

**Class I Ribonucleotide Reductases:  
overall activity regulation,  
oligomerization, and drug targeting.**

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**To my family and friends**

**“A gift is pure when it is given from the heart to the right person at the right time and at the right place, and when we expect nothing in return”**

**The Bhagavad Gita**



# Table of Contents

|   |    |
|---|----|
| List of publications:   | v  |
| Abbreviations:  | vi |
| Abstract:   | 1  |
| Aim:  | 3  |
| Introduction  | 3  |
| Mechanism:  | 4  |
| <i>Radical generation and transfer:</i>                       | 4  |
| <i>Substrate reduction:</i>                                   | 5  |
| RNR classification:   | 6  |
| Class I:  | 6  |
| Class Ia:   | 8  |
| Class Ib:   | 8  |
| Class Ic:   | 8  |
| Class II:   | 9  |
| Class III:  | 9  |
| Coexistence of different RNR classes and different variants:  | 9  |
| Evolution:  | 10 |
| Introduction to Class I RNR:                                  | 11 |
| Multilevel regulation:  | 12 |
| Allosteric regulation:  | 13 |
| Specificity regulation and oligomerization:                   | 15 |
| Overall activity regulation and oligomerization:              | 17 |
| Multiple mechanisms of overall activity regulation:           | 17 |
| Eukaryotic model of overall activity regulation               | 18 |
| Prokaryotic model of overall activity regulation:             | 20 |
| Medicine:   | 22 |
| Methodology:  | 25 |
| Gas-phase electrophoretic macromolecule analysis (GEMMA):     | 26 |
| Advantages and disadvantages:                                 | 28 |
| Results and Summary   | 30 |
| Paper I:  | 30 |
| Paper II:   | 34 |
| Paper III:  | 36 |
| Future plans:   | 38 |
| Targeting <i>P. aeruginosa</i> RNR.                           | 38 |
| Multiple mechanisms of allosteric overall activity regulation | 39 |
| Acknowledgements:   | 41 |
| References  | 45 |



## List of publications:

- I. **Jonna, V.R.**, Crona, M., Rofougaran, R., Lundin, D., Johansson, S., Brännstrom, K., Sjöberg, B.-M., and Hofer, A. (2015). Diversity in Overall Activity Regulation of Ribonucleotide Reductase. *J Biol Chem* 290, 17339-17348.
- II. Johansson, R., **Jonna, V.R.**, Kumar, R., Nayeri, N., Lundin, D., Sjöberg, B.M., Hofer, A., and Logan, D.T. (2016). Structural Mechanism of Allosteric Activity Regulation in a Ribonucleotide Reductase with Double ATP Cones. *Structure* 24, 906-917.
- III. Crona, M., Codo, P., **Jonna, V.R.**, Hofer, A., Fernandes, A.P., and Tholander, F. (2016). A ribonucleotide reductase inhibitor with deoxyribonucleoside-reversible cytotoxicity. *Mol Oncol*.
- IV. Chiruvella, K.K., Rajaei, N., **Jonna, V.R.**, Hofer, A., and Astrom, S.U. (2016). Biochemical Characterization of Kat1: a Domesticated hAT-Transposase that Induces DNA Hairpin Formation and MAT-Switching. *Sci Rep* 6, 21671.
- V. Sadanandan, S.A., Ekstrom, J.O., **Jonna, V.R.**, Hofer, A., and Hultmark, D. (2016). VP3 is crucial for the stability of Nora virus virions. *Virus Res* 223, 20-27.

Note: Publications IV and V are not part of the thesis.

## Abbreviations:

|             |  |
|-------------|--|
| RNR         | Ribonucleotide reductase   |
| NTP         | Nucleoside triphosphate  |
| ATP         | Adenosine triphosphate   |
| UDP         | Uridine diphosphate  |
| ADP         | Adenosine diphosphate  |
| CDP         | Cytidine diphosphate   |
| GDP         | Guanosine diphosphate  |
| dNTP        | Deoxyribonucleoside triphosphate   |
| dATP        | Deoxyadenosine triphosphate  |
| dGTP        | Deoxyguanosine triphosphate  |
| dCTP        | Deoxycytidine triphosphate   |
| Gemcitabine | Gemcitabine-5'-diphosphate   |
| NSC37375    | (redoxal, 2-[(4-{4-[(2-carboxyphenyl)amino]-3-methoxyphenyl}-2-methoxyphenyl)amino]benzoic acid) |
| GEMMA       | Gas-phase electrophoretic macromolecule analysis   |

## **Abstract:**

Ribonucleotide reductase (RNR) is a key enzyme in the *de novo* biosynthesis and homeostatic maintenance of all four DNA building blocks by being able to make deoxyribonucleotides from the corresponding ribonucleotides. It is important for the cell to control the production of a balanced supply of the dNTPs to minimize misincorporations in DNA. Because RNR is the rate-limiting enzyme in DNA synthesis, it is an important target for antimicrobial and antiproliferative molecules. The enzyme RNR has one of the most sophisticated allosteric regulations known in Nature with four allosteric effectors (ATP, dATP, dGTP, and dTTP) and two allosteric sites. One of the sites (s-site) controls the substrate specificity of the enzyme, whereas the other one (a-site) regulates the overall activity. The a-site binds either dATP, which inhibits the enzyme or ATP that activates the enzyme. In eukaryotes, ATP activation is directly through the a-site and in *E. coli* it is a cross-talk effect between the a and s-sites. It is important to study and get more knowledge about the overall activity regulation of RNR, both because it has an important physiological function, but also because it may provide important clues to the design of antibacterial and antiproliferative drugs, which can target RNR.

Previous studies of class I RNRs, the class found in nearly all eukaryotes and many prokaryotes have revealed that the overall activity regulation is dependent on the formation of oligomeric complexes. The class I RNR consists of two subunits, a large  $\alpha$  subunit, and a small  $\beta$  subunit. The oligomeric complexes vary between different species with the mammalian and yeast enzymes cycle between structurally different active and inactive  $\alpha_6\beta_2$  complexes, and the *E. coli* enzyme cycles between active  $\alpha_2\beta_2$  and inactive  $\alpha_4\beta_4$  complexes. Because RNR equilibrates between many different oligomeric forms that are not resolved by most conventional

methods, we have used a technique termed gas-phase electrophoretic macromolecule analysis (GEMMA). In the present studies, our focus is on characterizing both prokaryotic and mammalian class I RNRs. In one of our projects, we have studied the class I RNR from *Pseudomonas aeruginosa* and found that it represents a novel mechanism of overall activity allosteric regulation, which is different from the two known overall activity allosteric regulation found in *E. coli* and eukaryotic RNRs, respectively. The structural differences between the bacterial and the eukaryote class I RNRs are interesting from a drug developmental viewpoint because they open up the possibility of finding inhibitors that selectively target the pathogens. The biochemical data that we have published in the above project was later supported by crystal structure and solution X-ray scattering data that we published together with Derek T. Logan`s research group.

We have also studied the effect of a novel antiproliferative molecule, NSC73735, on the oligomerization of the human RNR large subunit. This collaborative research results showed that the molecule NSC73735 is the first reported non-nucleoside molecule which alters the oligomerization to inhibit human RNR and the molecule disrupts the cell cycle distribution in human leukemia cells.

## **Aim:**

The specificity regulation of RNR is well studied and documented, whereas the mechanism behind the overall activity regulation is only partially known. Our primary goal is to understand more about the oligomerization and allosteric overall activity regulation mechanisms of class 1 RNRs in different organisms. These studies can be an important step towards the identification of novel drugs to target RNRs of pathogenic organisms and give more knowledge about the evolution of different overall activity mechanisms.

## **Introduction**

It is amazing that the life starts with only four letters A, T, G, and C that are the bases in DNA. These DNA bases are the key elements in deoxyribonucleotides (dNTPs): dATP, dTTP, dGTP, and dCTP, which are building blocks to construct the DNA. The DNA is a genetic material, which carries the phenotypic information from one generation to the next. Every free-living organism, and many viruses as well, need DNA as a source to carry the genetic information. All four dNTPs are synthesized by salvage and *de novo* pathways. Ribonucleotide reductase (RNR) is a key enzyme in the *de novo* pathway, to reduce the RNA building blocks, ribonucleotides (NDPs/NTPs), to DNA building blocks, deoxyribonucleotides (dNDPs/dNTPs) (Reichard, 1993).

The first RNR activity was observed in the year 1950 by a Swedish researcher Peter Reichard and coworkers, where they observed the conversion of ribonucleotides to deoxyribonucleotides (Hammarsten et al., 1950; Reichard and Estborn, 1951). Later they found the enzyme involved in the conversion which is ribonucleotide reductase. Because RNR is important in DNA synthesis, soon after its discovery the field of RNR become well known and the study of RNR is extended in various

areas of science like biochemistry, biophysics, evolution and more significantly in the field of biomedicine. Seven decades after its discovery, RNR is still a popular field to study in the scientific community. Perhaps, it is no exaggeration to say that RNR is the most interesting enzyme to study as described in a recent publication (Mathews, 2016).

### **Mechanism:**

The enzyme RNR reduces RNA precursors NDPs/NTPs to the corresponding DNA precursors dNDPs/dNTPs by replacing the 2'-hydroxyl group in the ribose sugar of the nucleotide with a hydrogen atom (Figure 1). The mechanism is dependent on a free radical generation and transfer (Thelander, 1974). The reduction mechanism of the substrate is initiated by the oxidation of the active site-cysteiny radical (S<sup>•</sup>), which is in the large subunit ( $\alpha$ ) of the enzyme. The free radical required for the substrate reduction is generated by a di-iron-oxygen center present in the subunit  $\beta$ .

### **Radical generation and transfer:**

The small subunit  $\beta$  harbors a di-iron (Fe<sup>II</sup>/Fe<sup>II</sup>) center required for the free radical generation. The reaction is initiated by the oxidation of Fe<sup>II</sup>/Fe<sup>II</sup> to Fe<sup>III</sup>-O-Fe<sup>III</sup> with O<sub>2</sub>. One of the oxygen atom together with H<sup>+</sup> leaves as an H<sub>2</sub>O molecule and the other oxygen atom becomes part of the Fe<sup>III</sup>-O-Fe<sup>III</sup> center. Four electrons are needed for the above process. The first two electrons come from the oxidation of the two Fe<sup>II</sup> ions, and one electron comes from an external source, which is believed to come from tryptophan-48 in the  $\beta$  subunit (Baldwin et al., 2000). The fourth electron comes from the oxidation of tyrosine-122 of the  $\beta$  subunit, which generates an unusual stable tyrosyl radical (Y<sup>122•</sup>) (Eklund et al., 2001; Kolberg et al., 2004; Nordlund and Eklund, 1993; Nordlund et al., 1990).

The next task is to transfer the  $Y^{122\cdot}$  radical produced in the  $\beta$  subunit to cysteine-439, which is near to the active site of the large subunit  $\alpha$ , to generate a transient cysteinyl radical ( $C^{439\cdot}$ ). The distance between  $Y^{122}$  in the  $\beta$  subunit and  $C^{439}$  in the  $\alpha$  subunit is about 35 Å and this long-distance transfer of the  $Y^{\cdot}$  radical is governed by a long-range proton-coupled electron transport chain, where several intermediate amino acid free radicals are generated until the free radical is delivered to the  $C^{439}$  (Kolberg et al., 2004; Stubbe et al., 2003; Uhlin and Eklund, 1994).

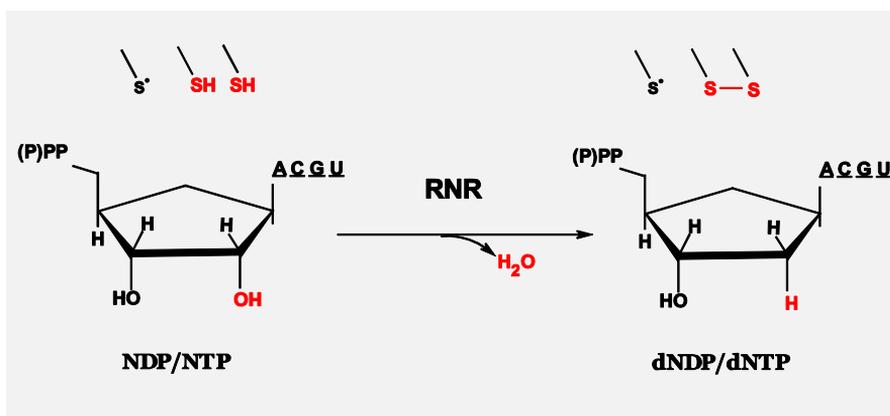


Figure 1. Schematic representation of the substrate reduction mechanism by the enzyme RNR.

The following reactions take place in both the subunits before the  $Y^{122\cdot}$  radical is transferred to  $C^{439}$ . The essential cysteine pair  $C^{225}$ - $C^{462}$  of the  $\alpha$  subunit will be reduced, followed by substrate binding, which is specifically controlled by the binding of allosteric effectors to the specificity site (s-site). Subsequent assembly of the RNR subunits and final conformational changes in the enzyme allow the free radical transfer.

### Substrate reduction:

The radical  $C^{439\cdot}$  which is near the active site of the  $\alpha$  subunit oxidizes the substrate by abstracting a 3'-hydrogen group, this process generates a substrate radical followed by the reduction of  $C^{439\cdot}$ . The subsequent reactions replace the 2'-hydroxyl group with hydrogen and the product is

formed. The final product is released by making the reduced C<sup>439</sup> to its oxidized form C<sup>439\*</sup> so the free radical is reintroduced (Figure 1) (Nordlund and Reichard, 2006).

### **RNR classification:**

The primary mechanism to reduce RNA building blocks to its DNA building blocks by radical chemistry is well conserved throughout the RNR evolution. The tertiary structure of the active site, where all RNRs share a common 10-stranded  $\alpha\beta$ -barrel and the specificity regulation are also evolutionarily well conserved (Nordlund and Reichard, 2006). Other than these similarities, the RNR family differ in the type of substrate used for the reduction and oxygen requirement during the reaction. The enzyme is grouped into three broad classes and several subclasses mainly depending on how the free radical is generated in each catalytic cycle (Table 1) (Nordlund and Reichard, 2006). All three major classes of RNR; class I, II, and III are grouped to several subclasses based on the RNR`s catalytic subunit amino acid sequences. The subclassification includes NrdAe, g, h, i, k, n and z of the class Ia and Ic, NrDE of the class Ib, NrdJd, f, and m of class II, and NrdDa, b, c, d, f, h, and I of class III. This type of extensive subclassification of the enzyme is important to predict the mechanistic differences in RNR`s family.

### **Class I:**

The class I RNRs are aerobic often referred as to the eukaryotic RNRs (Cotruvo and Stubbe, 2011a; Nordlund and Reichard, 2006), and this class is also present in bacteria, viruses and some archaea (Lundin et al., 2010; Torrents, 2014). Depending on the metal cluster needed for the free radical generation, the Class I RNR has been further subdivided into three subclasses; a, b, and c. All three subclasses need oxygen to form a metal-oxygen center for free radical formation.

Table 1. Different classes and subclasses of RNR and their characteristics.

|                                   | Class Ia   | Class Ib   | Class Ic   | Class II   | Class III                                       |
|-----------------------------------|--|--|--|--|---|
| <b>Distribution</b>               | Eukaryotes, several bacteria, viruses, and few archaea   | Bacteria and bacteriophages  | Eukaryotes, several bacteria, viruses, and few archaea | Mostly in Bacteria and in a few viruses and archaea. | Mostly in archaea, bacteria, and bacteriophages |
| <b>Oxygen requirement</b>         | Aerobic  | Aerobic  | Aerobic  | Independent  | Anaerobic                                       |
| <b>Catalytic subunit</b>          | NrdA ( $\alpha$ )  | NrdE ( $\alpha$ )  | NrdA ( $\alpha$ )                                      | NrdJ ( $\alpha$ )                                    | NrdD ( $\alpha$ )                               |
| <b>Radical generating factors</b> | NrdB ( $\beta$ )   | NrdF ( $\beta$ )   | NrdB ( $\beta$ )                                       | AdoCbl   | NrdG  |
| <b>Metal</b>                      | Fe <sup>III</sup> -O-Fe <sup>III</sup>   | Mn <sup>III</sup> -O-Mn <sup>III</sup><br>or<br>Fe <sup>III</sup> -O-Fe <sup>III</sup> | Mn <sup>IV</sup> -O-Fe <sup>III</sup>                  | Co   | 4Fe-4S  |
| <b>Radical</b>                    | Tyr*...Cys*  | Tyr*...Cys*  | Phe*/Leu*/Val*<br>...Cys*                              | dAdo*...Cys*   | Gly*...Cys*                                     |
| <b>Additional factors</b>         | Thioredoxin and glutaredoxin   | NrdI to generate Mn-o-Mn center  | Thioredoxin and glutaredoxin                           | Thioredoxin  | NrdG to generate Gly* radical                   |
| <b>Substrate</b>                  | NDP  | NDP  | NDP  | NDP/NTP  | NTP   |
| <b>ATP cone</b>                   | Present  | None   | Present  | Rarely present                                       | Present   |
| <b>Active complex</b>             | $\alpha_2\beta_2$ and $\alpha_6\beta_{2-6}$  | $\alpha_2\beta_2$  | Similar to class Ia                                    | $\alpha$ , and $\alpha_2$                            | $\alpha_2$                                      |
| <b>Inactive complex</b>           | $\alpha_6\beta_2$ (eukar.)<br>$\alpha_4\beta_4$ ( <i>E. coli</i> )<br>$\alpha_4\beta_2$ ( <i>P. aerug.</i> ) | None   | Similar to class Ia                                    | ?  | $\alpha_2$                                      |

**Class Ia:** This subclass is also referred to as canonical class 1 RNRs. Similar to other class I RNRs, it consists of two protein subunits needed for the enzyme to be active. The gene *NrdA* encodes for the large subunit R1 ( $\alpha$ ), and the gene *NrdB* encodes for the small subunit R2 ( $\beta$ ). The  $\alpha$  subunit harbors a catalytic site and in most of the cases two types of allosteric sites. The  $\beta$  subunit harbors a free radical generating machinery, where it generates the  $Y^{122\bullet}$  radical by creating a  $Fe^{III}\text{-O-}Fe^{III}$  center near the catalytic site. The radical is transferred to the catalytic site of the  $\alpha$  subunit via a long-range electron transport chain ( $\sim 35 \text{ \AA}$ ) consisting of aromatic amino acid residues to generate the  $C439\bullet$  radical, which initiates the reduction process. This is the most studied class of the RNR family and the *E. coli* RNR is often considered as a prototype.

**Class Ib:** In the class Ib enzymes, the large subunit R1 ( $\alpha$ ) is encoded by the *NrdE* gene and the small subunit R2 ( $\beta$ ) by the *NrdF* gene. The subunit  $\alpha$  harbors a catalytic site and in contrast to the class Ia enzymes, it harbors only one allosteric site (s-site). The most significant difference compared to the canonical class 1 RNRs is the type of metal oxygen center ( $Fe^{III}\text{-O-}Fe^{III}$  or  $Mn^{III}\text{-O-}Mn^{III}$ ) required to generate the  $Y^{122\bullet}$  radical. This class of enzymes needs an additional protein, *NrdI*, for the  $Mn^{III}\text{-O-}Mn^{III}$  center to generate the  $Y^{122\bullet}$  radical but there is no requirement of this extra protein for the  $Fe^{III}\text{-O-}Fe^{III}$  center. Recent studies indicate that the  $Mn^{III}\text{-O-}Mn^{III}$  center is the physiological form for the generation of  $Y^{122\bullet}$  radical (Cotruvo and Stubbe, 2011b; Cox et al., 2010; Crona et al., 2011b; Martin and Imlay, 2011).

**Class Ic:** Like the canonical subclass, the two essential  $\alpha$  and  $\beta$  subunits are encoded by the *NrdA* and *NrdB* genes, respectively. In class Ic the subunit  $\alpha$  shares similar features as in canonical class. This subclass has been classified entirely based on the subunit  $\beta$  (Hogbom et al., 2004). Unlike the other class I RNRs, the  $Y^{122\bullet}$  radical is replaced with a phenylalanine, leucine or valine, and the subunits are called *NrdB<sup>Phe</sup>*,

NrdB<sup>Leu</sup> or NrdB<sup>Val</sup> respectively. The metal center needed for the generation of the free radical is the Mn<sup>IV</sup>-O-Fe<sup>III</sup> center.

### **Class II:**

The enzyme activity is independent of oxygen presence in class II RNRs and present in all three domains of life and in some viruses (Lundin et al., 2015). The choice of substrates for this class of enzymes is either NDPs or NTPs. The enzyme has a single protein subunit ( $\alpha$ ), which is encoded by the gene NrdJ and the class has been divided into two subgroups: monomeric and dimeric class II RNRs. The enzymes from this class use the vitamin B<sub>12</sub>-derived coenzyme 5'-deoxyadenosylcobalamin (AdoCbl) to generate the cysteinyl radical.

### **Class III:**

The class III RNRs are anaerobic and present in bacteria, bacteriophages, archaea and some eukaryotes. The enzyme has one subunit, which is  $\alpha$ , and the NrdD gene encodes the protein. The class III proteins use a glycy radical for catalysis, which is generated by an additional protein called activase (NrdG). The activase is not a real subunit because the enzyme will remain active also after the activase has left the complex. The activity of the  $\alpha$  subunit can remain for several reaction cycles. The subunit  $\alpha$  harbors both the allosteric regulatory sites like in canonical class I RNR and uses NTPs as substrates.

### **Coexistence of different RNR classes and different variants:**

Coexistence of different classes is often an advantage for the organisms that live in a rapidly changing environment. Coexistence of all three classes of RNRs has been reported in more than five percent of fully sequenced bacterial genomes, one archeon, and in a few unicellular eukaryotes (Hofer et al., 2012; Lundin et al., 2010). One such example is *P.*

*aeruginosa*, which genome encodes all three classes of RNR, and each class RNR is differentially expressed depending on the external environment (Jordan et al., 1999; Sjöberg and Torrents, 2011; Torrents et al., 2005). Few eukaryotic genomes harbor alternative class Ia RNR isoforms. The mammalian genome encodes an alternative small subunit p53R2. The p53R2 protein was originally reported to be crucial for DNA repair (Tanaka et al., 2000), but later findings showed that the protein's primary function is to provide building blocks for mitochondrial DNA synthesis (Håkansson et al., 2006). The genome of *Saccharomyces cerevisiae* encodes two isoforms of each RNR subunit R1 and R3 are isoforms of subunit  $\alpha$  and R2 and R4 are isoforms of subunit  $\beta$ . The R4 subunit is inactive because it lacks some amino acids to form an iron center but the subunit is still needed for correct folding of the R2 subunit. Therefore, the active form of the  $\beta$  dimer consists of a R2-R4 heterodimer (Chabes et al., 2000; Wang et al., 1997). In contrast to the budding yeast, the class Ia RNR of the *Aeromonas hydrophilia* bacteriophage (Aeh1) has an  $\alpha$  subunit that consists of two polypeptides ( $\alpha_a + \alpha_b$ ) and the holoenzyme is a  $(\alpha_a + \alpha_b)_2\beta_2$  heterotetramer (Crona et al., 2011a).

## **Evolution:**

Perhaps life would not exist without the introduction of RNR to be able to replace the genetic material from RNA to DNA. The enzyme might have played a significant role in the transition from the RNA to DNA world. The common structural features in the active site, the mechanism governed by the free radical chemistry, and the similar allosteric specificity regulation in almost all classes of RNRs leads to the conclusion that the RNRs have evolved by a divergent evolutionary pathway with either class II or class III RNRs as a common ancestor for the present day RNRs (Reichard, 1993, 1997; Stubbe, 1990, 1998). The recent structural and bioinformatic studies of the ATP cone domain proposes that this domain is a jumping element

that has been lost and gained several times during the evolution, probably by recombination between the RNR genes (Jonna et al., 2015).

## **Introduction to Class I RNR:**

All class I RNR proteins have two subunits, subunit  $\alpha$  which is a large subunit and subunit  $\beta$  which is a small subunit. The  $\alpha$  subunit has two allosteric sites; the specificity site (s-site) and activity site (a-site) (Figure 2). In addition, it has one catalytic site, where the substrate binds. The specificity regulation via the s-site decides which of the four substrates to be reduced in the catalytic site to maintain the relative ratios between the four dNTPs in the cell. The allosteric specificity regulation is well conserved in all classes of RNRs, and it has been well studied (more details about the specificity regulation is discussed below). The a-site regulates the overall activity of the enzyme by switching it on and off. Binding of the effector ATP makes the eukaryotic RNR hyperactive, and the effector dATP inhibit the enzyme. The overall activity regulation from the a-site controls the absolute concentration of the dNTP pool in the cell. The a-site is located in the N-terminus of the  $\alpha$  subunit, which is called the ATP cone (Aravind et al., 2000), where the effectors can bind. More details about overall activity regulation are discussed in the overall activity regulation section.

The subunit  $\beta$  harbors the metal oxygen center ( $\text{Fe}^{\text{III}}\text{-O-Fe}^{\text{III}}$ ), which is required to generate and stabilize the  $\text{Y}^{122\bullet}$  free radical (Figure 2). The free radical is further transferred to generate a transient  $\text{C}^{439\bullet}$  radical in the active site of the  $\alpha$  subunit. The free radical transfer from subunit  $\beta$  to the active site of the  $\alpha$  subunit is governed by a long-range proton-coupled electron transport chain. More details about the free radical types are discussed in the above RNR classification section.

- A Activity site** : ATP and dATP
- S Specificity site** : ATP, dATP, dGTP and dTTP
- C Catalytic site** : CDP,UDP, ADP and GDP

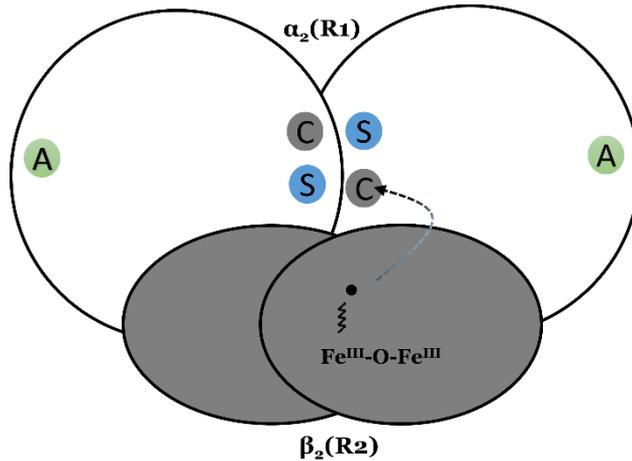


Figure 2. Schematic representation of the class I RNR `s active  $\alpha_2\beta_2$  heterotetramer complex. Each  $\alpha$  monomer shows two allosteric and one substrate binding sites. The subunit  $\beta$  dimer contains a metal-oxygen center with a free radical, which can be transferred to the catalytic site.

## Multilevel regulation:

Every living cell needs to have a balanced supply of all four dNTPs for the DNA replication and repair. Any imbalances in the supply lead to the accumulation of mutations in the DNA (Mathews, 2006). Therefore, the enzyme RNR must be regulated in a controlled way to make sure the cell gets a balanced supply of dNTPs. The controlled regulation is achieved by several strategies like transcriptional control, allosteric regulation and cell-cycle dependent proteolysis in mammalian cells and by small inhibitory proteins (sml 1, spd 1) in yeasts (Nordlund and Reichard, 2006). It is more uncertain if inhibitory proteins exist in metazoans although it has been suggested that IRBIT could be such a protein (Arnaoutov and Dasso, 2014). My major focus in the thesis is in allosteric regulation.

## Allosteric regulation:

As mentioned above, RNR is perhaps the most interesting enzyme in the world and its sophisticated allosteric regulation is one of the reasons why the RNR is interesting. The subunit  $\alpha$  has two allosteric sites; the specificity site (s-site) and activity site (a-site). Over the past few years, several RNR structures from different organisms including several bacteria and few eukaryotes have been resolved and given new insights into the mechanism of allosteric regulation (Ando et al., 2011; Fairman et al., 2011; Logan, 2011; Wang et al., 2007).

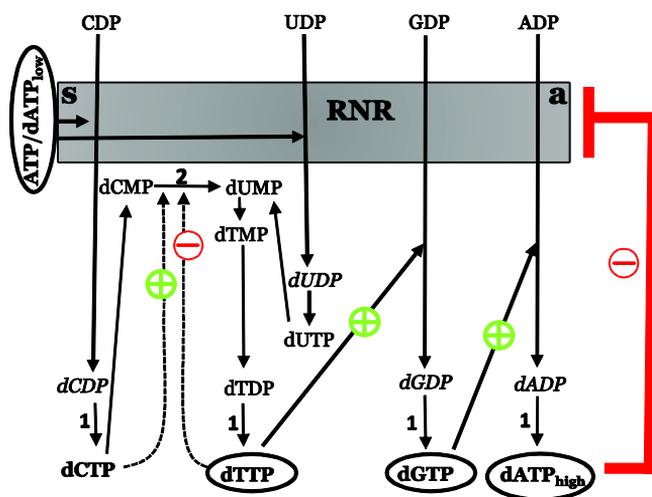


Figure 3. Allosteric regulation of class I RNR. Allosteric effectors are represented in the circles. a and s letters represent the a and s-sites respectively. The plus and minus circle signs represent the effector's activation and inhibition effects on the enzyme. The enzyme NDP kinase (1) is involved in the final reaction step of dNTP synthesis. The concentration ratio between dCTP and dTTP is controlled by an additional enzyme dCMP deaminase, or dCTP deaminase in some prokaryotes (2). Dotted arrows represent the regulation of dCMP or dCTP deaminase (2) by dCTP and dTTP.

### ***Specificity regulation:***

In contrast to PCR reaction mixtures, the four dNTPs are not in equimolar concentration in the cell. The relative ratios and the overall concentration of each of the four dNTPs differ from species to species. The physiological dNTP pools in mammalian cells are approximately 37  $\mu\text{M}$  dTTP, 29  $\mu\text{M}$  dCTP, 24  $\mu\text{M}$  dATP, and 5  $\mu\text{M}$  dGTP (Traut, 1994). The relative dNTP pool levels in *E. coli* follow the same trend as in mammals, except that the total dNTP pool is five to ten times higher (Buckstein et al., 2008). To maintain the relative dNTP pools, RNR has developed a strategy called allosteric specificity regulation. The s-site can bind to either of the effectors dATP, ATP, dTTP, or dGTP and decides what substrate to reduce in the catalytic site by a conformational change in loop 2, which is located between the s-site and the catalytic site. The effectors dATP or ATP promotes the reduction of CDP or UDP, dGTP stimulates ADP reduction, and dTTP stimulates the reduction of GDP (Figure 3) (Nordlund and Reichard, 2006).

The mechanism of allosteric specificity regulation is well conserved in all classes of RNRs except in a Herpesviridae class I RNRs, where the enzyme lacks specificity regulation altogether (Averett et al., 1983). The mechanism of specificity regulation has been well-studied by using biochemical, biophysical and structural studies and the regulation follows the scheme illustrated in Figure 3 (Hofer et al., 2012). When the cell is in the beginning of s-phase, the effector ATP binds to the s-site and promote the formation of dCTP/dTTP. The effector dTTP then promotes the production of dGTP, which further promotes the production of dATP and the dATP/ATP will start the cycle all over again by binding to the s-site. In mammalian cells, ATP or dATP hyperactivates or inhibits the enzyme respectively by binding to the a-site (Figure 3). Thus the dNTP levels are maintained in the cell. Since dCTP is not an s-site effector, an additional regulation by dCMP deaminase (dCTP deaminase in some prokaryotes) is

needed to maintain the ratio between dCTP and dTTP. The enzyme dCMP deaminase is allosterically regulated by dCTP (activator) and dTTP (inhibitor) as shown in Figure 3.

### ***Specificity regulation and oligomerization:***

The catalytic subunit  $\alpha$  is a monomer with no effector bound to the s-site. The binding of any of the specificity effectors to the s-site promotes the dimerization of the subunit  $\alpha$  with the s-sites and active sites being at the dimer interface. The subunit  $\alpha$  dimerization subsequently promotes the binding of the  $\beta$  dimer to form an active  $\alpha_2\beta_2$  tetramer.

### ***Overall activity regulation:***

Like the specificity regulation to maintain the relative ratios between the four dNTPs, the enzyme has developed a strategy called overall activity regulation to keep the total concentration of dNTPs in the cell. The ATP cone domain of the a-site regulates the overall activity regulation by turning the enzyme on and off. In mammalian RNR, the allosteric effectors ATP and dATP control the overall activity regulation. Binding of the effector ATP to the ATP cone domain makes the enzyme hyperactive, and the effector dATP inhibits the enzyme. Around 100 amino acids in the N-terminus of the  $\alpha$  subunit makes up the ATP cone, which is also called a-site (Aravind et al., 2000). More about the ATP cone is discussed in the ATP cone section. Both the effectors ATP and dATP can bind to either of the allosteric sites and regulates specificity and activity regulation. The effector ATP has a low affinity towards both the sites but can still compete with the other effectors because the physiological concentration of ATP is very high (3 mM) in mammalian and *E. coli* cells (Bochner and Ames, 1982; Buckstein et al., 2008). The effector dATP has 10 to 20 times lower affinity to the a-site than the s-site (Brown and Reichard, 1969b; Ormö and Sjöberg, 1990; Reichard et al., 2000). At low concentrations, dATP acts as a specificity regulator, and at high concentration, it acts as an

overall activator by binding to both s- and a-sites. The cellular concentration of dATP serves as a sensor to turn off the enzyme when it is enough dNTP pools for DNA synthesis. At high cellular dATP concentration, RNR senses the proper homeostasis between the four dNTPs and the dATP further binds to both allosteric sites where it will inhibit the enzyme via a-site regulation.

### ***ATP-cone:***

Bioinformatics studies reveal that the ATP cone distribution is common in all different classes of RNRs except class Ib which lacks the ATP cone and class II where it is only rarely present (Nordlund and Reichard, 2006). Surprisingly multiple ATP cones are commonly observed in all classes of RNR. Two such examples having multiples ATP cones are *P. aeruginosa* and *Chlamydia trachomatis* class Ia RNRs (Roshick et al., 2000; Torrents et al., 2006). Among the two ATP cones of the  $\alpha$  subunit of *P. aeruginosa* class Ia RNR, only the N-terminal ATP cone is functional (Torrents et al., 2006). The class Ia RNR of the bacteria *C. trachomatis* have instead three ATP cones, among that the first two N-terminal cones are functional (Jonna et al., 2015). Structural and bioinformatic studies show that the conserved KR(D/N) motif is crucial for the functionality of the ATP cone domain. Amino acid sequence studies revealed that the arginine (R) in the conserved motif is replaced by an alanine (A) residue in the nonfunctional ATP-cone domain. The amino acid arginine is critical for binding of the phosphate moiety of the overall activity effectors ATP and dATP to the ATP cone (Jonna et al., 2015). The structural studies of human RNR have revealed how the ATP cone discriminates between ATP and dATP binding (Fairman et al., 2011). The ATP cone is formed by four helices which are covered by a  $\beta$  hairpin at one end forming a pocket. The absence of 2'-hydroxyl group of dATP makes it bind deeper in the pocket and the residue Ile 18 seems to be important in the prevention of deeper binding of ATP by acting as a stereochemical barrier (Fairman et al., 2011).

### ***Overall activity regulation and oligomerization:***

Higher oligomerization has been noticed in the early studies of the allosteric regulation of class I RNR in *E. coli* (Brown and Reichard, 1969a), where they have observed a dATP-induced RNR complex which is double the size of the general active form,  $\alpha_2\beta_2$ . Nearly a decade later, higher oligomeric complexes were reported in the class I RNRs from calf thymus and human cells (Cory and Fleischer, 1982; Thelander et al., 1980). Although the higher oligomerization status of RNR was published, during the 70s - 80s, the importance of the higher order oligomerization in class I RNRs was not realized until 20-30 years later from a series of biochemical and structural studies (Fairman et al., 2011; Kashlan et al., 2002; Rofougaran et al., 2008; Rofougaran et al., 2006). The general active form of class I RNRs is an  $\alpha_2\beta_2$  tetramer, and it can form higher order oligomers, which is governed by overall activity regulation through the a-site.

Recent biochemical (Rofougaran et al., 2008; Rofougaran et al., 2006) and structural studies (Ando et al., 2016; Brignole et al., 2012; Fairman et al., 2011) on *E. coli*, budding yeast and mammalian class Ia RNRs give more insights into the oligomerization-mediated overall activity regulation. The overall activity regulation through oligomerization is common in class I RNRs with the notable exception of class Ib. Unlike specificity regulation, overall activity regulation is different in different classes of RNRs. Recent studies show that the mechanism by how the overall activity regulation is achieved is different in the *E. coli* class Ia RNR compared to the enzyme from mammalian cells (Rofougaran et al., 2008; Rofougaran et al., 2006).

### ***Multiple mechanisms of overall activity regulation:***

The overall activity regulation of RNR is observed only in RNRs with an ATP cone. Occupation of either ATP or dATP to the a-site of the subunit  $\alpha$

at physiological conditions (where the s-site is also occupied) initiates the overall activity regulation, by altering the RNR oligomeric status. Interestingly, *Trypanosoma brucei* and bacteriophage T4 class Ia RNRs do not have dATP-induced overall activity inhibition though they have an ATP cone which can bind dATP (Hofer et al., 2012). Recent structural, biochemical and bioinformatic data reveal that there are two different mechanisms (the *E. coli* and eukaryotic models) to achieve overall activity regulation. In my first publication, we have discovered a third way of overall activity regulation, which I will discuss in the section of results and summary.

***Eukaryotic model of overall activity regulation-S. cerevisiae, slime mold, and mammalian (mouse and human):***

Most of the details about the molecular and structural mechanisms behind the overall activity regulation of eukaryotic class Ia RNR come from the following published biochemical and structural data on yeast and mammalian RNRs (Ando et al., 2016; Crona et al., 2013; Fairman et al., 2011; Hofer et al., 2012; Logan, 2011; Rofougaran et al., 2006). The general active RNR complex governed by s-site effectors is an  $\alpha_2\beta_2$  tetramer. Overall activity regulation in eukaryotic class Ia RNRs is regulated by binding of either ATP (hyperactivation) or dATP (inhibition) to the a-site of the ATP-cone. Oligomerization-driven overall activity regulation was under debate until the studies of the mouse class Ia RNR, where our lab has used a novel method for oligomerization status called GEMMA. This study revealed that the effectors ATP and dATP induce higher complexes, which are active  $\alpha_6$  and inactive  $\alpha_6$  complexes respectively (Figure 4). Because the technique GEMMA is relatively new, the results were reconfirmed by using size exclusion chromatography and to get more exact results of the mass electrospray ionization mass spectrometry was used. The active and inactive  $\alpha_6$  complexes further bind to the  $\beta_2$  dimer and generate an ATP-induced active  $\alpha_6\beta_2$  complex and

dATP-induced inactive  $\alpha_6\beta_2$  complex (Figure 4). Thus both the a-site effectors induce the same quaternary complex with functionally different oligomers. In contrast to the published ATP-induced active  $\alpha_6\beta_2$  complex (Rofougaran et al., 2006), later gel filtration studies show that ATP induces an  $\alpha_6\beta_6$  complex in the presence of the substrate CDP analog gemcitabine-5'-diphosphate (Wang et al., 2007). Though this is not a natural substrate for RNR, the chances of the existence of an active  $\alpha_6\beta_6$  complex cannot be ruled out.

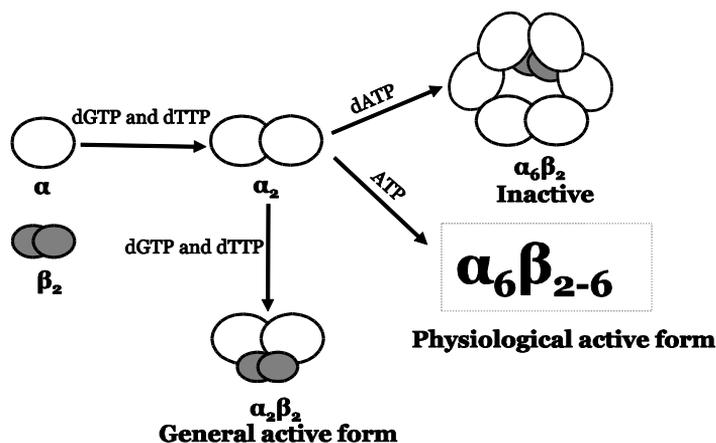


Figure 4. The eukaryotic model of overall activity regulation. The general active form is  $\alpha_2\beta_2$  complex induced by specificity site effectors binding to the s-site. Binding of either ATP or dATP to the a-site induces the active  $\alpha_{6a}$  or inactive  $\alpha_{6i}$  complexes respectively, which further binds up to one  $\beta$  dimer in the case of the inhibited  $\alpha_6$  complex and one to three  $\beta$  dimers in case of the activated  $\alpha_6$  complex.

The molecular mechanisms behind the induction of similar quaternary structures with functional differences by the effectors ATP and dATP remained a question until the advancements in solving the structure of *S. cerevisiae* class Ia RNR (Fairman et al., 2011). The x-ray crystal structure revealed that the dATP-induced  $\alpha_6$  complex is a ring-like structure formed by three  $\alpha$  dimers. dATP loses its ability to produce an  $\alpha_6$  ring formation when a point mutation (D16R) was introduced on each dimer surface. On the other hand, ATP could still induce the hexamerization even in the D16R mutant, which proves that  $\alpha$  hexamers induced by ATP and dATP

are structurally different. Further studies with cryo-electron microscopy (cryo-EM) revealed the details about the binding of the  $\beta$  dimer to the inactive  $\alpha_6$  ring, where the  $\beta$  dimer sits inside the ring in a way that the electron transport chain is interrupted between the  $\beta$  and  $\alpha$  subunits. Whereas it is known for sure that dATP induces  $\alpha_6\beta_2$  complexes, it is more uncertain how many  $\beta$  dimers can bind to the ATP-induced hexamer and the complex is therefore described as an  $\alpha_6\beta_n$  complex. The structural and molecular mechanisms behind why the complex is active are yet to be discovered (Fairman et al., 2011; Rofougaran et al., 2006).

### ***Prokaryotic model of overall activity regulation:***

*E. coli* class Ia RNR is the most studied and understood enzyme among all the classes, and it is often mentioned as a prototype of class Ia RNRs. During early years of RNR research, Reichard and coworkers investigated the oligomerization status of the *E. coli* class Ia RNR by using ultracentrifugation and found that ATP induces an active  $\alpha_2\beta_2$  complex and dATP induces an inhibited complex which is twice the size of the active complex (Brown and Reichard, 1969a). Recent biochemical and structural studies of the mammalian and *S. cerevisiae* proteins provided much more information on overall activity regulation (Fairman et al., 2011; Rofougaran et al., 2006). Further studies from our lab have started to unveil the overall activity regulation of the prototype class Ia enzyme from *E. coli*, and then proceeding studies from Drennan's group completely revealed the molecular mechanism of the *E. coli* class Ia enzyme overall activity inhibition by dATP by being able to crystalize the complex.

Though the outcome (to maintain the absolute concentration of dNTP pools) of the overall activity regulation is similar, the *E. coli* enzyme has many differences when compared to the yeast and mammalian enzymes (Figure 5 and 10). The differences are mostly about how the overall activity regulation is performed. Unlike the eukaryotic proteins, the *E. coli* enzyme

does not make ATP-induced active complexes which are larger than  $\alpha_2\beta_2$ . The enzyme uses a cross-talk strategy to become inhibited. This inhibition can occur by dATP alone or by a combination of ATP and dNTPs. dATP at high concentration binds to both the sites and inhibits the enzyme by  $\alpha_4\beta_4$  formation. A second way is that the effector ATP from the a-site can inhibit the enzyme by sensing the concentrations of dNTPs by their occupation of the s-site. Thus the *E. coli* class Ia RNR is cycling between active  $\alpha_2\beta_2$  and inactive  $\alpha_4\beta_4$  complexes by a cross-talk between the allosteric sites (Figure 5). In contrast, ATP has always an activating effect from the a-site in eukaryotic RNRs. The enzyme from *E. coli* also differs from the eukaryotic enzymes based on the requirements of the  $\beta$  subunit to form the inactive  $\alpha_4\beta_4$  complex. The *E. coli* enzyme cannot form an inactive  $\alpha_4$  complex. Instead, it forms an inactive  $\alpha_4\beta_4$  complex by dimerization of the  $\alpha_2\beta_2$  tetramer. However, the eukaryotic enzyme can form ATP-induced  $\alpha_{6a}$  complexes (active form) and dATP-induced  $\alpha_{6i}$  complexes (inactive form). Further binding of the  $\beta$  dimers leads to the formation of active  $\alpha_6\beta_n$  and inactive  $\alpha_6\beta_2$  complexes (Figure 4 and 10).

The above studies show the significant differences of how the RNR is inhibited by dATP-induced overall activity regulation in correlation with the oligomerization of its subunits (Figure 10). They clearly show that there are two different overall activity regulations to achieve the inhibitory effect. The eukaryotic model is well established and is studied in several organisms like baker's yeast, several mammal species, and *Dictyostelium discoideum*. Because of the limited information available about the overall activity regulation of other bacteria than *E. coli*, it has not been clear if the overall activity mechanism that the *E. coli* class Ia RNR uses is common to all the bacterial class Ia RNRs or if there are other types of mechanisms yet to be discovered in other types of bacteria. Therefore, it is important to study the overall activity regulation from different bacterial class Ia RNRs. It is clear that the overall activity regulation coupled with higher order oligomerization is present in the organisms with a functional ATP cone

and the ATP cone is believed to be lost and gained several times during the evolution. Therefore it is likely that there are several mechanisms of overall activity regulation, where each mechanism develops when the ATP cone is lost and gained.

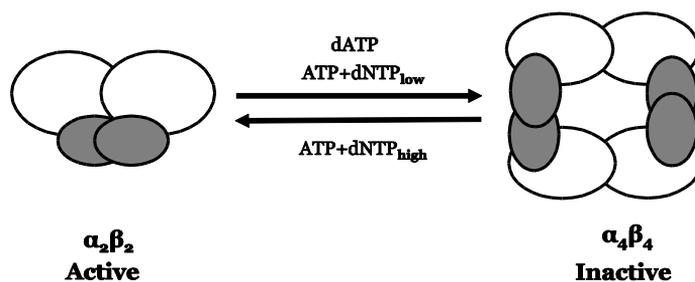


Figure 5. Overall activity regulation of *E. coli* class I RNR. The active  $\alpha_2\beta_2$  and inactive  $\alpha_4\beta_4$  complexes are induced by the cross-talk between a and s-sites.

## Medicine:

RNR is a potential drug target because it is a rate-limiting enzyme in the DNA synthesis. The enzyme has a multi-layered regulation, which contributes to several drug target points; transcriptional regulation, allosteric regulation (s-site, a-site and the associated oligomerization), regulatory proteins such as small inhibitory proteins like Sml 1 and Spd 1 in yeasts and the recently described IRBIT protein in metazoa (Arnaoutov and Dasso, 2014), and cell cycle dependent subunit  $\beta$  proteolysis to control the RNR activity (Eriksson and Martin, 1981). Other than the above-discussed drug target points, the free radical mechanism can also serve as a drug target, where the metal chelators and free radical scavengers such as triapine and hydroxyurea respectively can be used as drugs. Therefore, RNR is one of the potential candidates in drug targeting studies (Figure 6).

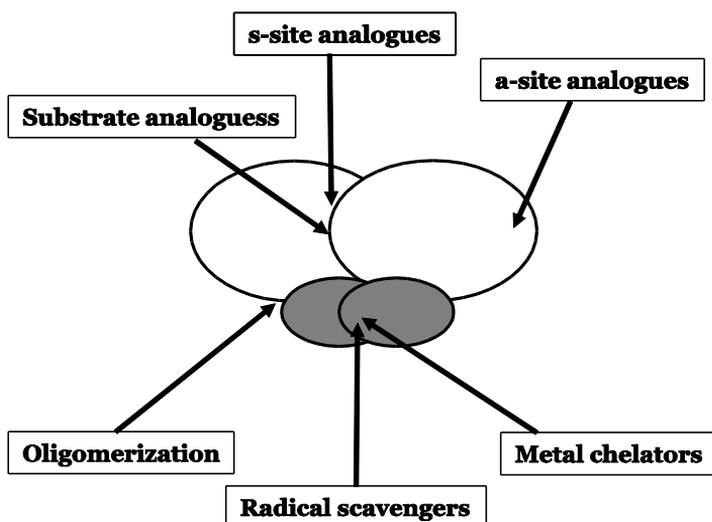


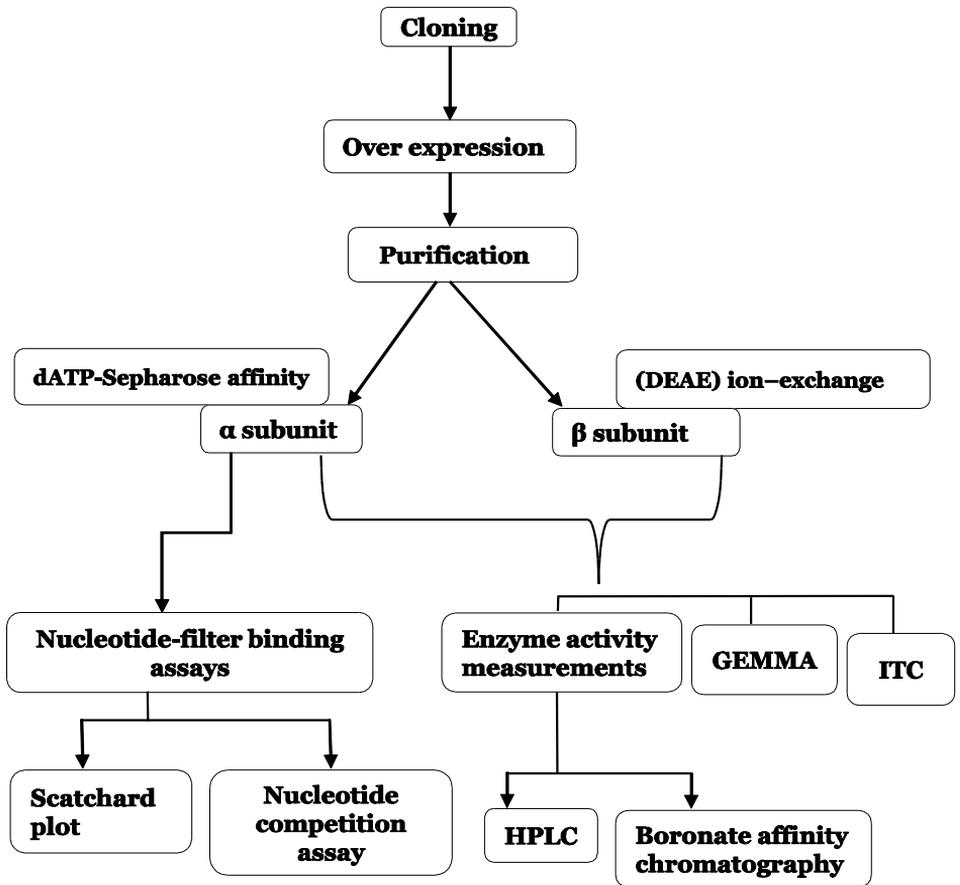
Figure 6. Different strategies to target the enzyme RNR

Although RNR is an obvious target for antimicrobial and antiproliferative drugs, there are only a few novel drugs that have been characterized so far (Table 2) because of the lack of a high throughput screening method for RNR activity until recently (Tholander and Sjöberg, 2012). The newly developed high-throughput PCR-based RNR activity study in microwell format allows the screening of several drugs at a time (Tholander and Sjöberg, 2012). With this technique, they screened several drugs and found 27 drugs which target the *P. aeruginosa* class Ia RNR (Tholander and Sjöberg, 2012).

Table 2. Established drugs in cancer therapy

|                      | <b>Inhibitor</b> | <b>Target type</b>                          | <b>Cancer type</b>                                     |
|----------------------|------------------|---|--|
|                      | Hydroxyurea      | Radical scavenger and metal chelator        | Myeloid leukemia, melanoma, and head and neck cancer   |
|                      | Triapine         | Metal chelator                              | In clinical trials                                     |
| Nucleoside analogues | Gemcitabine      | Alters the $\alpha$ subunit oligomerization | Lung, bladder, breast, ovarian and pancreatic cancers. |
|                      | Clofarabine      | Oligomerization                             | Refractory pediatric leukemia.                         |
|                      | Cladribine       | Oligomerization                             | Hematological cancers                                  |
|                      | Fludarabine      | Oligomerization                             | Hematological cancers                                  |

# Methodology:



## **Gas-phase electrophoretic macromolecule analysis (GEMMA):**

The technique GEMMA (Bacher et al., 2001; Kemptner et al., 2010) is used to determine the molecular mass of globular proteins and viruses and is relatively new in the field of biological science. The updated version of GEMMA is called MacroIMS-Macroion Mobility Spectrometer and the provider is TSI. The TSI company's expertise is in aerosol and particle research, and recently they have expanded their products to biological studies. The three units ESI, DMA, and CPC put together is called GEMMA (Figure 7). The underlying principle mechanism of GEMMA is the detection of the electrophoretic mobility of the charge-reduced ionized biopolymers.

## **Electrospray aerosol generator (Nano-electrospray ionizer) ESI:**

This unit contains a sample chamber, an electrode and a silica capillary. The aqueous solution with the biopolymer in question is transported through the capillary by atmospheric pressure and high voltage. When the sample reaches the capillary tip, it forms a cone jet of differentially charged monodisperse aerosol droplets with an average diameter of 160 nM. In principle, each droplet analyzed contains only one biomolecule. Then the monodisperse aerosol droplets are transported to the charge reducing chamber where each droplet is dried and charge reduced to single positive or negative charged aerosols (macroions) by means of a polonium source, which introduce neutralizing gas ions in the air/CO<sub>2</sub> mixture that enters the chamber.

## Differential mobility analyzer (DMA)

The macroions are then transported to the DMA, where they are separated based on the electrophoretic mobility (EM), which is directly proportional to the diameter of the particle diameter. This separation is under a continuous laminar airflow, using a differential voltage at atmospheric pressure. The macroions with a specific EM are selected by the voltage and travel through a slit towards the CPC. The macroions which could not go through the slit under the particular voltage exit from the DMA. Likewise, macroions with the voltage selected diameter will pass through the slit, and are transported to the CPC. By scanning the voltage over the size range of interest, a size distribution can be obtained for the sample.

**Note:** The electrophoretic mobility depends on the diameter of each macroion. There is a strong correlation between diameter and the molecular weight where the EM is directly proportional to the diameter which is calculated by the Millikan equation.

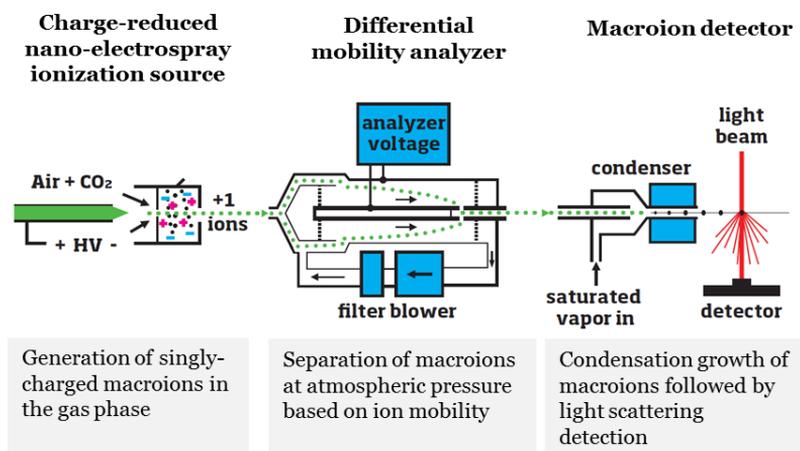


Figure 7. Schematic view of the method GEMMA with its three components which are nano-electrospray ionization source, differential mobility analyzer, and macroion detector. Reprinted with the permission from TSI.

## **Condensation particle counter (CPC)**

After the DMA, that the macroions reach the CPC for quantification. The macroions that reach the CPC are too small to be detected by the laser optical device. The *n*-butanol vapor condensation process makes the macroions large enough (10  $\mu\text{m}$ ) to be detected by the instrument for the quantification.

### ***Advantages and disadvantages:***

GEMMA is a relatively new mass spectrometry-related method to quantify biomolecules, and it certainly has many advantages compared to the methods that are already being used. The most important benefits are the small required sample quantity (0.01 mg/ml of protein in 20  $\mu\text{l}$ ), and the short time of analysis (2 min). Most mass spectrometry-based techniques like matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization are suitable methods to quantify accurate molecular masses of non-covalent high molecular weight biomolecules. But these two approaches have some limitations (Bacher et al., 2001); (i) method optimization for each sample, (ii) difficulty in charge determination of multiply charged ions at high mass range, (iii) complexes with hydrophobic interactions are very sensitive to decomposition under the high vacuum, and (iv) extremely low sensitivity for large complexes. The latter factor makes the method not suitable for quantification because of a strong underestimation of oligomerization. Size-exclusion chromatography has also limitations and we found that it was difficult to separate and quantify mixtures of different protein complexes which are in fast equilibrium between the complexes. On the other hand, GEMMA is an advantageous method to detect and quantify different oligomeric protein complexes which are in rapid equilibrium with noncovalent interactions.

The main limitation for GEMMA is a requirement of only using volatile salts and buffers such as ammonium acetate. In RNR research, GEMMA is proven to be a suitable method to study the oligomerization status of the subunits (Rofougaran et al., 2006), but the method has limitations dependent on the protein, where some proteins clog the capillary by binding. The problem with the clogging can be overcome by using soft detergents like Tween-20, or by using the updated version of GEMMA termed Macro-IMS, which seems to have fewer problems with the clogging due to that the capillary length is minimized.

## Results and Summary

### Paper I: Diversity in Overall Activity Regulation of Ribonucleotide Reductase

The mechanism behind the overall activity regulation of RNRs is only partially known. Previous studies of class I RNRs have revealed that the overall activity regulation is dependent on the formation of oligomeric complexes. Until now, there are two types of mechanisms to achieve overall activity regulations that have been reported, a eukaryotic model where the mammalian and yeast enzymes cycle between structurally different active and inactive  $\alpha_6\beta_2$  complexes (Fairman et al., 2011; Hofer et al., 2012; Rofougaran et al., 2006) and a prokaryotic model where *Escherichia coli* enzyme cycling between active  $\alpha_2\beta_2$  and inactive  $\alpha_4\beta_4$  complexes (Rofougaran et al., 2008; Uhlin and Eklund, 1994). These two mechanisms are here presented to be the outcome of the loss and gain of ATP cones during the evolution. One such example where an extra ATP cone is gained is the class Ia RNR of *P. aeruginosa*. From previous studies, it is known that *P. aeruginosa* has all three classes of RNRs and that the  $\alpha$  subunit of its class Ia RNR has two ATP cones, of which only the N-terminal ATP cone is functional (Torrents et al., 2006).

The quaternary structure of ATP-induced *P. aeruginosa* class I RNR analysis from the previous studies (Torrents et al., 2006) is ambiguous, which is a common limitation of gel filtration studies with rapidly equilibrating protein complexes. Therefore in this study, we have used a technique termed Gas-phase electrophoretic macromolecule analysis (GEMMA) to investigate the oligomerization status of the *P. aeruginosa* class I RNR. This method is an alternative method which is suitable to study different oligomeric complexes in fast equilibrium. In this project, the allosteric regulation of *P. aeruginosa* class I RNR is analyzed by using the following methods; GEMMA, ITC, enzyme activity assays, and nucleotide filter binding assays.

We have started with four substrate enzyme assays to see if this enzyme follows the same specificity regulation as other enzymes and we found it follows the same trend except for UDP, which is a very poor substrate for the RNR in this organism. It seems that most of the dTTP comes from an alternative pathway where CDP reduction is followed by deamination (explained in the specificity regulation section). This pathway is common in many organisms but likely to be especially crucial in *P. aeruginosa*.

We have examined the oligomerization of the subunits in the presence of different combinations of the allosteric effectors. The subunit  $\beta$  is mostly a dimer irrespectively of any effector presence. The subunit  $\alpha$  is mostly monomer with no effectors, but we could see minor populations of dimers and tetramers. The small amount of dimerization of the  $\alpha$  subunit with no effector is common among other characterized class Ia RNRs, but tetramerization is something new to this organism. Later, we repeated the experiments in the  $\sim\Delta 147$  N-terminal ATP cone deleted mutant and the results show that the mutant cannot form any tetramers. Therefore, it is clear that the tetramers that we have observed in the wild-type protein with no effectors are real. Subsequently, we have analyzed the effect of allosteric effectors on the oligomerization status of the subunit  $\alpha$  alone and in combination with the subunit  $\beta$ . The results show that all the effectors including s-site effectors induce  $\alpha$  tetramerization. The tetramers induced by s-site effectors dissociate into dimers in the presence of  $Mg^{2+}$  ions (physiologically relevant) and forms an active  $\alpha_2\beta_2$  tetramer by binding to the  $\beta$  dimer. However, the dATP-induced tetramers are stable also in the presence of  $Mg^{2+}$  and readily bind to the  $\beta_2$  dimer forming an  $\alpha_4\beta_2$  hexamer (Figure 8), which is inactive (enzyme activity assay data, Figure 7D in paper I).

The nucleotide filter binding and enzyme activity assay experiments gave us further details about how the allosteric effectors are binding to the allosteric sites and make the enzyme active or inactive. Perhaps the most

striking observation in this publication is that there are two dATPs binding to the functional N-terminal ATP cone. This is the first ever reported case where two dATPs bind to the a-site. Surprisingly, dGTP which is generally known as a s-site effector can also bind to the a-site in this organism. However, this binding has no physiological effect because ATP readily competes out the dGTP from the a-site and the dGTP-induced tetramers are dissociated to dimers.

To study the role of the ATP on overall activity regulation, we have performed enzyme activity assays. In a comparative study, we have checked the ATP function from the a-site in mouse and *P. aeruginosa* class Ia RNRs. We have performed GDP reduction with saturated amounts of dTTP (2 mM dTTP completely saturates the s-site) and analyzed the enzyme activity by adding increasing amounts of ATP (in this case ATP only binds to the a-site). As previously described (Eriksson et al., 1979; Rofougaran et al., 2006), the GDP reduction activity of the mouse enzyme raises 2-3 fold by increasing the ATP concentration with saturated amounts of dTTP, whereas ATP has no such effect on the the *P. aeruginosa* enzyme. This shows that ATP has an activating effect through the a-site on the mouse enzyme and no effect on the *P. aeruginosa* enzyme. Further experiments on the *P. aeruginosa* enzyme showed that although the ATP does not have any direct activation effect it will passively activate the enzyme by competing out the binding of dGTP and dATP to the a-site.

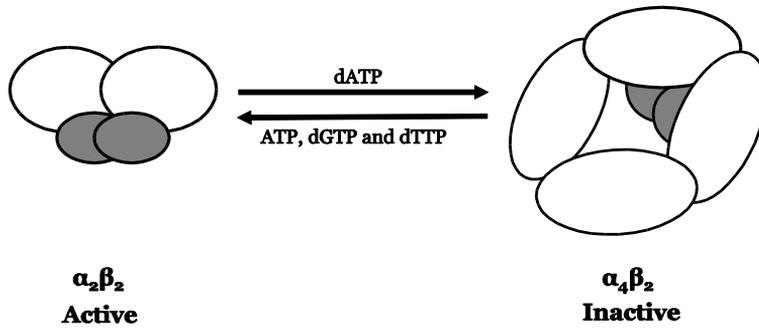


Figure 8. Overall activity regulation of *P. aeruginosa* class I RNR. The active  $\alpha_2\beta_2$  complex is induced by all the effectors except dATP. The inactive  $\alpha_4\beta_2$  complex is induced by dATP through a-site.

## Conclusions

As indicated before (Torrents et al., 2006), we confirm that there is a third kind of allosteric activity regulation in the *P. aeruginosa* class Ia RNR when compared to the *E. coli* and mammalian enzymes (Figure 10). These many mechanisms behind the overall activity regulation are interesting from an evolutionary viewpoint and from a drug development view, where the specific features of each pathogen can be targeted (Figure 9).

## **Paper II: Structural Mechanism of Allosteric Activity Regulation in a Ribonucleotide Reductase with Double ATP Cones**

In paper 1, we have used different biochemical methods to characterize the *P. aeruginosa* class I RNR overall activity regulation in correlation with the oligomerization. The second article is a natural continuation of my first article. Here we have studied the molecular mechanism behind the overall activity regulation of *P. aeruginosa* class I RNR with structural and biochemical techniques. Our coauthors have done the structural analysis of the enzyme by using crystallography and small-angle X-ray scattering (SAXS) and we have provided the biochemical evidence to strengthen their structural data.

The 2.3 Å resolution dATP-induced structure of subunit  $\alpha$  is a tetramer which is in agreement to our paper 1 results. The structure clearly shows the occupation of two dATP molecules neutralized by  $Mg^{2+}$  on the ATP cone 1 domain. We did biochemical studies on three mutants where the structurally defined interaction surfaces were altered to confirm the structural data with biochemical evidence. With site-directed mutagenesis, we have created three different point mutations on subunit  $\alpha$ , which are R119D, E106A/E126A, and H72A/D73A/Y830A. The biochemical studies include GEMMA, enzyme activity measurements, and nucleotide binding experiments to study the overall activity behaviors of the three mutant enzymes and compare with the wildtype. The Scatchard plot results show that each of all three mutants can bind up to three dATP molecules (Figure 5 in paper II). The GEMMA analysis shows that the R119D, and E106A/E126A mutants have lost their ability to tetramerize. Interestingly, the mutant H72A/D73A/Y830A has only a partial tetramerization ability. To check the oligomerization status of the mutant  $\alpha$  subunit in relations to the activity, we have performed enzyme activity

assays. These results show that the mutants R119D, and E106A/E126A have lost the ability to be inhibited by dATP which agrees with the GEMMA results where these two mutants cannot tetramerize (Figure 5 in paper II). The mutant H72A/D73A/Y830A could only partially be inhibited by dATP, which is in agreement with the GEMMA data.

The above results provide the structural evidence of a third kind of overall activity regulation in *P. aeruginosa* class I RNR, which is quite different from the previously two known eukaryotic and *E. coli* models (Figure 10). The sequence analysis data shows that the occurrence of multiple ATP cones is widely spread in different groups and subgroups of RNRs. The structural and sequence data helps us to predict the presence and functionality of multiple ATP cones in different organisms and at the same time, it gives us the information to predict the binding of more than one dATPs to the predicted functional ATP cone. Since we know the details about the molecular mechanisms behind the unusual binding of two dATPs to the functional N-terminal ATP cone, this information can lead us to develop new dATP analogues which target the *P. aeruginosa* class I RNR with minimized side effects on the host system (see the future plans and figure 9). One possibility could then be to connect two dATP analogues into one molecule.

### **Paper III: A ribonucleotide reductase inhibitor with deoxyribonucleoside-reversible cytotoxicity**

RNR controlled dNTP synthesis is crucial for DNA replication fidelity and repair. The characteristic of most cancer types is uncontrolled DNA replication and repair, which can be the consequence of RNR misregulation. Therefore the enzyme is undoubtedly a good target for anticancer drugs. There are several established drugs like gemcitabine and hydroxyurea, which are already being used in cancer therapy. The drug hydroxyurea is a metal chelator and radical scavenger. It removes the radical and radical generating di-iron center of the  $\beta$  subunit which makes the enzyme inactive (Aye et al., 2015; Gräslund et al., 1982). It has been reported that cells treated with the drug hydroxyurea become resistant by upregulating the  $\beta$  subunit (Aye et al., 2015; McClarty et al., 1987; Åkerblom et al., 1981) and its metal chelating property can target other metalloproteins like carbonic anhydrase (Temperini et al., 2006).

In a recent PCR-based drug screening study, novel inhibitors that target and inhibit the *P. aeruginosa* class I RNR have been found (Tholander and Sjöberg, 2012). These drugs are also inhibitors of the human RNR. Our paper III deals with the effect of non-nucleoside RNR inhibitors on human RNR and cellular proliferation. Out of several human inhibitors studied, the drug NSC73735 possesses many characteristics to be a potential lead drug candidate to target RNR.

Our coauthors initiated this study. The biological activity, RNR inhibition, binding properties to the RNR subunits, cytotoxicity, and effect on deoxynucleotide pools of the inhibitors were tested by using enzyme activity assays, thermal shift assays, FACS, and HPLC respectively. All the above analyses provided the drug candidate NSC73735 as the best hit among the 12 drugs analyzed. In this article, I was involved in the

oligomerization experiments by using GEMMA (Figure 1 and supplemental figure S2 in paper III). We have studied the mechanism of how NSC37375 inhibits the enzyme. From the thermal shift assays, it is evident that the drug binds to the  $\alpha$  subunit of the enzyme. Here, we have analyzed the effect of the drug on the oligomerization status of the enzyme. The experiments were performed either by incubating the  $\alpha$  subunit with ATP+NSC37375 or dATP+NSC37375. The results clearly show the destabilization of the ATP-induced active  $\alpha$  hexamers and dATP-induced inactive  $\alpha$  hexamer to dimers in the presence of the inhibitor NSC37375. The nucleotide pool studies show that the drug-driven RNR inhibition at physiological conditions is by destabilization of the ATP-induced hexamer complex.

### **Conclusion:**

The antiproliferative molecule NSC37375 is the first reported molecule that interferes the oligomerization of the large subunit of RNR to inhibit human RNR. The molecule NSC37375 is reported to have more specificity to human RNR than the currently used antiproliferative drug hydroxyurea.

## **Future plans:**

*P. aeruginosa* is a gram-negative opportunistic pathogen in immunocompromised patients and causes severe nosocomial infections like pneumonia and septic shock (Torrents et al., 2006). It develops resistance to most of the established antibiotics, and targeting nucleotide metabolism can be a new choice of making drugs. The studies of our first paper (Jonna et al., 2015) combined with the drug targeting studies (Tholander and Sjöberg, 2012) could be an important step towards the establishment of novel drugs to target RNRs of pathogenic organisms.

### **Targeting *P. aeruginosa* RNR.**

We have observed several differences in the allosteric regulation of *P. aeruginosa* RNR when compared to the mammalian RNR (paper I and II). In this project, we are planning to specifically target the a-site binding properties of the *P. aeruginosa* RNR since we know that it has broad specificity. In particular, the a-site of the  $\alpha$  subunit can bind dGTP, which is unique to this parasite and dGTP induces tetramer formation, which is the inactive RNR complex. A possibility could be that novel deoxyguanosine analogues can be developed, which specifically target the protein (Figure 9).

So far, we have only done studies on the isolated enzyme but our idea for the future is that the effect of the corresponding target studies will be evaluated on the growth and dNTP pools of *P. aeruginosa* cells with mammalian cells as a reference.

We are also planning to use GEMMA as a screening tool to check the oligomerization status of the *P. aeruginosa* RNR in the presence of inhibitors, which we know inhibit the *P. aeruginosa* RNR but the mechanism is not known. Here, we will study the ability of the inhibitors to inactivate the enzyme by altering the oligomerization status from an

active  $\alpha$  dimer to its inactive monomer or tetramer (Figure 9). Once we find a potential inhibitor that alters the oligomerization state of the enzyme, we will study the binding properties of the inhibitor to the enzyme.

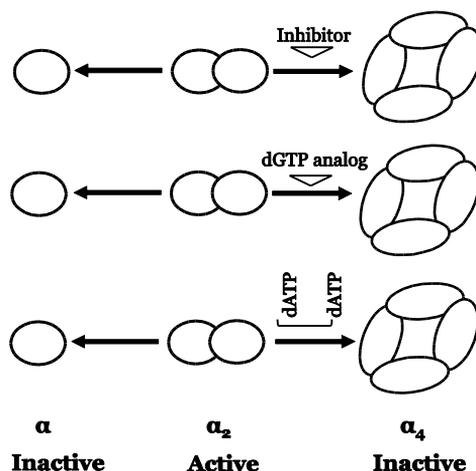


Figure 9. Three different strategies to target the *P. aeruginosa* class I RNR includes either by stabilizing the inactive complex  $\alpha_4$  or by dissociation of the active complex  $\alpha_2$  to its inactive monomers.

## Multiple mechanisms of allosteric overall activity regulation

In paper 1, we have observed several differences in the allosteric regulation and a novel kind of allosteric overall activity mechanism of *P. aeruginosa* RNR when compared to the two known regulation models primarily characterized in *E. coli* and mammalian RNRs (Figure 10). In future studies, our idea is to identify the possible occurrence of even more allosteric overall activity regulation mechanisms in the class I RNRs of different organisms. We are especially interested in RNRs from different pathogens to exploit the differences in overall activity mechanism to specifically target the pathogenic RNRs. We then start with bioinformatic analysis to find organisms, which are distantly related, based on the RNR protein sequence similarities and therefore more likely to contain new

allosteric mechanisms of overall activity regulation. Once we have selected RNR candidates from the bioinformatic analysis, each RNR candidate will be cloned for protein expression and purification. Allosteric overall activity regulation is studied by GEMMA, filter binding studies, enzyme activity assays, and other methods that our group has been using for to study oligomerization and allosteric regulation. This project will subsequently lead to several subprojects.

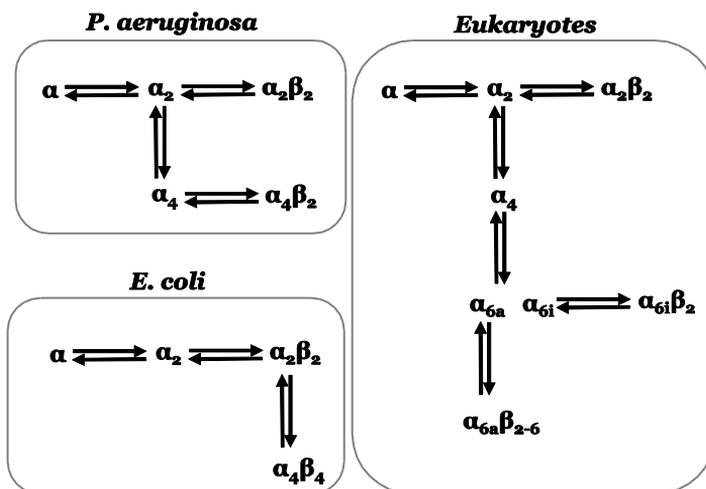


Figure 10. Three known mechanisms of overall activity regulation in class I RNRs

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