EXPANSION OF THE CLASS IC RIBONUCLEOTIDE REDUCTASE, CONVERSION OF A CLASS IC TO A CLASS IA, AND SUBUNIT SWAPPING BETWEEN THE CLASSES

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by
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ABSTRACT

The enzyme ribonucleotide reductase catalyzes the reduction of ribonucleotides to deoxyribonucleotides providing all organisms with the necessary precursors for DNA synthesis and repair. RNRs accomplish this chemically difficult reaction through the use a transient cysteine thiol radical (C•) which abstracts a hydrogen atom (H•) from the 3'-carbon of the bound nucleotide’s ribose moiety to initiate its reduction. Class I RNRs, the focus of this study, utilize either a homodinuclear Fe₂(III/III)/ or Mn₂(III/III)/ tyrosyl-radical (Y•) cofactor or a heterodinuclear Mn(IV)/Fe(III) cofactor.

Class I RNRs are comprised of two non-identical protein subunits, α and β. The α subunit contains the cysteine residue which is oxidized to form the C•, the site of nucleotide reduction and binding sites for effectors. The smaller β subunit assembles the metallocofactor, ~35 Å away from the site of catalysis in α, and houses the oxidizing potential. To initiate the reduction of ribonucleotides the oxidizing equivalent stored in β translocates to α to generate the C• in a reversible process.

The first part of this thesis addresses the need to expand the class Ic RNR beyond the RNR from Chlamydia trachomatis (Ct) and the ability of these new RNRs to assemble Mn(IV)/Fe(III) cofactors. In expanding the class Ic RNRs we have identified a large number of putative Ic RNRs that exhibit high levels of sequence similarity to putative class Ia RNRs. Using these similar enzymes, we addressed the question of whether the primary basis for the division of class I RNRs lies within the β subunit or if the α subunit has coevolved. Based on these high sequence similarity between the class Ia and class Ic RNRs we demonstrated that a class Ic RNR β subunit can be the re-engineered by site-directed mutagenesis into a class Ia β subunit capable of generating a Y• upon the addition of O₂ to a diferrous center. These findings further the understanding of the differences between the class Ia and class Ic RNRs.
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Chapter 1

Introduction to Ribonucleotide Reductase

1.1. Introduction to Ribonucleotide Reductases

The enzyme ribonucleotide reductase (RNR) catalyzes conversion of ribonucleoside di(tri)phosphates [ND(T)P] to deoxyribonucleoside di(tri)phosphates [dND(T)P], the required precursors for the de novo synthesis and repair of DNA. RNRs are found in all life forms that synthesize DNA (2, 3). Due to its presence and absolute functional necessity in a wide variety of organisms, it has been targeted as an antiviral and antibiotic target and its presence in humans has led to RNR being a target for anticancer drugs. Two of the most well-known RNR targeting drugs are gemcitabine, 2',2'-Difluoro-2'-deoxycytidine, used as an anticancer drug (4) and hydroxyurea, which in combination with antiviral drugs was effective in HIV treatments (5, 6).

The overall reaction catalyzed by RNRs is the replacement of the 2'-hydroxyl group on the ribose ring of the ND(T)P with a hydrogen atom (H•). This chemically difficult replacement enhances the stability of the phosphodiester linked 2'-deoxynucleotides as compared to the phosphodiester linked ribose units. by converting the 2’carbon to a more inert carbon-hydrogen bond rather than a carbon-oxygen bond (7). Nature accomplishes this difficult reaction via free radical chemistry, in which a cysteine radical (C•) that is unstable and thus is generated in situ abstracts the 3'-H• from the ribose ring of the ND(T)P (8, 9). Due to the necessity of this reaction among all life
forms in a wide range of habitats, nature has developed three distinct strategies for the generation of the \( \mathbf{C}^\cdot \). The mechanism used to generate the \( \mathbf{C}^\cdot \) is the basis for division of RNRs into three classes (2, 10).

Class I RNRs are found in all mammals, some viruses, and some bacteria. The most well-studied representative of this class is the RNR from the aerobically growing *Escherichia coli* (*Ec*). The tertiary structure of *Ec* RNR consists of two different protein subunits, designated \( \alpha \) and \( \beta \), each arranged as a dimer in a \( \alpha_2\beta_2 \) active configuration. Higher oligomeric states of class I RNRs, such as \( \alpha_2\beta_4 \) and \( \alpha_4\beta_4 \) (11, 12) and \( \alpha_6\beta_6 \), (13) have also been demonstrated to exist and are thought to be part of the complex regulation of their activity. The \( \alpha \) subunit contains the cysteine residue that is oxidized to form the transient \( \mathbf{C}^\cdot \) radical needed for substrate reduction, as well as sites for binding substrates and allosteric effectors (2). The \( \beta \) subunit holds a dinuclear metallocofactor, which is employed to reversibly generate the \( \mathbf{C}^\cdot \).

Class I RNRs are further divided into three subclasses based on the metal content of their dinuclear cofactors; subclasses Ia and Ib utilizing homodinuclear \( \text{Fe}_2(\text{III/III}) \) (14-16) and \( \text{Mn}_2(\text{III/III}) \)-tyrosyl radical (Y•) (17-20) cofactors, respectively, and subclass Ic employing a heterodinuclear Mn(IV)/Fe(III) cofactor (21-24).
The prototypical class II RNR comes from *Lactobacillus leichmannii*, and it is an O₂-independent RNR (25). Class II RNRs have either monomeric or homodimeric structures (26-28) and use adenosylcobalamin to generate the C• (25, 29). The carbon-cobalt bond of adenosylcobalamin is homolytically cleaved to produce a 5'-deoxyadenosyl radical that can directly generate the C• (8, 25).

Class III RNRs exist in both strictly and facultative anaerobic microorganisms (2, 30). The C• is generated by a glycyl radical (G•) (2) that is unstable in the presence of O₂. A Class III RNR requires two proteins for nucleotide reduction, a larger protein housing the catalytic site and an activase protein which generates the G•. The activase protein is a radical-SAM enzyme and uses its [4Fe-4S] cluster to cleave S-adenosylmethionine generating the 5′-deoxyadenosyl radical which abstracts a H• from the glycine on the large RNR protein (31).

1.2. Class I RNRs.

The most well-studied class I RNRs require two distinct dimeric proteins (α and β), each serving its own function. The α subunit houses the substrate and the cysteine residues necessary for catalysis, whereas the β subunit contains a carboxylate bridged di-iron cofactor. After reaction of the metal center with molecular oxygen, a tyrosyl radical (Y•) is generated. This Y• can engage in a radical translocation (RT) event to relay an oxidant through a series of aromatic residues to the cysteine residue ~ 35 Å away in the α subunit, thereby generating the initiating C• (10, 32-34).

1-2.1 Class Ia: *Ec* RNR Activation and Catalysis

In all known Class Ia RNRs, of which the RNR obtained from aerobically grown *Ec* is the most well studied example, the activation of the metallocofactor in β by reaction with
dioxygen occurs independently of α, substrate, or effectors. However, catalysis can only occur when substrate, allosteric effectors, and an activated β subunit are all present.

Much of what is known about the Class I RNRs arose from studies RNR used by aerobically grown Ec. In class Ia RNRs the activation to generate the required metallocofactor and catalysis to reduce the ND(T)P are effectively independent events.

Catalysis occurs in the larger (~ 85 kDa, monomer) α subunit. In addition to containing the substrate’s binding and reduction site, the α subunit also harbors binding sites for allosteric effectors that dictate substrate selectivity (2, 34). The allosteric regulation of the α subunit is accomplished through a specific site involved in the binding of adenosine-5'-triphosphate (ATP), deoxythymidine-5'-triphosphate (dTTP), or deoxyguanidine-5'-triphosphate (dGTP) as the effector.

These effectors do not regulate in a one to one fashion but instead ATP enhances selectivity for both cytidine-5'-diphosphate (CDP) and uridine-5'-diphosphate (UDP), dTTP enhances selectivity for guanidine-5'-diphosphate (GDP), and dGTP enhances selectivity for

Scheme 1-2. Mechanism of ND(T)P reduction occurring at the active site of Ec α. Taken from ref. (1).
adenosine-5'-diphosphate (ADP) (2). Without allosteric regulation, nucleotide reduction is minimal (35). The molecule dATP was found to be a general inhibitor of nucleotide reduction because it binds to another regulatory site that functions physiologically to prevent accumulation of dNDPs to cytotoxic levels (36). The complex allosteric control at the α subunit regulates the relative abundance of the four nucleotides used for DNA synthesis and repair (37, 38).

Site-directed mutagenesis and use of substrate analogs have shown five cysteine residues in α to be essential for turnover: C439 for the initial H-atom abstraction from the ribose ring, C225 and C462 to provide the reducing equivalents, and C754 and C759 for the re-reduction of C225 and C462 after their oxidation during catalysis (9, 39, 40). After each turnover, the disulfide bond between C225 and C462 is reduced by C754 and C759, which are in turn re-reduced by an external thioredoxin in vivo, or by a chemical reductant [e.g. dithiotreitol (DTT)] in vitro. The ND(T)P reduction begins with the abstraction of the 3'-H• from the bound ND(T)P by the C• (Ec C439•). Once the H• is abstracted, an unstable carbon radical is generated at the 3' position. This radical eliminates the 2'-hydroxyl group as water, taking a proton from cysteine 225 leaving a cysteine anion, while the glutamate E441 deprotonates the 3'hydroxyl, forming a 3'-keto-2'-deoxyribonucleotid-2'-yl radical. The 2' carbon radical then abstracts a hydrogen atom from a second cysteine and forms a disulfide radical anion, residing on C225 and C462. This radical reduces the 3'-ketone, which, upon protonation yields the 3'-centered product radical. This radical then re-abstracts the hydrogen atom from C439 to regenerate the C439•. C439• engages in RT to return the oxidizing equivalent to β.

The smaller, β subunit (~ 43 kDa, monomer) was first crystallized and its structure solved to 2.2Å resolution in 1990 (15). The structure revealed that β consists primarily of α-helices. The diiron (presumably diferric) site is buried in the protein, and the Y residue that becomes the Y• in the cofactor (Y122) is ~ 5 Å away from the metal site (41). The β subunit is a structural member of the large structural superfamily known as the ferritin-like non-heme dimetal
proteins, (42, 43) named after the prototypical ferritin, the iron storage protein (44). These proteins all incorporate two metals at a site that consists of two histidines and four carboxylates in addition to oxygen- and solvent-derived ligands. Other well-characterized members of this family are: the hydroxylase components of soluble methane monooxygenase (sMMOH) (45) and toluene/o-xylene monooxygenase (ToMOH) (46), the aryl-N-oxygenase (AurF), (47, 48) the stearoyl-acyl carrier protein Δ⁹ desaturase (Δ⁹D) (49) and the aldehyde-deformylating oxygenase (ADO) (50-52). These enzymes function with reduced divalent metals and typically use activate dioxygen to generate mid- or high-valent complexes that oxidize (or otherwise transform) their substrates (43, 45, 48, 49).

Addition of molecular oxygen to the diferrous cluster of the β subunit results in a μ-peroxo-diferric intermediate, characterized by $^{57}$Fe Mössbauer as having isomer shift, $\delta = 0.63$ mm/s and quadrupole splitting, $\Delta E_Q = 1.74\text{mm/s}$, (53, 54) imply the presence of two high-spin Fe(III) ions that couple antiferromagnetically (AF) to yield a diamagnetic ground state ($S=0$) (55, 53, 56). The peroxo-diferric intermediate is reduced upon cleavage of the O-O bond by addition of one electron from the diiron center and a second one form the near-surface tryptophan residue, W48. This leads to a state containing a Fe(III)/Fe(IV) cluster, termed X, (57, 58) and a tryptophan cation radical, W48•+ (59). The W48•+ can be reduced by ascorbate or a thiol compound (59, 60) to a state containing only the intermediate X. X is capable of oxidizing Y122 to form the μ-oxo-diferric cluster and stable Y122• (57, 61). The intermediate X was initially thought to be a diferroic
species with a ligand radical giving rise to $S=1/2$ state, but ENDOR spectroscopic characterization that provided a more accurate determination of the Fe hyperfine tensors permitted a better simulation of the Mössbauer spectrum. That work revealed that X harbors a Fe(III)/Fe(I V) center, with $\delta = 0.56$ mm/s and $0.26$ mm/s, and $\Delta E_Q = -0.9$ mm/s and -0.6 mm/s for the Fe(III) and Fe(IV) sites, respectively.

While the crystal structures of both the $\alpha_2$ and $\beta_2$ proteins have been solved, there is no crystal structure of the $\alpha_2\beta_2$ complex. Attempts to crystallize this state have yielded structures of higher order oligomeric states or of the subunits in close vicinity of one another but not actually bound together. Instead, a docking model (Figure 3), based on shape complementarity of the individual subunits, has been proposed. It has long been known that peptides containing the C-terminal amino acids of the $\beta$ subunit can inhibit RNR, as demonstrated for both the herpes simplex virus 1 and $Ec$ RNRs. Using this information $Ec$ $\alpha_2$ was co-crystallized with a peptide corresponding to the twenty C-terminal amino acids of the $\beta$ subunit. The last fifteen residues of this tail were determined to reside in a groove between two helices in the $\alpha$ protein. From this structural placement, combined with the solved $\beta_2$ structure, a computational model of the $\alpha_2\beta_2$ complex was created. The model revealed that the distance between the diiron-$Y^\ast$, in $\beta$, and the C$_{439}$, in $\alpha$, is $\sim 35$ Å. This distance was later verified through the use of pulsed electron-electron double resonance spectroscopy along with the RNR mechanism-based inhibitor 2'-azido-2'-deoxyuridine-5'-diphosphate ($N_3$-UDP), which

![Scheme 1-3. Mechanism of $O_2$ activation at the diiron center of $Ec$ $\beta$.](image)
allows the radical to be trapped on the substrate analog after H• abstraction from the ribose 3’-carbon (32, 33).

For electron transfer to take place in a single-electron tunneling step over such a long distance, the Marcus-Levich equation predicts a rate of product formation in the range of $10^{-4} – 10^{-9} \text{ s}^{-1}$, which is much slower than that experimentally observed in the Ec enzyme ($2 – 10 \text{ s}^{-1}$) (10). Nature has increased this rate by instead transferring the oxidizing equivalent through a series of “radical hopping” steps using a series of aromatic amino acid (tyrosine and perhaps a tryptophan, W) residues via a specific pathway that spans the subunits (Figure 4) (67-73). This process has recently been termed “radical translocation” (RT) (74). While these transiently oxidized Ys and/or W residues along the RT pathway have never been directly observed in the wild-type enzyme, due to unfavorable kinetics and thermodynamics, the insertion of unnatural analogs such as dihydroxyphenylalanine (DOPA), (71, 75) 3-amino tyrosine (NH$_2$-Y), (72, 76) and 3-nitrotyrosine (NO$_2$-Y) (77) in place of the cofactor and redox-active Y residues provided the first direct evidence for their transient oxidation. Insertion of the former two analogs, of which both have reduction potentials for their radical forms that are less than that of Y•, at positions along the RT pathway introduced a
thermodynamic depression along the pathway, thus allowing for accumulation of the oxidant at positions other than Y122 (71, 73, 76, 78, 79). Insertion of the latter analog, (NO2-Y), which has in its radical state an elevated reduction potential relative to Y•, at Y122, led to the formation of a NO2-Y122• in the activation reaction (77). This radical was found to rapidly generate a Y• in the presence of α, substrate, and effector (11, 77). The Y• generated is thought to reside on the RT pathway and to accumulate because it is incapable of re-oxidizing the NO2-Y122.

Based on these and numerous other studies, a mechanism for RT has been proposed. For the radical translocation event to occur, the α subunit must have both substrate and allosteric effector bound. Upon complexation of the β subunit to α•substrate•effector, (34) the β-Y122• is reduced through the RT pathway to generate α-C439• and begin NDP reduction (8).

1.3. Discovery of the Class Ic RNRs

In 2000, McClarty and coworkers, cloned and analyzed the genes encoding for the α and β subunits of RNR. When the sequence of the β subunit was analyzed, it was found to contain two otherwise highly conserved amino acid replacements. In place of the apparently essential radical Y residue (Ec Y122) was a redox-inert phenylalanine (F127) and the metal site one binding ligand aspartate (Ec D84) was replaced with a glutamate (Ct E89) (80). The sequence of Ct β showed a high degree of similarity to those of the other class Ia RNR βs, including the

![Figure 1-4. RT pathway of Ec RNR. Adapted from ref. (69).](image-url)
conservation of all other putative RT pathway and metal binding residues. Within the sequence of Ct β there is a Y residue located two residues downstream from F127. This Y residue was initially suspected of functioning as the Y•, with the protein requiring a slightly different fold to account for the two-residue difference in sequence.

In 2004, the crystal structure of the Ct β subunit (PDB: 1SYY) was solved, and it revealed a striking similarity to the class Ia Ec β. The crystal structure confirmed that the position occupied in Ec β by Y122, strictly conserved in all other Class Ia RNRs, is indeed occupied by F127 and the F is not post-translationally hydroxylated to yield Y. A second difference seen in the cofactor site is the replacement of an aspartate (D) ligand to the metal site proximal to Y122/F127 (site 1) with a glutamate residue (E). Regardless of these differences, initial experiments on Ct β with its α subunit supported its ability to perform nucleotide reduction. This initiated the proposal that the Ct RNR should define a new class of RNRs that requires a di-iron metal center but does not make use of a tyrosyl radical. Instead of generating a Y• as a part of its cofactor, it was proposed that Ct β’s intermediate X (X_Ct) was competent to generate the RT pathway radical directly. This idea was further supported by the formation of X_Ct upon addition of Fe(II) and O₂ to β, and the enhanced stability of the intermediate upon the addition of α, substrate and effector. However, the amount of product formed never correlated to the amount of X_Ct that

Figure 1-5. The diiron site of Ct β. Adapted from ref. (79).
accumulated. Moreover, incubation with the N3-UDP substrate analog, which generates a nitrogen-centered radical (N•) upon H• abstraction and can serve as a reporter for successful 3’-H• abstraction, did not result in formation of the characteristic radical nor accelerate decay of X_Ct, arguing against its catalytic competence.

In 2007, work from the Bollinger and Krebs laboratories revealed that the functional form of the Ct RNR does not contain a di-iron center but instead uses a heterodinuclear Mn/Fe cofactor for ND(T)P reduction in vitro (21, 22, 24). Jiang et al. showed that a stable Mn(IV)/Fe(III) cofactor is functional (21, 84) with the Mn(IV) ion providing the oxidizing equivalent for RT. These results solidified Ct RNR’s assignment as the founding member of a new subclass (Ic) of RNR.

1-3.1 Class Ic: Ct RNR Activation and Catalysis

The activation of the class Ic RNR proceeds in a manner similar to that of class Ia RNRs. Dioxygen adds to the divalent [Mn(II)/Fe(II)] metal cluster to generate a long-lived Mn(IV)/Fe(IV) intermediate (85). This high-valent intermediate, the first of its kind to be observed, was characterized by EPR and Mössbauer spectroscopies (85). Its EPR spectrum consists of a sextet, typical of coupling to a 55Mn nucleus (I=5/2). Mössbauer spectroscopy revealed the Fe to be in the formal Fe(IV) oxidation state, with an isomer shift of δ = 0.17 mm/s. Together, the spectroscopic characterization suggests that the intermediate exhibits a ground spin state of S=1/2 arising from AF coupling between an S=2 Fe(IV) ion and an S=3/2 Mn(IV) ion.

Scheme 1-4. Mechanism of O2 activation in Ct β.
The intermediate decays through a one-electron reduction of the Fe(IV), facilitated by a dyad of aromatic amino acids (Y222 and W51, see Figure 6) (86). Reduction of the intermediate yields the stable Mn(IV)/Fe(III) cofactor, (85) the class Ic functional equivalent to the class Ia Fe(III)/Fe(III)-Y• (21). The initial structural characterization of the cofactor was obtained via Mössbauer spectroscopy. With no externally applied magnetic field, the intermediate exhibits a quadruple doublet with $\delta = 0.52$ mm/s and $\Delta E_Q = 1.32$ mm/s, parameters typical of high-spin ferric ions. When a small magnetic field is applied (~ 53 mT), the quadrupole doublet broadens significantly, indicating an integer high-spin ground state with the Mn(IV) and Fe(III) AF coupled to yield $S_{\text{total}} = 1$. The cofactor was further characterized by extended X-ray absorption fine structure (EXAFS) spectroscopy (87). The studies revealed a Mn-Fe separation of 2.9 Å and a short Mn-O interaction at 1.74 Å and density function theory (DFT) calculations favored a $\mu$-oxo-$\mu$-hydroxo-Mn(IV)/Fe(III) core structure for the cofactor. The EXAFS characterization was unable to assign the site-location of the metal ions. In 2012 the question of metal assignment was answered by a study employing crystallography and Mn-and Fe-anomalous dispersion experiments correlated with protein activity on a per Mn basis. This work provided evidence for the formation of both possible Mn(IV)/Fe(III) clusters and that incorporation of Mn into metal site 1 results in optimal in vitro activity (88).

Similarly to the class Ia RNRs, after the activation of the β subunit and binding of allosteric effectors and substrate in the α subunit, a conformational change is thought to occur which then allows for RT culminating in nucleotide reduction. The RT process in the *Ct* RNR is very similar to that in the *Ec* class Ia RNR with many of the changes occurring in the direct vicinity of the dinuclear cluster. The replacement of the Y• with the Mn(IV) may have implications for the function of the RT pathway because, while in class Ia the electron and proton acceptor is the Y residue, the path of the proton in the presumptive PCET step that moves the hole toward a is not clear. Beyond the initial radical transfer, the remaining residues in the
pathway are known from the sequence alignment with the *Ec* RNR. The radical is proposed to “hop” through residues Y338 in $\beta$ to Y991, Y990 in $\alpha$ to the C672, which then initiates catalysis (see Figure 6) (74, 89).

1.4. Aims of this thesis

While a large number of class Ia and Ib representatives from multiple species have been documented, *Ct* RNR is thus far the only known example of a Mn-Fe dependent RNR. The uniqueness of *Ct* RNR, as well as the complexities associated with assembling its active heteronuclear cofactor (it can assemble catalytically inactive diferric and conditionally-active Fe(III)/Mn(IV) clusters *in vitro*), have raised doubts as to the physiological relevance of the Mn-Fe cofactor. Therefore, expanding class Ic by characterizing other members would shed light on whether Nature has indeed devised this heteronuclear cofactor as a different solution to perform this intriguing radical chemistry. Prior to my joining the Bollinger and Krebs laboratory, a phylogenetic search was carried out using the conserved glutamate/phenylalanine residues as the characteristic idiosyncrasies of other putative class Ic RNRs. On this basis, the RNR from the
bacterium *Saccharopolyspora erythraea* (Se) was identified as a putative class Ic RNR with a high sequence similarity (75%) to the Ct RNR. The genes from Se α and β were obtained, and the proteins were expressed and purified by Drs. Allen Easton and Laura Dassama, a former and current member, respectively, of the Bollinger and Krebs laboratory. Dassama and Easton showed that, like Ct β, Se β can assemble a Mn(IV)/Fe(III) cluster via a Mn(IV)/Fe(IV) intermediate. However, their reconstitution procedures did not yield enzyme with detectable CDP-reduction activity.

At the outset of my studies, Ct RNR thus remained the only demonstrated class Ic RNR with an active Mn-Fe cofactor. Towards the first aim of showing activity for another class Ic RNR using a heteronuclear cofactor, I employed a reconstitution method for Se β that was previously established to result in better metal incorporation (88) and activity for Ct β. This method yielded Se β with detectable but low activity, $k_{cat} = 2.0 \times 10^{-4} \text{s}^{-1}$. Performing a more careful analysis of the genome of Se, I found that Se encodes a second RNR in its genome, a class II RNR. The class Ic RNR of Se may therefore not be the functional RNR *in vivo*, which might complicate further study of this enzyme. In a new phylogenetic search with the goal of finding organisms that contain solely a class Ic RNR, I identified the sulfur-oxidizing chemoautotroph *Halothiobacillus neapolitanus* (Hn). In addition to encoding only a putative class Ic RNR, Hn can be cultured in the laboratory, which opens up the possibility of carrying out *in vivo* studies on a class Ic RNR. Such studies would provide the most definitive evidence for the physiological relevance of the Mn-Fe dependence of class Ic RNRs. Hn, as well as Se, RNRs belong to a group of class I RNRs containing highly similar amino acid sequences, but with members that are also assigned to class Ia because they possess the conserved Y and D residues.

This group of class I RNRs allows the identification of a pair of Ia and putative class Ic RNRs with extremely high sequence similarity to study differences, similarities and evolutionary
transitions between the two subclasses. In the second aim of this thesis, the putative Ic RNR from \textit{Hn} and the previously identified Ia RNR from \textit{Methylococcus capsulatus (Mc)} were characterized further with respect to cofactor activation and catalysis. Reconstitution of \textit{Hn} with Mn and Fe yields an EPR silent cofactor, as expected for a Mn(IV)/Fe(III) cluster, and reduction with dithionite results in an EPR spectrum attributable to a Mn(III)/Fe(III) cofactor. That the Mn-Fe cofactor in \textit{Hn} is active towards nucleotide reduction is suggested by the detection of a nitrogen-centered radical upon incubation of the Mn/Fe-reconstituted $\beta$ with the N$_3$-UDP substrate analog in the presence of the $\alpha$ subunit and allosteric effector. The genes for the $\alpha$ and $\beta$ subunits of the class Ia representative from \textit{Mc} were obtained by Easton and Dassama, who showed that the $\beta$ subunit incorporates iron and generates a Y•. However, they were unable to establish the ability of the Y• to reduce nucleotides. After joining the lab, I heterologously expressed the \textit{Mc} RNR subsunits, purified the proteins, and biophysically and biochemically characterized the enzyme. I confirmed formation of a Y• and was able to demonstrate nucleotide reduction of CDP to dCDP with an rate of 0.35 s$^{-1}$ at 37°C. In addition, I showed by freeze-quench Mössbauer spectroscopy that \textit{Mc} $\beta$ forms a peroxo intermediate similar to that in \textit{Ec} Ia RNR in the initial generation of the Fe(III)/Fe(III)/Y• cofactor.

An important issue that most of the studies so far have not addressed is if the subclass Ia and Ic differences are confined to the activation reaction in the $\beta$ subunits or if the $\alpha$ subunits in class Ic have co-evolved with their $\beta$ subunits to accommodate the different oxidizing equivalent. Addressing this question constitutes the third aim of this thesis. While it seems plausible to use \textit{Ec} and \textit{Ct} RNR, the C-terminal tails of the $\beta$ subunits of the two are very different. Because it is known that this tail is crucial for binding to $\alpha$, a lack of activity upon subunit swapping between the two subclasses would not answer the question. Instead, the class Ia RNR from \textit{Mc} and class Ic enzyme from \textit{Se} and \textit{Hn} have, in addition to their high sequence similarity, nearly identical C-
terminal tails. Interchanging subunits between these members with nearly identical binding
determinants for $\alpha$ should therefore allow the question of $\alpha$ adaptation to the different oxidizing
equivalent in $\beta$ to be addressed with greater certainty. Toward this goal, I have demonstrated the
ability of the class Ic $\alpha$ from $Se$ to carry out nucleotide reduction using the class Ia $\beta$ subunit from
$Mc$, though only at 60% the rate of the holoenzyme constituted from homologous $Mc$ subunits.
This subunit swapping demonstrates that the class Ic $\alpha$ can accommodate the oxidizing equivalent
from a Ia $\beta$ subunit. Further, it shows that the low activity seen in the $Se$ RNR results from the $\beta$
subunit.

The overall structure and sequence similarity between the class Ia and Ic RNRs is
intriguing and invokes a number of interesting questions: a) are the residues D/Y and E/F D/Y the
only residues necessary to functionally classify an RNR as a class Ia or Ic? b) is it possible to
convert a class Ic RNR into a class Ia RNR and $vice$ $versa$ by exchanging these specific amino
acid residues in the $\beta$ subunit to their counterparts? Addressing these questions in aim four of this
thesis should give a deeper understanding into how the two very similar $\beta$ subunits of Ia and Ic
accommodate radical translocation using different cofactors. Due to their high sequence
similarity, the class Ia $\beta$ from $Mc$ and the class Ic $\beta$ from $Se$ and $Hn$ provide a good starting point
for attempts to interconvert the two subclasses. By site-directed mutagenesis, I exchanged the two
conserved glutamate and phenylalanine residues of class Ic Se $\beta$ to their counterparts in class Ia
$\beta$, an aspartate and a tyrosine. Stopped-flow absorption spectroscopy of this double variant
(E$_{101}$D, F$_{139}$Y) revealed the transient formation of a Y•. Exchanging these two hallmark residues
is thus sufficient to generate the Y•; however additional, presumably more subtle changes seem to
be necessary to stabilize the Y• and enable efficient radical translocation.

Progress has been made in the expansion of the class Ic RNRs as well as in determining
the minimum residues in $\beta$ which determine whether it belongs to class Ia or Ic. The $in$ $vivo$
detection of the RNR in the bacterium *Hn* may provide the first proven example of an RNR that functions *in vivo* with a Mn/Fe cofactor.
1.5 References


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Chapter 2 Results and Discussion

ABSTRACT

In the first step of the reaction to convert ribonucleotides to deoxyribonucleotides, a class I ribonucleotide reductase (RNR) utilizes either a homodinuclear Fe$_2$(III/III)/ or Mn$_2$(III/III)/ tyrosyl-radical cofactor or a Mn(IV)/Fe(III) cofactor in its $\beta$ subunit to generate a cysteine thyl radical (C•) in its $\alpha$ subunit. The C• abstracts a hydrogen atom (H•) from the 3'-carbon of the bound nucleotide’s ribose moiety to initiate its reduction. To date, the only RNR demonstrated to employ a Mn(IV)/Fe(III) cofactor is the class Ic enzyme from Chlamydia trachomatis (Ct). In this work, we have identified other putative Ic RNRSs and have shown that they too can assemble Mn(IV)/Fe(III) cofactors. We have also identified a number of putative Ic RNRSs that exhibit high levels of sequence similarity to putative class Ia RNRSs. Using these similar enzymes, we have determined that the primary basis for the division of class I RNRSs lies within the $\beta$ subunit, as the $\beta$ subunit of a Ia enzyme is competent for nucleotide reduction with the $\alpha$ subunit of a Ic enzyme. Furthermore, we present mutagenesis studies that indicate that the re-engineering of a class Ic RNR into a class Ia RNR might require the substitution of only a few amino acid residues within the $\beta$ subunit. Together these findings establish that Ct RNR is not the only Mn(IV)/Fe(III)-employing RNR and further our understanding of this emerging subclass of RNRSs.
INTRODUCTION

Ribonucleotide reductases (RNRs) provide all organisms with the only established pathway for the de novo synthesis and repair of DNA (1, 2). All RNRs operate via a free-radical mechanism, utilizing a transient cysteine thiol radical (C•) to abstract a hydrogen atom (H•) from the 3'-carbon of the ribose moiety of a nucleoside di- or tri-phosphate [ND(T)P] substrate (3). The instability of the C• dictates its generation in situ, and the mechanism by which RNRs generate the C• is the primary basis for the division of these enzymes into classes I-III (1, 2).

A class I RNR, such as the enzyme from humans, mouse, or aerobically growing Escherichia coli (Ec), is comprised of two protein subunits, α and β, that function in a αβ2 complex (1, 2, 4-6). The α subunit binds ND(T)P substrates and contains the cysteine residue that is oxidized to the C• (6). The β subunit assembles a metallocofactor that generates the C• in a reversible fashion (4). The identity of the metallocofactor assembled by β further divides class I RNRs into three subclasses: Ia and Ib enzymes utilize homodinuclear Fe2(III/III)/tyrosyl-radical (5, 7, 8) and Mn2(III/III)/tyrosyl-radical (9) cofactors, respectively, and the class Ic enzyme from Chlamydia trachomatis (Ct) RNR employs a heterodinuclear Mn(IV)/Fe(III) cofactor (10).

The cofactor of the Ec class Ia RNR is formed in an auto-assembly process upon the addition of two equivalents of Fe(II) and O2 to the β protein (5). A near-surface tryptophan (W48) shuttles one electron to the metal cluster, and the metal cluster itself provides one electron so that the O–O bond is cleaved. Thus the reduction of the peroxo complex yields a tryptophan cation radical (W48+•) (11) and a Fe(III)/Fe(IV) cluster (termed X) (12). The reduction of the W48+• by ascorbate or a thiol leaves X to oxidize a nearby tyrosine (Y122) to generate the stable μ-oxo-Fe2(III)/(III) Y• cofactor (13). This cofactor can effect oxidation of the C residue in α via a
radical translocation (RT) process that utilizes a number of aromatic amino acids along a specific pathway that spans the subunits. These aromatic amino acids, tyrosines (Ys) and perhaps a W, are transiently oxidized to their radical forms as they relay the oxidant from β to α (14, 15).

The RNR from Ct is different in that its β subunit lacks the cognate of the class Ia cofactor Y residue (Y122 in Ec β is F127 in Ct β) (16, 17). Furthermore, an aspartate (D) ligand at one of the two metal sites (site 1, Figure 1B) is replaced with a glutamate (E) residue (16, 17). With these changes, Ct RNR defined a new subclass of RNRs (17). In vitro studies on Ct RNR established that the enzyme employs a heterodinuclear Mn(IV)/Fe(III) cofactor to effect formation of the C• in its α subunit (10). The Mn(IV)/Fe(III) cofactor of Ct β, akin to the Fe₂(III/III)-Y• cofactor of Ec β, self-assembles from the reaction of Mn(II)/Fe(II)-β with O₂ (10).

The first intermediate observed in the cofactor assembly and activation process is a high-valent Mn(IV)/Fe(IV) intermediate (18). An electron shuttled to the Fe(IV) site through Y222 (19), a residue that aligns with leucine, L234, in Ec β, and W51, the cognate of Ec W48, reduces the Mn(IV)/Fe(IV) intermediate to the stable Mn(IV)/Fe(III) cofactor. The Mn(IV)/Fe(III) cofactor effects C• formation through a RT process that is similar to that in Ec RNR (19).

Since the discovery of the Mn(IV)/Fe(III) cofactor of Ct β, all class Ic RNRs have been presumed to assemble Mn(IV)/Fe(III) cofactors. Presently, these RNRs are assigned to this subclass on the basis of the presence of (1) F at the position corresponding to the cofactor Y residue in the Ia and Ib orthologs and (2) E at the position of the unique metal site 1 D ligand of the Ia and Ib enzymes. To date, no other class Ic RNR has been shown to function with a Mn(IV)/Fe(III) cofactor. Thus, the ability to identify another Mn/Fe-dependent RNR would further establish this cofactor as a criterion for assigning RNRs to subclass Ic.

In this work, we have shown that two putative class Ic RNRs can indeed assemble Mn(IV)/Fe(III) clusters. For the class Ic RNR from Saccharopolyspora erythraea (Se), spectroscopic data show that the Mn(IV)/Fe(III) cofactor forms after the addition of Mn(II),
Fe(II), and O₂, to the β subunit. For the RNR from *Halothiobacillus neapolitanus* (*Hn*), the addition of Mn(II), Fe(II), and O₂ to the β subunit yields an EPR-silent species. In the presence of one equivalent of sodium dithionite, an EPR signal is seen which resembles the Mn(III)/Fe(III) of *Ct* β.

An important issue that remains to be addressed is whether the functionally important differences between subclass Ia and Ic are confined to the identity of the cofactor in the β subunit or if each α subunit has co-evolved with its β subunit to function with that cofactor. While the prototypical *Ec* and *Ct* RNRs might be used to address this question, their low sequence similarity (24 % identical, 47 % similar in the β subsints, and 32% identical and 45% similar in the α) makes it less likely that any additional important co-adaptations might be discerned from among the noise of evolutionary drift. In particular, the C-terminal tails of the β subunits, which are known to be important for binding the α subunits, are very different. However, our search to identify class Ic RNRs produced a number of putative class Ic enzymes (including those of *Se* and *Hn*) with much higher levels of sequence similarity to putative class Ia enzymes (66-70% identical, 80-85% similar). In this work, we show that the RNR from *Methylococcus capsulatus* (*Mc*), a putative subclass Ia enzyme, can indeed assemble a Fe₂(III/III)/Y• cofactor and use that cofactor to effect ND(T)P reduction. We also show that the β subunit of *Mc* RNR can bind to the α subunit of *Se* RNR to effect ND(T)P reduction. This “subunit swap” experiment provides the first evidence that all differences between the two subclasses lie within the β subunits of the two.

The overall structure and sequence similarity between the class Ia and Ic RNRs is intriguing and invokes a number of interesting questions: a) are the D/E and Y/F residues the only ones necessary to functionally classify an RNR class Ia or class Ic enzyme? b) is it possible to convert a class Ic RNR into a class Ia RNR and *vice versa* by exchanging these specific amino acid residues in the β subunit to their counterparts? Here we present results of site-directed
mutagenesis studies in which we exchanged the two conserved residues, E and F, of class Ic Se β to their counterparts in class Ia β, D and Y. Stopped-flow absorption spectroscopy with this double variant (E101D/F139Y) β reveals the formation of a transient Y•. Exchanging these two hallmark residues is sufficient to generate the Y• in Se β; however, additional, presumably more subtle changes seem to be necessary to stabilize the Y• and enable efficient RT.

RESULTS and DISCUSSION

2.1 Expansion of the Class Ic RNR

On the basis of sequence alignments and the presence of the Glu/Phe (E/F) dyad considered as the hallmark for Class Ic RNRs, the enzyme from the organism Se had been selected by previous members of the group as a putative class Ic RNR. Preliminary stopped-flow absorption experiments demonstrated its ability to form the high valent Mn(IV)/Fe(IV) intermediate, as evidenced by the appearance of the characteristic absorption band at 390 nm (see Figure 2) and the EPR spectrum in Figure 3. Mössbauer spectroscopy further confirmed formation of the stable Mn(IV)/Fe(III) species, the decay product of the Mn(IV)/Fe(IV) intermediate. The Mn(IV)/Fe(III) state has an integer-spin ground state (S=1), resulting from antiferromagnetic coupling of its Mn(IV) (S_Mn = 3/2) and high-spin Fe(III) (S_Fe = 5/2) constituents. The zero-field spectrum (Figure 4) is a sharp quadrupole doublet with parameters (δ = 0.53 mm/s and ΔE_Q = 1.37 mm/s) typical of high-spin Fe(III), which broadens in the presence of a weak applied magnetic field. The obtained parameters are very similar to those obtained for the case of Ct RNR (δ = 0.52 mm/s and ΔE_Q = 1.32 mm/s). These earlier results demonstrated that the Se β resembles Ct β in cofactor generation. However, activity measurements monitoring
its potency to support nucleotide reduction by its cognate α subunit had not been performed and, therefore, no firm conclusion that Se is indeed a class Ic RNR could be drawn.

Therefore, at the outset of my studies, I decided to purify the α subunit from Se, so as to demonstrate the catalytic activity of Se for nucleotide reduction. Purification of the His6-α subunit presented difficulties in its folding and binding to the Ni-NTA resin, resulting in impure eluates. In order to improve protein expression and folding, I overexpressed the α subunit along with the chaperonin protein complex, GroES-GroEL, which allowed for the successful purification of soluble protein using dATP-sepharose affinity-column chromatography. Having both subunits purified, I monitored the activity of Se RNR by mass spectrometric assays after employing a variety of methods, differing with respect to the type and stoichiometry of the metals added to the apo-protein, to reconstitute the Se β. The Se exhibited activity only when both manganese and iron were added to the β subunit. Figure 5 shows a correlation between substrate turnover and the metals added to the β subunit. The highest turnover number was seen when both Mn(II) and Fe(II) were added to the β subunit, resulting in 19 turnovers in 15 minutes, much greater than when only Fe(II) (5 turnovers) or only Mn(II) (4 turnovers) was added.

Performing a more careful analysis of the Se genome, I discovered that Se possesses a putative class II RNR in addition to the tentatively assigned class Ic RNR. The presence of a second RNR in Se might, in principle, have allowed the Ic RNR to have lost its in vivo functionality through evolution. A more targeted search for a class Ic RNR was then undertaken, with the requirement that the organism contain only a class Ic RNR. The Ic-like RNR from the sulfur oxidizing chemoautotroph, Halothiobacillus neapolitanus (Hn), was selected, because the Hn genome, like that of Ct, contains only the class Ic-like RNR.

The Class Ic RNRs from Hn and Se belong to a group of enzymes with high sequence similarity. Intriguingly, this group has members from both Ia and Ic subclasses. Initially, when the RNR from Se was found (in 2007), this group consisted of only fifteen RNRs,
of which ten were assigned as class Ia RNRs and five as class Ic RNRs. Since then, more bacterial genomes have been sequenced and made available through the NCBI Blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). At present, the group has expanded to include over one hundred members, with approximately equal representation of class Ia and class Ic RNRs, as classified on the basis of the characteristic D/Y and E/F dyads, respectively. Using this larger group of RNRs, I performed a more detailed and robust analysis of residue conservation. The comparison between the β subunits of class Ia and Ic RNRs within this group of very similar RNRs shows that, in addition to the two well-established residue replacements (i.e. D/Y \(\leftrightarrow\) E/F), several other residues showed a high degree of conservation within each subclass but differ between the two subclasses (highlighted in red in Figure 5). These residues include V\(_{28}\), T\(_{126}\), and F\(_{196}\) (\(Ct\) β numbering). Within this group of similar RNRs (which, incidentally, does not include the RNR from \(Ct\)), the residue corresponding to \(Ct\) β-196 is always phenylalanine in the class Ia RNRs but always a tryptophan in the class Ic orthologs. A close examination of the \(Ct\) β structure showed that most of these residues are localized on the protein surface and may thus have only an indirect effect on metal binding or the activation process. One of them, however, found in the position directly preceding the class-defining residue Y (or F) is strictly conserved within the two subclasses. In the Ia class, an alanine precedes the Y residue, whereas in the class Ic a threonine is found before the phenylalanine F. Due to its close proximity, this residue may have a functional implication in the stabilization of the tyrosyl radical.

2.2 Characterization of a pair of class Ia and class Ic with high sequence similarity

The identification of a pair of Ia and Ic representatives that share a high degree of similarity will allow the important differences between them to be defined by reciprocal re-engineering. In this context, the class Ic ortholog from \(Hn\) and class Ia ortholog from \(Mc\) were
selected for further study with respect to their cofactor identities, activation reactions, and catalytic activities.

The Hn RNR harbors the characteristic residue dyad (E/F) that is representative of the class Ic RNRS. As a first step, stopped-flow absorption spectroscopy was employed to monitor the initial activation of the cofactor in Hn β in the reaction of the Mn(II)/Fe(II) protein with O₂. No transient species with optical features in the visible regime could be detected, and therefore no evidence for the generation of the Mn(IV)/Fe(III) state could be obtained. I then tried to follow the activation reaction by EPR spectroscopy. In agreement with the fact that the activated Mn(IV)/Fe(III) state is EPR silent, no signals could be detected in samples of Hn β subjected to the activation reaction. However, it was anticipated that, upon treatment with the strong reductant, sodium dithionite, the characteristic spectrum of the one electron reduced Mn(III)/Fe(III) state might be formed. Indeed, the EPR spectrum of the dithionite-treated Hn β (Figure 7) reveals signals characteristic of a Mn containing species, suggesting that the Hn β subunit can undergo activation by dioxygen and assemble the active Mn(IV)/Fe(III) cofactor. The ability of the Mn-Fe cofactor in β to catalyze nucleotide reduction in the Hn α subunit was further studied by mass spectrometry. Preliminary assays at relatively low protein concentration did not show detectable conversion of the CDP to dCDP. Incubation of Hn β, α and effector with the substrate analog 2’-azido-2’-deoxyuridine 5’-diphosphate (N₃-UDP), which I synthesized following a literature procedure (20), does not generate the nitrogen centered radical, which suggests that the Hn β does not engage in translocation of the electron hole to the active site in α. The EPR spectrum of this sample (Figure 8) does not show the characteristic nitrogen centered radical known to be formed in other class I RNRs when they are treated with this analog, while the positive control using Ct RNR does show the radical, thereby verifying the quality of the N₃-UDP. The tentative conclusion is that a Mn(IV)/Fe(III) cofactor can form, but has no detectable
activity. This lack of activity may be rationalized, considering that the protein concentrations used for the experiments were not sufficiently high for detection of the species under investigation. Use of higher concentrations is planned. Toward this end, I have now obtained sufficient protein to permits systematic modification and optimization of the reconstitution procedures and other factors limiting proper assembly and activity of the holo-enzyme complex (e.g., removal of the His-tag, which could interfere with subunit association).

As a representative of class Ia, the enzyme from *Methylococcus capsulatus* (*Mc*) had previously been selected. It belongs to the group of very similar class Ia and Ic RNRs and has high sequence identity to Ic RNRs from *Hn* (63% identity in the β) and *Se* (67% identity in the β). *Mc* RNR had previously been expressed and purified by Easton and Dassama, who demonstrated by absorption and EPR spectroscopy the ability of the β subunit to form a Y•. However, they did not detect any nucleotide reduction.

The ability of *Mc* β to form a Y• suggests that it can form the active Y•/Fe(III)/Fe(III) cofactor in its initial reaction with O2. In the prototypical class Ia *Ec* RNR, the active cofactor is generated by addition of Fe(II) to the apo protein in the presence of O2, and the reaction proceeds via a peroxo-Fe5(III/III) intermediate, a state containing the Fe(III)/Fe(IV) cluster, X, and a tryptophan cation radical (W48•+), and a state containing only X. In the final step, X then oxidizes Y122 to form the active diferric/Y• cofactor. It was anticipated that *Mc* β would react via the same activation pathway, and I pursued detection and trapping of these intermediates.

The peroxo-diferric intermediate, the first intermediate in the activation pathway, has a broad absorbance at approximately 700 nm. Stopped-flow absorption kinetic experiments, in which the diferrous form of the protein was rapidly mixed with O2-saturated buffer, showed a fast (25 s⁻¹ at 5 °C) formation and slower decay (2.4 s⁻¹) of a species that absorbs at 700 nm. As shown in Figure 10, absorbance at 700 nm reaches its maximum value at 100 ms. Freeze-quench Mössbauer experiments were then carried out (Figure 12). A transient diferric species with
unresolved sites was observed, which accumulates maximally to approximately 35 - 40% at a reaction time of 100 ms. This conclusion is in agreement with the stopped-flow kinetic traces. This species has an isomer shift of $\delta = 0.58$ mm/s and a quadrupole splitting $\Delta E_Q = 1.71$ mm/s, values that agree well with those of the Ec $\beta$intermediate ($\delta = 0.63$ mm/s and $\Delta E_Q = 1.58$ mm/s) (21-23). The contribution from this species is diminished at a reaction time of 450 ms (Figure 10, trace C) in agreement with the stopped-flow kinetic traces. In addition, another species is now observable in the spectra of the sample quenched at 450 ms, signified from the pronounced shoulder at ~0.8 mm/s and the broadening at ~2 mm/s. This species can be well fitted using the parameters reported for the $\mu$-oxo-diferric product in Ec $\beta$ and is therefore assigned to that diferric product state (5, 24, 25). By 2.4 s, a time-point at which the amount of the tyrosyl radical formed is maximum, the amount of the $\mu$-oxo-diferric product state has increased (Figure 12, trace D). In addition to this species, another diamagnetic diferric state having a spectrum with unresolved Fe sites must be invoked so as to account for the residual intensity. This species increases with time, accumulating maximally after a reaction time of 15 min (Figure 12, trace F). The Mössbauer parameters of its spectrum, $\delta = 0.57$ mm/s and $\Delta E_Q = 1.75$ mm/s, are close to those of the tentatively assigned peroxy-diferric intermediate (Figure 12, traces B, C). However, the absence of the 700 nm band in the stopped-flow absorption spectra for the same timepoint, as well as the very slow increase in its intensity with time, indicate that it is a new diferric species, different from the peroxy-diferric intermediate observed in the earlier time points. Though more evidence is required to determine as to whether these two diferric species are different or the same, the observations that one species gradually decreases in intensity and the other one increases in intensity with time, might suggest that these diferric species are distinct. The isomer shifts and quadrupole splitting parameters of the $\mu$-oxo-diferric product in Mc $\beta$ are gradually shifting from the 2.4 s to the 15 min spectrum (Table 1). The observed shifts, though very subtle
and likely within the error of the analysis, can be perhaps correlated to the loss of a proton from the Fe1-OH₂ upon decay of the tyrosyl radical, which would slightly perturb the spectral features.

The decay of the peroxo-diferric intermediate is followed by the transient appearance of another chromophore having a sharp absorption band at 409 nm characteristic of a Y• on top of a shoulder (25) (Figure 11). The kinetics of the Y• were extracted by the well-known "drop-line correction" procedure, which relates the absorbance at 409 nm to the average of the absorbance at 404 and 414 nm, essentially measuring the peak height. Figure 10 shows the kinetic traces of the 700-nm and 409-nm bands. The signal associated with the Y• forms at 1.89 s⁻¹, as the peroxo-diferric signal decays at 2.4s⁻¹. Formation of a tyrosyl radical was confirmed by EPR spectroscopy (Figure 11). The tyrosyl radical signal is centered at g = 2.00 and shows ¹H hyperfine splittings that have been assigned to originate from six protons (the two methylene protons and the four ring protons, though two of the ring protons, named C2 and C6 have vanishing spin density and hardly contribute to the X-band spectrum) with the delocalized pi system (though not so-well resolved due to the experimental conditions at which it was measured). Overall, the breadth of the signal and its comparison with the cognate radical formed in Ec β shows that the tyrosine in Mc β has an orientation of its ring relative to the the β-hydrogens (which determines the magnitude of the hyperfine couplings and thus the spectral lineshape) that is similar to that in the Y• in Ec β. In addition, while the tryptophan cation radical is also expected to form in the activation reaction of the Mc RNR, its expected absorbance at 560 nm was not detected. The lack of transient absorption at 560 nm does not imply that the radical does not form, but rather that its kinetics are unfavorable for accumulation under the conditions used. Although formation of the Y• is, in the reactions of other β proteins, preceded by accumulation of intermediate X, the fact that only Y• could be detected in the Mc system suggests that X might not accumulate.
Having characterized the activation process in Mc β, I went on to demonstrate its capability to support nucleotide reduction by its α subunit, the ultimate proof that it is a functional class Ia RNR. The problem with the β subunit as expressed and purified by previous lab members was the presence of two His₆-tags, one located on the N-terminus and the other on the C-terminus of the protein, which prevented the interaction of the two subunits. Through modification of the plasmid, the C-terminal His₆-tag was eliminated. The expressed protein then contained only the N-terminal His₆-tag and a free C-terminus, which then allowed for the α and β subunits to associate, as required for nucleotide reduction. The Mc RNR was able to undergo multiple turnovers at a rate of 0.35 s⁻¹ per β₂ (Figure 14), which is 20% of the minimum value reported for than the Ec Ia RNR(4) and close to the rate of the Ct Ic RNR (0.4 - 0.6 s⁻¹)(26). Productive turnover shows that the goal of obtaining an active class Ia RNR from the group of very similar RNRs has been achieved.

2.3 Assessing co-evolution of the α subunits by subunit swapping between the class Ia and Ic

While the class Ic RNRs were not as active as expected, the small amount of activity seen can still allow for the determination of the presence of any additional modifications present in the α subunits by monitoring nucleotide reduction using subunits from different classes. The C-terminal tails of the β subunits of the Mc and Se RNRs are very similar, differing only in two amino acid residues. This high degree of similarity between the binding determinants improves the chances that the β subunits will be able to interact with the other organism’s α subunits. By removing the problem of binding determinants between the subunits, the only difference between the two β subunits will be the oxidizing agent stored in each.

A complex of the Se α subunit and the Mc β subunit is able to perform nucleotide reduction, demonstrating that there are no modifications within the α subunit of a class Ic RNR to
accommodate the different cofactor. Using the \( Mc \beta \), the rate of deoxynucleotide production by \( Se \alpha \) was less (0.16 s\(^{-1}\) or about 46\%) that observed with the homologous \( Mc \alpha \) (Figure 15). The multiple turnovers observed in the reaction demonstrate that the radical translocation pathway in the heterologous \( Se-\alpha^{\bullet}Mc-\beta \) complex is competent in both directions and not unidirectional, as seen in some RNRs with unnatural amino acids. Because RT can occur in both directions and deoxynucleotides are produced, it is likely that there are no co-adaptations associated with the use of the Mn/Fe cofactor in the class Ic \( \alpha \) subunits. The lack of deoxynucleotide production when using the \( Se \beta \) and the \( Mc \alpha \) does not necessarily imply that the Ia \( \alpha \) subunits are incompetent with Ic \( \beta \) subunits, because the \( Se \beta \) is not very active with its homologous \( \alpha \) subunit, and, therefore, the deficiency of the \( Se \) system resides in the \( \beta \) subunit.

### 2.4 Interconversion of a class Ic and a class Ia RNR

The results of the subunit swapping experiments suggested that the amino acids distinguishing a class Ia and Ic RNR are located primarily or exclusively in the \( \beta \) subunit. The residues that we have used to identify an RNR as either Ia or Ic are the D/Y dyad in Ia and E/F pair in Ic. Using these two replacements as a starting point, the variant \( Se \beta \ E_{101}D/F_{139}Y \) was created. If the D/Y-E/F residues represent the only important distinction, then this variant should resemble a class Ia RNR when it is loaded with ferrous iron and generate a stable diferric-\( \gamma^{\bullet} \) when reacted with \( O_2 \).

In the reaction of the manganese and iron loaded protein, there were no distinguishable intermediates. In the iron loaded protein, the reaction with \( O_2 \) shows the appearance of a peak at 409 nm, corresponding to a tyrosyl radical. While this radical is expected, it shows a different behavior than that of the \( Mc \beta \) activation. The radical forms with a rate constant of 0.17 s\(^{-1}\) and maximizes at 15 seconds, compared to the \( Y^{\bullet} \) in \( Mc \beta \), which forms at 1.7 s\(^{-1}\) and maximizes at
3.6 seconds (Figure 16). Holding all factors constant, the Y• in the Se β E101D/F139Y forms to half the intensity and has a much longer lag phase than the Y• in the Mc β. Due to the instability of the Y• in the variant, the activity assay was initiated by addition of iron to the mixture of α, β, substrate and effectors to be sure that the transient Y• was present, but no nucleotide reduction could be detected by mass spectrometry. These differences in formation and decay kinetics and incompetence for nucleotide reduction demonstrate that, while the presence of the tyrosine allows for creation of the Y•, it does not readily engage in radical translocation.

2.5 Conclusion

Progress has been made in expanding the class Ic family of RNRs, with the orthologues from Se and Hn demonstrating the ability to form the active Mn(IV)/Fe(III) cofactor in their β subunit and to mediate nucleotide reduction in their α subunits. Due to the activity of the α subunits, the problem resides with the β subunit. The most likely reasons are either incorrect placement of metals in the site or a different fold blocking the C-terminal tail is unknown. The activity of the α subunits with the β subunits of the class Ia RNR from Mc strongly suggests that only the β subunit determines whether an RNR is class Ia or Ic and the α subunit has not co-evolved to become specific to the oxidizing agent of its subclass.
Figure 2-1. The metal sites of class Ia *Ec β*(27) and class Ic *Ct β*(28). The metal ligands are largely conserved, with the exception of Y122→F127 and D84→E89. *Ct β* also incorporates a Mn ion at metal site 1 in its most active cofactor form.
**Figure 2-2.** Stopped-flow trace of the time evolution of the 390 nm optical feature denoting formation of the Mn(IV)-Fe(IV) intermediate in Se β2, after addition of 3 equivalents of Mn and 1.5 equivalents of Fe to the apo-protein. The second order rate constant for formation of the Mn(IV)-Fe(III) state is 4.8 mM\(^{-1}\)s\(^{-1}\) at 5 °C.
Figure 2-3. EPR spectrum of the apo-Se β overexpressed in Ec in which equivalents of Fe(II) and equivalent of Mn(II) were added anaerobically. The metal loaded protein was rapidly mixed with O₂ saturated buffer and frozen in . The spectrum resembles the Mn(IV)/Fe(IV) spectrum characterized in Ct β.
Figure 2-4. Mössbauer spectrum of $\text{Se} \beta$, which was loaded with 3 equivalents of Mn(II) and 1.5 equivalents of Fe(II) and allowed to react with O$_2$ in air for one hour on ice. The zero field spectrum of the $\text{Se} \beta$ is dominated by a quadrupole doublet with parameters $\delta = 0.53$ mm/s and $\Delta E_Q = 1.42$ mm/s, shown in red, and accounts for $\sim$69% of the $^{57}\text{Fe}$. This doublet arises from the Mn(IV)/Fe(III) cofactor and exhibits parameters similar to those from $Ct \beta$, $\delta = 0.52$ mm/s and $\Delta E_Q = 1.32$ mm/s. The remaining $^{57}\text{Fe}$ is from the Fe$_2$(III) form of $\text{Se} \beta$, shown in blue, is characterized by $\delta = 0.49$ mm/s and $\Delta E_Q = 1.01$ mm/s.
Figure 2-5. Metal dependent activity of the Se RNR. With both Mn and Fe present, 19 turnovers are completed in 15 minutes as compared to 5 turnovers with only Fe(II) added and 2 turnovers with only Mn(II) added. When no metals are added 4 turnovers are completed.
Figure 2-6. Comparison of the consensus sequences of the Class Ia and Ic RNRs from the group of very similar RNRs. The residues highlighted in yellow show a conservation >99% within the respective subclass, residues highlighted in green show a conservation >90% within the respective subclass. Residues highlighted in red show a 100% conservation within the subclass but differ between the two subclasses.
Figure 2-7. EPR spectrum of the apo-$H\text{n} \beta$ overexpressed in $Ec$ (grown in RLB with 100 µM 1,10 Phenanthroline) and in which 1.5 equivalents of Mn(II) and Fe(II), respectively, were added anaerobically. The reaction solution was allowed to react for 20 minutes in air and then was dialyzed once against 10 mM EDTA once, and then twice against 50 mM HEPES, 50 mM NaCl, 10% Glycerol, pH=7.6. An aliquot of this solution was transferred to an EPR tube and frozen in liquid N$_2$ (red trace). The same sample was subsequently thawed in air and 1.0 equivalent of sodium dithionite was added and allowed to react for 5 minutes; the sample was then frozen in liquid N$_2$ (blue trace). In the control (red trace) Mn(II) signals were hardly detectable, whereas in the sodium dithionite reduced sample signals characteristic of $^{55}$Mn (I=5/2) were observed around $g$=2.00.
Figure 2-8. EPR spectrum of the apo-Hn β overexpressed in Ec (grown in RLB with 100 µM 1,10 Phenanthroline) and in which 1.5 equivalents of Mn(II) and Fe(II), respectively, were added anaerobically. After exposure to air for 20 minutes nucleotide reduction was initiated by adding 100 µM β$_2$ to 150 µM α$_2$, 300 µM N$_3$-UDP, 300 µM ATP, 10 mM DTT, 12 mM Mg(II). The sample was then allowed to react in air for an additional 20 minutes before being frozen in liquid N$_2$. The spectrum of this sample is shown in red. As a positive control for the N$_3$-UDP inhibition the same reaction mixture was made containing Ct α$_2$ and β$_2$ instead of Hn α$_2$ and β$_2$. This spectrum is shown in black and shows splittings due to the $^{14}$N of the N$_3$-UDP and cysteine β protons. Experimental conditions: T=100K, microwave power 1 mW, microwave frequency 9.48 GHz, time constant .08 seconds, scan time 84 seconds, modulation amplitude 1.5 Gauss.
Figure 2-9. Absorption spectra of three time points of the reaction of Fe(II) loaded *Mc β*, with O₂. The spectra show the difference in absorbance between the time labeled and the initial spectrum. The loss of absorbance in the 700nm region of the spectrum coincides with an increase in absorbance in the region below 450nm. Of note is the appearance of the shoulder at 409 nm which is indicative of a Y•.(25)
Figure 2-10. Formation and decay of the 700 nm (red) and 409 nm (blue) species at 5°C as monitored by UV-Visible stopped-flow spectroscopy. The 700 nm absorbing complex, tentatively assigned as peroxo-Fe$_2$(III), forms at a rate of 25 mM$^{-1}$ s$^{-1}$ and decays at 2.4 s$^{-1}$. The absorption at 409 nm is characteristic of the Y$\bullet$ as seen in other class Ia RNRs, forms at 1.7 s$^{-1}$, which can be seen to being for form as the 700 nm species begins to decay.
Figure 2-11. Comparison of the two Y• formed of the Ec and Mc RNR β subunits. Mc protein concentration was 100 µM β2, 300 µM Fe, reacted at 5°C for 20 seconds before frozen in liquid N₂. Spectra were acquired at 14.0 ±0.2 K, microwave frequency of 9.48 GHz, a microwave power of 20 µW, a modulation frequency of 100 kHz, and a modulation amplitude of 10 G, a scan time of 167 s with a time constant of 167 ms.
**Figure 2-12.** Rapid freeze quench $^{57}$Fe Mössbauer spectra monitoring the reaction of Fe(II) loaded $Mc$ β with O$_2$. **A)** Anaerobic control **B)** 100 ms. The blue trace corresponds to the simulated spectrum assigned to the Fe$_2$(III)-peroxo intermediate. **C)** 450 ms Blue trace corresponds to the simulated spectrum assigned to the Fe$_2$(III)-peroxo which has now decreased almost 50% in intensity. A second spectrum with two resolved sites (red trace) is observed and assigned to the μ-oxo-Fe$_2$(III) product state, analogous to the one detected in $Ec$ RNR. **D)** 2.4 s A third diamagnetic species that has a spectrum with unresolved sites is also observed (green trace). **E)** 1 minute. After subtraction of the ferrous control from the spectrum (not shown), the spectrum the residual signals are characteristic of diamagnetic diferric clusters. The spectrum of the μ-oxo-Fe$_2$(III) product is now better described considering slightly different values than in C, which are more similar to those earlier reported(24). The subtle spectral changes may be related to loss of a proton from the Fe$_4$(III) upon decay of the tyrosyl radical. The parameters of the two sites of the μ-oxo-Fe$_2$(III) product state spectrum are now more resolved. This could be explained by the decay of the tyrosyl radical which is magnetically coupled to the diiron site. **F)** 15 minutes The
spectrum is dominated by the unknown Fe₂(III) species (green trace) and the \( \mu \)-oxo-Fe₂(III) state (red trace). There is a residual amount of Fe₂(II) species that corresponds to \( \sim30\% \). Experimental conditions \( T = 4.2 \text{ K}, B = 53\text{ mT} \) applied magnetic field parallel to the \( \gamma \) beam.
Table 1. Mössbauer parameters of the different species observed in the freeze quenched $^{57}$Fe Mössbauer time-course spectra monitoring the reaction of Fe(II) loaded Mc β2 with O$_2$. 

<table>
<thead>
<tr>
<th>Spectrum</th>
<th>Time(sec)</th>
<th>Peroxo</th>
<th>μ-oxo-diferric</th>
<th>Unresolved Diferric</th>
<th>Ferrous</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>$\delta$(mm/s)</td>
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<tr>
<td>A</td>
<td>00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.59</td>
<td>1.71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
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<td>0.59</td>
<td>1.71</td>
<td>0.54, 0.45</td>
<td>1.62, 2.16</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>0.54, 0.45</td>
<td>1.62, 2.19</td>
</tr>
<tr>
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<td>60</td>
<td>-</td>
<td>-</td>
<td>0.48, 0.45</td>
<td>1.56, 2.35</td>
</tr>
<tr>
<td>F</td>
<td>900</td>
<td>-</td>
<td>-</td>
<td>0.48, 0.48</td>
<td>1.43, 2.33</td>
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</table>
Figure 2-13. Depiction of different iron speciation observed in the freeze quenched $^{57}$Fe Mössbauer time-course spectra monitoring the reaction of Fe(II) loaded $Mc \beta 2$ with O$_2$. The parameters of the $\mu$-oxo-Fe$_2$(III) change slightly from the 2.4 second spectrum to the 1 minute and 15 minutes spectra, which may be related to the loss of a proton from the Fe$_2$(III) upon decay of the Y•. The Fe$_2$(III) species depicted in green bars has parameters similar to those of the tentatively assigned peroxo-Fe$_2$(III) intermediate. However, such an assignment would not be supported by the stopped flow experiments and from its gradual increase in intensity as observed in the Mössbauer time-course therefore it is more likely that it is another Fe$_2$(III) product state different from the peroxo-Fe$_2$(III).
Figure 2-14. Time dependence of deoxynucleotide production from Mc RNR. Deoxynucleotide production occurs at a rate of 0.35 s⁻¹ at 37 °C. Reaction conditions: 100μM α2, 10μM β2, 30μM Fe(II), 2mM ATP, 2mM CDP, 10mM DTT, 12mM Mg(II). Reaction was quenched with 2M Formic acid.
Figure 2-15. Comparison of deoxynucleotide production using the Mc β subunit with either the Se α or the Mc α. The exponential fits of the given rates of enzyme inactivation of 0.0016 s⁻¹ for the Mc α β reaction and a rate of inactivation of 0.00095 s⁻¹ for the Se α Mc β reaction. The rates of deoxynucleotide production are 0.35 s⁻¹ for the Mc α Mc β and 0.16 s⁻¹ for the Se α Mc β system. Reaction conditions: 100 µM α2, 10 µM β2, 30 µM Fe(II), 2 mM ATP, 2 mM CDP, 10 mM DTT, 12 mM Mg(II). Reaction was quenched with 2 M formic acid.
Figure 2-16. Formation and decay of the Y• in the β subunit from three different proteins. The *Mc* β protein shows a formation rate of the Y• of 1.7 s⁻¹ with a decay of 0.009 s⁻¹ while the engineered *Se* β protein shows a formation rate of 0.17 s⁻¹ and a decay of 0.022 s⁻¹, all at 5°C. The Fe(II) loaded wild type *Se* β protein does not show a 409 nm absorbance.
2.6 References.


Chapter 3 Outlook

3.1 Establishing catalytic competence of RNRs from \textit{Se} and \textit{Hn}

3.1.1 Assessing the stability of the Mn(IV)/Fe(III) cofactor in \textit{Se} \(\beta\)

The \(\beta\) subunit of \textit{Se} RNR has been shown to assemble a Mn(IV)/Fe(III) cluster that is spectroscopically similar to the stable Mn(IV)/Fe(III) cofactor of \(Ct\) \(\beta\). However, the rate at which CDP is converted to dCDP is by Se RNR is only \(\sim 3\%\) that of \(Ct\) RNR. A number of factors could contribute to the seemingly low activity of Se RNR, and each will need to be thoroughly investigated. For one, the stability of the Mn(IV)/Fe(III) cluster in Se has not been reported. The Mössbauer spectrum reported in Figure 2 is the product of a reconstitution procedure that spanned 1-2 days. While the Mn(IV)/Fe(III) cofactor of \(Ct\) \(\beta\) is stable over a similar period of time, Mn(IV)/Fe(III) cluster of \(Se\) \(\beta\) may not be. The 4.2-K/53-mT spectrum of \(Se\) \(\beta\) shows a paramagnetic species at \(\sim 0.53\) mm/s. This indicates the presence of high-spin Fe(III) ions, inactive Mn(III)/Fe(III) clusters, or both. To determine and quantify the species, the spectra will need to be acquired over a wider range of Doppler velocities.

The second factor that could contribute significantly to the low activity observed is simply the quantity of Mn(IV)/Fe(III) clusters relative to \(\beta\). To date, all activity measurements on \textit{Se} have reported turnover numbers on a per-enzyme basis, as opposed to a per-cofactor basis. Work on the \(Ct\) \(\beta\) cofactor assembly has shown that there is at most \(\sim 1.2\) cofactors per dimer of \(\beta\). Furthermore, the assembly of heterogeneous cofactor forms in \(Ct\) \(\beta\), in which Mn can occupy site 1 or site 2 and couple to Fe, results in lower catalytic activity. Thus, a more rigorous analysis, including a quantitative analysis of the metals incorporated and spectroscopic characterization of all the cluster forms is necessary to ascertain whether the low activity is inherent to the enzyme or it is a consequence of poor cofactor assembly.
The third factor that might contribute to the comparatively low activity of Se RNR could be the presence of the N-terminal His-tag on the β subunit. The His-tag might affect cofactor assembly and stability, and radical translocation by impairing proper protein folding, dynamics and subunit-subunit interactions. To assess disturbance of the His6-tag, the tag can be removed by proteolytic cleavage using the thrombin site of the construct. Alternatively, the β subunit can be expressed and purified without a tag. The subunits of Ec RNR are produced and purified without any tag, the purification procedure for Se β can therefore be based on this established procedure.

3.1.2. Kinetic and spectroscopic characterization of cofactor assembly in Hn

The addition of Mn(II), Fe(II), and O₂ to Hn β yields an EPR-silent cluster. This cluster is presumed to be an $S=1$ Mn(IV)/Fe(III) form because treatment of the protein with a chemical reductant (e.g., sodium dithionite) results in the formation of an EPR-active Mn species that is coupled to Fe. Ultimately, the presence of the Mn(IV)/Fe(III) cluster will be probed using Mössbauer spectroscopy. Quantitative analyses, similar to those proposed for the Mn(IV)/Fe(III) cluster in Se RNR and studies with the untagged β subunit will need to be carried out to ascertain the source of the lack of catalytic activity.

3.2 Detection of the use of a Mn/Fe cofactor in vivo

In vitro characterization of Ctr RNR suggests that during catalysis the Mn(IV) is reduced by one electron, generating the EPR active species Mn(III)/Fe(III). This electron is believed to originate from a radical-translocation (RT) pathway tyrosine (Y) or tryptophan (W), generating a radical (Y• or W•) on the RT pathway. Studies have shown that the $\alpha_2\beta_2$•substrate•effector complex is rapidly inactivated by hydroxyurea (HU). Presumably, this is due to HU interception of a RT pathway radical, leading to irreversible RT and causing the metal cluster to remain as Mn(III)/Fe(III). From this knowledge, as well as the use of HU in antiviral therapies, the in vivo
detection of the characteristic EPR signal of the Mn(III)/Fe(III) state upon addition of hydroxyurea is feasible.

Seβ has been demonstrated to assemble a Mn(IV)/Fe(III) cluster and utilize this cluster for nucleotide reduction in vitro. For Hn, the product of divalent metals and O₂ addition to the protein are inconclusive. Unlike Ct RNR, these enzymes originate from organisms that can be cultured under normal laboratory conditions. Therefore the two offer the opportunity to ascertain the use of the Mn(IV)/Fe(III) cofactor for nucleotide reduction in vivo, thereby providing the most definite evidence that the functional cofactor in class Ic RNRs is a Mn-Fe cluster.

Preliminary results from the in vivo studies on Se are inconclusive. The organism was cultured and grown but initial whole cell EPR investigations did not show formation of the expected Mn(III)/Fe(III) signal. Samples of the cell mass both with and without hydroxyurea, concentrations up to 10mM, did not show any difference. The discovery of the gene for a class II RNR in the Se genome make it less attractive for further in vivo studies because the class II RNR may be the preferential RNR of the organism.

The organism Hn is well studied due to extensive work on carboxysomes and the RuBisCO system. From these studies methods to genetically engineer Hn have been developed (1). Previous work has demonstrated that Hn is capable of heterologously expressing proteins from other organisms, including antibiotic resistance proteins(1). This knowledge can be used to add affinity tags to the β subunit of its class Ic RNR, while also introducing antibiotic resistance, to then allow for easier purification of the protein from host organism. Purification of the β subunit from the native organism would allow for detection of the cofactor used in vivo. Crystallization of this protein should then reveal the location of each metal ion in the physiologically functional cofactor. Preliminary results show that I am able to grow Hn both in small cultures as well as in bigger scale using a fermentor. An initial 1 L culture is slowly diluted
with fresh media over the course of 24 hours to 5 Liters while pH is kept constant at 6.4 and with under a constant supply of air. The 5 L culture yields approximately 10 grams of cell paste. While the growth of *Hn* require more work than *Ec*, its ability to be grown as well accept genes for antibiotic resistance, makes it a good target for *in vivo* study.

### 3.3 Rational reprogramming of a class Ic RNR into a class Ia RNR, and vice versa

The attempt to rationally reprogram the class Ic *Se* β into a class Ia β by exchange of the conserved residues F and E to Y and D yields an *Se* double variant that is able to form a Y•. However, more changes seem to be necessary to allow the Y•/diferric cluster within this class Ic protein scaffold to initiate nucleotide reduction. Sequence analysis of the group of highly similar class Ia and class Ic RNRs revealed that an additional seven residues show a high degree of conservation within each subclass. Whereas most of these residues are on the protein surface, the residue directly preceding the class defining Y or F is highly conserved within the two subclasses of that group and might directly influence cofactor assembly and stability and radical translocation. Therefore, as a continuation of the rational reprogramming of the *Se* β subunit, residue T₁₃₈, the amino acid residue preceding Y/F, will be the first target towards further mutagenesis. T₁₃₈ will be changed to alanine, the corresponding residue in the Ia RNRs of that group. In addition, the effect of the other six conserved residues will be assessed by stepwise and combined exchange of the residues in Ic *Se* to their counterpart in class Ia RNRs. Alternatively the β subunit from *Hn* can be used as a starting point.

The *Mc* RNR has shown to be the most active RNR of the group of very similar RNRs and creation of the D₁₄₄E and Y₁₈₂F double variant will be attempted to determine if a known class Ia RNR can be converted into a class Ic RNR. In addition to these two mutations, substitution of the residue directly preceding the Y/F (A₁₈₁T) and the additional conserved
residues will be performed. The effect of these substitutions on Mn(IV)/Fe(III) cofactor generation and stability, and nucleotide turnover will be assessed towards the goal of converting a class Ia into class Ic RNR.
3.4 References