in E. Coli) in R2. Structural studies, in conjunction with site-directed mutagenesis studies (6-8), suggest that the Cys439 residue in the R1 subunit is located 35Å away from the Tyr122 residue in the R2 subunit, making it too far removed to directly transfer an electron. It has been proposed that the electron is transferred between these two residues via a pathway involving conserved amino acid residues (Figure 1).
Information about the mechanism of tyrosyl radical formation in the R2 subunit could be used to inhibit DNA synthesis, and thereby restrict the growth of tumors in cancer or bacterial and virus replications with inhibiting drugs. The proposed electron transfer pathway consists of residues (mouse numbering) Tyr177-Asp139-His173-Asp266-Trp103-Tyr370 in R2 (8). Aspartate 266 (D266) in mouse R2 is a conserved residue involved in electron transfer. Residue D266 will be changed as a probe of the oxygen activation mechanism. Definition of the altered mechanism occurring in the variant protein as a consequence of the substitution will inform as to the tuning mechanism and importance of the electron transfer step determining the reaction outcome. Aspartate, a residue capable of electron transfer, is substituted with alanine, a small hydrophobic residue incapable of fulfilling the same function as aspartate. Investigating mouse R2 variant D266A, in which electron transfer to the iron center is blocked, the transient kinetics and mechanism involved in the formation of tyrosyl radical can be examined through the use of stopped-flow absorption spectroscopy, rapid freeze-quench electron paramagnetic resonance spectroscopy, and rapid freeze-quench Mössbauer spectroscopy.

**Preparation of vector**

Plasmid mR2 contains the mouse R2 gene in the pET 22b-expression vector. This plasmid was constructed by polymerase chain reaction (PCR), which makes it possible to amplify the mutated mouse gene rapidly (without the need for a living cell) for insertion into pET 22b. Primers used in PCR are complementary to each DNA strand. A silent mutation was also introduced, changing the coding sequence from *cct* to *ctc*, but not altering the amino acid, leucine. This mutation allowed the construction of a restriction site that will permit rapid identification of clones that contain the desired substitution. The D266A primers used were:

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ccaatgagctcattacagagCcgaggtttac 5’ SacI mR2 D266A
ggtggtgtgtcagttagaagtcagcatcacaaggg 3’ XhoI mR2
ggagatatacatatgctctccgcaccc 5’ NdeI mR2
cctcggctcgtcaatgagctcattggaatg 3’ SacI mR2 D266A
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Underlined regions represent restriction sites and bolded letters indicate a mutation in residue.
Once the DNA strand is heated, the double helix will separate and each single strand will then anneal with its primer at the complementary region. DNA polymerase then continues adding bases to the primer strand to produce double stranded DNA, then template amplification stops (9). PCR continues for many cycles yielding exponentially increasing amounts of double stranded DNA.

**Purification of DNA**

Purification is achieved through agarose gel electrophoresis. The PCR products are loaded into wells of an agarose gel and eluted via application of a potential of 90V. DNA has a net negative charge, so the DNA fragments will migrate towards the positive electrode. 100 bp and 1 kb ladders are DNA fragments that run with samples to establish a standard curve to assess the size of the DNA produced in the PCR reaction. Under an UV lamp, the DNA bands are visible in the gel by ethidium bromide staining. The DNA is extracted from the gel by using a Qiagen QiaQuick kit.

*FIGURE 3-DNA is visualized by the use of ethidium bromide (EtBr) added to gel. EtBr intercalates into DNA, causing the DNA bands to glow under a long UV light. Figure provided by [http://dlab.reed.edu/projects/vgm/vgm/VGMProjectFolder/VGM/RED/RED.ISG/gel6.html](http://dlab.reed.edu/projects/vgm/vgm/VGMProjectFolder/VGM/RED/RED.ISG/gel6.html)*

*Restriction Digest*
After purification, the DNA is cleaved with restriction endonucleases at specific base sequences to generate smaller fragments. The first fragment was created by a cleavage at the 5’ end with Sacl at the 3’ with XhoI. Similarly the second fragment was cleaved with NdeI at the 5’ end and Sacl at the 3’ end. After purification, the fragments produced are then joined in a ligation reaction.

3-point Ligation Reaction and control

In a ligation reaction, the fragments produced can be isolated and joined with pET 22b cloning vector, which has been digested with the two restriction endonucleases at the end (NdeI and XhoI). T4 DNA ligase catalyzes the joining reactions. This involves a control (restriction digested vector without insert) and multiple trials with varying amounts of both the fragment and plasmid vector. Once the insert is ligated into the pET 22b it is then introduced into a host *E. coli* DH5*α* cells via transformation.

Transformation and Expression

During transformation, 2 µL of ligation reaction mixture is incubated with 20 µL of competent DH5*α* cells on ice for 30 minutes followed by heat shock at 37 °C for 1 minute and incubation at 37 °C for 1 hour after addition of 250 µL of LB media, before being plated on LB agar. Uptake of the plasmid DNA is confirmed by growth of colonies on LB agar plates with ampicillin, since the β-lactamase gene is encoded for on the pET 22b plasmid. Through transformation, DNA plasmids become cloned as individually transformed cells undergo many divisions creating colonies.

Mini Preps of single colonies

This process verifies that the correct plasmid was transformed into DH5*α* by removing one colony from each LB agar plate. The colony is placed in a tube with 5 mL LB agar and 5 µL ampicillin then incubated for 12-16 hours. The plasmid DNA is purified using Wizard Plus SV Mini Prep kits (Promega) to determine if the plasmid contains the gene of interest by performing a diagnostic restriction digest. In this restriction digest, the DNA is cleaved with Sacl, Sacl and NdeI, XhoI and SphI. These restriction digests will show fragments with the correct sizes if the cloned gene is present in the vector. The DNA is sequenced at the Nucleic Acid Facility in Wartik laboratory at the Pennsylvania State University to verify the presence of the mouse R2 D266A gene and a final transformation into BL21 (DE3) Rosetta cells (Novagen) is performed.

Preliminary Results

Preparation of Vector-
Agrose gel after PCR shows sizes of amplified DNA product.
Rows 1, 2 are standard markers. 1kb std, 100bp std
Rows 3, 4 were created with a Sacl/XhoI primer. The fragment is ~783bp
Rows 5, 6 were created with a NdeI/SacI primer. The fragment is ~393bp

1             2                3             4             5                  6

FIGURE 3- picture of PCR products taken by Eagle Eye camera in Althouse laboratory at the Pennsylvania State University

Transformation-
Visible single DH5α colonies on LB agar plates.

Diagnostic Restriction Digest-
Gel shows 2 fragments of DNA from each product.

Discussion
The variant mouse R2 will be useful for understanding the complexities of tyrosyl radical formation. Mutation in D266 primarily provides a foundation for probing the kinetics of electron transfer into the iron center of R2. PCR, restriction digest, ligation, and transformation are the molecular biology techniques necessary to create D266A.

After PCR, the bands in the agarose gel indicated DNA amplification was successful (Figure 3). A restriction digest and ligation reaction prepared D266A for transformation into competent DH5α cells. After transformation colonies appeared on each LB agar plate, but this did not ensure that our gene was present in the plasmid. The plasmid was removed from the cells using Wizard mini-prep kits and then digested with enzymes to produce DNA fragments. Fragment sizes:

Sacl-6540 bp
Sacl/Ndel-5757 bp, 783 bp
Sacl/SphI- 5459 bp, 1081 bp
Ndel/XhoI-5364 bp, 1176 bp

Unfortunately, after transformation only single fragments for each digestion were produced, thus indicating the absence of the mouse R2 D266A gene in the plasmid. In
order to understand why the transformation did not work, the initial restriction digest and subsequent steps that follow were performed again. Once mouse R2 D266A is transformed, the plasmid DNA will be sequenced in the Nucleic Acid Facility in Wartik Laboratory at the Pennsylvania State University.

After a final transformation of BL21 (DE3) expression strain with the desired plasmid, protein produced from these cells will be purified and used to evaluate the mechanism of tyrosyl radical formation in the R2 subunit and the consequences of disabled electron transfer in the variant mouse R2. Given the complexity of this study and remarkable implications of R2 tyrosyl radical formation, complete comprehensive results and analysis are not available at this time.

**Works Cited**


**References**