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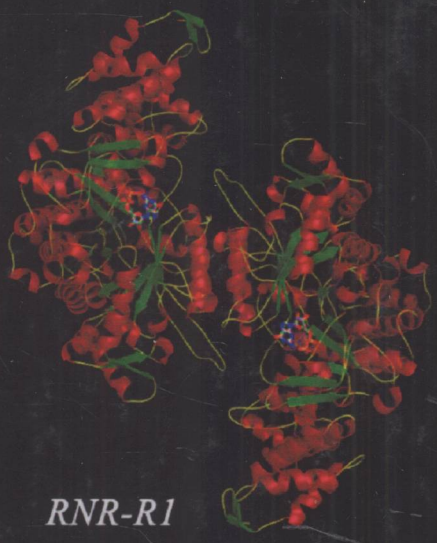

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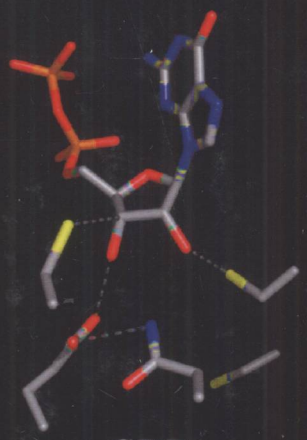

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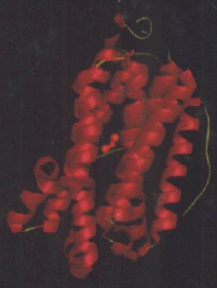
Ribonucleotide Reductase

K. Kristoffer Andersson
Editor



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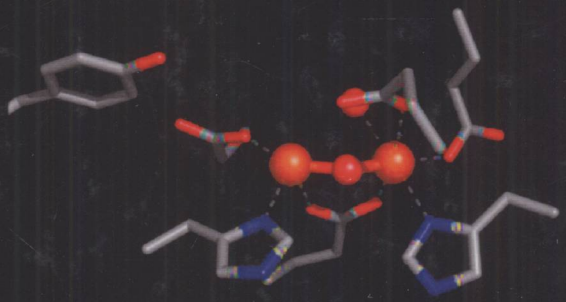

Iron-oxygen cluster

Molecular Anatomy and Physiology of Proteins
Vladimir N. Uversky - *Series Editor*

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A Volume in Molecular Anatomy and Physiology of Proteins Series

RIBONUCLEOTIDE REDUCTASE

K. KRISTOFFER ANDERSSON
EDITOR

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Contents

In: Ribonucleotide Reductase
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Chapter I

General Short Overview of RNR and Introduction

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Introduction

The subject of this book is the amazing enzyme ribonucleotide reductase (RNR), the enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides. The prerequisite for DNA-synthesis and DNA-repair in all living cells is the supply of the four deoxyribonucleotides. Such molecules result from the enzymatically difficult radical-induced reduction of ribonucleotides, a multistep chemical process catalyzed by RNR. RNR was the first enzyme in which the presence of an amino acid radical (a tyrosyl) in *E. coli* Class Ia RNR has been proven [1]; since then several other biological amino acid radical species have been found on e.g. tryptophan, glycine, cysteine, lysine residues and on amino acid derived small cofactors like 2 tryptophanes in thryptophan-trypthanyl-radical or cysteine-tyrosyl-radical in other enzymes. As all known cellular life forms store their genetic information as DNA, RNR is likely to be found in all growing cells of every living organism, a fact that is confirmed by a rapidly increasing number of genomic screenings. In addition, several species of viruses and bacteriophages evolved in such a way to carry their own copy of RNR proteins, probably to ensure faster proliferation in the infected host cell. Understanding how the RNR enzymes work was pioneered by the group of Peter Reichard and collaborators at the Karolinska Institute in Sweden. Nowadays RNR research constitutes a multidispinary field which includes studies on whole cells and organisms, transgene mice, 3D protein structure, magnetic spectroscopy analysis, chemical kinetically mechanism and reactivity, and quantum mechanical studies to mention some.

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Studies of RNR serves as models for the radical enzymes group and long range electron/radical transfer proteins. Generally free radicals contain one unpaired electron and they usually exhibit high reactivity, which can promote fatal damages to the living organisms by destroying biological materials. During evolution the major challenge of controlling the biological free radical chemistry of the highly reactive as well as short-lived radical units led Nature to form three major Classes of RNR (Table 1) depending on the cells living conditions. The formation of 2'-deoxyribonucleotides begins (see description below in Figure 2) with the hydrogen-atom abstraction from the 3'-ribonucleotides by a cysteine derived thiyl radical located in the active site of RNRs. The three main Classes of RNR known so far use different types of cofactors with different strategy to generate the thiyl radical (Table 1) in order to carry out the reduction process and they are called Class I (Chapters 2-8, II (Chapters 2,9) and III (Chapters 2,9). Class I enzymes contains a diiron-oxygen/tyrosyl radical cluster and/or Fe-Mn/FeFe cluster in Class Ic; Class II uses a cobalt containing deoxyadenosyl-cobalamin cofactor (vitamin B_{12}); Class III contains an 4Fe4S iron-sulfur cluster, one S-adenosylmethionine (SAM or AdoMet) and a glycyl-radical (see Table 1 and Chapter 2 for definitions). The generation, storage and transport of the radicals (thiyl, tyrosyl, glycyl, tryphophanyl and deoxyadenosyl) are also dependent on these cofactors.

Table 1. The Classes of Ribonucleotide Reductase in Overview

	Class Ia	Class Ib	Class Ic	Class II	Class III
Metal/ cofactors	Fe-O-Fe	Fe-O-Fe	Fe-O-Fe and/or Mn-Fe	Deoxyadenosyl-Cobalamin, (B_{12})	4Fe-4S SAM/AdoMet
Subunit structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	α or α_2	$\alpha_2\beta_2$
Radicals involved in turnover	Tyrosyl, Cysteinyl	Tyrosyl, Cysteinyl	Fe(IV)Fe(III), Mn-Fe (also ?) Cysteinyl	Deoxyadenosyl Cysteinyl	Deoxyadenosyl Glycyl, Cysteinyl
Oxygen dependence	Aerobic	Aerobic	Aerobic	No dependency	Anaerobic
Substrates [a]	NDP	NDP	NDP	NDP/NTP	NTP
Reductant	Thioredoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	Thioredoxin	Formate
Allosteric sites	2	1	1	1	2
Occurrence	Eukaryotes Eubacteria, e.g. *Escherichia coli* Bacteriophages Viruses	Eubacteria	Eubacteria	Archaebacteria Eubacteria, e.g. *Lactobacillus leichmannii*	Archaebacteria Eubacteria, e.g. *Escherichia coli* Bacteriophages

[a] N represents any of the four ribonucleotide bases, A, C, G, or U.

In Class Ia and b RNR in the smaller subunit R2 the tyrosyl-radical is generated via oxygen reaction with 2 Fe(II) + one electron yielding a mixed valent Fe(IV)Fe(III) species, called intermediate X, that finally abstracts an hydrogen atom (one electron + a proton) from

the tyrosine, yielding formation of both neutral tyrosyl-radical and a μ-oxy bridged oxygen-Fe(III)Fe(III) cluster (a iron-oxygen cluster) in active R2. Class Ic does not have this tyrosine residue. The active Fe(IV)Fe(III) species coordinate two O(H) groups bridging the Fe(III) and Fe(IV) metal cores, hence forming a structure like diamond label in poker cards named a diamond core structure. The intermediate X in this case functions alone and/or in combination with a MnFe cluster (see Chapter 6) as the initial radical generator instead of the tyrosyl-radical. In Class II the Co(III)deoxyadenosyl-cobalamin the metal-carbon bond is homolytically cleaved. This reaction provides both one electron that reduces the Co(III) to Co(II) and a deoxyadenosyl-radical. In Class III the S-adenosylmethionine binds to one open iron-site in the Fe4S4 cluster and then SAM is homolytically cleaved to a deoxyadenosyl-radical and one electron reduced iron cluster (the Class III RNR belong to the super family of radical SAM enzymes). The initially cofactor formed radicals then abstracts hydrogen-atoms (or possibly an electron) yielding other new radicals. The mechanism of radical transport or coupled radical-proton transport to and back to radical source can occur over conserved amino acids. The process can provide a transient amino acid radicals transport chain up to 30-35 Å long pathway in Class I RNR (Chapters 2,3), while deoxyadenosyl-radical in Class II RNR is in the active site with the thiyl radical (see Figure 1).

The RNR represents both the beginning and the rate limiting step in the DNA–synthesis, and the process is highly allosterically regulated, especially in higher organisms. Therefore subtle differences exist between RNRs depending on organisms such as presence of different hydrogen bonds in mammalian RNR missing in most simpler organisms. Because the reduction of ribonucleotides represents the rate-limiting step of DNA synthesis, it renders RNR an important target for cell growth control, and several RNR inhibitors have been used (see Chapter 2 for structures of several different groups of drugs), or have been proposed, as drugs. For example RNR inhibitors can be found in chemotherapeutic treatments of cancer, sometimes in combination with other drugs, and could potentially be used in a range of infections such as tuberculosis, sexually transited diseases (e.g. AIDS and Chlamydia), sleeping sickness and malaria (Chapters 2-5).

Recently the RNR enzymes exhibit even more complex structure and properties in higher organisms such as mammalian cells, where at least two iso forms of the smaller subunit R2 exist with different functions. The R2 subunit first discovered is active during cell division, mainly in S-phase of cell cycle, where it carries de novo synthesis of deoxynucleotides for general DNA syntheses. The second subunit called p53R2 is a target gene for the (tumour) p53 protein (Tp53) as well as important in the mitochondrial DNA synthesis. Recent findings have shown that most of the p53R2 knockout mice died by the age of 11–12 weeks and these mice had abnormalities in the kidney and several other organs. In addition living cells lacking p53R2 were more susceptible to oxidative stress by hydrogen peroxide than cells containing p53R2 thus further strengthening the role of p53R2 in DNA repair and the mitochondrial DNA depletion was found in various tissues of the Rrp53R2_/_ mouse (Chapter 2,5).

Within the realm of novel drugs development, very recently a novel metal Mn-Fe cluster was reported in 2007. The presence of the Mn-Fe cluster increased activity in RNR Class Ic. These important findings open up new ways of specific inhibition of the human parasite *Chlamydia trachomatis* RNR (Chapter 6). Not all aspects of RNR can be comprehensively treated in this book, hence we have chosen to report mainly studies on RNR of Class I

enzymes which are more suited examples for the Molecular Anatomy and Physiology of Proteins series, for some general references describing RNR see ref [2-11].

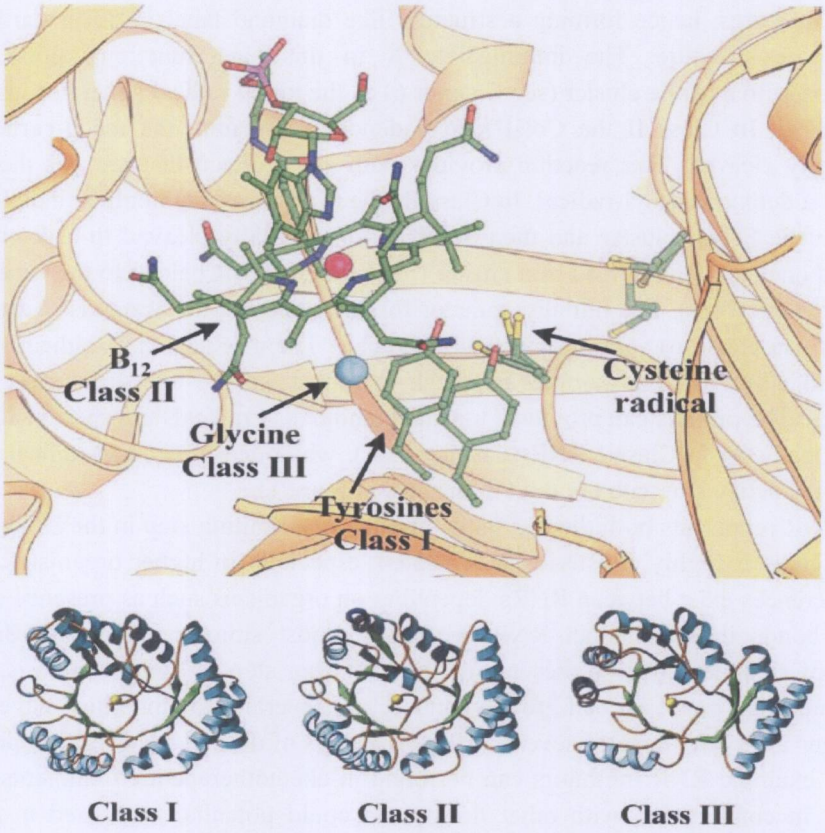

Figure 1. Active sites in three Classes of RNR. The top figure shows an overlay of the substrate binding active sites in the three Classes of RNR. The common cysteine radical sites are indicated, and the relative positions of the different radical generation systems in the three Classes are indicated by the last tyrosines of the radical transfer pathway for Class I, the B_{12}-derivative adenosylcobalamin for Class II (magenta sphere: cobalt), and the glycyl radical site for Class III (cyan sphere). The lower figure shows the similarity of the substrate-binding domain of the three Classes of RNR, the ten-stranded α/β-barrel. The position of the cysteine radical at the tip of the internal loop is indicated by a yellow sphere. The figures are taken from Catherine L. Drennan and coworkers; reference [12] with permission.

Similar Thiyl Radical and Reaction Mechanism in the Three Classes of RNR

Over the last few years, the crystal structures of the active site subunits of all three Classes of RNR have been solved; first, the R1 subunit of Class I RNR from *E. coli* in 1994 in Uppsala, then the Class III protein from Bacteriophage T4 in 1999, and finally in 2002, the monomeric Class II protein from *L. leichmannii*, which was crystallized as an apo enzyme, as well as in complex with the vitamin B_{12} analogue adeninylpentylcobalamin [12]. Despite large differences in protein sequences (less 10% based on sequence alignment), the three

Classes have very similar 3D structure of the (substrate reduction) active site (see Figure 1 from [12]), confirmed by the constant increasing number of new crystal structures of RNR enzymes (see Chapter 2, 3, 4, 8 and 9). Although the three different Classes of RNR enzymes depend on different metal cofactors for the catalytic activity, all three Classes have as mentioned earlier a conserved cysteine residue at the active site located on the tip of a protein loop in the centre of common 10-stranded α/β barrel with postulated thiyl radicals site close to the turning points. This cysteine residue is believed to be converted into a thiyl radical that initiates the substrate turnover in all three Classes of RNR [5-11]. Figure 1 gives an overview of the active site loop of Class I, II and III, where the thiyl radical is located at Cys439 in Class Ia R1 from *E. coli* (it is similar to Cys388 in Class Ib R1E from *Salmonella typhimurium* and a yeast R1 see Chapter 2 and 4), Cys408 in Class II RNR from *Lactobacillus leichmannii*, and Cys290 in Class III RNR from a mutated bacteriophage T4. The functional and structural similarities suggest that the present-day RNRs have all evolved from a common ancestral reductase; you can read more about the evolutionary aspects of the RNRs in Chapter 2.

The reaction mechanism of the three Classes is similar as well (Figure 2), in addition to the structurally conserved cysteine (Cys 439) forming the thiyl-radical in Class I and II is a conserved cysteine pair (Cys 462 and Cys 225, *E. coli* numbering) that reduce the 2'-suger derivative the deoxy form with formation a disulfide bond, the one of these (probably Cys 225) is replaced with formate in Class III RNR (see last chapter 9 for the reaction mechanism of Class III RNR). For a complete turnover, the active site disulfide bridge between C225 and C462 must be reduced. The electron needed for this reduction is provided via a redox chain, which involves a cysteine pair on the surface of R1 (C754 and C759, part of the flexible C-terminal not seen in the X-ray structure), thioredoxin (or glutaredoxin), and finally the NADPH binding flavoprotein thioredoxin reductase. Thus, NADPH ultimately provides the reducing power of Class I RNR. During this regeneration of the cysteine pair no free-radical (thiyl or other radicals) can be present in the active site as any radical (which always have a high redox potential close to one Volt) would be then be reduced before any of cysteine-disulfide pair with low redox potential and consequently enzyme will would die as it would lose the activity dependent radical. Thus the activity dependent radical property is transported away from the active site in Class I and III or deoxyadenosyl-cobalamin-Co(III) is reformed in Class II before any regeneration of the cysteine pair.

In the first step of the substrate turnover cycle (see Figure 2), the thiyl radical (C439 in *E. coli* in the Figure 2) (state 1) will abstract the 3'-hydrogen atom from the ribose ring of the substrate and thereby generate a substrate radical (state 2). The ribose radical makes the 2'-OH-group more accessible to acid catalysis, and the 2'-OH-group is therefore protonated by C225 and then leaves as a water molecule. The substrate is thereby converted to a 2'-ketyl radical (state 3). The two cysteine residues, C225 and C462, are then oxidized to a disulfide anion radical when they transfer a hydrogen atom to the substrate (state 4). The excess radical electron is then transferred via a chain of hydrogen bonded active residues, N437 and E441, to the substrate 2'-postion, thereby regenerating a substrate radical (state 5). This substrate radical then abstracts the hydrogen from C439, which results in completion of the deoxyribonucleotide product and a thiyl radical on C439 (state 6).

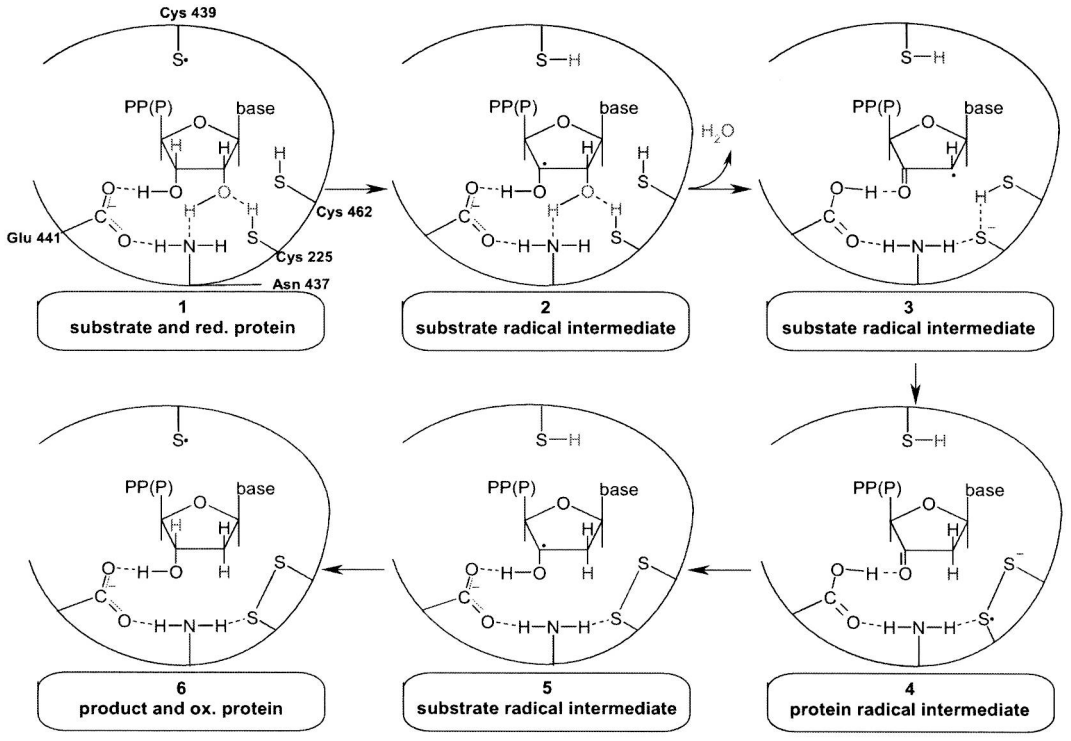

Figure 2. Reaction mechanism of Class 1a RNR. The postulated reaction mechanism for reduction of a ribonucleotide by Class I RNR. The involved amino acid residues are given with *E. coli* numbering. The reaction scheme is believed to be very similar for Class II RNR. For Class I, the substrate is a 5'-diphospho (PP) ribonucleotide, whereas Class II mainly utilizes 5'-triphospho (PPP) ribonucleotides. The thiyl radical abstract the ribose 3'-H-atom forming substrate radical intermediate (step 1); then the ribose 2'-hydroxide is protonated and leaves as water generating a substrate 2'- ketyl-radical (step 2); followed substrate reduction on 2'-possition with formation di-sulfide radical on the protein (step 3); later the protein radical generate new 3'-substrate/product radical (step 4); finally the thiyl radical is regenerated by a hydrogen atom abstraction as well as the end product (step 5). In the end the thiyl-radical is converted to cysteine when the radical is transported back to radical storage/generator center. The figure is taken from reference [10] with permission.

The radical is then transferred via the long range radical transfer chain back in Class I RNR. The active site of Class II RNR contains all the conserved residues found in Class I RNR, indicating a similar reaction mechanism as Class I. However, there is one significant difference and that is the generation of the cysteine radical in Class II is believed to be formed directly at the active site using the vitamin B12 derivative, 5'-deoxyadenosylcobalamin. This cofactor therefore seems to replace the role of the β2 subunits of Class I and III as the radical generator. For detailed mechanism for Class I and II RNR see Chapter 3 and reviews [4-11], and Chapter 9 for Class III RNR [7-11].

RNR has several allosteric sites (Chapters 2-5,9) that contribute to a very highly tuned activity for the enzyme, especially in higher organisms, to obtain a balanced pool of the four deoxyribonucleotides needed for DNA synthesis in the cell. The allosterically regulated RNRs show differences between Classes. Class Ia has an activity site A-site (that regulates overall activity by increasing activity with e.g. ATP binding and decreasing activity with e.g.

dATP binding), and a specificity binding site (S-site) that regulates substrate specificity in complex manor as it can bind many different ligands in addition to the active site (with the thiyl radical). Furthermore, interactions between subunits are also important and complex because hexamerization of the H-site been suggested (see details in Chapters 2, 3 and 4). The location of the allosteric sites in the three Classes of RNR has been identified by site-directed mutagenesis studies and by cocrystallisation of allosteric effectors with the respective proteins. Interestingly, Class Ia and Class III enzymes, but not Class Ib and II, have the activity site (A-site), while the location and function of the specificity site (S-site) seems to be more comparable between the three Classes of RNR. These differences in allosteric regulation of the different Classes of RNR are discussed in detail in Chapter 9.

Spectroscopic and Structural Studies of the Diiron-Oxygen Radical Site of RNR R2

The multi-meric Class I RNR has active and allosteric sites in the larger subunit called R1 (Chapters 2-4). The smaller dimeric subunit has a heart shape is called R2 and contains a μ-oxo bridged Fe(III)Fe(III) cluster plus a semi-stable tyrosyl –radical in the active form (Chapters, 2-5,7,8). Figure 3 shows one subunit of mouse R2 and a blow up of the Fe(III)Fe(III) cluster with an oxygen-atom bridging the two irons 3.3 Å apart in mouse R2 protein. The iron-radical center is arranged in a so called 4 α-helix bundle, which has similar overall protein fold to the 2UW2.pdb of the human R2 protein. Combination of magnetic electron paramagnetic resonance (EPR) techniques and protein crystallography demonstrated that the tyrosyl-radical moves in the active radical form compared to the Fe(III)Fe(III) form (see Chapter 8) [10,11]. Pulsed EPR spectroscopy enabled to detect weak magnetic interactions among sites located more than 40 Å apart to each other and showed that tyrosyl-radicals can be present at the same time in both R2 subunits. Substrate analog radicals located in the active site of R1 RNR protein can have magnetic interaction with the tyrosyl-radical in the R2 protein (see Chapter 8). Both the iron cluster and the radical unit are buried inside the R2 protein backbone, making the radical stable at +4C for months in *E. coli*, the radical has a weak exchange coupling with the iron-oxygen cluster. The radical properties need to be transported to and from the active site by a pathway composed of several conserved of amino-acids in holo-RNR. Part of this pathway is shown in Figure 4 for the case of mouse R2 protein. The tyrosyl-radical is interacting to one iron center by very weak exchange coupling, then the iron is linked via hydrogen bonds to an histidine-carboxylate-tryptophane triad, similar to triad that one have found in cytochrome c peroxidases (see Chapter 2 and 3). In Chapter 3 is a description of a model for the conserved pathway in *E. coli* holo-RNR plus the structure of a holo-RNR complex is reported. However, probably this structure does not represent the catalytically active holo-RNR conformation. In one structure of the mouse R2 protein an extra non biological active tri-nuclear Fe cluster could be formed accidentally (see Chapter 8); several unsuccessful trials were performed further on in order to reproduce this unusual cluster (Åsmund K. Røhr K.K. Andersson unpublished results also on http://www.esrf.eu/exp_facilities/BM1A/site/report/report0304.pdf p. 4-6).

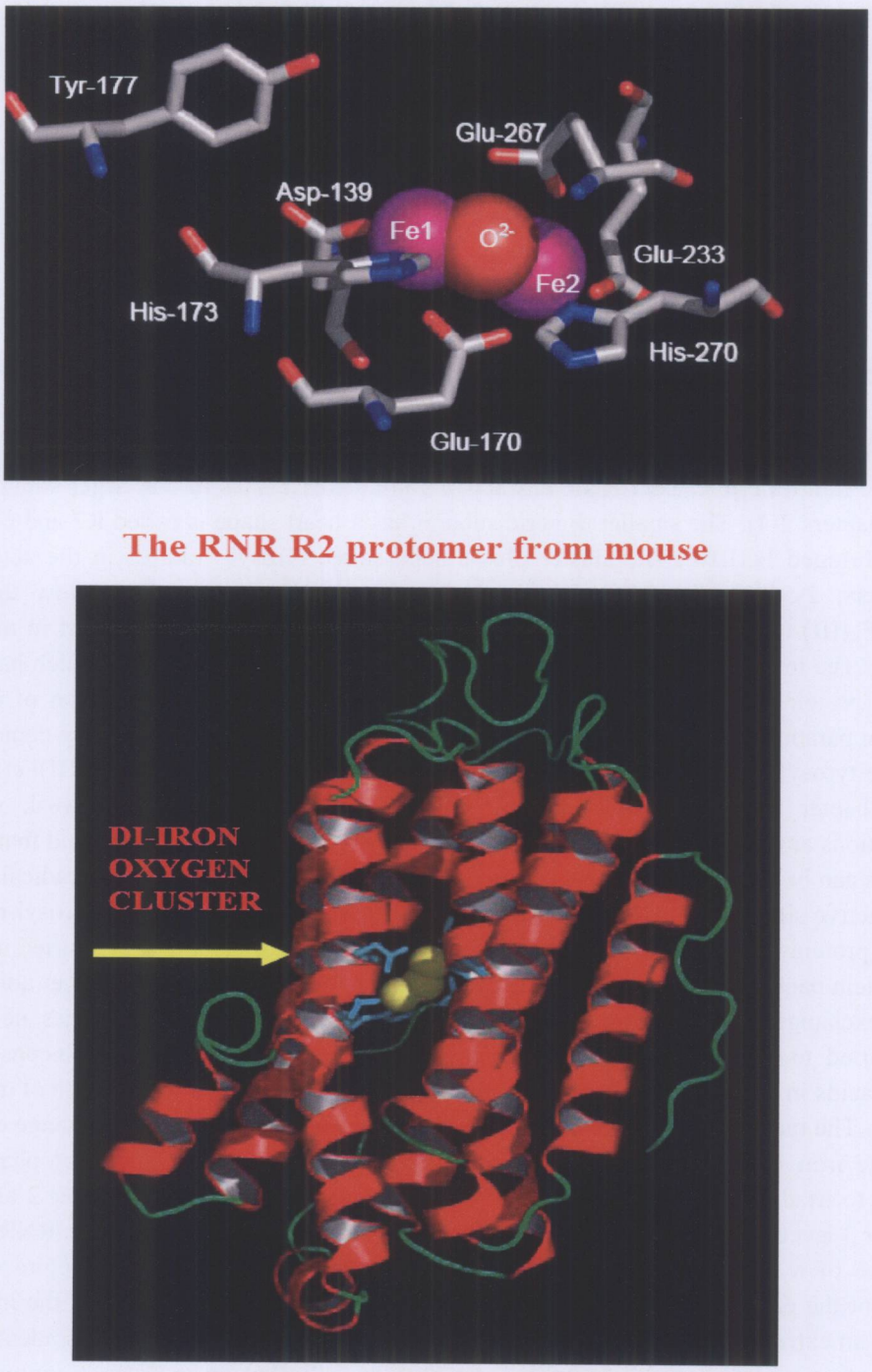

Figure 3. Structure of mouse di-ferric RNR R2 protein. Top panel shows the tyrosyl-radical site with the ferric iron-oxygen cluster. Lower panel shows one subunit of R2 with ferric iron oxygen cluster. (Figures Kari R. Strand and Åsmund K. Røhr).

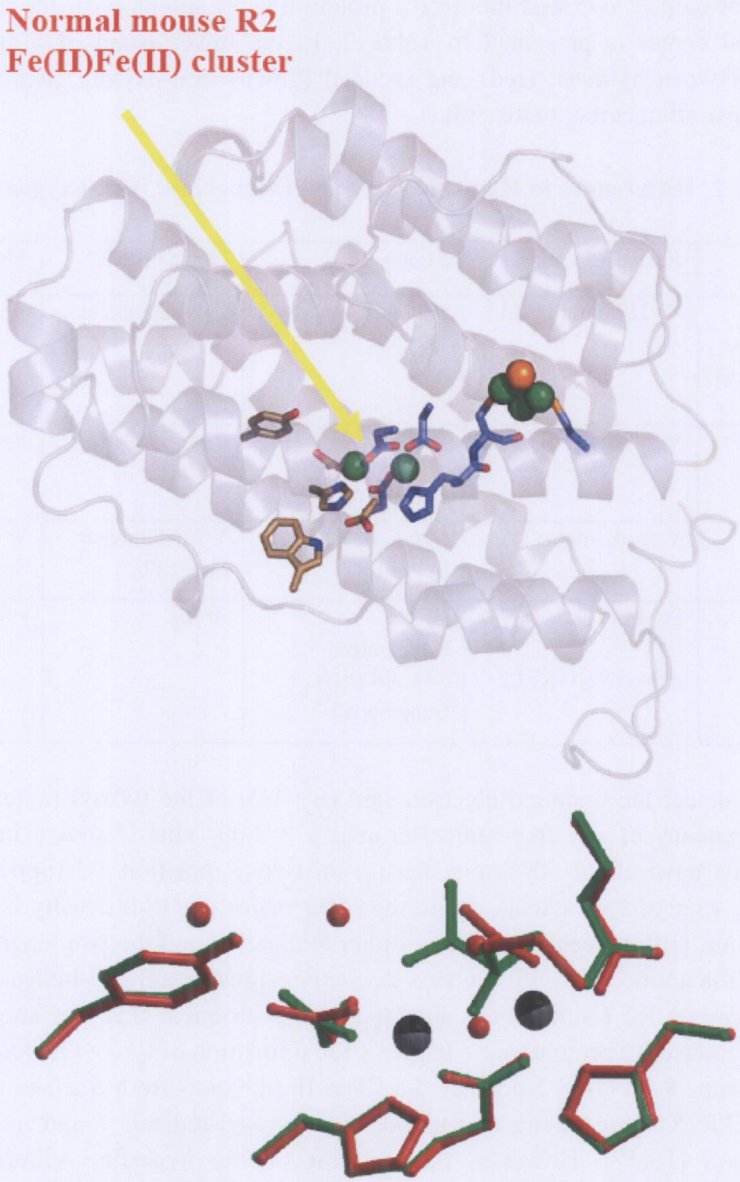

Figure 4. Location of iron clusters, conserved radical pathway and effect of oxidation/reduction of cluster in mouse RNR R2 protein. Upper panel; One mouse R2 subunit with high lighted conserved radical pathway as well as the diferrous -iron cluster and location of a tri-nuclear iron cluster (Åsmund K. Røhr) (Addapted from http://www.esrf.eu/exp_facilities/BM1A/site/report/report0304.pdf). Lower panel: An overlay of oxidized (red) and reduced (green) structures of diiron center in mouse R2. The diferrous form has an additional acetate from buffer. (Kari R. Strand PhD Thesis, University of Oslo).

However, one tri-nuclear different cluster was earlier observed by the Nordlund group in *Corynebacterium ammoniagenes* R2, but these trinuclear clusters are probably not biological active. The R2 iron site can be substituted by different metals such as Mn(II)Mn(II) where three known structures were obtained or Co(II)Co(II). The Co(II)Co(II) cluster show

differences between *E. coli* and mouse R2 protein a short summary of the iron-oxygen and tyrosyl radical center is presented in Table 2. In the lower panel of Figure 4 is shown differences between oxidized (red) and reduced (green) iron-oxygen cluster of mouse R2 protein demonstrating carboxylate shift.

Table 2. Differences in R2 protein tyrosyl radical and iron-oxygen cluster

	E. coli R2	Mouse R2	Human p53R2	Mouse p53R2
Active Fe(III)Fe(III) Tyrosyl radical	No H-bond radical	H-bonds	H-bond radical	
Mixed valent Fe(III)Fe(II)	Very small	Yes	Yes	Yes
Diferrous Fe(II)Fe(II)	V weak integer spin	Strong Integer Spin EPR	V weak Integer Spin EPR	V weak Integer Spin EPR
Fe(II) Binding Co(II) binding	Strong mono Co(II) S=3/2	Weak Cooperative S=3 di-Co(II) Cooperative	strong	strong

EPR can detect the unpaired electron spin (S= 1/2) of the tyrosyl radicals close to the resonance frequency of any free radical around g = 2.00. This is shown in Figure 5. The tyrosyl radicals have about 50% spin density on tyrosyl position 1C (opposite site of the oxygen in the ring of the radical), while the other major electron density is located on the phenol C-O unit. Differences in the phenol-plan orientation and the two magnetic (I= 1/2) β-protons from the amino acid split the first derivative signal of tyrosyl-radical. This is shown for *Bacillus cereus* R2 (with a very similar EPR spectrum to that one shown in *Bacillus antracis)* and mouse R2 protein (see Chapter 8 for simulation of tyrosyl radical EPR) at usual X-band frequency 9-10 GHz. Normally the Class Ib like ones from *Bacillus* and *Salmonella* species has EPR X-band spectra resembling most tyrosyl-radicals found in photosystem II and peroxidases [13-17]. However, they exhibit smaller hyperfine splittings than those observed in Class 1a radicals like in mouse R2. The unusual large hyperfine splittings in Class Ia radical is nearly unique to R2 Class Ia in proteins (but it can be found in irradiated compounds).

The spin density changes on the tyrosyl radical phenol C-O unit can be tuned by interaction with a close positive charge. For example, in presence of an hydrogen bond to the tyrosyl oxygen, a clear difference is seen in the g_1-component of the resolved g-tensor at lowest magnetic field, upon using high frequency/high field EPR (Figure 5 lowest panel), which is not resolved and overlapped by the two so called hyper-fine β-proton couplings at X-band frequencies (9-10 GHz).